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The ARID domain protein dril1 is necessary for TGFβ signaling in *Xenopus* embryos☆

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Abstract

ARID domain proteins are members of a highly conserved family involved in chromatin remodeling and cell-fate determination. *Dril1* is the founding member of the ARID family and is involved in developmental processes in both *Drosophila* and *Caenorhabditis elegans*. We describe the first embryological characterization of this gene in chordates. *Dril1* mRNA expression is spatiotemporally regulated and is detected in the involuting mesoderm during gastrulation. Inhibition of dril1 by either a morpholino or an engrailed repressor–dril1 DNA binding domain fusion construct inhibits gastrulation and perturbs induction of the zygotic mesodermal marker *Xbra* and the organizer markers *chordin*, *noggin*, and *Xlim1*. *Xenopus tropicalis* dril1 morphants also exhibit impaired gastrulation and axial deficiencies, which can be rescued by coinjection of *Xenopus laevis dril1* mRNA. Loss of dril1 inhibits the response of animal caps to activin and secondary axis induction by smad2. Dril1 depletion in animal caps prevents both the smad2-mediated induction of dorsal mesodermal and endodermal markers and the induction of ventral mesoderm by smad1. Mesoderm induction by eFGF is uninhibited in dril1 morphant caps, reflecting pathway specificity for dril1. These experiments identify dril1 as a novel regulator of TGFβ signaling and a vital component of mesodermal patterning and embryonic morphogenesis.

Keywords: ARID; Dril1; TGFβ; *Xenopus*; Mesoderm; Embryo

Introduction

Elucidation of the molecular mechanisms controlling embryogenesis has revealed the parsimony of evolution: a few transcriptional motifs are used repeatedly in different contexts to control many aspects of developmental gene expression. Given the redundancy of nature's transcriptional toolbox, discovery of new DNA binding domains may identify novel developmental regulators.

We have begun a developmental investigation of one such recently identified family of transcriptional regulators: the ARID family, which is highly conserved across eukaryotes. The ARID domain, an acronym for "AT-rich interacting domain," was first identified in mouse Bright, a B cell regulator of immunoglobulin heavy chain (IgH) transcription (Herrscher et al., 1995). Binding of the Bright protein to its target DNA sequence was abolished when part of the ARID domain was deleted. Almost contemporaneously, the *Drosophila* orthologue of Bright, named *dead ringer* (*dri*), was identified and shown to have 75% amino acid identity with mouse Bright over its DNA binding domain (Gregory et al., 1996).

Several proteins containing an ARID domain of approximately 100 amino acids are found in yeast as well as higher eukaryotes, indicating that this is an ancient protein module. The *rum1* gene of the corn smut fungus *Ustilago maydis* controls spore development by repressing specific targets (Quadbeck-Seeger et al., 2000). The *S. cerevisiae* ARID protein SWI1 is a component of the SWI/SNF chromatin remodeling complex that mediates both transcriptional activation and repression (Martens and Winston, 2002).

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The ARID protein osa has been shown to associate with the SWI/SNF complex in both *Drosophila* and humans, indicating that the ancient function of ARID proteins in chromatin remodeling may have been retained in metazoans (Collins et al., 1999; Hurlstone et al., 2002; Kozmik et al., 2001; Vazquez et al., 1999). *Drosophila* osa represses wingless targets and *osa* mutants have defects in embryonic segmentation and wing development (Collins and Treisman, 2000; Treisman et al., 1997). An additional *Drosophila* ARID gene implicated in chromatin regulation is *little discs* (*lid*) (Gildea et al., 2000). Like *osa*, *lid* is classed as a trithorax group gene because it is involved in maintenance of homeotic gene expression (Gildea et al., 2000; Vazquez et al., 1999). *Lid* is a homologue of the retinoblastoma binding protein, RBP2, which is down-regulated in human melanomas (Vogt et al., 1999). Other ARID proteins are associated with cancer; plu-1 is a transcriptional repressor up-regulated in breast cancer (Lu et al., 1999), and RBP1L1 is associated with several malignancies (Cao et al., 2001). Additionally, murine fibroblasts transformed with *dril1*, the human orthologue of *Bright*, are rescued from ras-induced senescence and become highly oncogenic (Peeper et al., 2002).

Only two vertebrate ARID mutants have been studied: *jumonji*, involved in mouse neurulation and heart development (Lee et al., 2000; Takeuchi et al., 1995), and *desrt*, loss of which results in reduced growth and abnormal reproductive organs in mice (Lahoud et al., 2001). We decided to focus on the founding member of the ARID family, *Bright/dri*, as both *Drosophila* and *Caenorhabditis elegans* mutants exhibit defects in embryogenesis. Fly *dri* maternal/zygotic mutants have abnormal axial patterning and muscle development (Shandala et al., 1999). In addition to its role in both anteroposterior and dorsoventral patterning, *dri* functions in neuronal migration of the longitudinal glia in flies (Shandala et al., 2003). Its orthologue in *C. elegans*, called *CFI-1*, also plays a role in nervous system development as a regulator of neuronal subtype identity (Shaham and Bargmann, 2002). *Spdeadringer*, the echinoderm orthologue, is involved in gastrulation and primary mesenchyme cell patterning (Amore et al., 2003).

The dril protein performs both activatory and repressive functions. Fly dri recruits the repressor Groucho into a complex with Dorsal, resulting in repression of the Dorsal targets *zerknüllt* and *huckebein* (Hader et al., 2000; Valentine et al., 1998), mouse Bright is a transactivator of the intronic enhancer of IgH genes (Herrscher et al., 1995), and human dril1 stimulates E2F-dependent transcription (Suzuki et al., 1998). There are two copies of *dri* in vertebrates, *dril1* and *dril2*. Mouse *Bright* is orthologous to dril1. *Dril2* binds the retinoblastoma protein, as do several other ARID proteins (Numata et al., 1999), but little else is known about the function of this protein.

Because ARID genes are highly conserved genes with developmental functions in protostomes, it is surprising that little is known about their role in chordate embryogenesis. Therefore, we have begun a developmental analysis of ARID genes in *Xenopus*. Here we describe the isolation and analysis of *Xenopus dril1*. We show that *dril1* is developmentally regulated in *Xenopus* and is expressed in the involuting mesoderm during gastrulation. Dril1 depletion by morpholino treatment or by an engrailed repressor–dril1 DNA binding domain fusion construct causes inhibition of gastrulation. Maternal signaling pathways appear intact in these embryos but zygotic mesoderm induction is inhibited by dril1 depletion. Loss of dril1 prevents the elongation of animal caps and the induction of mesoderm in response to activin. We demonstrate that dril1 is necessary for both smad1 and smad2-mediated transcriptional activation but not for mesoderm induction in response to eFGF.

Materials and methods

Cloning of X. laevis dril1 and creation of fusion constructs

A BLASTN search identified an incomplete *X. laevis* EST (gi:7697621) possessing 82% identity with *Homo sapiens dril1*. This EST was used to probe a lambda ZAPII cDNA library derived from NF28 embryonic heads (Hemmati-Brivanlou et al., 1991). Two full-length dril1 clones were isolated, corresponding to a 2.2-kb RNA/ 539 amino acid protein. One clone was sequenced completely and the sequence was deposited in GenBank (AY787401). Dril1 was subcloned into pCS2 (Turner and Weintraub, 1994) for embryonic expression. Fusion constructs were created by inserting the dril1 DNA binding domain N-terminal to either the engrailed repressor domain or the VP16 activator domain (Kessler, 1997). The dril1 DNA binding domain was amplified using the following primers (5′–3′): CTCTCGAGCCATGGAGACTGGACA (EnR–dril+); CTCTCGAGCATGGAGACTGGACA (VP16+); ACTCTAGAGGATGAAAGCATGC (EnR–dril− and VP16–dril−). Underlined sequences indicate restriction sites used to facilitate cloning. A C-terminally myc-tagged dril1 rescue construct lacking the morpholino target site but containing an optimal Kozak sequence (CS2-Δ5′ dril-MT) was constructed in CS2-MT using the following primers (5′–3′): ACTTAGATCTGCCGCCATGAAGCTGCAAGC (+); ACTTATCGATTGGGAGAGGGGTTATTAG (−).

Embryo experiments

Xenopus embryos were staged according to Nieuwkoop and Faber (1994). *X. laevis* microinjections were performed in 3% Ficoll/0.5× MMR and embryos were subsequently transferred to 0.1× MMR containing 10 μg/ml gentamicin. *Xenopus tropicalis* injections were performed in 4% Ficoll/0.1× MMR and embryos were transferred to 0.01× MMR before gastrulation. RNAs for microinjection were synthesized using mMessage mMachine (Ambion). RNA was synthesized from linearized DNA templates of CS2–dril1 (*Not*I), CS2-Δ5′dril-MT (*Not*I), CS2–EnR–dril (*Sac*II), and

CS2–VP16–dril (*Not*I) using SP6 RNA polymerase. RNA was transcribed from the following plasmids as previously described: CS2-smad1 (Thomsen, 1996), CS2-FLAG-hsmad2 (Eppert et al., 1996), and pSP35-chordin-myc (Piccolo et al., 1996). The dril1 morpholino sequence designed by Gene Tools (Philomath, Oregon) used was 5′-GGTGGGGCAGGCGAGCGGGCACAG-3′. The 5-bp mismatch morpholino used was 5′-GGTTGGACAGGC-TAGCGTGCAGAG-3′ (substituted bases are underlined). The sequence of the Gene Tools control morpholino used was 5′-CCTCTTACCTCAGTTACAATTTATA-3′. For animal cap assays, caps were excised at Nieuwkoop and Faber (NF) stage 8.5 and cultured in 1× MMR/0.1% BSA/10 μg/ml gentamicin. RNA from *smad*-injected caps was isolated at NF11.5 for real-time PCR analysis. For activin experiments, the culture medium was supplemented with 20 ng/ml human recombinant activin A (R&D Systems) and caps were cultured until NF12 for real-time PCR or until neurula stages for morphological analysis.

In situ hybridization and antibody staining

In situ hybridization was performed according to standard techniques (Harland, 1991) using digoxigenin-labeled probes. BM purple (Roche) was the substrate for the colorimetric reaction. Sagittal bisections were performed after MEMPFA fixation but before hybridization. Bisected embryos were not proteinase K treated. Sense controls were included to monitor nonspecific staining. Plasmids used for probe synthesis were as follows: Xbra-pXT1 (Smith et al., 1991); pBS-frzb-1 (Leyns et al., 1997); CS2-Fz7 (Djiane et al., 2000); CS2-sprouty2 (Nutt et al., 2001); pBS-cerberus (Bouwmeester et al., 1996); and CS107-prickle (Wallingford et al., 2002). The *Eco*R1–*Hin*cII fragment from pSP35-chd-myc (Piccolo et al., 1996), subcloned into pGEM4Z, was used to make the chordin probe. The dril1 probe was synthesized from pBS–dril1 linearized with *Not*I using T7 polymerase. Antibody staining with αPH3 (Upstate) was performed according to Saka and Smith (2001).

Real-time PCR

Embryonic RNA was extracted according to Marikawa et al. (1997) but was precipitated with ammonium acetate/ethanol rather than LiCl. After DNAse I treatment for 1 h at 37°C, followed by phenol–chloroform–isoamyl alcohol extraction, RNA was precipitated with sodium acetate/ethanol and dissolved in DEPC-treated water. cDNA was

Fig. 1. Multiple sequence alignment of *Xenopus* dril1 with related proteins. Abbreviations: X.l. (*X. laevis)*; M.m. (*Mus musculus*); H.s. (*H. sapiens*); D.m. (*Drosophila melanogaster*); C.e. (*C. elegans*). BOXSHADE coloring indicates completely conserved residues (green), identical residues (yellow), and similar residues (cyan). The ARID domain is overlined (pink).

synthesized at 42°C by superscript II (Invitrogen) using 0.5–1 μg RNA as template. The cDNA was diluted fourfold with deionized water before real-time PCR analysis. Real-time PCR using the LightCycler (Roche) was performed according to Kofron et al. (1999), except that 2.5 μl of the diluted cDNA reaction was used as template. Relative amounts of PCR product were determined based on interpolation from a four-point standard curve, derived from dilutions of the sample predicted to give the highest expression (control embryos or activin/smad-induced caps). Duplicates of cDNA samples were amplified in most LightCycler reactions to ensure reproducibility. Typically, the standard deviation was about 10% of the mean. Expression levels were normalized relative to *ornithine decarboxylase* (*ODC*) levels in the samples. The following LightCycler primer sequences and reaction conditions have been published previously: *chordin*, *Xbra*, *xnr1*, *xnr2*, *xnr3* (Kofron et al., 1999); *bix4*, *endodermin*, *mix1*, *mixer*, *ODC*, *sox17*, *Xlim1* (Xanthos et al., 2001); *BMP4*, *noggin, siamois*, *sizzled* (Xanthos et al., 2002); and *goosecoid*, *vent1*, *vent2* (Kofron et al., 2001). *Xhox3* primers (Rupp and Weintraub, 1991) were adapted for real-time PCR using a 63°C/5-s annealing step, a 72°C/13-s elongation step, and a 90°C/3-s acquisition step.

Results

Dril1 is a developmentally regulated member of the ARID family

Two full-length dril1 clones were isolated from a library screen. The predicted protein sequence had an overall amino acid identity of 63% with human dril1 and 42% with human dril2 (Fig. 1). Alignment of the *Xenopus* sequence with dril homologues from other species identified the ARID domain between amino acids 194 and 318; the *Xenopus* protein shares 99% identity with its human orthologue over this region. The *Drosophila* sequence is more divergent than its orthologues in other species, containing several unique stretches.

The spatial localization of *dril1* mRNA during development was analyzed by in situ hybridization (Fig. 2). In whole embryos, *dril1* expression was detected in the ectoderm of various embryonic stages, including the animal half of the blastula, gastrula ectoderm, nonneural ectoderm of the neurula (Fig. 2A), and the tadpole epidermis (Fig. 2B). Expression in the neural plate was low throughout neurulation, indicating a down-regulation of *dril1* in neural tissue (Fig. 2A). To determine whether *dril1* was expressed in any of the other germ layers, embryos were sagittally bisected at various stages of gastrulation and then subjected to in situ hybridization. In the early gastrula, *dril1* expression was seen in the mesendoderm above the dorsal lip (Fig. 2D). By midgastrulation, *dril1* expression in the mesoderm had intensified in the involuting mesodermal layer (Fig. 2E). Towards the end of gastrulation, *dril1* was detected throughout the involuted mesoderm, with particularly high levels of expression in the newly involuted mesoderm comprising the dorsal and ventral lips of the closing blastopore (Fig. 2F). Expression of *dril1* was also observed in the presomitic mesoderm of neurulae (Fig. 2G). Northern analysis corroborated the in situ data, detecting low *dril1* expression during early embryogenesis and highest mRNA levels between NF19 and NF28 (data not shown).

Dril1 is necessary for gastrulation

To determine whether dril1 has a developmental role, 2 ng *dril1* mRNA was microinjected into embryos. Reduced or abnormally shaped eyes were evident in 13/15 tadpoles scored at NF39 (Fig. 3B). Gastrulation was normal in 70/72 cases and no obvious axial defects were observed. Animal

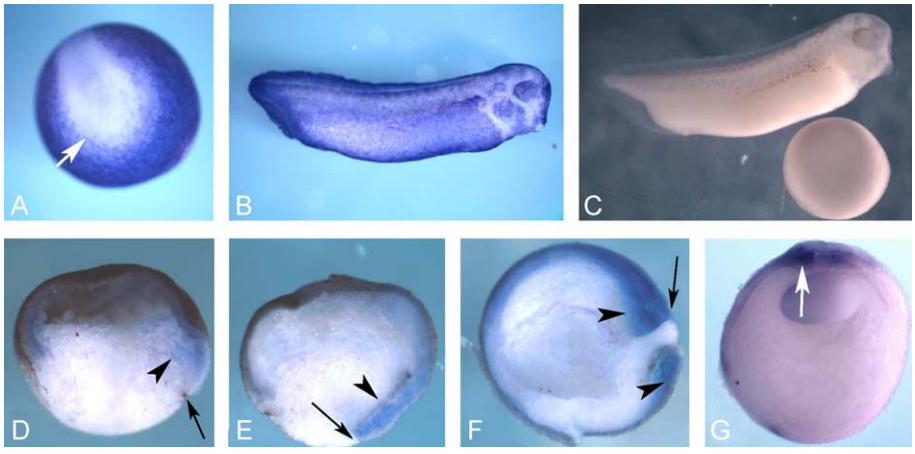

Fig. 2. *Dril1* in situ hybridization. Expression is high in the nonneural ectoderm of the early neurula, but it is excluded from neural plate; boundary marked with an arrow (A). Late tailbud embryo stained throughout the epidermis (B). Sense controls (C). Sagittal bisections of NF10 (D), NF11 (E), and NF12.5 (F) gastrulae, showing mesodermal expression (black arrowheads). Arrows mark the dorsal lip of the blastopore. Transverse cut of neurula (G) showing staining in presomitic mesoderm (white arrow).

Fig. 3. Effects of dril1 overexpression and depletion on *X. laevis* development. Uninjected controls (A); tadpoles exhibiting eye defects (white arrowheads) but unperturbed axes after injection with 2 ng *dril1* mRNA (B) or 2 ng *VP16–dril* (C). Gene Tools control morpholino (D and H), 30 ng dril1 morpholino (E and I), 200 pg *EnR–dril* RNA (F), 600 pg *EnR–dril* RNA (G), and 30 ng 5 bp mismatch dril1 morpholino (J).

caps injected with 2 ng *dril1* mRNA did not undergo morphogenetic movements ($n = 30$) and did not express the pan-mesodermal marker *Xbra* (data not shown). The VP16 activation domain was fused to the DNA binding domain of *dril1* in an attempt to enhance possible transcriptional activation by the protein. Injection of 2 ng of the *VP16–dril* fusion construct mimicked the effect of the full length RNA, causing eye defects in 12/19 NF39 embryos (Fig. 3C). However, gastrulation was normal in 65/74 cases. These overexpression results indicate that expression of *dril1* mRNA alone is not sufficient to cause major morphogenetic or fate changes in early embryogenesis.

To investigate whether dril1 is necessary for development, both cells of 2-cell embryos were injected equatorially with a morpholino designed to prevent translation of the dril1 protein. Morphant embryos had profound gastrulation defects, especially at the highest injection amount of 30 ng, which resulted in embryos with gaping blastopores in 105/108 embryos, whereas only 3/104 embryos injected with the control morpholino failed to complete gastrulation when wild-type siblings reached stage NF13 (Figs. 3D, E, H, and I). A BLASTN search of the NCBI EST database using the morpholino sequence did not reveal any other mRNAs containing the morpholino target site. Injection of 30 ng of a 5-bp mismatch dril1 morpholino (5-mis MO) did not perturb gastrulation, providing further evidence of morpholino specificity (Fig. 3J). We attempted to perturb dril1 function in an independent manner by utilizing an engrailed repressor–dril1 DNA binding domain fusion construct, EnR–dril. Injection of 400 pg *EnR–dril* RNA recapitulated the morphant phenotype, resulting in embryos that failed to complete gastrulation in 55/81 cases. The effect of *EnR–dril* RNA injection was dose dependent, 600 pg causing severe gastrulation deficiencies (Figs. 3F and G).

To confirm that the dril1 morpholino inhibits translation specifically, in vitro transcription/translation reactions incorporating ^{35}S-methionine were performed using the Promega TNT kit according to the manufacturer's instructions, and the products were analyzed by SDS-PAGE and subjected to autoradiography (Fig. 4). When CS2–dril1 was used as a template, the amount of protein produced from a reaction containing 160 ng dril1 MO (lane 2) was significantly reduced relative to that derived from a reaction containing either 160 ng (lane 1) or 800 ng (lane 3) of 5-mis MO, proving that the morpholino can inhibit translation of dril1 protein, and that it acts with sequence specificity. Furthermore, when Δ5′dril-MT, which lacks the target site, was used as template, there was no inhibition of translation by the dril morpholino, relative to the 5-mis MO, even at 800 ng, indicating that the dril1 morpholino does not inhibit general translation (lanes 4–8).

The finding that two independent methods used to disrupt dril1 function produced similar embryonic defects provides a strong indication of the specificity of these

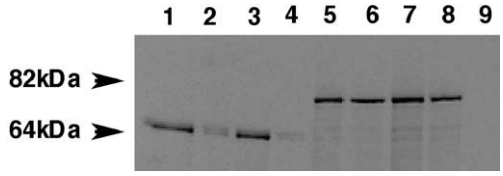

Fig. 4. Dril1 morpholino specificity. Autoradiogram of in vitro transcription/translation products derived from CS2–dril1, which contains the morpholino target site (lanes 1–4), or CS2-Δ5′ dril-MT, which lacks the morpholino target site (lanes 5–8), plus the following combinations of morpholino: (1 and 5) 160 ng 5 bp mismatch morpholino (5-mis MO); (2 and 6) 160 ng dril1 MO; (3 and 7) 800 ng 5-mis MO; and (4 and 8) 800 ng dril1 MO. Lane 9 contains reticulocyte lysate without vector or morpholino. Comparisons cannot be made between lanes 1–4 and 5–8 because products are derived from different templates. The Δ5′ dril-MT product is of a higher mass than dril1 protein because of the addition of the 6-myc tag.

treatments. However, as we were unable to rescue the embryonic defects in *X. laevis*, we searched the closely related *X. tropicalis* genome to determine whether the morpholino could be targeting a different gene. The search identified the *X. tropicalis dril1* orthologue as the top hit, revealing that there is only one variant base between the species in the region where the morpholino binds. The next nearest hit had a four-base mismatch and an insertion and was not in the vicinity of a translation start site, indicating the dril1 morpholino should not inhibit translation of any other gene in the *X. tropicalis* genome. Injection of the dril1 morpholino into *X. tropicalis* embryos elicited the same morphological defects as observed in *X. laevis*; 20 ng inhibited gastrulation in 52/53 cases, whereas embryos injected with an equivalent amount of control morpholino gastrulated normally in 29/30 cases (Figs. 5A and B). Embryos injected with 3 ng dril1 morpholino were able to complete gastrulation but developed into tadpoles with shortened anteroposterior axes, reduced dorsal tissue, and eye defects (Fig. 5D). Coinjection of *X. tropicalis* embryos with both 3 ng of the morpholino and 300 pg *Δ5′dril-MT* RNA, encoding a version of *X. laevis* dril1 lacking the morpholino target site, was sufficient to significantly rescue the morphological defects induced by morpholino treatment alone (Fig. 5E). The rescued embryos had better-developed eyes and dorsal axes than their morphant siblings. The mean lengths of morphants and rescued NF31 embryos were 1.83 and 2.12 mm, respectively. These means were significantly different when subjected to a *t* test ($P = 0.005$). While the rescued embryos were motile, the morphant embryos did not exhibit aversive behavior when lightly touched with forceps, likely due to their defective axial muscles. These results demonstrate that dril1 is necessary for gastrulation,

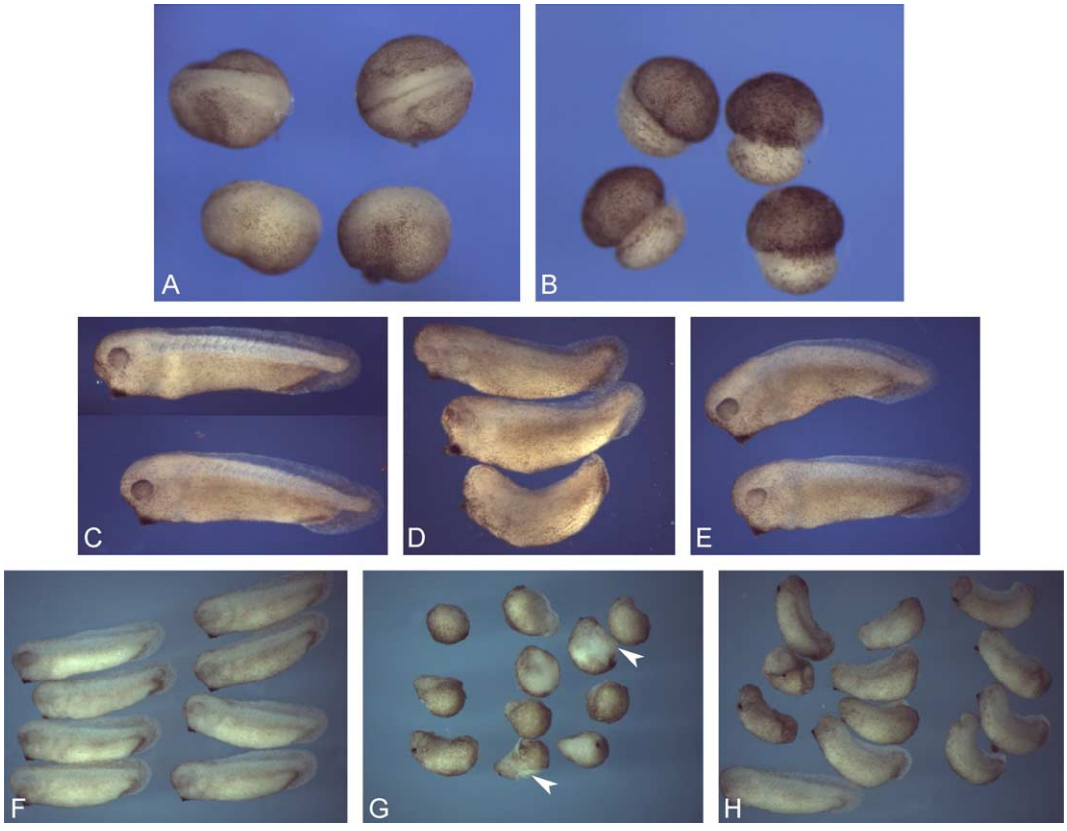

Fig. 5. Effects of dril1 morpholino on *X. tropicalis* development. NF21 embryos injected with 20 ng of a control morpholino, anterior to left (A), or dril1 morpholino (B). The latter embryos failed to complete gastrulation. Uninjected tadpoles (C), tadpoles injected with 3 ng dril1 morpholino (D), morphants rescued by coinjection with 300 pg Δ5′ dril-MT RNA (E). Rescue of a more severe morphant phenotype (F–H). Uninjected embryos (F), embryos injected with 6 ng dril1 morpholino (G), embryos rescued with 260 pg Δ5′ dril-MT RNA (H); note elongated anteroposterior axes, and more developed head structures, as evidenced by presence of cement gland. White arrowheads in G mark epidermal lesions.

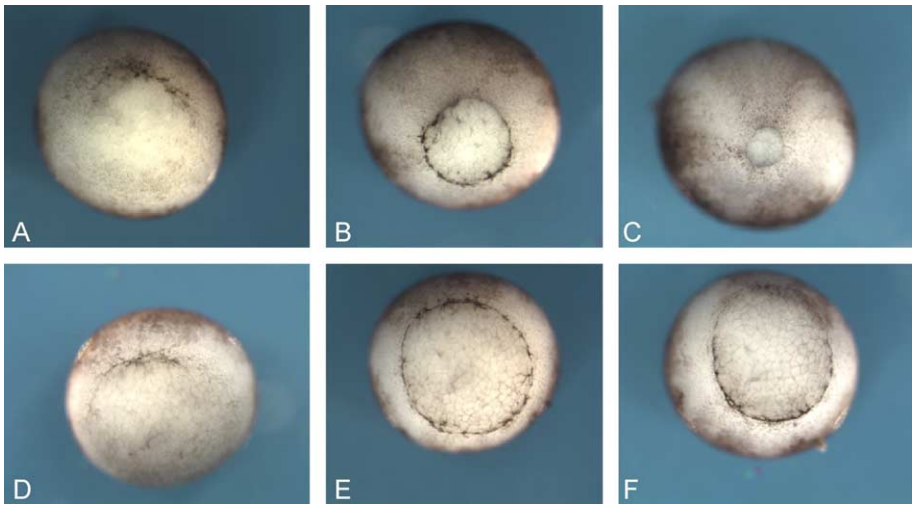

Fig. 6. Time lapse of gastrulation in dril1 morphants. Embryos were injected with 30 ng control morpholino (A–C) or dril1 morpholino (D–F) and photographed at NF10 (A and D), NF11 (B and E), and NF12.5 (C and F).

axial patterning, and eye development and are further substantiated by the rescue of a more severe morphant phenotype, as shown in Figs. 5F–H. Injection of *X. tropicalis* embryos with 6 ng dril1 morpholino resulted in severe axial deficiencies (Fig. 5G). Morphant embryos did not undergo axial elongation in the anteroposterior axis and appeared as round blobs deficient in dorsoanterior structures. Additionally, many embryos had lesions by the tailbud stage, resulting from a combination of incomplete gastrulation and possibly effects of dril1 loss on epidermal integrity. In contrast, embryos coinjected with 6 ng dril1 morpholino and 260 pg *Δ5′dril-MT* RNA exhibited substantially more axial development, with better anteroposterior elongation, more developed head structures, and greater epidermal integrity (Fig. 5H), indicative of substantial phenotypic rescue. These results provide clear evidence that dril1 is important in axial patterning.

Gastrulation defects can result from the perturbation of several developmental pathways. Possible reasons for abnormal gastrulation include problems with dorsal determination, mesoderm induction, or planar cell polarity (Conlon et al., 1996; Gerhart, 2001; Wallingford et al., 2000). To gain a more detailed understanding of the gastrulation deficiency in dril1 morphants, individual embryos were observed during gastrulation. The first visible sign of gastrulation is the formation of the pigmented dorsal lip at NF10, and this occurred normally in dril1 morphants (Figs. 6A and D). This suggests that the defect is not likely to result from problems with dorsal specification because in ventralized embryos there is a delay in lip formation. During gastrulation, the diameter of the circular blastopore normally decreases as cells invaginate. In morphant embryos, this did not happen and a clear difference was visible between control and dril1 morphant embryos by midgastrulation (Figs. 6B and E). This difference became more pronounced as the control embryos completed gastrulation (Figs. 6C and F).

When the control siblings neared the end of gastrulation, expression patterns of several marker genes were

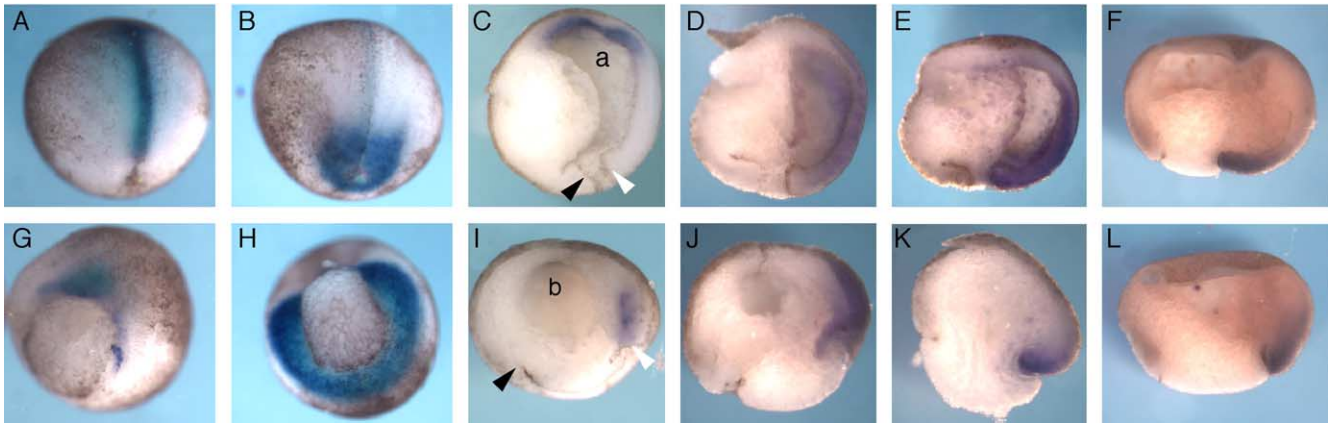

Fig. 7. In situ hybridization of dril1 morphants. Embryos were injected with either control morpholino (A–F) or dril1 morpholino (G–L). Whole NF13 embryos injected with 20 ng morpholino were stained for *chordin* (A and G) and *Xbra* (B and H). Sagittally bisected embryos injected with 30 ng morpholino were stained for *frzb-1* (C and I), *Fz7* (D and J), and *prickle* (E and K) at NF13, and for *sprouty2* at NF12 (F and L). Symbols in C and I represent archenteron (a), blastocoel (b), dorsal lip of blastopore (white arrowhead), and ventral lip of blastopore (dark arrowhead).

analyzed by in situ hybridization. *Chordin* marks the dorsal mesoderm (Sasai et al., 1994), and in control embryos *chordin* was expressed in a long thin stripe of cells running along the dorsal midline (Fig. 7A). In dril1 morphant embryos, *chordin*-expressing cells were confined to the area around the open blastopore lip and the intensity of staining appeared reduced (Fig. 7G). In control embryos, the pan-mesodermal marker *Xbra* was detected in a ring around the closed blastopore at the end of gastrulation (Fig. 7B) and was robustly expressed around the open blastopore of dril1 morphant embryos (Fig. 7H). To examine the internal morphology of dril1 morphants in more detail, In situ hybridizations were performed on sagittally bisected embryos. The gross morphology of the bisected embryos was clearly abnormal, with the archenteron absent and the blastocoel still visible in some embryos (Fig. 7I). In situ hybridization revealed further abnormalities. Normally, expression of *frzb-1* initiates in cells near the dorsal lip of the early gastrula organizer and these *frzb-1*-positive cells undergo extensive anterior migration during gastrulation, marking the most anterior mesendoderm, namely, the prechordal plate and foregut (Fig. 7C) (Leyns et al., 1997). The *frzb-1* expression domain in dril1 morphants was confined to the area around the dorsal lip, indicating that while some cells are specified as anterior mesoderm in the dril1 morphants, the positioning of these cells is greatly perturbed (Fig. 7I). Likewise, expression of the anterior endoderm marker, *cerberus*, was detected in dril1 morphants but the *cerberus*-positive cells did not migrate to their appropriate anterior position (data not shown). Fz7 is involved in noncanonical wnt signaling and germ layer separation during gastrulation (Winklbauer et al., 2001). At the end of gastrulation, *Fz7* was detected in an extended area above the archenteron, marking the dorsal mesoderm and neurectoderm of controls (Fig. 7D). In morphants, *Fz7* was intensely expressed on the dorsal side but again the migration of *Fz7*-expressing cells was impaired (Fig. 7J). Expression of the planar cell polarity gene, *prickle*, a component of the noncanonical wnt pathway, was also detected in morphant embryos, but in a domain confined to the area immediately surrounding the blastopore (Figs. 7E and K). The FGF antagonist, sprouty2, is necessary for gastrulation cell movements but not for mesoderm formation (Nutt et al., 2001). A comparison of *sprouty2* expression at stage NF12 did not show any marked difference between control and dril1 morphants, with the most intense expression observed in the posterior mesoderm abutting the dorsal lip (Figs. 7F and L). Overall, the expression analysis demonstrated that while there is some degree of specification of several different cell populations, as identified by the various markers, the positioning of these cells during gastrulation is severely abnormal.

While there were no overtly recognizable differences in cell size between dril1 and control morphant cells, we investigated the possibility that the morphant phenotype could result from a general inhibition of cell division. Because involuting dorsal mesoderm is not mitotically active (Saka and Smith, 2001), we examined the degree of mitotic activity in the animal pole ectoderm of NF10.25 embryos by immunostaining with αPH3, which recognizes the phosphorylated form of histone H3, and is a marker of mitotic cells (Hendzel et al., 1997). Fifteen dril1 morphants, injected with 30 ng morpholino, were compared with uninjected embryos, and no difference in the number of mitotic cells was obvious. A representative embryo from each of the control and morphant samples is depicted in Supplementary Fig. 1. While this does not exclude a potential role for dril1 in the mitotic progression of a specific cell population during embryogenesis, it does eliminate the possibility that the morphant exerts its effects through a general inhibition of cell division.

As mentioned above, morphological defects in dril1 morphants were first obvious around NF10.5. To determine

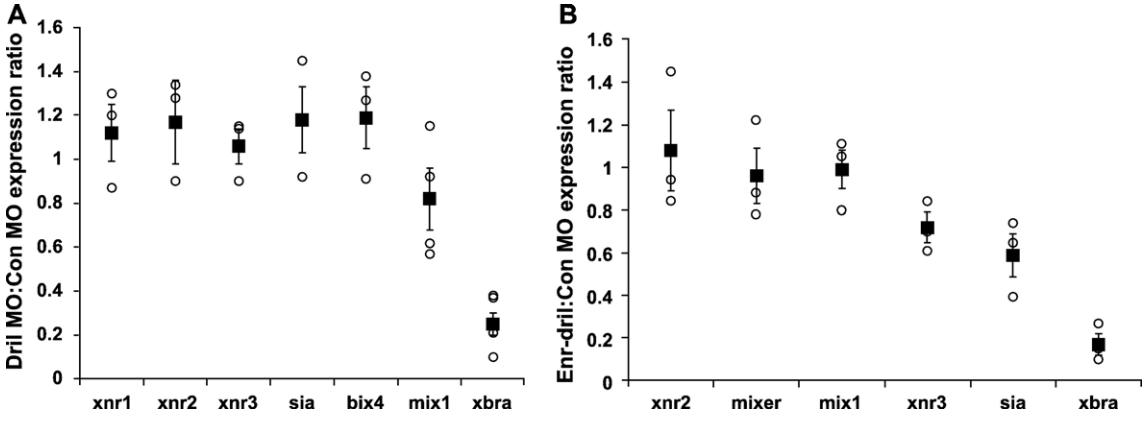

Fig. 8. Real-time PCR analysis of dril1-depleted embryos at the onset of gastrulation. (A) Effect of morpholino treatment. The expression levels of NF10 embryos injected with 30 ng of either the dril1 morpholino or the control morpholino were normalized relative to the loading control, ODC. These normalized values were used to calculate the dril morpholino–control morpholino expression ratio (plotted as a circle). A value of 1 indicates that expression levels are the same in both control and dril1 morphant embryos. Each circle represents the ratio derived from an independent experiment. For each gene assayed, the mean (±SE) of the independent replicates is plotted as a black square. (B) The effect of 500 pg *EnR–dril* RNA overexpression. Ratios were calculated as in A.

whether these midgastrulation anomalies were the result of signaling defects earlier in development, gene expression levels indicative of signaling in various pathways were measured by real-time PCR, using cDNA derived from NF10 embryos, at the onset of gastrulation (Fig. 8A). The transcription factor VegT is vital for the induction of mesoderm and endoderm (Zhang et al., 1998), activating transcription of *xnrs*, the *Xenopus* nodal-related proteins, which are zygotic TGFβ ligands (Jones et al., 1995; Kofron et al., 1999). Interference with VegT function should result in loss of expression of its target genes. *Bix4* and *xnr1* are immediate targets of VegT (Casey et al., 1999; Kofron et al., 1999), and expression of these genes was not affected in dril1 morphants (Fig. 8A). VegT is also necessary for the expression of *xnr2* (Kofron et al., 1999), and expression of this gene was also unaffected by morpholino treatment. There was a slight decrease in *mix1* expression in two out of four cases. A possible role for dril1 in VegT-controlled transcription during early embryogenesis cannot be excluded because both *dril1* and *VegT* mRNAs are maternally provided; hence, the morpholino will not prevent VegT-dependent transcription that is mediated by maternal proteins. What can be definitively concluded from these experiments is that the profound defects observed in dril1 morphants are not due to inhibition of VegT.

A second pathway critical in early development is the wnt signaling pathway, which specifies the dorsal axis (Moon and Kimelman, 1998). Zygotic target genes of this maternal wnt pathway include *siamois* and *xnr3* (Carnac et al., 1996; McKendry et al., 1997). Neither of these genes was inhibited in dril morphants (Fig. 8A), demonstrating that canonical wnt signaling was not abrogated. Hence, morphant gastrulation defects do not result from a lesion in the canonical wnt pathway.

The gene expression analysis of early gastrulae indicates that induction of *xnrs* is intact, and thus the signaling lesion in dril1 morphants occurs downstream of zygotic TGFβ ligand production. To determine the status of mesoderm induction, expression of the pan-mesodermal T-box gene *Xbra* was measured. Morpholino treatment resulted in a fourfold reduction of *Xbra*, demonstrating that dril1 is necessary for the correct expression of this key mesodermal gene (Fig. 8A).

Gene expression in *EnR–dril*-injected embryos was also examined at NF10 to investigate whether the morpholino and the EnR–dril fusion construct inhibited gastrulation by the same mechanism. VegT-dependent genes, including

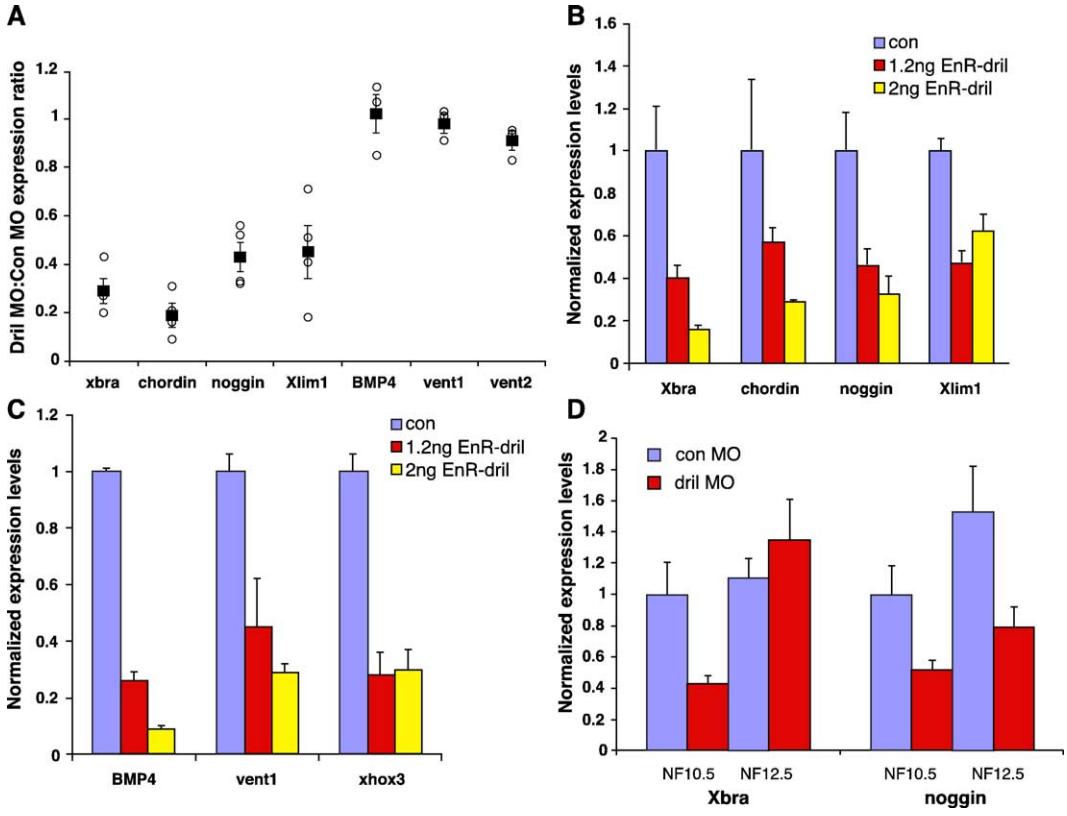

Fig. 9. Effect of dril1 depletion on mesoderm induction. (A) Expression of various mesodermal markers in NF10.5 morphant embryos was calculated as described in Fig. 8 legend. Embryos were injected with 30 ng morpholino. (B and C) Gene inhibition in response to increasing amounts of *EnR–dril* mRNA. A single experiment is shown. Expression in *EnR–dril*-injected embryos is plotted relative to control levels, which are set at 1. Error bars represent the SEM of Light Cycler duplicates of the same cDNA. Inhibition of gene expression by *EnR–dril* was duplicated in an independent experiment. (B) Pan-mesodermal and dorsal mesodermal markers. (C) Ventral mesodermal markers. (D) Expression of *Xbra* and *noggin* in NF10.5 and NF12.5 morphants.

Xnr2, *mix1*, *mixer*, and *bix4* were not inhibited by *EnR–dril* (Fig. 8B, data not shown). Levels of the wnt targets *siamois* and *xnr3* were mildly lowered; a possible reason for this reduction will be discussed later. *Xbra* expression was reduced by a factor of six, indicating that, as with the morpholino, *EnR–dril* injection also causes aberrations in zygotic mesoderm formation.

To determine the extent of mesodermal defects, several additional markers were scored at NF10.5. In addition to *Xbra* inhibition, the organizer markers *chordin*, *noggin*, and *Xlim1* were all reduced by dril1 morpholino treatment (Fig. 9A). Expression of the ventral markers *vent1*, *vent2*, and *BMP4* was not affected in these embryos, indicating that the gastrulation defects in the morphants are not due to ventralization. Expression of several organizer genes was abrogated in a dose-dependent manner by *EnR–dril* injection; a representative experiment is shown in Fig. 9B. Additionally, *BMP4* and *vent1* levels were reduced by *EnR–dril* injection in three independent experiments, and *xhox3* expression was inhibited in two out of three experiments (Fig. 9C). The differential responsiveness of embryonic BMP targets to the two methods of dril1 depletion will be considered in the Discussion section.

To explain the discrepancy between the in situ analysis showing that *Xbra* was robustly expressed in dril morphants at the end of gastrulation (Fig. 7H), yet its levels were decreased in early gastrulae, *Xbra* levels in sibling embryos were measured at NF10.5 and NF12.5. While *Xbra* expression was depressed at the midgastrula stage, it had recovered by the end of gastrulation (Fig. 9D). In contrast, *noggin* levels were still decreased in these embryos. Inhibition of *Xbra* by the morpholino at NF11 was observable by in situ analysis of sibling embryos (data not shown).

Dril1 is required for the response of animal caps to activin

The finding that dril1-depleted embryos express *xnrs* yet have impaired mesoderm induction is consistent with the possibility that these embryos are defective in TGFβ signal transduction. To test this hypothesis, animal caps from embryos injected with either control morpholino, dril1 morpholino, or *EnR–dril* mRNA were excised at NF8.5 and treated with the TGFβ ligand, activin, until neurula stages when they were observed for signs of elongation. Untreated caps remained round (Figs. 10A and B), whereas caps injected with the control morpholino underwent morphogenetic elongation movements in response to activin, and in most cases formed prominent proboscides (Figs. 10C and D). Caps injected with either the dril1 morpholino (n = 60) or *EnR–dril* (n = 31) showed no such morphogenetic transformation after activin treatment, remaining in most cases as rounded balls, with a few caps showing slight elongation (Figs. 10E and F). To confirm that the inhibition of activin-mediated morphogenesis was due to defects in mesoderm induction in the animal caps, levels of *Xbra* and *chordin* were measured in the activin-treated caps. Induction of both genes was reduced by inhibition of dril1

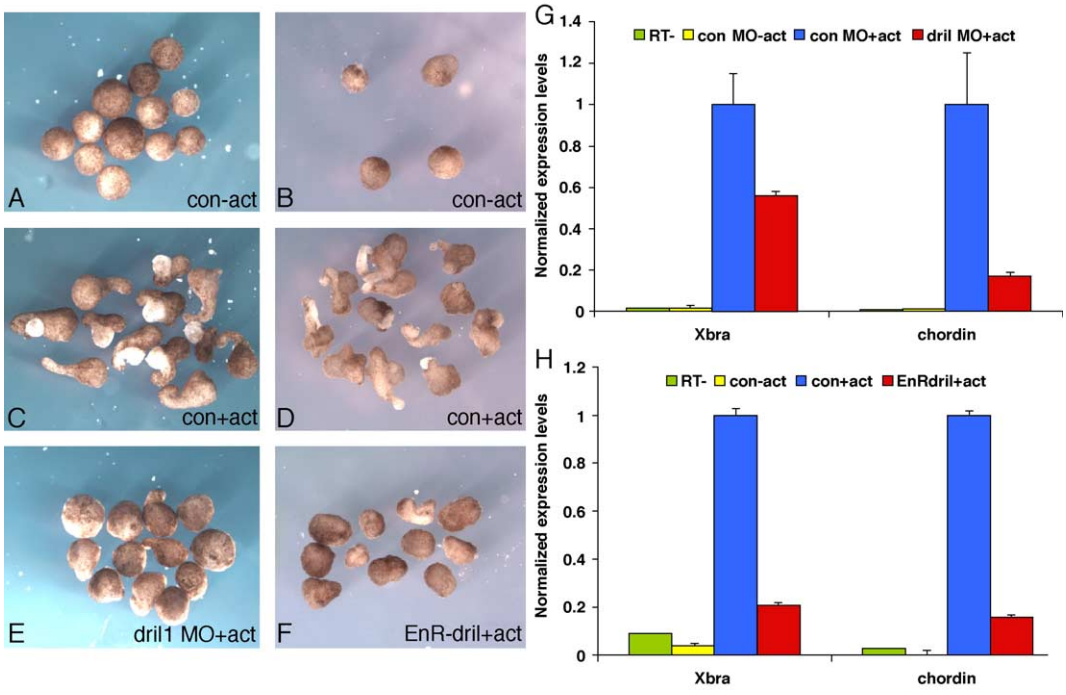

Fig. 10. Dril1 depletion inhibits the response of animal caps to activin. Control caps minus activin (A and B). Control caps plus activin (C and D). Activin-treated caps from dril1 morphants (E) and *EnR–dril*-injected embryos (F). Gene induction in activin-treated caps injected with 30 ng dril1 morpholino (G) or 600 pg *EnR–dril* (H).

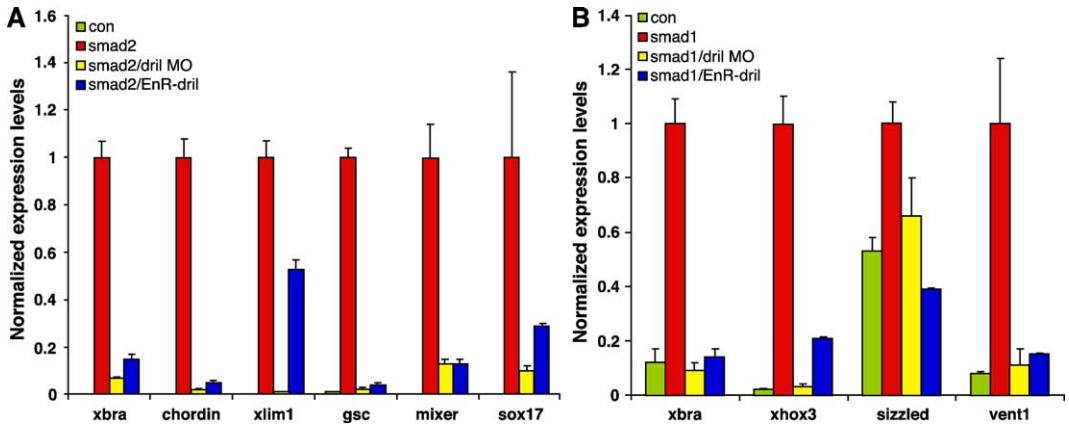

Fig. 11. Effect of dril1 depletion on smad-mediated gene induction in animal caps. Coinjection of either 30 ng dril1 morpholino or 500 pg *EnR–dril* inhibits response to 1 ng *smad2* (A) and 2 ng *smad1* (B).

by either method (Figs. 10G and H), implicating dril1 in activin-mediated mesoderm induction.

TGFβ signaling through both smad1 and smad2 requires dril1

TGFβ signaling is transduced intracellularly through the smad proteins, which shuttle from the cytoplasm to the nucleus and activate transcription of target genes in response to TGFβ stimulus (Derynck and Zhang, 2003). Activin signals through the smad2 pathway, and in *Xenopus* embryos, smad2 signaling is necessary for the production of the dorsoanterior axis (Hoodless et al., 1999). As a transcription factor, dril1 could be acting either upstream of smad, perhaps to stimulate production of one of the cytoplasmic components of the smad signaling pathway, or in parallel/downstream of smad, as a necessary component of the smad transcriptional response. To distinguish between these possibilities, animal caps derived from embryos that were coinjected with *smad2* mRNA alone or *smad2* and either the dril1 morpholino or *EnR–dril* were analyzed by real-time PCR at NF11.5. The induction of a wide array of activin-responsive genes, including *Xbra*, *chordin*, *goosecoid*, *Xlim1*, *mixer*, and *sox17* was inhibited by both methods of dril1 depletion, proving that dril1 plays an obligatory role in the transduction of smad2 signaling in animal caps (Fig. 11A). *Smad2* injection into a ventral vegetal blastomere of the 8-cell embryo is capable of inducing a partial secondary axis (Baker and Harland, 1996). Coinjection of *smad2* and the dril1 morpholino completely inhibited secondary axis formation (Table 1; Figs. 12A and B), demonstrating the necessity of dril1 in *smad2*-mediated axis formation.

TGFβ ligands comprise a large and diverse family of molecules that signal through two main pathways: the activin/smad2 pathway, as described above, and the BMP/smad1 pathway. Some of these components, such as smad1 and smad2, are pathway specific; others, such as smad4, function in both pathways. To determine whether the role of dril1 is confined to activin-type signaling, or whether it is also important for BMP-type signaling, the ability of dril1-depleted animal caps to respond to *smad1* mRNA was assessed. Induction of *Xbra*, *vent1*, *sizzled*, and *Xhox3* was inhibited by both the morpholino and the *EnR–dril* fusion, showing that dril1 is also a vital component of the smad1 response (Fig. 11B).

In the embryo, BMP signaling is inhibited by extracellular antagonists in the presumptive neural ectoderm. Because dril1 is expressed in a complementary pattern to these BMP antagonists during neurulation, we investigated whether modulation of dril1 activity can regulate neuralization of animal caps. Whereas caps injected with the 100 pg of the BMP antagonist *chordin* showed a marked upregulation of the neural markers NRP1, NCAM and the cement gland marker, XAG1, injection of 500 pg to 1 ng *EnR–dril* did not elicit neuralization in three independent experiments (Fig. 13A). Because inhibition of smad1 can induce neuralization (Zhu et al., 1999), this result indicates

Table 1
Dril1 depletion inhibits smad2-mediated secondary axis induction

Injected RNA	2° Axis formation
Smad2 (500 pg)	40.6% (28/69)
Smad2 (500 pg) and dril1 morpholino (15 ng)	0% (0/46)
Uninjected controls	0% (0/67)

Embryos were injected in one ventral vegetal blastomere at the 8-cell stage and scored for secondary axes at NF35.

Fig. 12. Dril1 morpholino inhibits smad2-mediated secondary axis formation. (A) Representative secondary axes induced by injection of 500 pg smad2 into a ventral vegetal blastomere at the 8-cell stage. (B) Inhibition of smad2-induced secondary axes by coinjection of 15 ng dril1 morpholino.

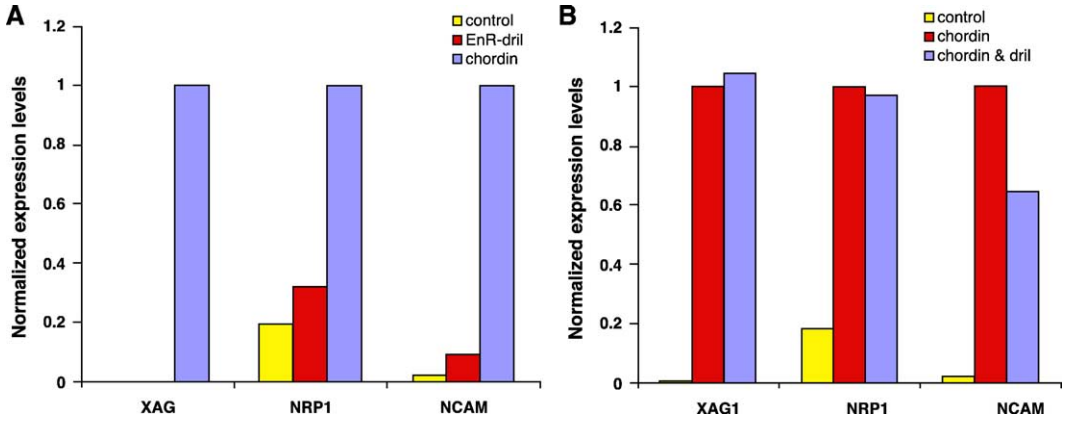

Fig. 13. Animal cap fate is not affected by perturbation of dril1 expression. (A) EnR–dril does not induce neural markers in animal caps. Graph depicts a representative experiment showing expression of cement gland and neural markers in NF21 caps injected with either 100 pg chordin or 1 ng EnR–dril. (B) Compilation of three experiments showing the effect of dril1 RNA overexpression on the ability of chordin to neuralize animal caps. Caps were injected with 100 pg chordin and 2 ng dril1 RNA and harvested at NF21-22. Expression of all markers was normalized relative to ODC.

that EnR–dril does not completely abolish endogenous smad1 activity in animal caps. As the amount of EnR–dril used in this assay is sufficient to impair BMP signaling in the gastrula (Fig. 9B), and to inhibit gastrulation (Figs. 3F and G), it is conceivable that the mesoderm is more sensitive than the ectoderm to inhibition of dril1. To determine whether overexpression of dril1 can counter the neuralizing effects of chordin, we analyzed expression of *XAG1*, *NRP1*, and *NCAM* in caps injected with 100 pg *chordin* and 2 ng *dril1* RNA. Induction of *XAG1* and *NRP1* was unaffected by coinjection of 2 ng *dril1* RNA, whereas *NCAM* was moderately reduced in two out of three experiments. These data indicate that expression of *dril1* RNA is insufficient to counteract the inhibitory effects of extracellular BMP antagonists through potentiation of intracellular smad1 signaling. This is reminiscent of the inability of dril1 to induce mesoderm and suggests that transcriptional modulation by dril1 may require obligatory cofactors. Given that chordin can induce neuralization in caps containing endogenous dril1, this lack of effect of dril1 overexpression on neural induction may not be surprising. However, the slight down-regulation of *NCAM* is intriguing because it has been shown that *NCAM* is much more sensitive to smad1-mediated inhibition than is *XAG1* (Wilson et al., 1997). It is possible that the decrease in *NCAM* (by 30% and 63% in the two out of three experiments in which it was reduced) is due to a slight potentiation of smad1 signaling by dril1. This effect would not be seen on a gene such as *XAG1*, whose transcription is not inhibited by moderate levels of smad1 and therefore would be less affected by a slight increase in smad1 signaling (Wilson et al., 1997).

Induction of mesoderm by FGF is independent of dril1

Members of the TGFβ family are not the only growth factors capable of inducing mesoderm; FGF is also able to induce the formation of this germ layer. To determine whether dril1 is necessary for the induction of mesoderm in response to FGF, the effect of dril1 morpholino on the induction of the eFGF targets *Xbra* and *myoD* was examined (Fig. 14). Animal caps injected with either 30 pg *eFGF* or 500 pg *smad2* mRNA and harvested at NF12.5 expressed both *Xbra* and *myoD*. As expected, the induction of these genes by *smad2* was greatly reduced when coinjected with 15 ng dril1 morpholino. In contrast, induction of these genes by *eFGF* was not inhibited by the same amount of dril1 morpholino in two independent experiments. These results demonstrate that rather than acting as a general regulator of mesodermal gene expression, dril1 exerts some degree of pathway specificity in its actions, selectively inhibiting TGFβ signaling while leaving the eFGF response unimpaired.

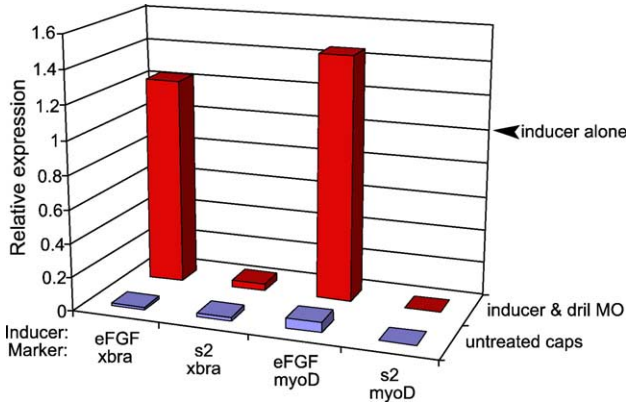

Fig. 14. Dril1 morpholino does not inhibit mesoderm induction in FGF-treated animal caps. The extent to which dril1 morpholino inhibited induction of *Xbra* or *myoD* was determined by calculating the level of expression in caps coinjected with inducer (smad2 or eFGF) and morpholino as a fraction of expression levels in caps stimulated with the inducer alone (red bars). A value of 1 indicates that the morpholino has no effect on the pathway tested. All values were normalized relative to ODC. Expression level ratios of untreated caps relative to induced caps are shown in blue.

Discussion

The ARID domain proteins belong to an ancient family, members of which have been implicated in chromatin remodeling and cell fate specification in both the fungal and animal kingdoms (Kortschak et al., 2000). Because a number of ARID family mutants exhibiting developmental abnormalities have been isolated from various protostome phyla, the potential role of various ARID proteins in chordate development is a topic worthy of investigation. We have selected the founding member of the ARID family, dril1, as the topic of our initial study. To determine whether dril1 is necessary for normal germ layer patterning, we performed a loss-of-function analysis. We employed two independent methods: firstly, using a morpholino, which prevents translation of dril1 protein; secondly, by constructing an engrailed repressor–dril1 DNA binding domain fusion protein (EnR–dril), which should bind to and repress dril1 target genes. Both of these methods demonstrated that dril1 is necessary for completion of gastrulation movements in *Xenopus* and that its depletion profoundly perturbs zygotic mesoderm induction. Additionally, we have identified dril1 as a necessary component of the smad1 and smad2 transcriptional response.

The morphologically normal appearance of the blastopore lip at the onset of gastrulation belies the fact that zygotic mesoderm induction has been perturbed in dril1-depleted embryos, as evidenced by *Xbra* inhibition. *Xbra* is the *Xenopus* orthologue of the T-box gene *brachyury* (*T*), first identified in a mesoderm-deficient mouse mutant (Gluecksohn-Schoenheimer, 1938; Herrmann et al., 1990). *Xbra* is necessary and sufficient for mesoderm induction (Conlon et al., 1996; Cunliffe and Smith, 1992) and its expression is regulated by TGFβ signaling (Hemmati-Brivanlou and Melton, 1992; Smith et al., 1991). Our discovery that dril1 is necessary for induction of smad-responsive genes in animal caps identifies dril1 as an obligate partner in TGFβ-mediated mesoderm induction. As *Xbra* expression is dependent upon activin-type TGFβ signaling, loss of dril1 results in *Xbra* inhibition. *Xbra* inhibition due to dril1 depletion is transient, as expression returns to wild-type levels by the time the control embryos complete gastrulation, suggesting that either inhibition of dril1 is incomplete or that the embryos regulate to activate *Xbra* by a dril1-independent mechanism. Phenotypically, the embryos are not able to recover from even this temporary ablation of *Xbra*, further underscoring the importance of this gene. Given that *Xbra* is necessary for the convergent extension movements that drive involution of the mesoderm during gastrulation (Conlon et al., 1996; Conlon and Smith, 1999), the loss of *Xbra* caused by dril1 depletion is a likely explanation for the failure of dril1 morphants to undergo the normal cell movements of gastrulation.

To determine whether the inhibition of *Xbra* caused by dril1 depletion was due to disruption of gene networks active in early embryogenesis, we investigated whether activation of VegT or wnt targets were affected. *VegT*, a T-box gene encoding a vegetally localized maternal mRNA, is involved in specification of both the mesoderm and the endoderm in the *Xenopus* embryo (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Zhang et al., 1998). VegT is necessary for activation of many genes, including *mix1*, *mixer*, *xnr2*, and the known direct targets *xnr1* and *bix4* (Xanthos et al., 2001). Dril1 morphants had normal expression levels of VegT-dependent genes, including *xnr1*, *xnr2*, and *bix4*. Dril1 morpholino treatment caused a slight decrease in *mix1* expression but there was no inhibition of this gene or of *mixer* or *xnr2* in *EnR–dril*-injected embryos. Because *dril1* mRNA is present in the egg, we cannot exclude the possibility that maternal dril1 protein functions in VegT-mediated transcription. However, we can conclude that the mesoderm defects we see in our dril1-depleted embryos are not due to abrogation of VegT-dependent transcription. The *xnr* genes are TGFβ ligands involved in zygotic mesoderm specification (Jones et al., 1995; Onuma et al., 2002; Osada and Wright, 1999). Given that the VegT-dependent *xnrs* are not inhibited by dril1 depletion, any signaling lesion in mesoderm induction caused by dril1 depletion must occur downstream of zygotic TGFβ ligand production.

Another maternal signaling pathway exists in the *Xenopus* embryo: the wnt/β-catenin pathway. *Siamois* and *xnr3*, which are wnt/β-catenin targets, were unaffected by dril1 morpholino treatment. *EnR–dril* mRNA injection mildly decreased *xnr3* expression and halved *siamois* expression. This effect may be explained by the synergistic role played by smad2 in expression of wnt-target genes in the early *Xenopus* embryo. Crease et al. (1998) found that *smad2* coexpression with wnt pathway activators enhanced *siamois* induction in animal caps. Furthermore, *siamois* and *xnr3* levels were reduced by greater than 50% in embryos injected with mRNA encoding the dominant-negative *activin receptor*, which inhibits smad-mediated transcription. Transcriptional activation of *Xenopus twin*, a gene related to *siamois*, also requires smad activity, mediated by complex formation between smad4, β-catenin, and Lef1/Tcf (Nishita et al., 2000). As dril1 is necessary for smad1- and smad2-mediated gene induction, *siamois* and *xnr3* down-regulation caused by *EnR–dril* injection is likely attributable to abrogation of the smad transcriptional input. A plausible explanation for the greater efficacy of EnR–dril compared to the morpholino is that whereas EnR–dril can compete with maternal dril1 protein for dril1 transcriptional targets, maternal dril1 protein can still function in morphants, allowing normal expression of wnt/TGFβ targets in the early embryo.

The Spemann organizer, responsible for generating both the anteroposterior and dorsoventral axes, arises in the dorsal equatorial region in response to the combination of

wnt/β-catenin and activin/nodal/Vg1-type TGFβ signaling (reviewed by Gerhart, 2001). Loss of dril1 disrupted organizer formation, as evidenced by reduced expression of the organizer markers *noggin*, *chordin*, and *Xlim1*. Because *noggin* and *Xlim1* are direct activin targets, and *chordin* is indirectly induced by activin (Sasai et al., 1994; Tadano et al., 1993), the inhibition of these genes is consistent with diminished TGFβ signaling.

BMPs are TGFβ family ligands that transduce their signal through smad1. In the gastrula, BMPs function in ventral mesoderm specification, and their subsequent expression in the epidermis acts as an antineural and proepidermal signal (Wilson and Hemmati-Brivanlou, 1995). *Dril1* expression during neurulation is reminiscent of *BMP4*, which is expressed throughout the nonneural ectoderm but excluded from the neural ectoderm (Hemmati-Brivanlou and Thomsen, 1995). However, whereas inhibition of BMP4 signaling is sufficient to neuralize animal caps (Xu et al., 1995), *EnR–dril* overexpression does not induce neuralization. This finding indicates that *EnR–dril* does not reduce BMP signaling below the threshold required for maintenance of epidermal cell fate. *Dril1* mRNA is abundantly expressed in the animal cap ectoderm, and thus it may be difficult to inhibit activity of the endogenous protein to the extent where neuralization occurs. A role for dril1 in BMP signal transduction is indicated by the down-regulation in *EnR–dril*-treated embryos of *vent1*, *xhox3*, and the *BMP4* gene itself, all of which are regulated by the BMP4 ligand. Interestingly, BMP signaling in dril1 morphant gastrulae was unimpaired. *Xenopus* eggs contain *BMP4* mRNA (Dale et al., 1992; Hemmati-Brivanlou and Thomsen, 1995), and while the BMP pathway cannot be activated prior to the midblastula transition (MBT), smad1 protein is present in an unphosphorylated inactive state at this stage (Faure et al., 2000). After MBT, BMP4 can induce phosphorylation of smad1 in the absence of transcription (Faure et al., 2000). As *dril1* mRNA is present in eggs, it is possible that maternal dril1 protein acts in conjunction with other maternally supplied components of the BMP pathway. Depletion of only zygotic dril1 by the morpholino is insufficient to prevent the activation of BMP-responsive genes, whereas the efficacy of the EnR–dril construct may result from interference with both maternal and zygotic protein. In contrast, zygotic ligand synthesis is necessary for activin/nodal-mediated mesoderm induction through smad2, and activation of smad2 protein requires zygotic transcription (Faure et al., 2000; Kofron et al., 1999; Zhang et al., 1998). This zygotic requirement may explain the effectiveness of the dril1 morpholino in inhibiting endogenous TGFβ signaling in the organizer.

Our results provide direct evidence for the necessary function of dril1 in smad signaling. Both activin-induced morphogenetic movements and gene expression are inhibited by dril1 depletion in animal cap assays. Dril1 functions after the TGFβ signal has been transduced from the cytoplasm into the nucleus, as its depletion prevents the transcription of genes in response to both *smad1* (*Xbra*, *vent1*, *sizzled*, *xhox3*) and *smad2* (*Xbra*, *chordin*, *goosecoid*, *Xlim1*, *mixer*, *sox17*). The prevention of *smad2*-induced secondary axis formation by inhibition of dril1 demonstrates its necessity in embryonic patterning that is controlled by smad2. As our experimental perturbations targeted the mesoderm, we have concentrated our analysis on this germ layer for the present study. However, the discovery that induction of the endodermal genes *sox17* and *mixer* in the animal cap depends on dril1 suggests that an examination of the role of dril1 in endodermal patterning is a worthwhile area for future research, especially considering that TGFβ signals are known players in specification of this germ layer (Joseph and Melton, 1998; Osada and Wright, 1999).

How exactly does dril1 regulate mesodermal gene expression? Our finding that an EnR–dril1 DNA binding domain fusion construct can inhibit gene activation indicates that it is acting directly on DNA to modulate transcription. Replication of the morpholino phenotype by EnR–dril is consistent with the hypothesis that the normal function of dril1 in gastrulation is transcriptional activation. However, overexpression of dril1 did not induce mesoderm, demonstrating that dril1 alone is not sufficient to activate TGFβ targets. Overexpression of a VP16 activator–dril1 DNA binding domain fusion construct did not perturb gastrulation, so that if dril1 is involved in transactivation, other regions of the protein in addition to the DNA binding domain may be required. Our results demonstrate that dril1 is an essential cofactor in smad-mediated transcription. Dril1 could recruit, or be recruited by activators, such as smads, to the promoters of target genes. Alternatively, given that other ARID proteins are involved in chromatin remodeling, it is possible that dril1 modulates chromosomal architecture, thereby controlling the access of transcriptional activators to the promoter. Because both *Xbra* and *myoD* exhibit dril1-dependent activation in response to smad2, yet their response to eFGF is dril1-independent, it is clear that dril1 plays a specific rather than a general role in mesodermal gene regulation.

A DNA binding consensus sequence for murine dril1 has been identified (Herrscher et al., 1995), and we used the MatInspector program (Genomatix) to investigate whether several TGFβ-responsive *Xenopus* promoters contain this putative dril1 binding site (Table 2). The activin-inducible gene, *Xbra*, which is down-regulated in dril morphants, has three putative dril1 binding sites in its promoter and is thus a promising candidate for direct regulation by dril1. The induction of *Xlim1* by smad2 is also dril1 dependent, and the intronic region of *Xlim1* that mediates activin responsiveness also contains two possible dril1 binding sites. A third activin-inducible gene, *HNF1α*, contains six sequences matching the dril1 bind-

Table 2
In silico analysis of various TGFβ-responsive promoters for the presence of putative dril1 binding sites

Gene	DNA length (bp)	Description	No. putative dril1 sites (No. nonoverlapping)	Reference
Xbra[a]	1565	Promoter	3 (3)	Artinger et al. (1997)
Goosecoid[a]	360	Promoter	0	Watabe et al. (1995)
Mix2[a]	783	Promoter	0	Vize (1996)
Eomes[a]	571	ARE	0	Ryan et al. (2000)
XFD-1′[a]	60	ARE	0	Kaufmann et al. (1996)
HNF1α[a]	886	Promoter	6 (4)	Zapp et al. (1993)
Xlim1[a]	1932	Intron containing ARE	2	Rebbert and Dawid (1997)
Bambi[b]	199 and 300	BRE and proximal promoter	0	Karaulanov et al. (2004)
Smad7[b]	1466	Promoter	1	Karaulanov et al. (2004)
Vent-2B[b]	124	BRE	0	Henningfeld et al. (2000)

Abbreviations: activin-responsive element (ARE); BMP-responsive element (BRE).
[a] Promoters can be characterized as activin responsive.
[b] Promoters can be characterized as BMP responsive.

ing consensus, four of which have no overlapping sequence, so it will be interesting to investigate whether dril1 is involved in transcriptional regulation of this gene. Interestingly, putative dril1 binding sites were not identified in several promoters that contain either activin- or BMP-responsive elements (AREs or BREs), including the *goosecoid*, *mix2*, and *bambi* promoters. As seen in Fig. 11, dril1 is required for induction of *goosecoid* by smad2, and we found that both the dril1 morpholino and EnR–dril impaired the activation of *mix2* by smad2 (data not shown). How might dril1 regulate the expression of these genes if no binding sites are identified in their promoters? If dril1 acts as a regulator of chromatin architecture, it may bind regulatory elements further upstream than the promoter sequences analyzed here. Alternatively, the dril1-dependent genes whose promoters lack a dril1 binding site may be indirect targets whose transcription is activated by an intermediary protein. A third possibility is that dril1 can bind to sequences other than the canonical consensus identified by the MatInspector program; however, it is also possible that the consensus sequences identified in *Xbra*, *Xlim1*, and *HNF1α* may not function as dril1 binding sites in vivo. Therefore, it is important to note that silico analysis, while a useful preliminary step, cannot substitute for an empirical investigation of promoter binding.

Considering that the regulation of gastrulation by *brachyury* is conserved among vertebrates, it is likely that dril1 plays a role in gastrulation throughout this group. The involvement of dril1 in gastrulation may be conserved throughout deuterostomes because dril1 has recently been shown to be necessary for gastrulation movements in the echinoderm *S. purpuratus* (Amore et al., 2003). However, there is little similarity between the regulatory networks modulated by dril in the two deuterostome groups: dril depletion has no effect on *brachyury*, *lim1*, or *bmp4* expression in the echinoderm or on the battery of endomesodermal patterning genes assayed. Even within the echinoderms, the presence of *brachyury* in the presumptive mesoderm is quite variant; its roles in endoderm patterning and invagination appear more ancient (Gross and McClay, 2001). A contributory factor in the failure of gastrulation in dril-depleted sea urchin embryos may be the inhibition of *goosecoid*, which is required for gastrulation and greatly reduced by dril depletion (Amore et al., 2003; Angerer et al., 2001). In vertebrates, smad2 is involved in activation of the *goosecoid* promoter (Labbe et al., 1998). The role of smad signaling in echinoderm development is unknown so it is not possible to determine whether dril mediates its effects on this deuterostome group through inhibition of these transcription factors. However, because components of the TGFβ regulatory pathway are known to function in flies and worms, it will be interesting to determine whether dril also modulates this pathway in protostomes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.11.017.

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