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Klf4 is required for germ-layer differentiation and body axis patterning during *Xenopus* embryogenesis

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SUMMARY

Klf4 is a transcription factor of the family of Kruppel-like factors and plays important roles in stem cell biology; however, its function during embryogenesis is unknown. Here, we report the characterization of a Klf4 homologue in *Xenopus laevis* during embryogenesis. *Klf4* is transcribed both maternally and zygotically and the transcript is ubiquitous in embryos during germ-layer formation. Klf4 promotes endoderm differentiation in both Nodal/Activin-dependent and -independent manners. Moreover, Klf4 regulates anteroposterior body axis patterning via activation of a subset of genes in the Spemann organizer, such as *Noggin*, *Dkk1* and *Cerberus*, which encode Nodal, Wnt and BMP antagonists. Loss of Klf4 function leads to the failure of germ-layer differentiation, the loss of responsiveness of early embryonic cells to inducing signals, e.g. Nodal/Activin, and the loss of transcription of genes involved in axis patterning. We conclude that Klf4 is required for germ-layer differentiation and body axis patterning by means of rendering early embryonic cells competent to differentiation signals.

KEY WORDS: Kruppel-like factor 4 (Klf4), Germ-layer differentiation, Body axis patterning, Transcriptional regulation, *Xenopus laevis*

INTRODUCTION

During *Xenopus* early embryogenesis, Nodal/Activin, Wnt, BMP and FGF signaling pathways play key roles in promoting germ-layer formation. Nodal/Activin is the primary signal to induce mesoderm and endoderm in a dose-dependent fashion. FGF signaling also participates in mesoderm formation (Amaya et al., 1991; Amaya et al., 1993), mainly through providing competence for the embryonic cells to Nodal/Activin. BMP and Wnt pathways are active at the ventral side of embryo (Christian et al., 1991; Dale and Wardle, 1999) and are principally responsible for ventro-posteriorization of germ layers (Maéno et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995; Dale and Wardle, 1999). At the dorsal side, they are blocked by antagonists secreted from the Spemann organizer: notably Noggin, Chordin, Cerberus, Dkk1, Xnr3, etc. (De Robertis et al., 2000). Thus the two groups of signals establish a balance for patterning body plan.

In *Xenopus*, the Nodal ligand genes, *Xnr1-6*, are induced by the maternal transcription factor VegT in vegetal cells (Clements et al., 1999; Hyde and Old, 2000; Takahashi et al., 2000; Hilton et al., 2003). Upon ligand gene transcription, Nodal signal is transmitted downstream and induces transcription of mesoderm- and endoderm-specific genes: *Xbra*, *Mix1*, *Mix2*, *Goosecoid*, *Milk*, *Mix.1*, *Mixer*, *Sox17* and *GATA4-6*, for example (Xanthos et al., 2001; Shivdasani, 2002; Zorn and Wells, 2007). Endoderm-specific genes, meanwhile, inhibit mesoderm genes such that mesoserm and endoderm formation is restricted within correct locations. Maternal β-catenin signaling is enriched in dorsal-vegetal cells and induces *Siamois* transcription in the Nieuwkoop centre (Wodarz and Nusse, 1998), which subsequently induces gene transcription in the Spemann organizer (Wessely et al., 2001) to antagonize ventral signals. β-Catenin also works in synergism with VegT to enhance transcription of Nodal-related genes (Agius et al., 2000; Takahashi et al., 2000), hence establishing a gradient of Nodal signal, with higher activity dorsally and lower activity ventrally. In addition, complex autoregulatory loops play important roles in the regulation of the activity of Nodal signaling (Schier, 2003).

Differentiation of early embryonic cells into germ layers is accompanied by the loss of pluripotency, which is maintained by pluripotency factors. In mammals, these factors are typically Oct4, Sox2, Nanog, cMyc and Klf4 (Niwa et al., 2000; Zaehres et al., 2005; Avilion et al., 2003; Fong et al., 2008; Nakatake et al., 2006). *Xenopus* Oct4 homologous factors Oct60, Oct25 and Oct91 inhibit mesendoderm germ-layer formation via inhibition of the activities of VegT, β-catenin and Nodal (Cao et al., 2006; Cao et al., 2007; Cao et al., 2008). Sox2 is well known for its role in neural fate specification. Although these factors are crucial for the maintenance of pluripotency and self-renewal of embryonic stem (ES) cells, they exhibit distinct functions in ES cell differentiation assays and in embryonic development. Here, we report the identification and characterization of Kruppel-like factor 4 (Klf4) during *Xenopus* early embryogenesis. It promotes endoderm differentiation in both Nodal/Activin-dependent and -independent mechanisms. Moreover, it is involved in body axis patterning via activation of a subset of Spemann organizer genes, which code for Nodal/Activin, Wnt and BMP antagonists. In addition, loss of Klf4 function leads to failure of germ-layer differentiation. Thus we propose that Klf4 confers the competence of early embryonic cells to the activities of inducing signals such as Nodal/Activin so that embryonic cells can differentiate properly. Our results gain novel insights into the functions of Klf4 and the regulatory network for germ-layer differentiation and axis patterning in *Xenopus* embryos.

MATERIALS AND METHODS

Embryos and explants

Xenopus laevis embryos and embryonic explants were obtained and cultured using conventional methods. To block endogenous Nodal activity, uninjected or injected embryos were incubated in culture medium

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containing 100 μM SB431542 (Sigma) from the four-cell stage until gastrulation. To block protein translation, uninjected or injected embryos were incubated in medium containing cycloheximide (CHX) at 25 μg/ml from stage 7 until stage 10.5. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975).

Cloning of *Xenopus laevis* Klf4 cDNA and plasmid construction

By searching databases with mouse Klf4, we found the Klf4 homologue in *Xenopus tropicalis* (XtKlf4). Further search of databases with *XtKlf4* cDNA revealed two *Xenopus laevis* expressed sequence tags (ESTs) (GenBank accession numbers: BI445569 and CB196881) that encode two peptides sharing highest identities with XtKlf4. One EST contains the translational start site and the other contains the stop site. The cDNA containing the complete open reading frame (ORF) was amplified from a pool of cDNAs derived from stage 1 to stage 26 embryos. To make expression plasmid of *Xenopus laevis* Klf4, the ORF was subcloned to pCS2+ to generate pCS2+Klf4. The N-terminal region aa 1-304 with C-terminal zinc fingers missing was PCR amplified to make construct pCS2+Klf4ΔZF. The C-terminal DNA-binding domain (DBD) aa 270-404 was subcloned to make pCS2+Klf4(DBD). For the test of efficiency of the antisense morpholino against Klf4, the ORF including the morpholino binding site was ligated to pCS2+eGFPmcs and pCS2+6MTmcs vectors to make pCS2+Klf4-eGFP and pCS2+Klf4-MT, respectively. The repression and activation form of Klf4 were made by ligating Klf4 DNA binding domain to pCS2+evemcs and pCS2+VP16mcs (Cao et al., 2008), thus resulting in plasmids pCS2+eve-Klf4(DBD) and pCS2+VP16-Klf4(DBD). A plasmid containing complete cDNA of mouse Klf4 (mKlf4) was purchased from IMAGE Consortium (Berlin) and the coding region was subcloned to make pCS2+mKlf4.

Whole-mount in situ hybridization

Whole-mount in situ hybridization on whole embryos or animal caps was carried out essentially as described (Harland, 1991).

In vitro transcription, antisense morpholino oligonucleotides (MOs) and microinjection

Antisense RNA probes for whole-mount in situ hybridization and mRNAs for microinjection were prepared as described (Cao et al., 2006). To prepare antisense RNA probes for whole-mount in situ hybridization, plasmids for Cerberus, Chordin, Dkk1, Gsc, Klf4, Mix2, Mixer, Noggin, Siamois, Sox17α, Sox2, XAG2, Xbra, Xnr1, Xnr5 and Xvent2 were linearized and transcribed with T7 RNA polymerase. To prepare mRNAs for microinjection, plasmids pCS2+Klf4, pCS2+Klf4-eGFP, pCS2+Klf4-MT, pCS2+mKlf4, pCS2+Klf4ΔZF, pCS2+Klf4(DBD), pCS2+VP16-Klf4(DBD), pCS2+eve-Klf4(DBD), pCS2+dnTCF3, pCS2+NLS-LacZ, pSP64T-activinβB, pSP64T-dnXAR1, tBR-64T, pXFD (dnFGFR) and pSP64T-Xnr2 were linearized and transcribed with Sp6 mMessage mMachine kits (Ambion). All probes and mRNAs were cleaned up with an RNeasy Kit (Qiagen). An antisense morpholino oligonucleotide (MO), K4MO: TTCCCTCCACCTCTCATTAATCTGG – which targets 36/–12bp of 5′UTR – was designed to knock down endogenous Klf4 in *Xenopus laevis*. A six-base mismatched MO, K4MO6mis: TTCtCTCgACCTaTCATgAATaTGc (mismatched bases are in lowercase), and the standard control MO (ctrlMO), CCTCTTACCTCAGTTACAATTTATA, were used as controls. All MOs were purchased from GeneTools. Injected doses of mRNAs or MOs are described in the text.

Quantitative RT-PCR

Total RNAs and cDNAs were prepared using exactly the same procedure as described (Cao et al., 2006). Quantitative RT-PCR (qPCR) was performed on an ABI 7300 system and primers are listed in supplementary material Table S1. Amplification parameters were as follows: one cycle of predenaturation at 95°C for 10 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing and extension at 60°C for 31 seconds and an additional cycle for the melting curve. Crosspoints were calculated using ABI 7300 system SDS software. Final results were presented as histograms with relative units.

Luciferase assays

Luciferase assays were carried out with embryos or cells. In embryos, promoter reporter plasmid DNAs and mRNAs were injected into the equatorial region of all blastomeres at the two- or four-cell stage. Embryos were collected at gastrula stage and the method for measuring luciferase activity was as described (Cao et al., 2007). HEK 293T cells were grown in 24-well plates and cells in each well were transfected with 100 ng of reporter plasmid together with 100 ng of various expression plasmids. In each well, 1 ng of Renilla luciferase reporter plasmid was co-transfected as internal control and the total amounts of transfected plasmids were normalized using pCS2+ empty vector. Luciferase activity was measured using the Dual-Luciferase Assay System (Promega). Each measurement was repeated with at least four independent transfections.

Western blotting

Uninjected and injected embryos were collected at stage 10.5, homogenized in cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40) supplemented with protease inhibitors (Roche). Homogenates were incubated on ice for 10 minutes and centrifuged at 12,000 rpm for 10 minutes, and supernatants were transferred to fresh tubes, boiled in 1× Laemlli buffer, and centrifuged again at 12,000 rpm for 5 minutes. Supernatants were collected and 10 μl of each sample were loaded into SDS-PAGE for electrophoresis. Western blotting was performed using the conventional method. A myc-tag antibody was used to detect the expression of Klf4-MT and an α-actin antibody was used for detection of actin.

X-gal staining

Embryos injected with *lacZ* mRNA were fixed in HEMFA and subjected to X-gal staining (Coffman et al., 1990). After staining, embryos were washed in PBS, fixed again in HEMFA and stored in 100% ethanol at –20°C, until processed for whole-mount in situ hybridization.

RESULTS

Spatial-temporal expression of *Klf4* during *Xenopus* embryonic development

The identified cDNA encodes a protein of 404 amino acids. The sequence has the highest similarities to Klf4 in other species: for instance, 94% in *Xenopus tropicalis*, 51% in zebrafish and 55% in mouse (supplementary material Fig. S1A,B). Three classical zinc-finger motifs are present at the carboxyl terminus, which are typical for Kruppel-like factors (Pearson et al., 2008) and nearly identical among Klf4 proteins in different species (supplementary material Fig. S1A). There is a record for *Xenopus laevis Klf4* under accession number NM_001086359 in GenBank; however, this gene product shares the highest identity to *Xenopus* Klf17 [or Neptune (NM_001088664)] and mouse Klf2, but not Klf4. In the genome of *Xenopus tropicalis*, *Klf4* gene locates upstream sequentially to *rad23b*, *znf462* and *tmem38b*. When the order of these genes is reversed, the arrangement is identical to that in both zebrafish and mouse (*Zfp462* is synonymous with *znf462*) (supplementary material Fig. S1C). These comparisons suggested that the sequence we identified is orthologous to *Klf4* in other species.

Klf4 is maternally transcribed as it is present in the animal region of early cleavage stages, e.g. stages 3 and 6.5 (supplementary material Fig. S2A,B). During midblastula, *Klf4* was detected ubiquitously in embryos but slightly enriched at one side of the embryos (supplementary material Fig. S2C). Later, the enrichment was found in the dorsal marginal zone in gastrula embryos (supplementary material Fig. S2D,E). Bisection of a gastrula embryo showed that Klf4 was present in ectoderm and the marginal zone, but enriched slightly in the dorsal margin of the organizer, prechordal mesoderm and endomesoderm (supplementary material Fig. S2F). During neurulation, *Klf4*

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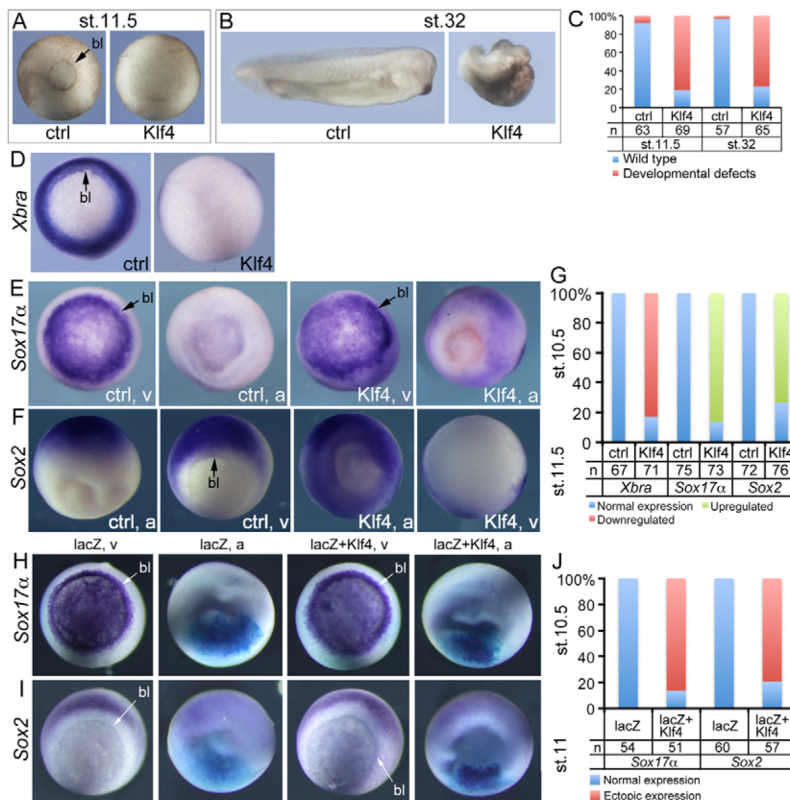

Fig. 1. Overexpression of *Klf4* in *Xenopus* early embryos. (**A**,**B**) The effect of *Klf4* mRNA injection on gastrulation (A) and on body axis formation (B). (**C**) Quantification of phenotypes shown in A and B in triplicate. (**D-F**) The influence of *Klf4* mRNA injection on mesoderm (D), endoderm (E) and ectoderm (F). Embryos in D were placed in vegetal views; those in E and F were placed in vegetal view (v) and animal view (a), separately. (**G**) Quantification of embryos with gene expression observed in D,E,F. In these experiments, 400 pg of *Klf4* mRNA were injected into the equatorial region of all blastomeres of two-cell or four-cell embryos. (**H**,**I**) *lacZ* labeling of targeted injection into one animal-ventral blastomere at the eight-cell stage and whole-mount in situ hybridization detection of *Sox17α* (H) and *Sox2* (I) expression. Embryos were also placed in animal (a) and vegetal (v) views, respectively, as indicated at the top of the panels. (**J**) Quantification of embryos with normal or ectopic gene expression in H and I. *lacZ* mRNA was injected at 20 pg/nl; *Klf4* mRNA was injected at 40 pg/nl. The arrows indicate the blastopore (bl). In all the panels, dorsal is up for *Sox2*-stained embryos.

localizes to two narrow lines within the neural folds (supplementary material Fig. S2G) and the anlage of cement gland (supplementary material Fig. S2H). Neural expression of *Klf4* soon disappears but the cement gland expression persists until the tadpoles hatch (supplementary material Fig. S2I,J). *Klf4* is also specifically present in trigeminal nerve and lung primordium at stage 34 (supplementary material Fig. S2J), and the prospective duodenum/stomach at stage 43 (supplementary material Fig. S2K). During embryogenesis, maternal *Klf4* is more abundant than zygotic *Klf4* in gastrulae and neurulae. During the tailbud stages, the expression level rises up again (supplementary material Fig. S2L). *Klf4* transcript is present in both animal and vegetal blastomeres at the eight-cell embryo stage (supplementary material Fig. S2M). At stage 8.5 when zygotic transcription and germ-layer differentiation starts, transcript was detected in animal, equatorial and vegetal regions (supplementary material Fig. S2N). Therefore, *Klf4* transcription is ubiquitous in early embryos. In summary, spatiotemporal expression patterns of *Klf4* suggest that it might be involved in early embryonic development.

Klf4 gain-of-function analyses in *Xenopus* embryos

The blastopore formed normally in uninjected control embryos and tended to close at stage 11.5. In embryos injected with *Klf4* mRNA, gastrulation was severely interrupted, as there was no clear blastopore formation (Fig. 1A,C). At stage 32, the majority of these embryos showed severely reduced anteroposterior body axis, pronounced belly protrusion with heavy pigmentation and seemingly exaggerated cement glands (Fig. 1B,C). In injected embryos, expression of the pan-mesoderm marker *Xbra* was strongly inhibited, suggesting that mesoderm formation was blocked (Fig. 1D,G). The endoderm gene *Sox17α* was detected only in the vegetal area of normal embryos, but it was ectopically activated in equatorial and animal regions in injected embryos (Fig. 1E,G). The neuroectoderm gene *Sox2* was expressed at the dorsal side of control gastrula embryos; however, *Klf4* RNA injection led to expansion of the *Sox2* expression domain to the ventral side, thus suggesting an increment in neuroectoderm (Fig. 1F,G). Furthermore, we injected one ventral-animal blastomere at the eight-cell stage with *lacZ* RNA alone or *lacZ* and *Klf4* RNAs together. Klf4-induced ectopic expression of *Sox17α* or *Sox2* occurred within the *lacZ*-labeling regions (Fig. 1H-J), implying an autonomous effect of Klf4.

Isolated *Xenopus* blastula ectoderm, i.e. the animal caps, differentiates into epidermis. It can be induced to adopt different cell fates by inducers. At the gastrula stage, animal caps without *Klf4* injection did not exhibit any discernible *Xbra* and *Sox17α* expression (Fig. 2A,B). Caps injected with *Klf4* showed no difference from uninjected caps with respect to *Xbra* expression. However, there was strong activation of *Sox17α* in caps injected with *Klf4* (Fig. 2B). We observed repeatedly weak *Sox2* expression in uninjected caps, but *Klf4* overexpression clearly led to an increase (Fig. 2C). These results are in agreement with the data observed in whole embryos. In addition, *Mixer*, another gene that is required for endoderm induction (Henry and Melton, 1998), was also strongly stimulated by *Klf4* overexpression in both whole embryos and animal caps (Fig. 2D). Therefore, Klf4 is capable of inhibiting mesoderm while promoting endoderm and neuroectoderm formation. At neurula stage, Klf4-injected caps still showed higher levels of genes that specify neural precursors, e.g. *Sox2*, *Sox3* and *SoxD*, but no neural tissue differentiation was observed, as revealed by *NCAM* expression (Fig. 2E). Epidermal differentiation was nearly completely blocked in Klf4 caps (Fig. 2E). Genes marking mesodermal tissues, *α-globin* and *α-actin*, were detected only in background levels in both control and Klf4 caps (Fig. 2E). Instead, significant increases in expression of the

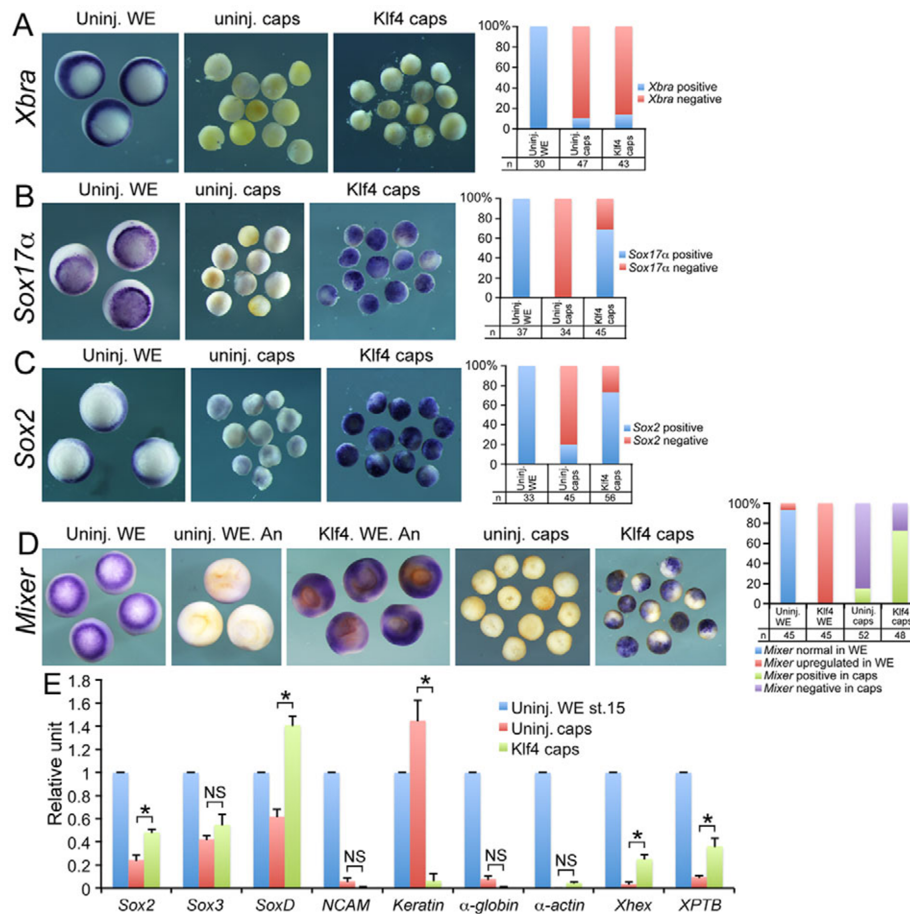

Fig. 2. Assays on Klf4 function with animal caps. (**A-C**) Uninjected control whole embryos (Uninj. WE), uninjected control animal caps (uninj. caps), and caps injected with *Klf4* mRNA (Klf4 caps), were assays for the expression of *Xbra* (A), *Sox17α* (B) and *Sox2* (C), and their respective quantification. (**D**) *Mixer* expression in uninjected control whole embryos (Uninj. WE), *Klf4* mRNA-injected whole embryos (Klf4. WE), uninjected control animal caps (uninj. caps) and injected caps (Klf4 caps), and respective quantification. In A-D, embryos were placed vegetally to view normal expression of marker genes; except those in D, embryos were also orientated to animal view (An) to show the staining for *Mixer* in animal pole. Graphs represent the numbers of WE or caps with (positive) or without (negative) gene expression in three experiments. (**E**) qPCR detection of gene expression to analyse tissue differentiation in animal caps injected with *Klf4* RNA. Error bars represent s.d. in triplicate. A Student's *t*-test was conducted to compare the changes in gene expression between uninjected (Uninj. caps) and *Klf4*-injected (Klf4) caps. Asterisks indicate $P<0.01$. NS: not significant. In the experiments above, 400 pg of *Klf4* mRNA was injected close to the animal pole of all blastomeres of four-cell embryos, and animal caps were removed at stage 8.5. For whole-mount in situ hybridization assays, caps were cultured until sibling control embryos reached stage 10.5. For qPCR assays, caps were cultured until sibling control embryos reached stage 15.

liver marker genes *Xhex* and *XPTB* (Chen et al., 2003) demonstrated that endodermal tissue differentiation occurred in Klf4 caps (Fig. 2E). The result suggested that Klf4 is capable of promoting the formation of neural precursor cells, but is not able to induce neural tissue differentiation on its own.

Klf4 loss-of-function analyses

We designed an antisense morpholino oligonucleotide (K4MO) to knock down *Xenopus laevis* Klf4 by targeting the 5′UTR of its mRNA. K4MO could efficiently inhibit translation of the mRNA for the fusion protein Klf4-GFP (Fig. 3A,B) and mRNA for Klf4-MT fusion protein in embryos (Fig. 3C). By contrast, both the six-base mismatched control MO (K4MO6mis) and the standard control MO (ctrlMO) did not inhibit protein translation (Fig. 3A-C), showing the specificity of K4MO.

At the tailbud stage, the Klf4 morphant displayed a severely reduced anteroposterior body axis and head size (Fig. 3D,E). This phenotype was rescued by co-injection of 10, 20, 30 or 40 pg *Klf4* mRNA, as co-injection of the mRNA reversed the shortening of the body axis to different degrees, with a better rescuing effect at higher doses (Fig. 3D,E). The rescued embryos were obviously better in body axis formation than the Klf4 morphant. Moreover, co-injection of 10 or 30 pg mouse *Klf4* RNA (mKlf4) also resulted in a similar rescuing effect (Fig. 3D,E), suggesting a conserved function of *Xenopus* and mouse Klf4.

Injection of ctrlMO or K4MO6mis in embryos didn't affect expression of *Xbra*, *Sox17α* and *Sox2*. However, they were inhibited in the Klf4 morphant (Fig. 3F,G). In addition, the mesoderm genes *Chordin*, *Xvent1*, *Xvent2* and *Wnt8*, the endoderm genes *Mixer*, *FoxA2*, *GATA4*, *GATA5* and *GATA6*, and the ectoderm genes *Sox2*, *Sox3*, *SoxD* and *XEMA* were all repressed (Fig. 3H). The repression effect was specific for these genes because other genes such as *Goosecoid* (*Gsc*), *Oct25*, *Oct60*, *Oct91*, *KMT5C*, *Cbx4* and the germ cell genes *Nanos* and *Xpat* were not significantly altered or even upregulated (Fig. 3H). These results implied that Klf4 is a prerequisite for the differentiation of early embryonic cells to germ layers.

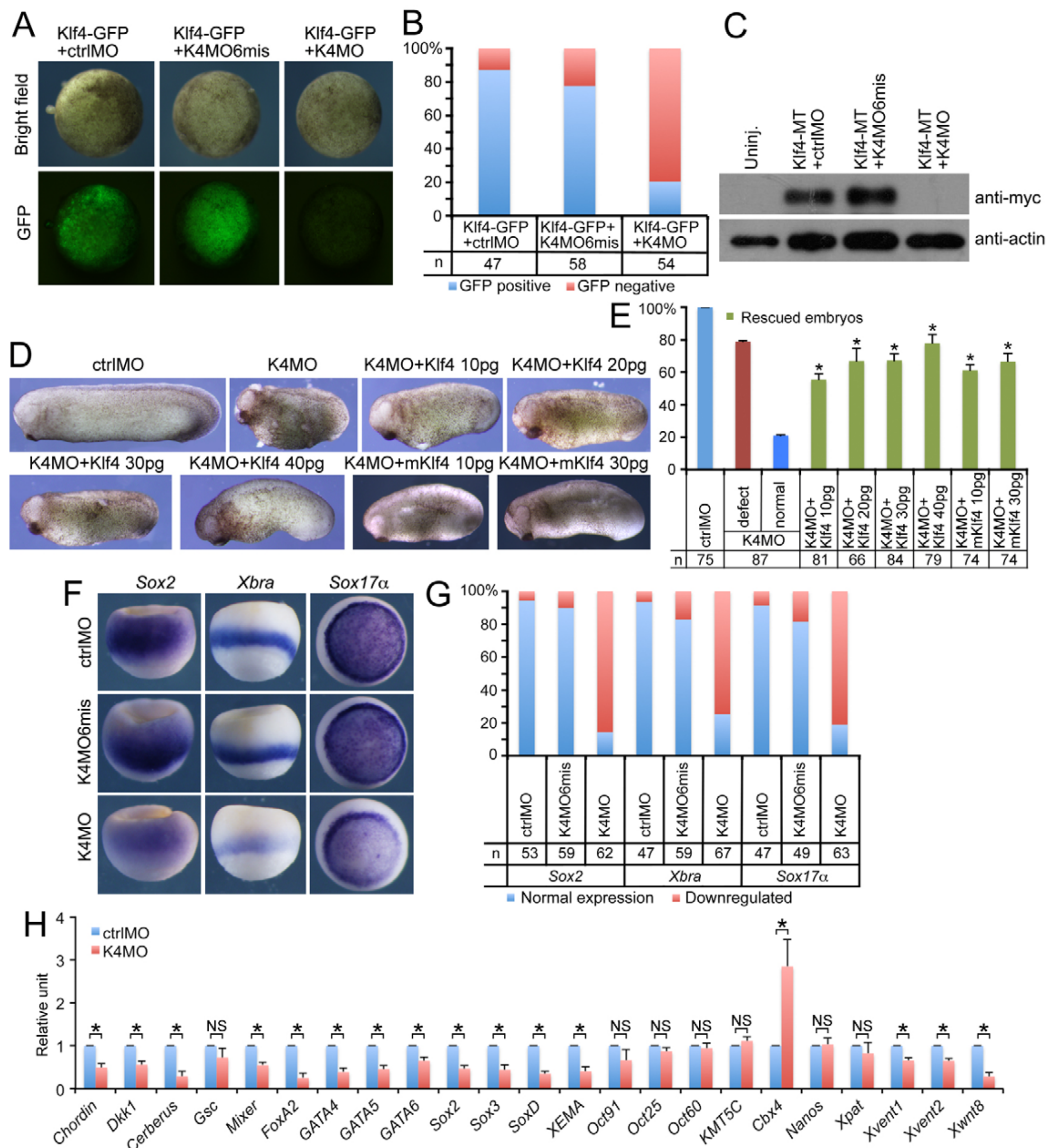

Fig. 3. Loss-of-function analyses on Klf4. (**A**) Effect of ctrlMO, K4MO6mis or K4MO on the translation of co-injected mRNA for Klf4-GFP in embryos. Embryos were observed at stage 11 under brightfield or green fluorescent protein (GFP) and photos were taken using the same exposure parameters. (**B**) Quantification of embryos without (GFP-negative) or with (GFP-positive) green fluorescence. (**C**) Effect of ctrlMO, K4MO6mis and K4MO on the translation of Klf4-MT using western blotting (WB). Uninjected (Uninj.) and injected embryos were collected at stage 10.5 for WB. A myc-tag (MT) antibody (anti-myc) was used to detect the Klf4-MT fusion protein. α-Actin was used as a loading control. In A and C, each MO was injected at 10 ng, *Klf4-GFP* or *Klf4-MT* mRNA was injected at 400 pg. (**D**) The rescuing effect of injected *Xenopus Klf4* mRNA (Klf4) or mouse *Klf4* (mKlf4) mRNA on the Klf4 morphant. MOs were injected at 10 ng each. The dose of mRNA was indicated at the top of each panel. (**E**) Quantification of normal, Klf4 morphant and rescued embryos in D. Error bars represent s.d. in three experiments. Student's *t*-test was used to compare the ratio of rescued embryos against the ratio of unaffected embryos among K4MO-injected embryos. The asterisks indicate $P<0.01$. (**F**) Effect of injection of ctrlMO, K4MO6mis and K4MO on expression of *Sox2*, *Xbra* and *Sox17α*. Embryos for *Sox2* expression were orientated to a dorsolateral view; those for *Xbra* to a lateral view, and *Sox17α* to a vegetal view. (**G**) Statistical numbers of embryos in three experiments with normal or downregulated gene expression in F. (**H**) Gene expression analysis with qPCR. In F and H, 20 ng of ctrlMO or K4MO was injected into the equatorial region of all blastomeres at the four-cell stage and collected at gastrulation for whole-mount in situ hybridization or qPCR. Error bars represent s.d. in triplicate. A Student's *t*-test was used to compare the change in gene expression between ctrlMO- and K4MO-injected embryos. Asterisks indicate $P<0.01$. NS: not significant.

Klf4 regulates Nodal/Activin pathway

In the classic point of view, the Nodal/Activin pathway is the primary inducing signal for endoderm specification. Thus we have investigated whether Klf4 has any influence on Nodal/Activin activity. Overexpression of *Klf4* induced ectopic transcription of *Xnr1* and *Xnr5* (Fig. 4A,B,D). Accordingly, the Nodal/Activin direct target gene, *Mix2*, was stimulated (Fig. 4C,D). In HEK293T cells, transfection of the Xnr5 expression plasmid slightly

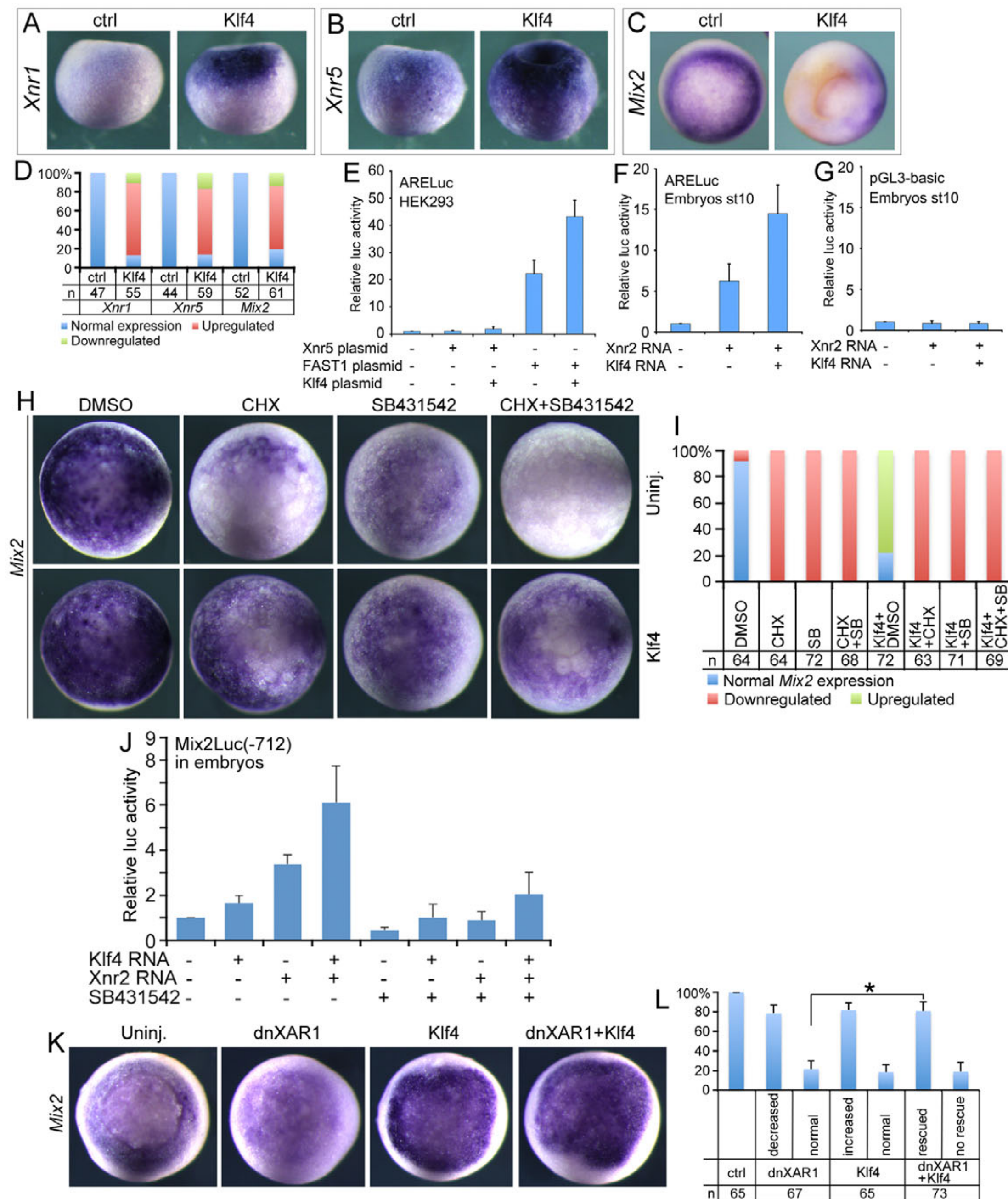

Fig. 4. The regulatory effect of Klf4 on the Nodal/Activin pathway. (**A-C**) The effect of *Klf4* overexpression on transcription of *Xnr1* (A), *Xnr5* (B) and *Mix2* (C). *Klf4* mRNA (300 pg) was injected into the equatorial region of all blastomeres at the four-cell stage. Control and injected embryos at stage 8.5 were collected for detection of *Xnr1* and *Xnr5* expression, while embryos at stage 10 were collected for detection of *Mix2* expression. In A and B, embryos were in lateral view. In C, vegetal view was shown for 'ctrl' to reveal normal expression of *Mix2*, and animal view was shown for 'Klf4' to reveal ectopic *Mix2* expression in ectoderm. (**D**) Quantification of embryos in A-C in three experiments. (**E**) Luciferase assays in HEK293T cells transfected with ARELuc reporter plasmid and expression plasmids for Xnr5, FAST1 or Klf4. (**F**) Luciferase assays in embryos injected with ARELuc reporter plasmid and mRNAs for Xnr2 or Klf4. (**G**) Luciferase assays on pGL3-basic vector in embryos injected with *Xnr2* or *Klf4* mRNAs. Error bars represent s.d. in eight experiments. In F and G, plasmids were injected at 40 pg, *Xnr2* mRNA was injected at 10 pg, and *Klf4* mRNA was injected at 300 pg. (**H**) *Mix2* expression in uninjected (Uninj.) and *Klf4* RNA-injected (Klf4) embryos, and treated separately with different chemicals as indicated. All embryos are in vegetal view. *Klf4* mRNA (400 pg) was injected vegetally at the four-cell stage, treated with chemicals and collected at stage 10.5 for whole-mount in situ hybridization. (**I**) Quantification of embryos in H with normal, downregulated or upregulated *Mix2* expression in three experiments. (**J**) Mix2Luc(–712) luciferase assay with untreated embryos or embryos treated with SB431542. Plasmid was injected at 40 pg, *Xnr2* mRNA was at 10 pg, and *Klf4* mRNA was injected at 300 pg. Error bars represent s.d. in seven experiments. (**K**) The rescuing effect of Klf4 on *Mix2* expression. All embryos were in vegetal view. *dnXAR1* RNA (1.5 ng) and *Klf4* RNA (400 pg) were injected separately or together into the vegetal pole of four-cell embryos. Embryos were collected at stage 11 for whole-mount in situ hybridization. (**L**) Quantification of embryos with normal or altered *Mix2* expression observed in K. Error bars represent s.d. in triplicate. Student's *t*-test showed the significance of the ratio of rescued *Mix2* expression in embryos with *dnXAR1+Klf4* RNA injection compared with background (ratio of embryos with unaffected *Mix2* expression in *dnXAR1*-injected embryos). Asterisks indicate $P<0.01$.

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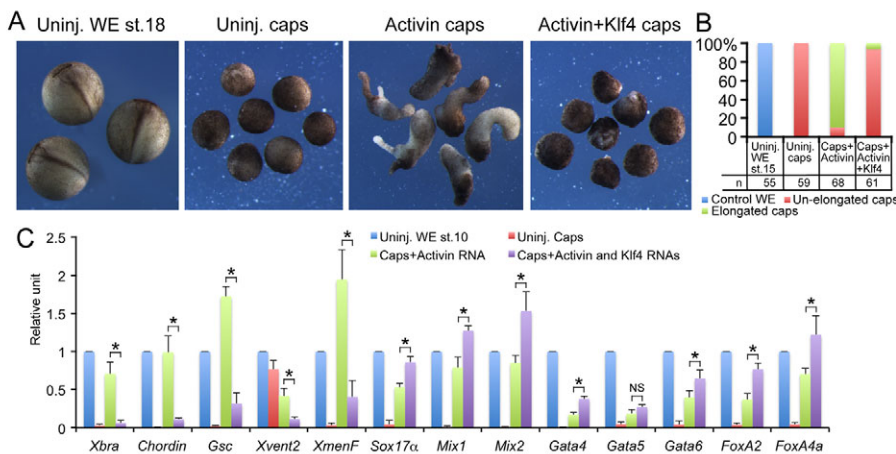

Fig. 5. Influence of *Klf4* overexpression on tissue differentiation induced by Activin. (**A**) *Activin βB* mRNA (0.2 pg) was injected alone or together with 300 pg of *Klf4* mRNA into the animal pole of all blastomeres of two-cell or four-cell embryos. At stage 8.5, animal caps were removed from uninjected or injected embryos and cultured until the neurula stage. (**B**) Quantification of embryos and caps in A. (**C**) Gene expression analysis with qPCR on the caps described in A, but collected at stage 10. Error bars represent s.d. in three experiments. Asterisks indicate $P<0.01$. NS: not significant.

stimulated ARELuc, the Nodal/Activin responsive luciferase reporter (Pierreux et al., 2000; Germain et al., 2000). Co-transfection of the Klf4 expression plasmid stimulated the reporter somewhat more strongly. FAST1, a key nuclear signal transducer for the Nodal/Activin pathway, could stimulate ARELuc significantly. Addition of Klf4 plasmid to the cells led to much stronger stimulation of the reporter (Fig. 4E). Stimulation of ARELuc reporter by Klf4 was recapitulated nicely in embryos via injection of the Nodal ligand mRNA alone or together with *Klf4* mRNA (Fig. 4F). Injection of these mRNAs had no significant effect on the pGL3-basic plasmid (Fig. 4G) that was used for constructing the ARELuc reporter, excluding the unspecific stimulation of ARELuc by Klf4. Therefore, Klf4 promoted Nodal/Activin activity. In uninjected embryos, blocking protein translation with CHX treatment or blocking Nodal/Activin with SB431542, a specific chemical inhibitor of the Nodal/Activin type I receptor, led to dramatic inhibition of *Mix2* expression. It was completely eradicated in embryos treated with both CHX and SB431542 (Fig. 4H,I). Vegetal injection of Klf4 RNA enhanced *Mix2* transcription compared with uninjected embryos. Treatment of injected embryos with CHX, SB431542 or both resulted in significant reduction in *Mix2*; however, the expression levels were much higher than in uninjected embryos, respectively (Fig. 4H,I). Therefore, even in the absence of protein translation and/or the Nodal feedback loop, Klf4 was still able to stimulate Nodal target gene expression. Klf4 exhibited the same effect on *Mix2* promoter activity. A luciferase reporter, Mix2Luc(–712), which contains –712/+13 fragment of *Xenopus Mix2* promoter (Cao et al., 2008), was stimulated in embryos by *Klf4* or *Xnr2* overexpression. The stimulation grew much stronger when Klf4 and Xnr2 were simultaneously overexpressed (Fig. 4J). SB431542 treatment dampened the stimulation, however, the promoter activity in embryos with *Klf4* overexpression was still much higher than the background level in treated embryos without *Klf4* overexpression (Fig. 4J). Both gene expression and promoter analyses demonstrated that *Klf4* directly regulates Nodal/Activin target gene expression. As a support, *Klf4* could rescue the decrease of *Mix2* expression resulting from injection of *dnXAR1* (Fig. 4K,L), the dominant-negative *Xenopus* Activin receptor I (Hemmati-Brivanlou and Melton, 1992).

Klf4 promotes transcription of genes responsible for endoderm differentiation

We tested the effect of Klf4 on mesoderm and endoderm differentiation induced by Nodal/Activin in animal caps. At the neurula stage, control animal caps differentiated into epidermis and showed no elongation; however, those injected with Activin mRNA showed obvious elongation (Fig. 5A,B). By contrast, caps injected with both Activin and *Klf4* mRNAs did not elongate (Fig. 5A,B). qPCR revealed that, at the gastrula stage, animal caps injected with Activin mRNA showed high levels of mesoderm genes such as *Xbra*, *Chordin*, *Gsc*, *Xvent2* and *XmenF*. Meanwhile, the Nodal target and endoderm genes such as *Mix1*, *Mix2*, *Sox17α*, *Gata4-6*, *FoxA2* and *FoxA4a* were also induced in these caps (Fig. 5C). When *Klf4* mRNA was co-injected, the mesoderm genes were dramatically inhibited, whereas the Nodal target genes and endoderm genes were enhanced (Fig. 5C). In summary, these analyses showed that Klf4 promotes endoderm differentiation while inhibiting mesoderm differentiation.

Crosstalk between Klf4 and Nodal/Activin signaling in germ-layer differentiation

Activation of Nodal via injection of *Xnr2* mRNA or inhibition of Nodal activity via injection of *dnXAR1* mRNA led to different developmental defects (Fig. 6A,B), as previously reported (Hemmati-Brivanlou and Melton, 1992). Ventral injection of *Klf4* resulted in belly protrusion with heavy pigmentation, resembling an anteriorized phenotype. K4MO injection generated embryos with a significantly shortened anteroposterior axis (Fig. 6A,B). Interestingly, co-injection of *Xnr2* RNA and K4MO led to nearly normal embryos. By contrast, co-injection of *Klf4* and *dnXAR1* brought about an extremely anteriorized phenotype, which showed a severely decreased anteroposterior body axis but with hugely exaggerated cement glands (Fig. 6A,B). Finally, when *dnXAR1* RNA and K4MO were injected together ventrally, embryos bent towards the ventral side, probably owing to a lack of tissue differentiation (Fig. 6A,B).

In congruence with the phenotypes above, injection of *Klf4* or *dnXAR1* RNA alone resulted in ectopic expression of *XAG2*, an anterior marker gene. When they were injected together, much stronger *XAG2* expression was observed (Fig. 6C,D). This confirms the idea that overexpression of *Klf4* in the absence of Nodal/Activin leads to extreme anteriorization of the body axis. The effect was also observed in embryos with *Klf4* overexpression and inhibition of either BMP, FGF or Wnt (supplementary material Fig. S3A,B). Therefore, Klf4 anteriorizes body axis and enhances anteriorization in response to inhibition of posteriorization signals.

We examined how the crosstalk between Klf4 and Nodal/Activin affected germ-layer differentiation. First, injection of *dnXAR1* mRNA resulted in decreased *Sox17α* in the vegetal region.

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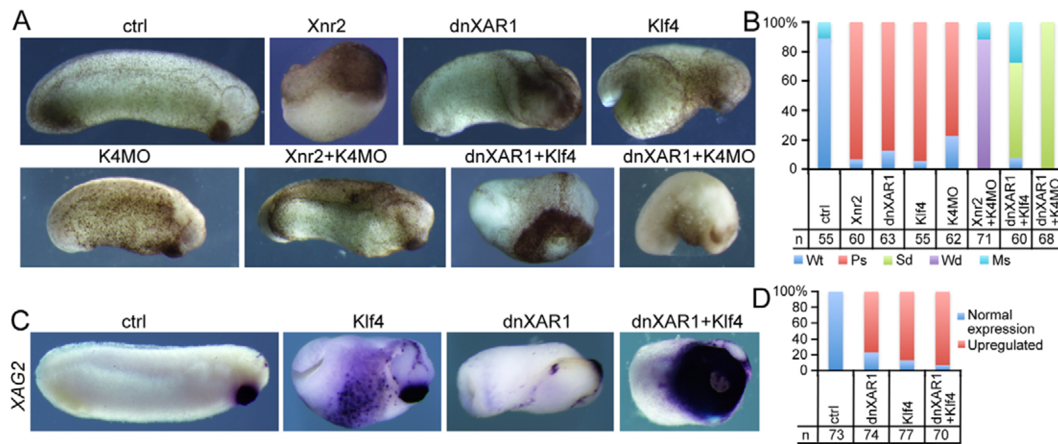

Fig. 6. Correlation between Nodal and Klf4 during embryonic development. (**A**) Injection of *Xnr2* mRNA, *dnXAR1* mRNA, *Klf4* mRNA, or K4MO individually or in different combinations, as labeled above each panel, generated different effects on embryogenesis. (**B**) Quantification of phenotypes observed in A in three experiments. Wt, wild type; Ps, phenotypic changes after single injection; Sd, stronger phenotype after double injection; Wd, weaker phenotype after double injection; Ms, other phenotypes, such as those dead, or similar to phenotypes after single injection. (**C**) Expression of *XAG2* in uninjected control embryos at stage 28 and injected embryos as indicated. (**D**) Quantification of *XAG2* expression in embryos in C in four experiments. In all experiments above, 1.5 pg *Xnr2* mRNA, 1.5 ng *dnXAR1* mRNA, 300 pg *Klf4* mRNA or 5 ng K4MO was injected. Injections of *Xnr2* alone or *Xnr2* plus K4MO together were radial, while others were ventral injections made at the four-cell stage.

Overexpression of *Klf4* again activated ectopic *Sox17α*. When both *Klf4* and *dnXAR1* were injected together, *Sox17α* was still ectopically stimulated in the animal region (Fig. 7A,B). Such an effect could be reproduced in embryos in which Nodal/Activin was blocked with SB431542 (Fig. 7A,B). *Mixer*, another gene involved in endoderm differentiation, could also be induced by overexpression of *Klf4* in embryos with SB431542 treatment (supplementary material Fig. S4A,B). These effects are similar in that *Klf4* still activates *Mix2* expression in the absence of Nodal/Activin. By contrast, *Klf4* knockdown compromised the upregulation of mesendoderm genes *Xbra*, *Chordin* and *Sox17α* resulting from *Xnr2* RNA injection (Fig. 7C,D). This result was in agreement with the fact that Klf4 knockdown was able to rescue the *Xnr2* overexpression phenotype (Fig. 6A). Finally, blocking either Nodal/Activin or *Klf4* led to a decrease in *Xbra*, *Sox17α* and *Cerberus* expression. When both signals were inhibited, gene expression was nearly totally lost (Fig. 7E,F). This means that there is a synergistic effect between the two signals and both are a prerequisite for germ-layer formation.

Klf4 induces anteriorizing signals

Injection of *Klf4* alone or meanwhile in the absence of Nodal/Activin activity led to anteriorization of embryos. Accordingly, *Klf4* overexpression stimulated transcription of *Siamois* (Fig. 8A,B) at midblastula, which is required for induction of the organizer precursor (Nieuwkoop centre) and organizer gene transcription. Organizer genes such as *Cerberus*, *Dkk1* and *Noggin* were activated prematurely at midblastula, when there were no or very weak transcription of these genes (supplementary material Fig. S5A,B). During gastrulation, these genes were expressed in much broader or ectopic regions in *Klf4*-injected embryos (Fig. 7A,B). Activation of the organizer genes was a direct effect. In uninjected embryos, CHX treatment totally eliminated gene expression. By contrast, *Klf4* overexpression was able to activate the genes in both DMSO- and CHX-treated embryos (supplementary material Fig. S6A,B).

Klf4 did not stimulate all organizer genes, as *Chordin* and *Gsc* were inhibited in response to *Klf4* overexpression (Fig. 8C,D). Moreover, the ventral gene *Xvent2* was also significantly inhibited in *Klf4*-injected embryos (Fig. 8C,D). Similar to *Xenopus Klf4*, mouse *Klf4* (mKlf4) likewise induced *Dkk1* activation in *Xenopus* embryos, supporting the idea that they are functionally homologous (Fig. 8C,D). Opposite to the effect of overexpression, *Klf4* knockdown resulted in downregulation of *Dkk1*, *Cerberus* and *Noggin* (Fig. 8E,F). Upregulation of ventral genes such as *Xvent2* was not detected in Klf4 morphant (Fig. 8E,F), this was possibly due to the failure of germ-layer differentiation (Fig. 7E). Hence, *Klf4* is sufficient and necessary for the transcription of the subset of organizer genes, which are known as anterior fate inducers. Overexpression of *Klf4* enhanced anteriorization when Nodal, BMP or Wnt was blocked. Accordingly, expression of *Dkk1* was also strongly augmented in such embryos during gastrulation (Fig. 8G,H).

Regulation of genes in germ-layer differentiation and axis patterning by *Klf4* gave rise to the question of whether it acts as a repressor or an activator. Klf4 consists of the transcriptional regulation domain at the N-terminus and the DNA-binding domain (DBD) of three zinc fingers at the C-terminus (supplementary material Fig. S7A). Injection of mRNA for the N-terminal region without zinc fingers (Klf4ΔZF) led to no significant change in embryogenesis. Injection of mRNA for the C-terminal region [Klf4(DBD)] had a minor effect on embryonic development. The affected embryos showed decreased tail region and slight belly protrusion (supplementary material Fig. S7B,C). Therefore, both regions are required for *Klf4* function. As the C-terminus is responsible for DNA binding, we replaced the N-terminus of *Klf4* with VP16 transcriptional activation domain or Even-skipped transcriptional repression domain to make VP16-Klf4(DBD) and eve-Klf4(DBD) fusion constructs, respectively (Fig. 9A). Injection of mRNA for VP16-Klf4(DBD) led to stimulation of *Dkk1* and *XAG2*, similar to the wild-type *Klf4*. However, eve-Klf4(DBD) caused an opposite effect (Fig. 9B,C). Luciferase assays in cells exhibited that VP16-Klf4(DBD) acted similarly to Klf4 to enhance FAST1 stimulated ARELuc activity, but eve-Klf4(DBD) could not exert such an effect (Fig. 9D). As CHX treatment showed that *Mix2*

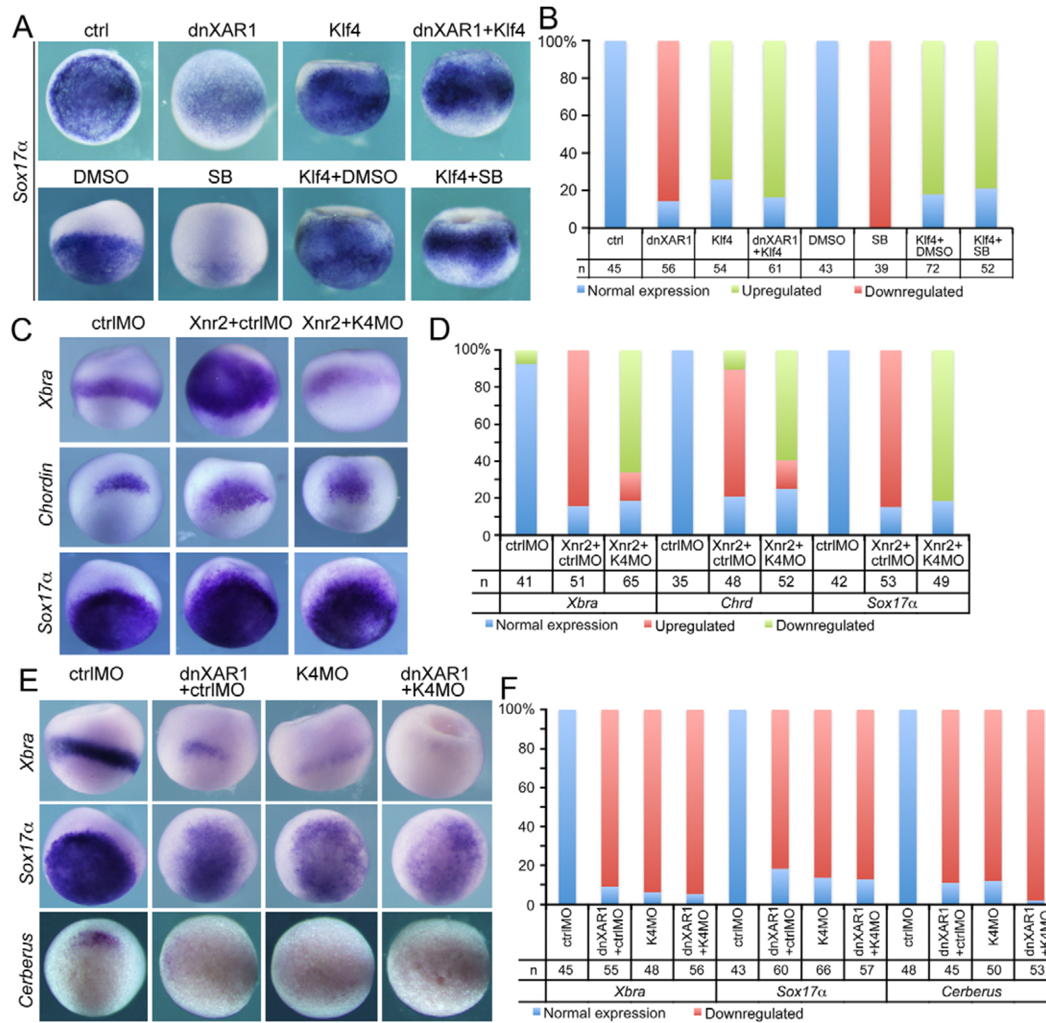

Fig. 7. Combinatorial effects of Nodal/Activin and Klf4 on germ-layer differentiation. (**A**) The effect of blocking Nodal/Activin via injection of *dnXAR1* or treatment with SB431542 (SB) on *Klf4*-induced endoderm gene expression. The 'ctrl' and 'dnXAR1' embryos were orientated in vegetal view; the others were in lateral view to show staining in both vegetal and animal regions. (**B**) Quantification of embryos with normal or altered *Sox17α* expression in A in triplicate. (**C**) The effect of K4MO or/and *Xnr2* mRNA injection on mesoderm and endoderm gene expression. The embryos are in lateral view. (**D**) Quantification of embryos with normal or altered gene expression in C in triplicate. (**E**) The effect of injection of *dnXAR1* or/and K4MO on mesendoderm gene expression. The embryos for '*Xbra*' staining are in lateral view, whereas the rest are in vegetal view. (**F**) Quantification of embryos with normal or altered gene expression in E in three experiments. In all experiments above, 1.5 ng *dnXAR1* mRNA, 300 pg *Klf4* mRNA, 15 ng ctrlMO or K4MO, 2 pg *Xnr2* mRNA were injected. Injected or treated embryos were collected at stage 10.5 for whole-mount in situ hybridization.

is a direct target of *Klf4*, a fragment of *Mix2* promoter –221/+13 that contains the FoxH1 and Smad binding sites (the Activin response element) and a fragment of –142/+13 that does not contain the binding sites (Cao et al., 2008) were also used for testing the activity of the fusion constructs in embryos. VP16-Klf4(DBD) showed strong stimulating effect on both reporters; by contrast, eve-Klf4(DBD) was repressive (Fig. 9E,F). These experiments demonstrated that *Klf4* is an activator for transcription of these genes.

DISCUSSION

We have observed for the first time in the present study that during *Xenopus* embryogenesis: (1) *Klf4* promotes endoderm differentiation; (2) *Klf4* functions in pattern formation of the body axis; and (3) *Klf4* acts as a competence factor for early embryonic cells to differentiate.

Klf4 is required for germ-layer differentiation

We identified the cDNA for the mammalian *Klf4* orthologue in *Xenopus laevis*. This is supported not only by sequence comparison and synteny analysis, but also by the fact that mouse *Klf4* can rescue the *Xenopus* Klf4 morphant and that overexpression of *Xenopus* or mouse *Klf4* generates a similar effect on gene expression. *Xenopus Klf4* is maternally expressed. In mammals, there is little maternal transcript of *Klf4* according to microarray data (Hamatani et al., 2008); however, significant maternal inheritance of *Klf4* is observed in medaka and zebrafish (Wang et al., 2011; Li et al., 2011; Luo et al., 2011). This might be because of the divergence of gene regulation between lower and high vertebrates during evolution. Zygotic transcription of *Klf4* is detected in both lower vertebrate and mammalian early embryos, but the abundance of zygotic transcripts is lower than maternal

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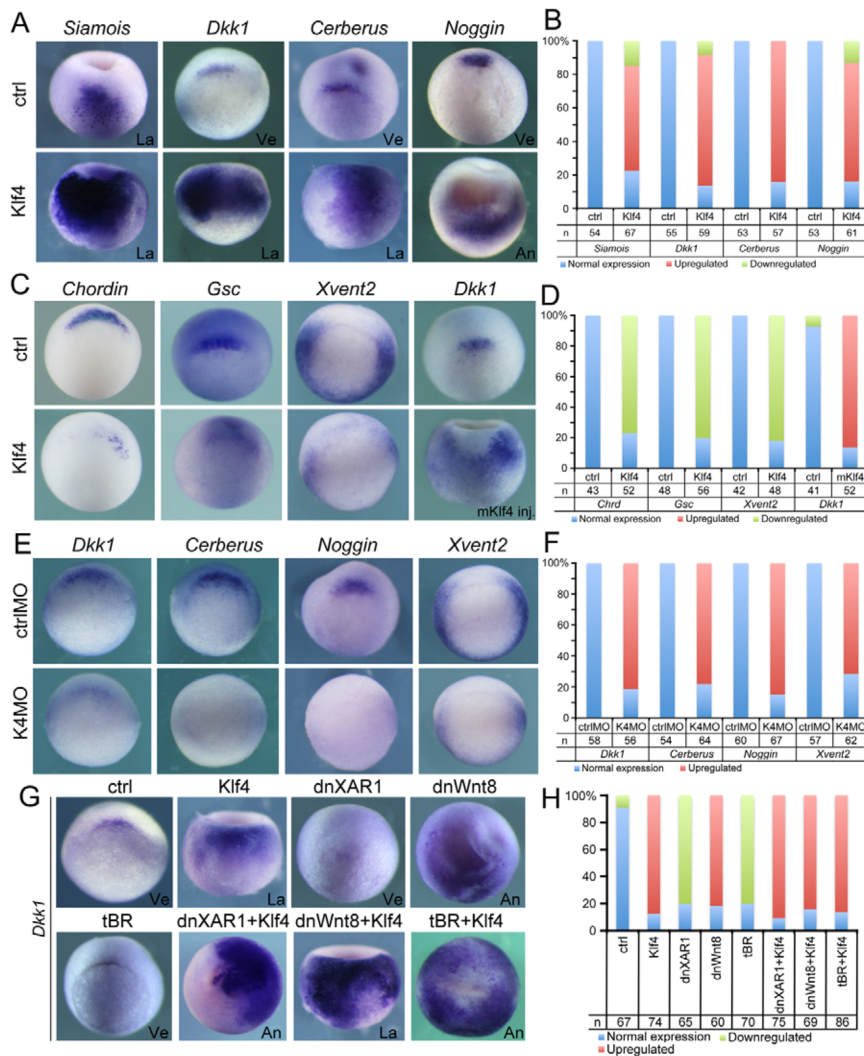

Fig. 8. The effect of Klf4 on anteriorization signals. (**A**) The effect of *Klf4* injection (Klf4) on *Siamois*, *Dkk1*, *Cerberus* and *Noggin* transcription. (**B**) Quantification of embryos with normal or altered expression in A in three experiments. (**C**) The effect of *Klf4* mRNA injection (Klf4) or mouse *Klf4* (mKlf4) injection on *Chordin*, *Gsc*, *Xvent2* and *Dkk1*. All embryos are in vegetal view except the *mKlf4*-injected one (mKlf4), which is in lateral view to show ectopic *Dkk1* transcription. (**D**) Quantification of embryos with normal or altered expression in C in triplicate. For experiments in A and C, *Klf4* or *mKlf4* mRNAs were injected at 300 pg into all blastomeres at the four-cell stage. Embryos were collected at stage 10.5 for whole-mount in situ hybridization except that embryos at stage 8.5 were collected for detecting *Siamois*. (**E**) *Dkk1*, *Cerberus*, *Noggin* and *Xvent2* expression in response to K4MO injection. Embryos were in vegetal view. ctrlMO and K4MO were injected at 20 ng and embryos were collected at stage 10.5 for whole-mount in situ hybridization. (**F**) Quantification of embryos with normal or altered expression in E in triplicate. (**G**) The effect of injecting *Klf4* (at 300 pg), *dnXAR1* (1.5 ng), *dnWnt8* (1 ng) or *tBR* mRNA (1 ng) alone or in combination on *Dkk1* expression, as indicated above each panel. Embryos were collected at stage 10.5 for whole-mount in situ hybridization. (**H**) Quantification of embryos with normal or altered expression in G in triplicate. An: Animal view; La: lateral view; Ve: vegetal view.

ones, as revealed in *Xenopus* and medaka fish (Wang et al., 2011). Ubiquitous transcription of *Klf4* in early cleavage, blastula and gastrula embryos fits well with its function in germ-layer formation described in the present study. Besides, the localized expression of *Klf4* in later stages suggests that *Klf4* might also function in tissue differentiation or organ formation.

Overexpression and knockdown experiments in whole embryos and animal caps demonstrate that *Klf4* plays critical roles for germ-layer differentiation and body axis patterning. In animal caps, *Klf4* promotes neuroectoderm and endoderm; however, only endodermal tissue differentiation and not neural tissue differentiation occurs. Thus, Klf4 can drive endodermal differentiation and maintain the identity of neural precursors. The fate choice should be context-dependent. Although *Klf4* induces transcription of *Dkk1*, *Cerberus* and *Noggin*, which neuralize ectoderm, the promotion of neuroectoderm by *Klf4* seems to be cell-autonomous. In dissociated animal caps with *Klf4* knockdown, neuralization of cap cells is not affected significantly (data not shown). In ES cells, Klf4 and Sox2 are components of the core regulatory circuitry for the maintenance of pluripotency via protein-protein interaction, thus promoting transcription of each other (Orkin et al., 2008; Wei et al., 2009). By analogy, Klf4 may directly interact with Sox2 in *Xenopus* embryos to promote neuroectoderm formation.

Klf4 enhances the activity of the Nodal pathway. The effect is reflected by the fact that Klf4 activates transcription Nodal ligand genes and target genes, and enhances promoter/reporter activity. This provides at least in part a mechanism for how Klf4 promotes endoderm differentiation. Luciferase assay with ARELuc in cells and embryos suggests that Klf4 might regulate the Nodal pathway indirectly, possibly via a Nodal feedback loop. Blocking the activity of Nodal receptor leads to a decrease in *Klf4*-induced *Mix2* expression and promoter activity. Therefore, the feedback loop plays a role in *Klf4*-regulated Nodal activity. Meanwhile, *Mix2* expression and promoter activity in response to CHX or/and SB431542 treatment also imply that *Klf4* regulates Nodal target genes directly. Hence, both direct and indirect effects play a role in the regulation of Nodal/Activin by *Klf4*. This is consistent with the idea that *Klf4* still induces ectopic endoderm gene expression, but the endogenous endoderm genes are repressed when Nodal receptor is blocked. As endoderm genes, e.g. *Sox17α* and *Mixer*, also inhibit mesoderm gene expression, it is plausible that endoderm differentiation dominates over mesoderm in both animal caps and whole embryos with overexpression of *Klf4*.

Klf4 is involved in body axis patterning

Overexpression of *Klf4* caused an anteriorized phenotype resembling the effect of the simultaneous blocking of BMP, Wnt and Nodal

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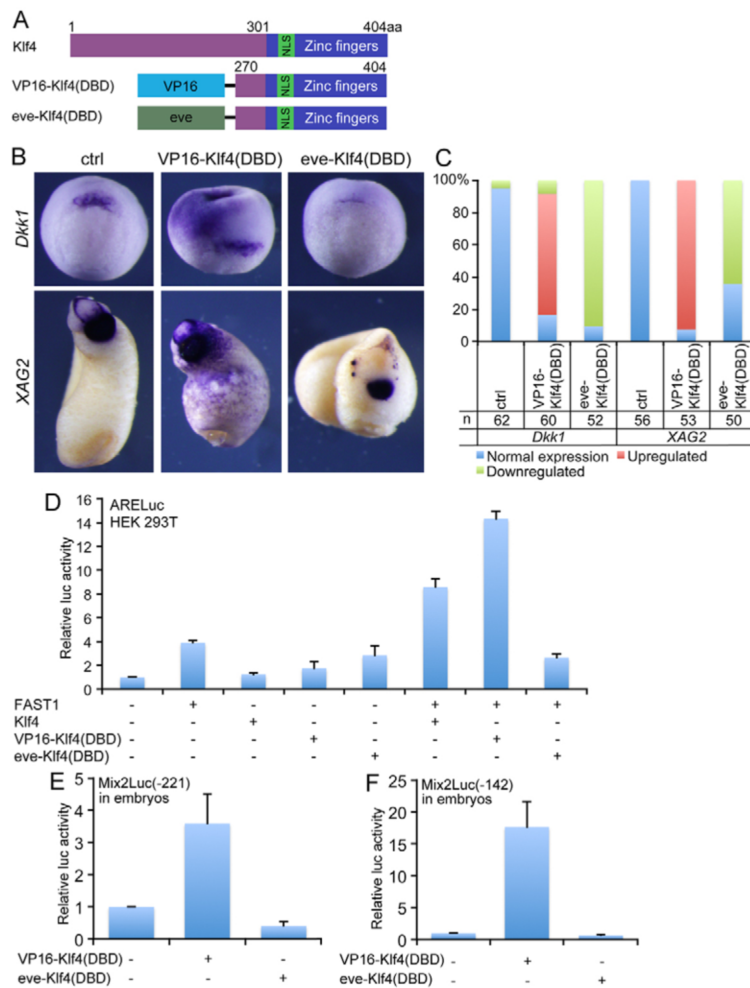

Fig. 9. Assays on the transcriptional activity of Klf4. (**A**) Construction of fusion constructs of Klf4. (**B**) The effect of the injection of 150 pg of *VP16-Klf4*(*DBD*) or *eve-Klf4*(*DBD*) mRNA on gene expression. Embryos at stage 10.5 were used for detection of *Dkk1* and embryos at stage 26 were used for detection of *XAG2*. In the panels for *Dkk1* expression, the middle one was lateral view and the other two were vegetal view. In the panels for *XAG2* expression, the first two from the left are ventral view and the third is anterior view. (**C**) Quantification of embryos with normal or altered gene expression observed in B in triplicate. (**D**) The effect of fusion constructs on ARELuc using luciferase assays in HEK 293T cells. (**E**,**F**) The effect of fusion constructs on *Mix2* promoter/luciferase reporters in embryos. *VP16-Klf4*(*DBD*) or *eve-Klf4*(*DBD*) mRNA (150 pg), and 40 pg of each reporter plasmid were injected. Embryos were collected at stage 10 for luciferase activity measurement. Error bars represent s.d. in eight (D) or six (E,F) experiments.

(Piccolo et al., 1999). The effect can be explained by the idea that *Klf4* is sufficient and necessary for the activation of a subset of organizer genes, e.g. *Dkk1*, *Cerberus* and *Noggin*, which code for antagonists for BMP4, Xnrs and Wnt8. However, *Klf4* is not able to induce a complete secondary body axis. This is probably due to the fact that Klf4 induces some organizer genes but at the same time represses others; hence it is insufficient to drive the formation of a complete secondary axis. This can be supported by the idea that the anteriorized phenotype grows much stronger in embryos with *Klf4* overexpression and Nodal, Wnt or BMP inhibition.

The mechanism of differential regulatory effects of *Klf4* on organizer genes remains to be elucidated. Previous studies demonstrated that *Klf4* can function as both a transcriptional activator and a repressor, depending on the transcriptional corepressors or coactivators it recruits (Ai et al., 2007; Evans et al., 2007; Evans et al., 2010). Thus *Klf4* might regulate different organizer genes in cooperation with different transcriptional corepressors or coactivators. In addition, organizer genes and Nodal, BMP or Wnt pathways regulate each other and consist of a regulatory network for axis patterning. Disturbance of one or more signals in the network by Klf4 will inevitably lead to changes in other signals. According to the present knowledge, there are more than a dozen of genes expressed in the organizer. It will be interesting to investigate thoroughly the differential regulatory effects of *Klf4* on these genes, which will give us more insights into the molecular mechanisms that control body axis patterning.

Klf4 functions as a competence factor

Loss of Klf4 function results in failure of the differentiation of germ layers, suggesting that *Klf4* is required for the initiation of a differentiation program. This is because, in the absence of Klf4, Nodal/Activin activity is impaired. Therefore, target gene expression and mesendoderm differentiation is inhibited. Blocking both Klf4 and Nodal/Activin activity results in more severe inhibition. By contrast, Klf4 activates Nodal/Activin target gene expression and promoter activity. Thus, a synergistic effect exists between Klf4 and Nodal/Activin to induce target gene transcription. This dual regulation might exemplify a model for the correlation between Klf4 and other signaling, especially the Wnt pathway. *Dkk1* is a known Wnt target, and Klf4 is also required for *Dkk1* transcription: likewise Wnt and Klf4 might cooperate to regulate *Dkk1*. The model remains an intriguing topic and the detailed mechanisms need further investigation. In summary, we propose that Klf4 serves as a competence factor and enables early embryonic cells to be responsive to inducing signals for germ-layer differentiation and body axis patterning.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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