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., 1993; Schmidt et al., 1995; Dale and Wardle, 1999) and are principally responsible for ventro-posteriorization of germ layers (Maeno 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 mesoderm 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).

Differentialiation 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; Zabers 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.
containing 100 μM SB431542 (Sigma) from the four-cell stage until
gastrulation. To block protein translation, un.injected 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+Klf4(DBD). For the test of efficiency of the
expression plasmid of 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
binding site was ligated to pCS2+eGFPmcs and pCS2+6MTmcs vectors to
make pCS2+Klf4-eGFP and pCS2+Klf4-MT, respectively. The repression
activation form of Klf4 were made by ligating Klf4 DNA binding
domain to pCS2+evcems 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 morpholin 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,
Sox17a, 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+Klf4(DBD), pCS2+VP16-Klf4(DBD), pCS2+eve+Klf4(DBD),
pCS2+VP16-Klf4(DBD), pCS2+6MTmcs vectors to
make pCS2+Klf4-eGFP and pCS2+Klf4-MT, respectively. The repression
activation form of Klf4 were made by ligating Klf4 DNA binding
domain to pCS2+evcems 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.

**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 in zebrafish and mouse (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

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× Laemmli 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.
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 mRNA 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 Sox10, 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. 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) 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.
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 (ctrlMO) and the standard control MO (ctlMO) 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, Sox2. However, they were inhibited in 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.

**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.
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
Klf4 is required for germ layers

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. Embryos were injected with or without dnXAR1 and Klf4 mRNA (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.
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 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 (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). 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 towards the ventral side, probably owing to a lack of tissue differentiation (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.
Overexpression of 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 Chordin 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 Xnr2 mRNA (Fig. 8A,B) at midblastula, which is required for induction of the organizer precursor (Nieuwkoop centre) and organizer gene transcription. Organizer genes such as Chordin, 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-terminal 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 of Xenopus Klf4, similar to the wild-type 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.
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
neuralize ectoderm, the promotion of neuroectoderm by embryos to promote neuroectoderm formation. By analogy, Klf4 may directly interact with Sox2 in transcription of each other (Orkin et al., 2008; Wei et al., 2009). Ubiquitous transcription of Klf4 seems to be cell-autonomous. In dissociated animal caps, Klf4 promotes neuroectoderm and endoderm; 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 neutralize ectoderm, the promotion of neuroectoderm by Klf4 seems to be cell-autonomous. In dissociated animal caps with Klf4 knockdown, neutralization 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 signals.
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.

**Acknowledgements**

We thank Drs W. Wu, Y. L. Chen, M. Taira and D. Melton for gifts of plasmids.

**Funding**

This work was supported by the Ministry of Science and Technology (2011CB943804) and the National Science Foundation of China (30971649) to Y.C.
Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/doi/10.1242/dev.082024/-/DC1

References


Stem Cells 26, 1931-1938.


Stem Cells 26, 1931-1938.


Stem Cells 26, 1931-1938.


Stem Cells 26, 1931-1938.


Stem Cells 26, 1931-1938.


Stem Cells 26, 1931-1938.
Fig. S1. Identification of *Xenopus laevis* Klf4. (A) Multi-alignment of sequences of *X. laevis* Klf4 (XlKlf4, present study), *X. tropicalis* Klf4 (XtKlf4, Acc. No.: NP_001017280), zebrafish Klf4 (zKlf4, Acc. No.: NP_001106955) and mouse Klf4 (mKlf4, Acc. No.: NP_034767). Identical residues are marked by asterisks underlined. Gaps are introduced to achieve optimum alignment. (B) Percentages of identity between Klf4 proteins. (C) Genomic organization comparison using Ensembl genome browser (http://asia.ensembl.org).
Fig. S2. Expression patterns of Klf4 during embryonic development in Xenopus laevis. (A-G) Spatial expression pattern of Klf4 during embryogenesis. (A,B) Klf4 transcript is obvious in the animal pole of early cleavage embryos. (C) Animal view of a midblastula embryo; transcript is widely distributed in animal region with slight enrichment at one side, which is putatively the dorsal side. (D,E) Vegetal view and lateral view of a gastrula embryo, signal in dorsal marginal zone is stronger. (F) Longitudinal bisection of an embryo at stage 10.5. Outlined is the signal in dorsal marginal zone. (G,H) Dorsal and ventral views of a neurula embryo; Klf4 is present as two narrow lines in the neural plates at dorsal side and localized to cement gland in the anterior. (I) In early tailbud stage, transcription is only localized to cement gland. (J) At stage 34, Klf4 is transcribed in cement gland, the future trigeminal nerve and lung primordium. (K) In tadpoles, Klf4 is localized to the prospective duodenum/stomach. bl, blastopore lip; nf, neural fold; cg, cement gland; tg, trigeminal nerve; lp, lung primordium; dd, duodenum/stomach. (L) Temporal expression as detected by qPCR. Quantification was normalized to the expression level in egg. (M,N) Relative distribution of Klf4 transcripts in eight-cell (M) and midblastula (stage 8.5) (N) embryos. The four animal and vegetal blastomeres of eight-cell embryos, the animal, equatorial and vegetal parts of midblastula embryos were manually separated. Klf4 transcript was detected with qPCR and quantification of expression level in each part was normalized to the respective expression level in whole embryos. Error bars in L-N represent s.d. in three measurements.
Fig. S3. Effect of overexpression of Klfl4 and inhibition of BMP, FGF or Wnt pathway on embryonic development. (A) Detection of \textit{XAG2} expression in embryos injected with Klfl4, tBR, dnFGFR or dnTCF3 mRNAs individually or together, as indicated in each panel. tBR is truncated BMP receptor that blocks BMP pathway, dnFGFR is dominant-negative FGF receptor blocking FGF pathway, and dnTCF3 is dominant-negative TCF3 that inhibits Wnt/\beta
d-catenin pathway. Control or injected embryos were collected at tailbud stage for whole-mount in situ hybridization. 300 pg Klfl4, 1 ng tBR, 1 ng dnFGFR or 300 pg dnTCF3 mRNA was injected. (B) Quantification of embryos with normal or altered gene expression in A in three experiments.
Fig. S4. Effect of blocking of Nodal/Activin signaling on Klf4 induced *Mixer* expression. (A) *Mixer* expression in embryos treated with chemical or/and injected with *Klf4* mRNA, as indicated atop each panel. 300 pg of *Klf4* mRNA was injected into each embryo. (B) Quantification of embryos with normal or changed gene expression in A in three experiments.
Fig. S5. Premature activation of genes by Klf4. (A) Control (ctrl) and injected (Klf4) embryos were collected at midblastula (stage 8.5) and assayed for Dkk1, Cerberus and Noggin expression using whole-mount in situ hybridization. Embryos are all in lateral view. 300 pg of Klf4 mRNA was injected into all blastomeres of four-cell embryos. (B) Quantification of embryos with normal or changed gene expression in A in three experiments.

Fig. S6. Activation of gene expression in the absence of protein translation. (A) Dkk1 and Noggin expression in embryos treated with chemical or/and injected with Klf4 mRNA, as indicated atop each panel. 300 pg of Klf4 mRNA was injected into each embryo. Embryos were collected at stage 10.5 for whole-mount in situ hybridization. (B) Quantification of embryos with normal or changed gene expression in A in three experiments.
Fig. S7. Contribution of the N- and C-terminal regions to the activity of Klf4. (A) Domain structure and construction of two deletion mutants. (B,C) Effect of the two deletion mutants on embryogenesis (B) and their quantification (C) in three experiments. 500 pg Klf4ΔZF or Klf4(DBD) mRNA was injected into all blastomeres at the four-cell stage. Phenotypes were documented when control embryos reached at stage 34.
<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Sequences (5’→3’)</th>
<th>References*</th>
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| α-actin      | F: ACGGTCAGGTCATCACCATTTG  
R: ATACCGGCAGATTCCATACCAA |            |
| α-globin     | F: GCTGCAAGACACATCAATGA  
R: CCAGGATCCACTCTCAGGTTGTA |            |
| Cbx4         | F: AAAGACAAGAGCAGATCATGGGATAAC  
R: CGAGCAAACGATGCGATAGAA |            |
| Cerberus     | F: AAGAGGAGCAGCTAGGAGCAAG  
R: GCCAAAATACCCATGCCC | Cao et al., 2006 |
| Chordin      | F: AACTGCCAGGACTGGATGGT  
R: GGCAGGATTAGATGGCTTC | Cao et al., 2006 |
| Dkk1         | F: ATGCTGATCATGCAAAATGGAT  
R: TTAAAGGGCTGCATGCTAGTG |            |
| FoxA2        | F: GGGAGGCAAGAAACTTCTAGAAG  
R: TGAGCTTTTCAGAGCTGTGTT |            |
| FoxA4a       | F: GTTCCATGCCTCAAGGAATG  
R: GCAATAGCCCAAATGGATAAGCA |            |
| GATA4        | F: GTGTGGCCCTTACATGAAGCTACA  
R: TGCCTGTGGATCCCTCCTCCTC |            |
| GATA5        | F: CCACTTTATCAAGGCCACAGAAAAGAC  
R: GTGTGACAGTTGCGAGCAAAG |            |
| GATA6        | F: TGACCCECCAGCTAAACATCTG  
R: TTGTGCTCTCTCGACTTGTC |            |
| Gsc          | F: GATGCCGCCAGTGCCGCTC  
R: TGCAGCTCAGGTCGACAAA | Cao et al., 2006 |
| Keratin      | F: CATTGTGAGGAGGTGTGTTGA  
R: TTTGTGACATTATTTTCGACATTAGT |            |
| Klf4         | F: GAGAGGTTGGAGGAGATCCA |            |
KMT5C
F: AGCCGAATTGTGTGAACACTTGT
R: GGATTTGCGGCTCATAGAAAGAG

Mix1
F: CCAATAGGAGAGAAGTCACCAAGATC
R: TAATTGCGTCTTTGGGTCTGACA

Mix2
F: GAGGAGCTAGCGAGGCACATTTA
R: CGTCTGACCTTTGCTCTTCTGTTC

Mixer
F: GCCCCTAGGATGGACACGTT
R: CTGGACCTGGCAGAGAGGA

Nanos
F: AGGCCAAGATGGGACGTTTTA
R: CTTTGTGTCACCCACAGACTCATTACTG

NCAM
F: AGCATTGGACATGAGTCTTCTGAGT
R: AGAATATGGTTCCTATTTACGAATG

Oct60
F: TGTGTAACCTCAAACCCCTATGGAA
R: TCTGGGCTTTGTGGATCATC

Oct25
F: CCCAGCCTTCGCTTTCAAC
R: AACCCGTGTAACCCCCAAA

Oct91
F: GCAGATAATTCCTCAGGTCAGAAGA
R: TCTCCAAGGGTACCTTCACGTT

ODC
F: CAAAGCCTGTTCTACGCATAGCA
R: GGTGGCACCACCATTTCTACTCT

Sox17α
F: GCCGCAGATGGAAAGGATGT
R: ACCTGGCCGTGGAGTAAG

Sox2
F: CTGCCTCAACAAAAACGAAATTA
R: CCCCTGAGGCATTACCACAG

Sox3
F: GGGTTATGGTTTGGTCCCGGG
R: AGCGCCCAATCTTTTGCTGC

SoxD
F: AGTACAGCGGATACCAGGCT
R: AAAGCCATAAAGCGCAGACAC

Wnt8
F: GATGTGATGACTCCAGAAATGGC
R: CGAGATCCGCTCACCAAATT  
Cao et al., 2006

Xbra  
F: AGCTCACCAACGAGATGATCG

R: AACCGTATACATTGCATTGGGAT  
Cao et al., 2006

XEMA  
F: AAAGCTTCTTACATCCCCAGACAA

R: AGGGATTTTCAGTGTTGCTG

Xhex  
F: GGAGACCCACAGGGAAATAAAGAA

R: CGCTCAAACACCTCTCTTGACT

XmenF  
F: CAACTAAACCACGGATACGGGATAT

R: TGCATTCACCAATCTTTTCAA

Xpat  
F: GCATGGAGGGTACAAATCATTTTC

R: GCACCTGCAACAATTTTCCTCATC

XPTB  
F: GTGTGGGAATTGGATGATGGA

R: ATATCGAGGACTTGAGGTGTGTTC

Xvent1  
F: TGAAGAGGCAGATACAAGACAAGC

R: GGACAGGAAAGCCACCAGG

Xvent2  
F: CAGAACCGAAGGATGAAATACAA

R: AACTGGGCTGGGTGGTATGAG

*All primers without a reference were newly designed in the present study.