cipitated beads were resuspended in 23 μl lysis buffer and aliquoted as indicated.

Histone deacetylase assays. Assays were done as described[4]; where indicated, ATP was added to 2.5 mM in the presence of 5 mM $MgCl_2$, and trapoxin was added to 200 nM.

8-azido-adenosine-γ-^{32}P-ATP crosslinking. NRD antibody-immobilized bead complexes were incubated with 2 μCi 8-azido-adenosine-5′-γ-^{32}P-triphosphate (ICN) for 3 min at room temperature in 50 μl buffer containing 20 mM HEPES, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 1 μm $ZnSO_4$, 0.1% Tween-20. The reaction mixture was irradiated for 5 min with 254-nm UV light (1,120 μW cm^{-2} from 3 cm away). Beads were washed three times with 1 ml 20 mM HEPES, pH 7.6, 100 mM KCl, 0.1% Tween-20. Bound proteins were resolved by SDS–PAGE and imaged using a phosphorimager.

Nucleosome-disruption assays. the 155-bp pTPT *Mlu*I/*Eco*RI fragment was prepared and footprinted as described[13]. 0.2, 1.0 and 5.0 μl HDAC/CHD immunoprecipitates, 1.0 and 5.0 μl peptide-blocked HDAC/CHD immunoprecipitates, and 0.04, 0.16, 0.6 and 2.4 μl of hSWI/SNF were tested for disruptive activity. 2 mM ATP/$MgCl_2$ was added where indicated; after 45 min, reactions were treated with 0.12 U DNase I (0.012 U for bare DNA).

Plasmid chromatin reconstitution. HeLa cells were radiolabelled as described previously[4] and hyperacetylated histones were purified by hydroxyapatite chromatography as described[14]. 16 μg of a 9-kb plasmid was linearized, purified, and mixed with an equal mass of radiolabelled, hyperacetylated core histones in 50 μl of 15 mM HEPES, pH 7.5, 1 M NaCl, 0.2 mM EDTA and 0.2 mM PMSF. Stepwise dilution of salt was done by addition of the same buffer without NaCl over 3 h at 10-min intervals until the final concentration of NaCl was 100 mM. Non-nucleosomal histones were then separated by repeat Centricon-500 (Amicon) filtration. Nucleosomal structure was confirmed by micrococcal nuclease digestion of the product.

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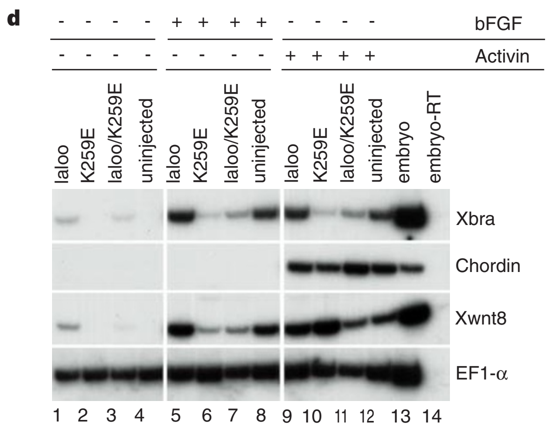
erratum

FGF-mediated mesoderm induction involves the Src-family kinase Laloo

Daniel C. Weinstein, Jennifer Marden, Francesca Carnevali & Ali Hemmati-Brivanlou

Nature **394,** 904–908 (1998)

In Fig. 4d of this Letter, it was wrongly indicated that activin was added to samples 5–8. Activin was not added to these samples but to samples 9–12 as shown here.



described[28]. IL-2 production was measured by enzyme-linked immunosorbent assay (ELISA) with the monoclonal antibodies JES6-1A12 and JES6-5H4 (Pharmingen). Purified naive splenic CD4 cells were differentiated into Th1 cells as described[29] or with PMA plus A23187. Briefly, 10^6 CD4 cells were stimulated with 5 ng ml^{-1} PMA (Calbiochem), 100 ng ml^{-1} A23187 (Calbiochem) and 1.5 ng ml^{-1} IL-12 (R&D, Wiesbaden). After 4 days of culture, cells were restimulated with PMA plus A23187 and analysed for intracellular cytokine expression[29].

Western blot analysis and *in vitro* kinase assays. Cells were lysed in 1% NP-40 lysis buffer (10 mM Tris/HCl, pH 7.8, 150 mM NaCl, 4 mM EDTA, 10% glycerol, 2 mM Na_3VO_4, 100 mM NaF, 2 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, 1 mM PMSF). Lysates were resolved by 10% SDS–PAGE and transferred to PVDF membrane (Immobilon-P, Millipore). Membranes were incubated with anti-Csk (Santa Cruz), anti-α-tubulin (DM1A, Sigma), anti-Lck (CT, UBI, Lake Placid) or anti-Fyn (FYN3, Santa Cruz) antibodies and proteins were detected by chemiluminescence (SuperSignal, Pierce). For *in vitro* kinase assays, NP-40 lysates of 5×10^7 thymocytes were precipitated with anti-Lck (3A5, UBI) or anti-Fyn (FYN15, Santa Cruz) antibodies and protein-A sepharose. Immune complexes were incubated in kinase buffer (10 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$) with 15 μCi[γ-^{32}P]ATP for 2 min at 37 °C and resolved by 10% SDS–PAGE. The conditions of the kinase assay were tested to fall within the linear range of the kinase reaction. Autophosphorylation of Lck and Fyn was quantified by phosphorimage analysis of dried gels with Fuji Bas1000.

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FGF-mediated mesoderm induction involves the Src-family kinase Laloo

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During embryogenesis, inductive interactions underlie the development of much of the body plan. In *Xenopus laevis*, factors secreted from the vegetal pole induce mesoderm in the adjacent marginal zone; members of both the transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF) ligand families seem to have critical roles in this process[1]. Here we report the identification and characterization of *laloo*, a novel participant in the signal transduction cascade linking extracellular, mesoderm-inducing signals to the nucleus, where alteration of cell fate is

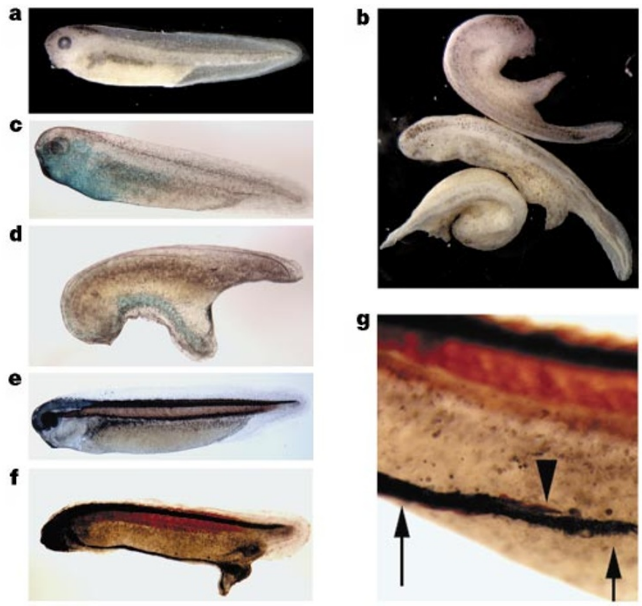

Figure 1 Effects of injection of *laloo* into embryos. Embryos were injected with 100 pg β-galactosidase RNA (**c**, **d**) and/or 750 pg (**b**, **d**, **f**, **g**) 27A1JA (*laloo*) RNA. All embryos are stage 33–35. **a**, Uninjected embryo. **b**, Dorsal view of embryos injected with 27AIJA. **c**, Embryo injected with β-galactosidase (β-Gal) RNA and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as a substrate. **d**, Embryo co-injected with 27AIJA and β-Gal RNA, and stained with X-gal as a substrate. **e–g**, Lateral views of embryos probed with neural-specific (blue stain) (6F11 (ref. 27)) and somite-specific (red stain) (12/101 (ref. 28)) antibodies. **e**, Uninjected embryo. **f**, **g**, Embryo injected with 27AIJA RNA. Panel **g** is a detail of the trunk of embryo in **f**; arrows indicate ectopic neural tissue, arrowhead indicates ectopic mesoderm. Embryos in **c–g** were cleared in 2:1 benzyl benzoate/benzyl alcohol.

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driven by changes in gene expression. Overexpression of *laloo*, a member of the Src-related gene family, in *Xenopus* embryos gives rise to ectopic posterior structures that frequently contain axial tissue. Laloo induces mesoderm in *Xenopus* ectodermal explants; this induction is blocked by reagents that disrupt the FGF signalling pathway. Conversely, expression of a dominant-inhibitory Laloo mutant blocks mesoderm induction by FGF and causes severe posterior truncations *in vivo*. This work provides the first evidence that a Src-related kinase is involved in vertebrate mesoderm induction.

In an attempt to isolate factors involved in patterning of the body axis, we constructed and screened a *Xenopus* gastrula expression library. Early cleavage stage embryos were injected in the animal pole with RNA from fractionated library pools. One clone, 27AIJA, generated ectopic structures that resemble tails in over 90% of injected embryos ($n = 102$) (Fig. 1a, b). In addition, a reduction of anterior structures, including eyes, was often observed (Fig. 1, and not shown). Co-injection of 27AIJA and β-Gal RNA revealed that injected cells contribute directly to ectopic structures (Fig. 1c, d). Whole-mount immunohistochemistry demonstrated that these ectopic structures often contain neural tissue (17 out of 32 embryos) and paraxial mesoderm (5 out of 32 embryos) (Fig. 1e–g). Thus, overexpression of 27AIJA generates ectopic, posterior structures that frequently contain axial and paraxial tissue.

The 27AIJA cDNA contains an open reading frame of 496 amino acids (Fig. 2a); this sequence shows homology with the Src family of intracellular tyrosine kinases[2], and includes putative SH3, SH2 and kinase domains (Fig. 2b). Several lines of evidence indicate that 27AIJA encodes a novel member of this gene family. First, the unique, amino-terminal domain of 27AIJA resembles that of two amniote family members, Lyn and Hck, almost equally (26% and 31% similarity, respectively)[2]. Second, the complete coding sequence of 27AIJA is more than twice as divergent from its closest amniote relative (Hck, 38–40%) than are the sequences of other cloned *Xenopus* Src-family genes (*Xsrc*, *Xyes*, *Xfyn*, *Xlyn*) from their amniote homologues (3–18% (ref. 2; Xlyn sequence ID no. 2114076)). Finally, 27AIJA is less closely related to amniote Hck (38–40% divergence) than is the putative *Xenopus* homologue of the related Lyn gene (31–34% divergence). Thus, 27AIJA encodes a novel factor that can induce posterior, ectopic axes; we have named this gene *laloo*, after a nineteenth-century circus performer who had a small, headless twin protruding from his breastbone.

To better define the embryonic function of *laloo*, we tested its activity in ectodermal explant (animal cap) assays. At midgastrula stages, reverse transcription in conjunction with polymerase chain reaction (RT-PCR) revealed that *laloo*-injected caps express both *Xbra*, a pan-mesodermal marker, and *Xwnt8*, a marker of ventrolateral mesoderm[3,4], but not *chordin*, a dorsal mesodermal marker[5] (Fig. 3a, lanes 1–4). At late neurula stages, *laloo*-expressing caps show strong expression of *HoxB9* (*XlHbox6*), which at this stage is

a

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ATGGGCTGCATCAAGTCAAAGGATTCAAATACGACTGGCAAAAGTCTGGGACCTCCGGAAAGCACCCAAACCCATTATGTGAAGGACCCA
 M  G  C  I  K  S  K  D  S  N  T  T  G  K  S  L  G  P  P  E  S  T  Q  T  H  Y  V  K  D  P     30
ACATCTACAGTAACTATGACTAAACCTGAAAGATCATCTAAGCACCCCAGAGAGGAAGGGCAAGAAGAAGTGGTCCTGCTGGCTTTGTAT
 T  S  T  V  T  M  T  K  P  E  R  S  S  K  H  P  R  E  E  G  Q  E  E  V  V  L  L  A  L  Y     60
GACTATGATGGAGTCCACCCTGGGGATCTGACTTTTAGGAAAGGGGACCATCTCCTGCTAAAGAAAGAGTCAGGGGAGTGGTGGGAAGCA
 D  Y  D  G  V  H  P  G  D  L  T  F  R  K  G  D  H  L  L  L  K  K  E  S  G  E  W  W  E  A     90
TGTCTAATTTCCACTGGTGAAGAAGGCTTTGTTCCCAGTAACTATGTAGCGTATTTCAATTCCCTGGAATCTGAAGAGTGGTACTTTAAA
 C  L  I  S  T  G  E  E  G  F  V  P  S  N  Y  V  A  Y  F  N  S  L  E  S  E  E  W  Y  F  K    120
GGCATGAGCCGGAAGGAAGCTGAAAGGCAGCTGCTATCTCCTGTTAATAAAAGTGGGGCTTTCATGATCCGAGACAGTGAGACAATGAAA
 G  M  S  R  K  E  A  E  R  Q  L  L  S  P  V  N  K  S  G  A  F  M  I  R  D  S  E  T  M  K    150
GGTTGTTTCTCCCTCTCTGTGCGAGACTCAGGGGACACTGTGAAACATTACAAAATTCGCACACTCGATGATGGAGGTTTCTTCATTTCT
 G  C  F  S  L  S  V  R  D  S  G  D  T  V  K  H  Y  K  I  R  T  L  D  D  G  G  F  F  I  S    180
ACACGGATCCCTTTTCCTTCTTTGCCAGAGCTGGTACGCCATTATCAAGGTAAAGTGGATGGCTTGTGTCAGTGCCTTACAATACCATGC
 T  R  I  P  F  P  S  L  P  E  L  V  R  H  Y  Q  G  K  V  D  G  L  C  Q  C  L  T  I  P  C    210
CAAACTGTGCGTCCAGAGAAACCATGGGAAAAGGATGCCTGGGAGATCCCGCGCGAGTCACTGTCACTGCAGAAGAAGCTTGGAGCTGGA
 Q  T  V  R  P  E  K  P  W  E  K  D  A  W  E  I  P  R  E  S  L  S  L  Q  K  K  L  G  A  G    240
CAGTTTGGGGATGTTTGGTTGGCCATGTACAATGGACACACAAAAGTAGCTGTAAAAACAATGAAGCCAGGCAGCATGTCCCCCGGTGCC
 Q  F  G  D  V  W  L  A  M  Y  N  G  H  T  K  V  A  V  K  T  M  K  P  G  S  M  S  P  G  A    270
TTCCTTGAAGAGGCAAATCTGATGAAGAGCTTGCAGCATGACCGGCTGGTGCGGTTGCATGCCGTTGTGACTCAGGGGGAACCAATATAT
 F  L  E  E  A  N  L  M  K  S  L  Q  H  D  R  L  V  R  L  H  A  V  V  T  Q  G  E  P  I  Y    300
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 I  I  T  E  Y  M  Q  K  G  S  L  L  D  F  L  K  S  E  E  G  S  D  Q  P  L  I  Q  L  I  D    330
TTCTCTGCCCAGATTGCAGAAGGAATGTGGTTTATTGAGCAAAGGAATTATATTCACCGTGATCTGAGGGCAGCAAACTGCCTGGTATCA
 F  S  A  Q  I  A  E  G  M  W  F  I  E  Q  R  N  Y  I  H  R  D  L  R  A  A  N  C  L  V  S    360
GAAACTTTGTTGTGCAAAATAGCAGACTTTGGGCTGGCCCGAGTGATAGAGGACAGCGAGTATACTGCCAGGGAAGGTACCAAATTTCCC
 E  T  L  L  C  K  I  A  D  F  G  L  A  R  V  I  E  D  S  E  Y  T  A  R  E  G  T  K  F  P    390
ATCAAGTGGACATCCCTGGAGGCTGCCAATTATGGCTCTTTTACTATCAAGTCAGATGTATGGTCATTTGGTGTATTGCTAACTGAAATA
 I  K  W  T  S  L  E  A  A  N  Y  G  S  F  T  I  K  S  D  V  W  S  F  G  V  L  L  T  E  I    420
ATAACATATGGGAGGACTCCATATCCAGGTATGTCCAACTCGGAGGTAATTACAGCCCTTGAGCGTGGTTATCGCATGCCGTGTCCCAGC
 I  T  Y  G  R  T  P  Y  P  G  M  S  N  S  E  V  I  T  A  L  E  R  G  Y  R  M  P  C  P  S    450
ACTTGTCCAAAAGAGCTCTACAGCATCATGCTCCAGTGTTGGCAGCAGGACCCTGAGCAACGGCCAACGTTTGAATATTTACAGAGCATC
 T  C  P  K  E  L  Y  S  I  M  L  Q  C  W  Q  Q  D  P  E  Q  R  P  T  F  E  Y  L  Q  S  I    480
CTAGAGGACTTCTTTACTGCCACTGAAACACAGTACCAGGCACAACCTTAA
 L  E  D  F  F  T  A  T  E  T  Q  Y  Q  A  Q  P  .                                           496
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b

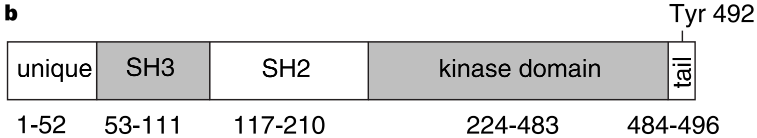

Figure 2 *laloo* is a novel Src-related gene. **a**, Nucleotide sequence and predicted open reading frame of *laloo*. Residues mutated in this study (K259, Y492) are boxed. **b**, Schematic of the Laloo protein tyrosine kinase. The *Xenopus* Laloo protein is drawn approximately to scale, showing the positions of domains conserved throughout the Src gene family.

expressed in both lateral mesoderm and the spinal cord[6] (Fig. 3b, lanes 1–4). Because NCAM, a pan-neural marker[7], is not induced, we conclude that the *HoxB9* expression induced by Laloo at this stage represents mesodermal tissue. At high doses, Laloo induces the expression of *muscle actin*, a marker of paraxial mesoderm[8] (Fig. 3b; lane 1); this result demonstrates that high levels of Laloo expression give rise to more dorsal fates than do lower doses. Figure 3c shows that Laloo also induces mesodermal markers in the context of the whole embryo at gastrula stages. Whereas control embryos express *Xbra* in a ring at the marginal zone at midgastrula stages, animal pole injections of *laloo* RNA give rise to ectopic *Xbra* expression in the animal pole (39%, $n = 18$; Fig. 3c). These data indicate that overexpression of Laloo induces mesoderm, both in the animal cap assay and *in vivo*.

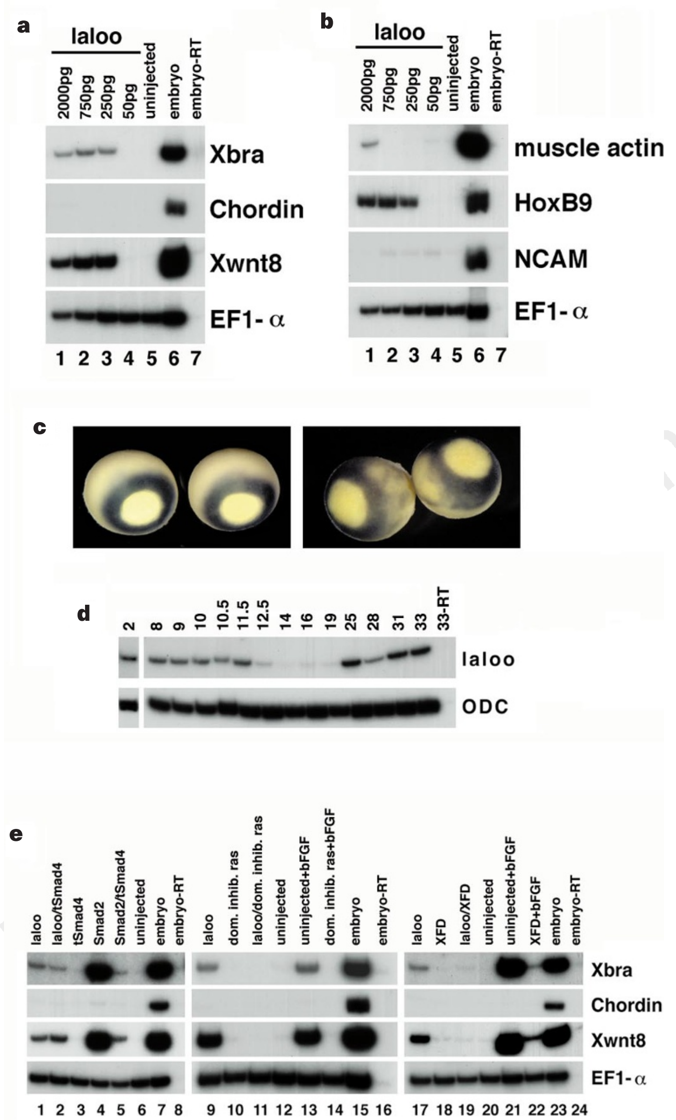

Figure 3 Ectopic Laloo induces mesoderm. RT–PCR analysis of animal caps dissected at late blastula stages and cultured until the stages listed. EF1-α is used as a loading control[29]. The –RT lane contains all reagents except reverse transcriptase, and is used as a negative control. **a**, Analysis of animal caps cultured until midgastrula stages. Doses of *laloo* RNA over 2 ng were lethal. **b**, Analysis of animal caps cultured until late neurula stages. **c**, Whole-mount *in situ* hybridization of midgastrula stage embryos using an antisense *Xbra* probe. The embryos in the right panel were injected with 800 pg *laloo* RNA in the animal pole. **d**, Timing of *laloo* expression during early development. Ornithine decarboxylase (ODC) is used as a loading control[30]. **e**, Induction of mesoderm by Laloo is unaffected by inhibition of Smad1 and Smad2, but is blocked by inhibition of the FGF signalling pathway. Animal caps were cultured until midgastrula stages. 750 pg each of *laloo*, *Smad2* and *tSmad4* RNA, 1.0 ng dominant-inhibitory *Ras* RNA and 1.5 ng *XFD* RNA were injected, as listed. Basic FGF (bFGF) was added to a final concentration of 25 ng ml^{-1}.

Mesoderm induction in *Xenopus* occurs between cleavage and early gastrula stages[1]; *laloo* is expressed throughout this period (Fig. 3d). *laloo* RNA is present maternally; this early expression is, however, greatly diminished by late gastrula stages. Zygotic *laloo* expression initiates after late neurula stages. Using a combination of whole-mount *in situ* hybridization and microdissection techniques, we observed no tissue-specific localization of *laloo* RNA through neurula stages (not shown). The ubiquitous, early expression of this gene is consistent with a role for *laloo* in endogenous mesoderm induction.

Members of both the TGF-β and FGF ligand families have been shown to be capable of inducing mesoderm[1]. We sought to determine whether mesoderm induction by Laloo is mediated through the signal transduction pathways downstream of these ligands. The intracellular Smad proteins transduce signals from activated TGF-β receptors; Smad4 has a central role in this process[9]. A truncated Smad4 molecule (tSmad4) has been shown to block mesoderm induction by both Smad1 and Smad2, signal transducers of the bone morphogenetic proteins and activin, respectively[9]. To test whether mesoderm induction by Laloo requires signalling through the Smad pathway, we co-expressed Laloo and tSmad4. As expected, co-injection of tSmad4 inhibits mesoderm induction by Smad2 (Fig. 3e, lanes 4 and 5). In contrast, tSmad4 does not block induction by Laloo (Fig. 3e, lanes 1 and 2). Thus, we conclude that the induction of mesoderm by Laloo acts downstream, or independently, of the Smad proteins.

The other intracellular pathway known to be involved in mesoderm induction acts through stimulation of the Ras/MAP kinase cascade, downstream of the FGF receptor[10–17]. To determine whether Laloo induces mesoderm independently of the FGF pathway, we first co-expressed Laloo and a dominant-inhibitory form of Ras[11]. Dominant-inhibitory Ras entirely blocks mesoderm induction by FGF (Fig. 3e, lanes 13 and 14), and also blocks induction by Laloo (Fig. 3e, lanes 9 and 11). This result indicates that mesoderm induction by Laloo requires signalling through the wild-type Ras protein. We then challenged Laloo activity with a truncated form of the FGF receptor (XFD), also shown to act as a dominant-inhibitory molecule[10]. We reasoned that, as a putative intracellular signalling molecule, Laloo might bypass an inhibition by XFD at the cell surface. XFD blocks *Xbra* and *Xwnt8* induction by FGF, as expected (Fig. 3e, lanes 21 and 22). Interestingly, XFD also blocks the induction of *Xbra* and *Xwnt8* by Laloo (Fig. 3e, lanes 17 and 19). These results indicate that Laloo is either part of the FGF pathway or in a parallel pathway that requires signalling through the FGF receptor and Ras.

All Src-family proteins contain a C-terminal tyrosine that, when phosphorylated, markedly inhibits the activity of the protein[2]. To examine whether similar regulation of Laloo might occur during early *Xenopus* development, we constructed a mutant form of Laloo in which we replaced the putative negative regulatory tyrosine residue with phenylalanine (Y492F). Y492F is indeed a more potent mesoderm inducer than wild-type Laloo. At midgastrula stages, 50 pg of Y492F RNA is sufficient to induce *Xbra* and *Xwnt8* (Fig. 4a, lanes 1–4), whereas 250 pg of *laloo* RNA is required to induce expression of these markers (Fig. 3a, lanes 1–4). At late neurula stages, 50 pg of Y492F RNA induces *HoxB9* expression, and 250 pg induces expression of *muscle actin* (Fig. 4b, lanes 1–4); 250 pg and 2 ng, respectively, of *laloo* RNA is required to induce *HoxB9* and *muscle actin* expression (Fig. 3b, lanes 1–4). Thus, the mesoderm-inducing activity of Laloo is modulated through a C-terminal tyrosine residue. As shown earlier, both dominant inhibitory Ras (Fig. 4c, lanes 1 and 3) and XFD (Fig. 4c, lanes 1 and 4) block mesoderm induction by wild-type Laloo. On the other hand, while mesoderm induction by Y492F is blocked by dominant-inhibitory Ras (Fig. 4c, lanes 2 and 5), it is largely unaffected by the co-expression of XFD (Fig. 4c, lanes 2 and 6). These results indicate that the requirement for FGF receptor activity is mediated through Tyr 492 of Laloo; furthermore, these data implicate Laloo as an integral component of the FGF signal transduction pathway, acting downstream of the FGF receptor and upstream of Ras.

In cell culture studies, kinase-defective mutants of Src-family members have been shown to act as dominant-negative molecules, blocking signalling through the wild-type kinase[2]. We constructed a kinase-defective Laloo mutant by disrupting the putative ATP phosphotransferase site[2]. This mutant, K259E, does not induce either *Xbra* or *Xwnt8* in mid-gastrula ectoderm explants (Fig. 4d, lane 2), demonstrating that mesoderm induction by Laloo requires a functional kinase domain. K259E expression inhibits mesoderm induction by Laloo at four-fold concentrations over wild-type (Fig. 4d, lanes 1 and 3); moreover, this mutant can inhibit mesoderm induction by both bFGF and activin in animal cap assays. Expression of K259E blocks induction of both *Xbra* and *Xwnt8* by bFGF (Fig. 4d, lanes 6 and 8), and blocks induction of *Xbra*, but not *Xwnt8* or *chordin*, by activin (Fig. 4d, lanes 10 and 12). Coexpression of Laloo moderately but consistently rescues mesoderm induction by FGF (Fig. 4d, lanes 6 and 7) and activin (Fig. 4d, lanes 10 and 11). This suggests that K259E acts by competition with wild-type Laloo. Rescue can also be achieved by coexpression of constitutively active Ras (not shown; ref. 11), supporting the placement of Laloo upstream of Ras in mesoderm induction. Marginal zone injections of K259E result in a reduction of trunk and tail structures *in vivo* similar to that seen after injection of other molecules that disrupt the FGF signal transduction pathway[10,12,14–16,18]; coexpression of either Laloo or constitutively active Ras partly rescues axis formation (Fig. 4e). These results indicate that Laloo, or a related factor inhibited by K259E, is required for mesoderm induction by FGF or activin and for normal development of the body axis.

Our results are consistent with a model placing Laloo as an essential intermediate in the FGF signalling pathway, downstream of the FGF receptor and upstream of Ras. The block of activin-induced *Xbra* expression by K259E suggests that Laloo might also mediate aspects of activin signalling; we believe, however, that this inhibition is indirect. Although FGF signalling is required for the full range of induction by activin, some activin-inducible genes are 'FGF-independent'; for example, *Xwnt8* is induced in XFD-expressing animal caps treated with activin[13,19]. We have demonstrated both that K259E fails to block the induction of *Xwnt8* by activin and that Laloo activity is unaffected by coexpression of tSmad4; thus, the inhibition of activin-induced *Xbra* expression by K259E is likely to be secondary to a block of FGF signalling.

Experiments with XFD and the hyperactive Laloo construct Y492F suggest that mesoderm induction by Laloo requires the FGF-mediated dephosphorylation of Y492. Interestingly, inhibition of SH2-containing protein tyrosine phosphatase 2 (SH-PTP2) blocks mesoderm induction by FGF[20]. Thus, *in vivo*, the activated FGF receptor might activate SH-PTP2, or a related phosphatase, which in turn could activate Laloo via dephosphorylation of Y492.

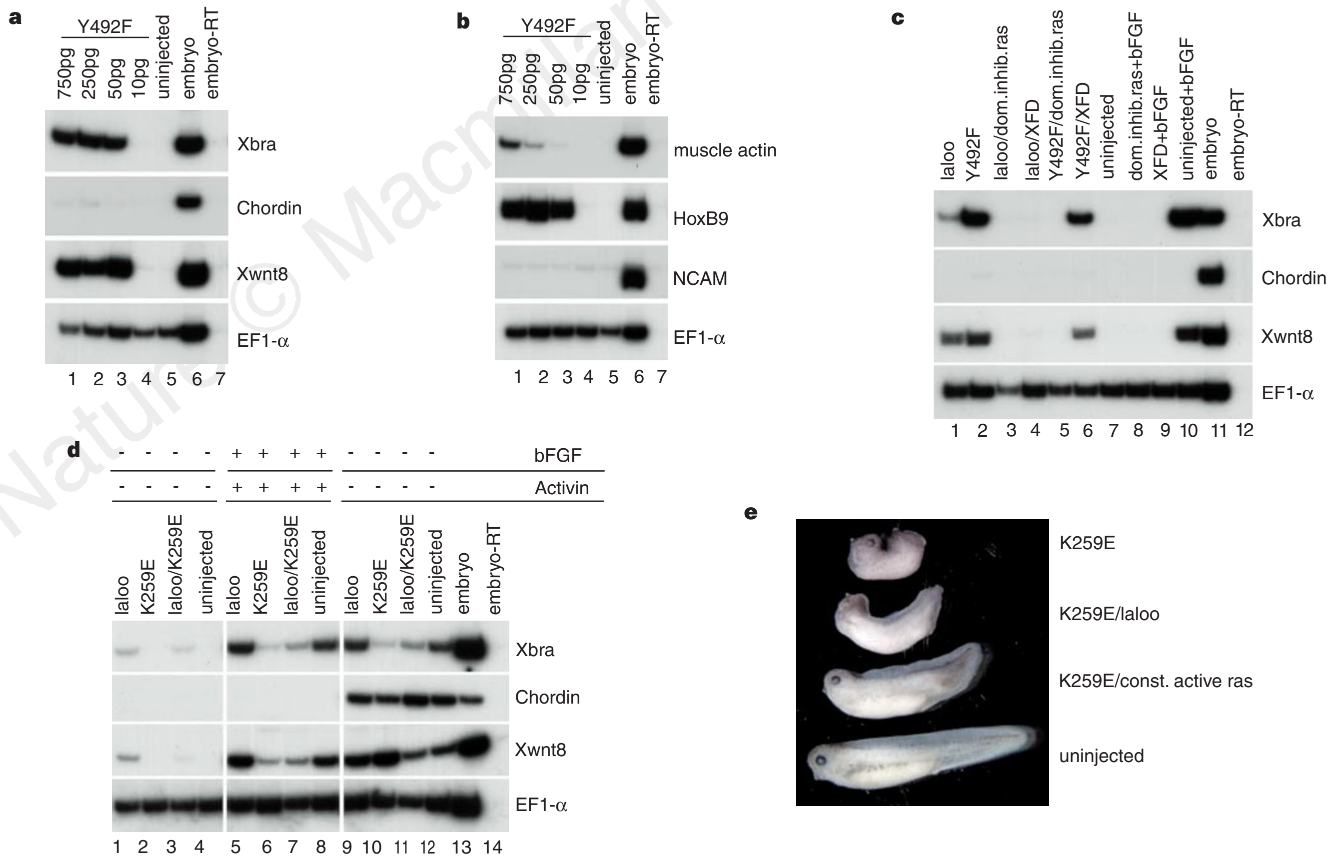

Figure 4 Y492F bypasses inhibition by XFD, and K259E inhibits the activity of mesoderm-inducing growth factors and normal development. Animal caps were cultured until midgastrula (**a**, **c**, **d**) or late neurula (**b**) stages. **a**, **b**, Y492F is a more potent mesodermal inducer than wild-type laloo. **c**, A hyperactive laloo mutant bypasses inhibition by the truncated FGF receptor. 750 pg laloo, 250 pg Y492F, 1.0 ng dominant-inhibitory Ras and 1.5 ng XFD were injected. **d**, K259E inhibits mesoderm induction by bFGF and activin. 800 pg laloo and 3.2 ng K259E RNA were injected. Inhibition by K259E required doses of 3.2 ng or more. **e**, K259E perturbs normal development of the body axis. Lateral view of stage 37 embryos; 2 ng K259E, 400 pg *laloo* and 40 pg activated *Ras* RNA were injected radially, as listed, at early cleavage stages. Top, embryo injected with K259E RNA. 81% of these embryos display a failure of blastopore closure and a complete loss of tail structures ($n = 78$). Second from top, embryo injected with both K259E and *laloo* RNA (56% with posterior truncations; $n = 70$). These embryos often show a reduction of anterior structures. Second from bottom, embryo injected with both K259E and constitutively active *Ras* RNA (33% with posterior truncations, $n = 64$). Bottom, uninjected control embryo.

Our results also suggest that the activity of ectopic Laloo is dependent upon a basal level of signalling through the FGF receptor. In support of this, it has been shown that FGF receptor-dependent MAP kinase activity is present throughout the early *Xenopus* embryo[21]. It has also been demonstrated that an autoregulatory loop involving FGF is required for mesoderm maintenance[18,22,23]; Y492F might thus generate ectopic mesoderm in the presence of XFD by bypassing the requirement for continued signalling through the FGF receptor.

The positioning of Src-related factors between the FGF receptor and Ras has precedents in other studies. Src-family kinases have previously been shown to transmit signals through Ras; the molecular interactions proposed to link these factors include phosphorylation of Shc and/or Ras GTPase-activating protein[2]. Functional association between Src-related proteins and receptor tyrosine kinases has also been demonstrated[24]. Our studies do not, however, address whether signalling upstream of Laloo is mediated solely through the FGF receptor; Laloo might also receive input from other cell-surface receptors. Furthermore, redundancy has been demonstrated among Src-family genes in other biological contexts[2]; mesoderm induction *in vivo* might thus involve additional Src-related factors. Although a constitutively active form of chicken Src has been reported not to induce mesoderm in *Xenopus* explants[13], this apparent difference might be due to cross-species incompatibility as well as to differences in assay conditions. Efforts are currently in progress to address these possibilities. This report provides strong evidence of a critical role for a Src-family kinase in early vertebrate development, acting as a required component of the FGF signal transduction pathway. □

Methods

Expression library construction and screening. Early gastrula (stage 10) *Xenopus laevis* embryos were homogenized with RNAzol B solution and processed in accordance with the manufacturer's instructions (Tel-Test). 4.5 μg poly(A)$^+$ RNA was selected from 2.4 mg total RNA using the Oligotex mRNA midi kit (Qiagen). cDNA synthesis and linker addition was performed using the Superscript II unidirectional kit (Gibco BRL). After second-strand synthesis, cDNAs were size-selected by gel filtration (average size 2.0 kb) and directionally subcloned into the *Sal*I and *Not*I sites of a modified pCS2 vector. 2×10^6 transformants were obtained after electroporation in ElectroMAX DH10B cells (Gibco BRL).

The library was plated to obtain initial fractions of approximately 200 clones. Similar functional screens in *Xenopus*, using small pools of RNAs, have been described elsewhere[25]. For subsequent sib selection, 10 pools of fivefold fewer clones were screened (e.g. 10×40, 10×8). Pooled plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen) and linearized with *Asc*I. Capped RNA was synthesized using the mMessage mMachine kit (Ambion). Embryos were injected with 10 nl/blastomere of 0.5 mg ml^{-1} RNA into animal poles of both blastomeres at the two-cell stage, and cultured until tadpole stages.

RNA preparation, explant dissection and cell culture. *laloo* RNA and all mutant derivatives contained the *laloo* open reading frame, as well as 84 nucleotides of 5′ and 34 nucleotides of 3′ untranslated sequence. All mRNA was synthesized *in vitro* in the presence of cap analogue using the mMessage mMachine kit. Microinjection, explant dissection and culture were performed as described[26]. Recombinant bFGF was obtained from Boehringer Mannheim.

Whole-mount immunohistochemistry, β-Gal detection and *in situ* hybridization. Whole-mount antibody staining was performed as described[26]. The 6F11 antibody was used at 1:1 dilution, and the 12/101 antibody was used at 1:500 dilution. Secondary antibody was a donkey anti-mouse IgG coupled to horseradish peroxidase (Jackson Laboratories) and was used at 1:250 dilution. Colour reactions were performed using the DAB and Vector SG kits (Vector Laboratories). Whole-mount β-Gal detection and hybridization *in situ* were performed as described[4]. The antisense *Xbra* probe was synthesized in the presence of digoxigenin-11-UTP (Boehringer Mannheim).

RT–PCR. RT–PCR was performed as described[9]. Primers constructed for this study were as follows: *laloo* (forward, 5′-TGGCTCTGTACTGTGATC; reverse, 5′-GTCATACAAAGCCAGCAG); *chordin* (forward, 5′-CAGTCAGATGGAGCAGGATC; reverse, 5′-AGTCCCATTGCCCGAGTTGC); *ODC* (forward, 5′-AATGGATTTCAGAGACCA; reverse, 5′-CCAAGGCTAAAGTTGCAG). All other primer sequences were as described[26]. PCR for *laloo*, *Xwnt8*, *HoxB9* and *NCAM* were performed for 25 cycles; PCR for *EF1-α*, *Xbra*, *chordin*, *muscle actin* and *ODC* were performed for 21 cycles.

Preparation of *laloo* mutant constructs. The *laloo* mutants K259E and Y492F were generated by PCR. For K259E, we introduced a point mutation (A → G) that resulted in a lysine (AAA) to glutamic acid (GAA) mutation in the resulting construct. The oligonucleotide used for this mutagenesis was as follows: 5′-GTA GAA ACA ATG AAG CCA GGC AGC. For Y492F, we introduced a point mutation (A → T) that resulted in a tyrosine (TAC) to phenylalanine (TTC) mutation in the resulting construct. The complementary strand oligonucleotide thus includes a T → A mutation: 5′-TTA AGG TTG TGC CTG GAA CTG.

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Correspondence and requests for materials should be addressed to A.H.-B. (e-mail: brvnlou@rockvax.rockefeller.edu). The GenBank accession number for *laloo* is AF081803.