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Swift Is a Novel BRCT Domain Coactivator of Smad2 in Transforming Growth Factor β Signaling

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Transforming growth factor β (TGFβ) signaling is transduced via Smad2–Smad4–DNA-binding protein complexes which bind to responsive elements in the promoters of target genes. However, the mechanism of how the complexes activate the target genes is unclear. Here we identify *Xenopus* Swift, a novel nuclear BRCT (BRCA1 C-terminal) domain protein that physically interacts with Smad2 via its BRCT domains. We examine the activity of Swift in relation to gene activation in *Xenopus* embryos. *Swift* mRNA has an expression pattern similar to that of *Smad2*. Swift has intrinsic transactivation activity and activates target gene transcription in a TGFβ-Smad2-dependent manner. Inhibition of Swift activity results in the suppression of TGFβ-induced gene transcription and defective mesendoderm development. Blocking Swift function affects neither bone morphogenic protein nor fibroblast growth factor signaling during early development. We conclude that Swift is a novel coactivator of Smad2 and that Swift has a critical role in embryonic TGFβ-induced gene transcription. Our results suggest that Swift may be a general component of TGFβ signaling.

Transforming growth factor β (TGFβ) superfamily signaling mediates a very diverse array of biological processes, including immune function, growth control, cell differentiation, patterning the embryonic body, sexual reproduction, and skeletal formation (reviewed in reference 29). The TGFβ family member activin elicits several different gene expression and cell fate pathways in a concentration-dependent way in early *Xenopus* development (15–17). The study of activin response genes in early *Xenopus* development has provided an excellent tool to elucidate the general mechanism of the TGFβ signaling pathway.

TGFβ-activin signals through serine-threonine kinase receptors I and II at the cell surface and initiates the following sequence of events (reviewed in reference 30). Binding of activin induces the formation of heteromeric complexes of these receptors, and signaling is initiated when receptor I is phosphorylated and activated by receptor II. Activated receptor I phosphorylates Smad2. Activated Smad2 forms a complex with Smad4 (Smad2/4 complex) in the cytoplasm and then enters the nucleus. In the nucleus, the Smad2/4 complex interacts with specific DNA-binding cofactors that help to select target genes. In *Xenopus* development, FAST, Mixer, and Milk have been identified as Smad2-recruiting DNA-binding cofactors (4, 13). FAST, which contains a winged helix DNA-binding domain, binds to an activin-responsive element and is required for activation of *Mix.2* gene in response to activin. FAST bound to DNA alone does not appear to activate transcription (41). However, recruitment of an activated Smad2/4 complex to the activin-responsive element by FAST results in activation of *Mix.2* gene expression (5). Mixer and Milk are paired-like homeodomain transcription factors of the Mix family (7, 19). In a mechanism similar to FAST, they bind to the activin-inducible distal element of the *Xgsc* promoter, recruit Smad2, and initiate transcription of *Xgsc* (13).

Recently, it has been demonstrated that the Smad2/4 complex recruits the transcriptional coactivators p300 and CREB-binding protein (CBP) (10, 21, 32). However, little is known about how the Smad2/4 complex with DNA-binding proteins activates gene expression. Here we describe the identification and function of a novel *Xenopus* Smad2 coactivator which we call Swift. Interestingly, Swift contains six BRCT (BRCA1 C-terminal) domains, first described in the breast cancer suppressor protein BRCA1. Swift binds to Smad2 via its BRCT domains. We show that *Swift* mRNA is maternally expressed and that its expression pattern during early development is similar to that of *Smad2*. Swift synergistically activates gene expression with Smad2 in an activin signaling-dependent manner. Swift has intrinsic transactivation activity that is enhanced by activin signaling. Using two different dominant interfering approaches, we show that Swift function is required for TGFβ-activin-induced gene expression in vivo and for normal mesendoderm formation. Our results suggest that Swift is a necessary component of the embryonic TGFβ signaling pathway. Swift may have a role in many instances of TGFβ signaling.

MATERIALS AND METHODS

Isolation of *Xenopus* Swift. For the yeast two-hybrid screening, the linker region and a part of the MH2 domain of Smad2 (amino acids 180 to 432) was subcloned into a pBTM116 bait vector; the expressed fusion protein contains a LexA DNA-binding domain (40). The cDNA library, consisting of poly(A) mRNA and poly(A)$^+$ mRNA random primed from *Xenopus* eggs, was cloned into the *Eco*RI site of the pACTII library vector that contains a GAL4 transactivation domain. Yeast two-hybrid screening was performed using *Saccharomyes cerevisiae* strain L40 as described previously (40). A screen of 10^7 clones yielded

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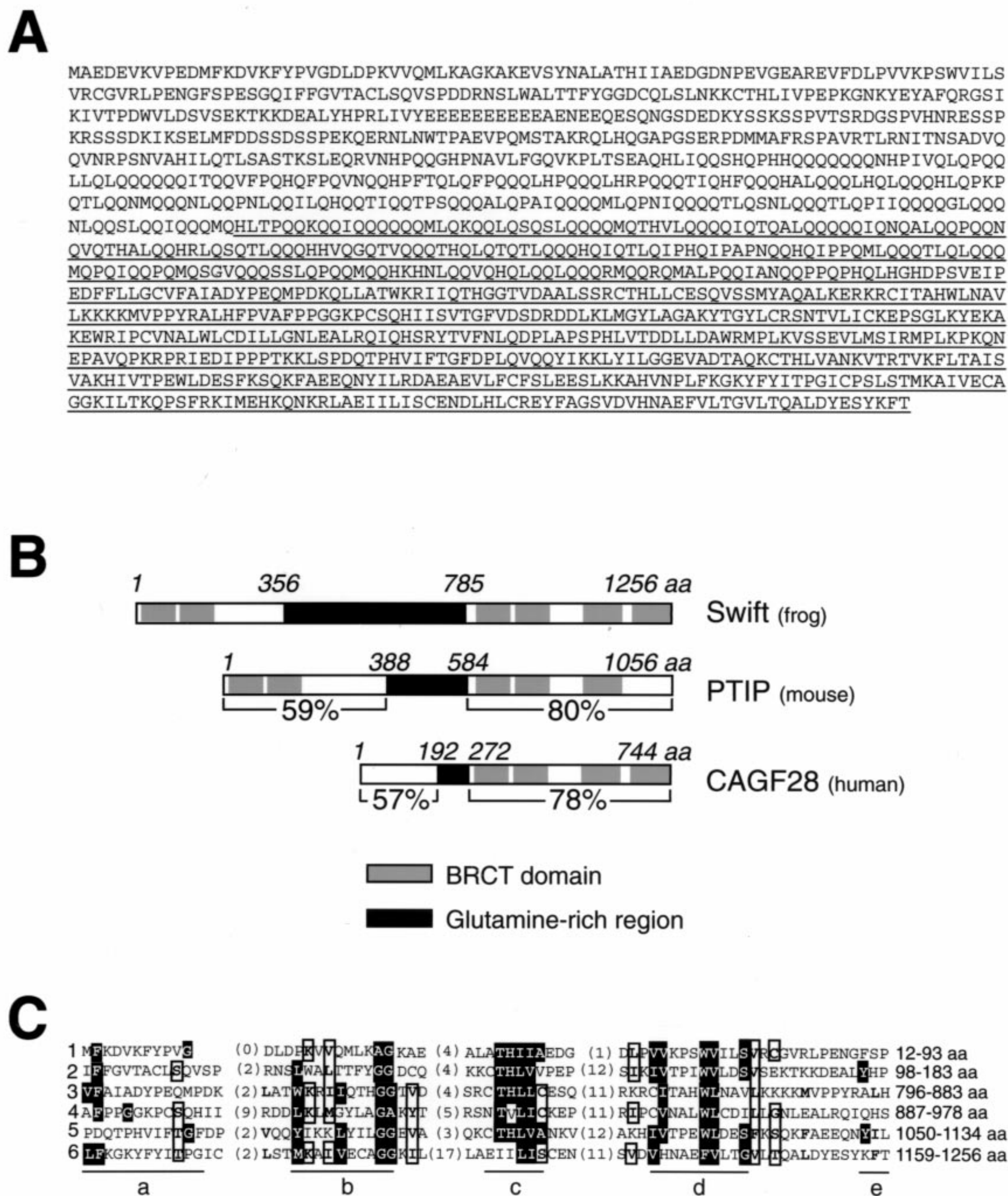

FIG. 1. Cloning of Swift. (A) Deduced amino acid sequence of Swift. The cDNA fragment isolated from the yeast two-hybrid screen is underlined. (B) Comparison of the primary structure of Swift with those of PTIP and CAGF28. The percentage values in the N-terminal regions of PTIP (amino acids 1 to 388) and CAGF28 (amino acids 1 to 192) show amino acid sequence identity with that of Swift (amino acids 1 to 356 and 207 to 356 respectively). The percentage values in the C-terminal regions of PTIP (amino acids 584 to 1056) and CAGF28 (amino acids 272 to 744) show amino acid sequence identity with that of Swift (amino acids 785 to 1256). (C) Consensus sequence for the six BRCT domains of Swift. Five aligned blocks (designated a to e) constituting the five conserved regions of the domain are separated from each other as described elsewhere (3). Matches to the consensus are in black boxes. Bulky hydrophobic residues (I, L, V, M, F, Y, W) and small residues (G, A, S, T, C) are grouped; residues conserved in four out of six sequences are in white boxes (24).

31 positive clones, 6 of which contained identical 2.8-kb cDNAs. A full-length Swift was obtained by a 5′ primer extension on a *Xenopus* egg cDNA library (33).

RNA expression constructs. For Swift expression constructs, the open reading frame was amplified by PCR using *Pfu* Turbo DNA polymerase (Stratagene) and subcloned into expression vectors. The capped mRNAs were synthesized in vitro (33). pT7TSHA-HA (hemagglutinin)-Swift was constructed by inserting full-length Swift cDNA into pT7TSHA-HA (36), linearized by *Xba*I, and transcribed with T7. pT7TSHA-HA-En^{R}-Swift ΔN was constructed by inserting a DNA fragment encoding the *Engrailed* sequence into the C terminus of Swift ΔN (amino acids 567 to 1256) in pT7TSHA-HA-Swift ΔN, linearized by *Xba*I, and transcribed with T7. pActRIB* was linearized by *Hin*dIII and transcribed with T7. pT7TSHA-HA ActRIB* was constructed by inserting an ActRIB* cDNA into pT7TSHA-HA, linearized by *Xba*I, and transcribed with T7. pT7TS-VP16^{A}-HA-HA was constructed by inserting a DNA fragment encoding a VP16 trans-

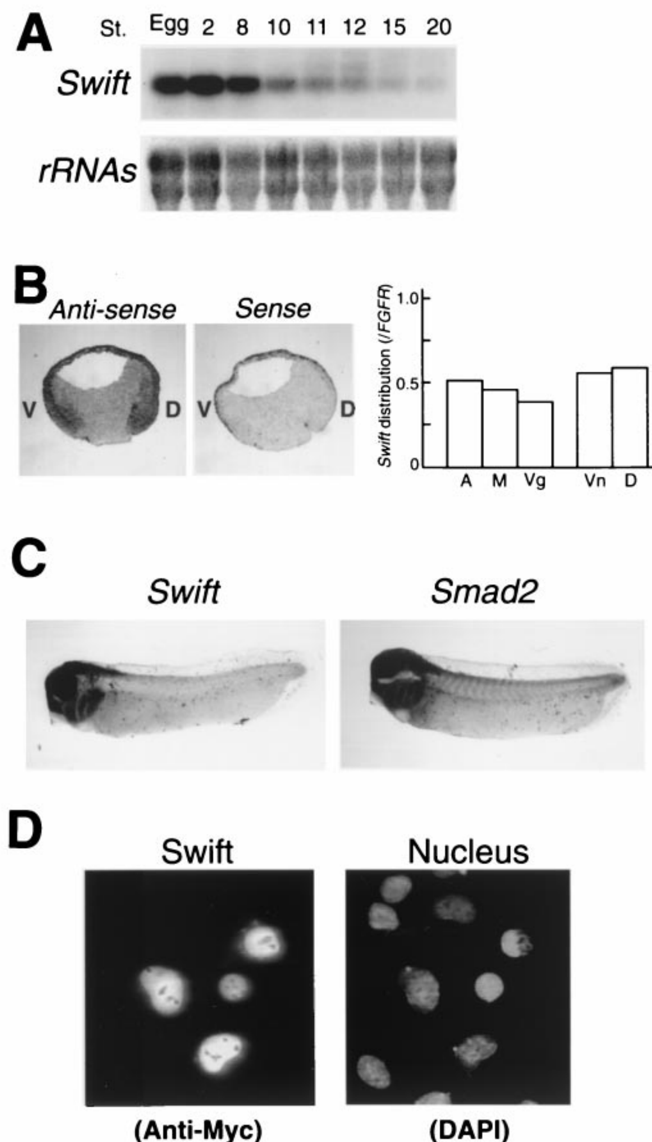

FIG. 2. Developmental expression of Swift. (A) Temporal expression of *Swift* mRNA during *Xenopus* development. Northern blots were probed with random-prime-labeled Swift cDNA (nucleotides 2991 to 3768). The bottom panel shows ethidium bromide staining of rRNAs. (B) Distribution of *Swift* mRNA at the gastrula stage. Left, in situ hybridization to sections cut vertically through the dorsoventral axis of stage 11 embryos, using full-length antisense and sense *Swift* mRNA probes. Ventral (V) and dorsal (D) are marked. Grey staining shows the expression of *Swift* mRNA. Right, quantitation of *Swift* mRNA. Stage 11 embryos were dissected into roughly thirds (animal [A], marginal [M], or vegetal [Vg]) or halves (ventral [Vn] or dorsal [D]), and total RNA was harvested. The level of *Swift* mRNA was quantitated by RNase protection assays, and *FGFR* was used as a loading control. (C) Whole-mount hybridization with full-length *Swift* and *Smad2* mRNA probes, using *Xenopus* embryos at stage 28. (D) Swift is a nuclear protein. U20S human osteosarcoma cells were transiently transfected with pcDNAmyc-Swift (full length). Cells expressing Myc-tagged Swift were analyzed by indirect immunofluorescence using an anti-Myc monoclonal antibody and fluorescein-conjugated rabbit anti-mouse immunoglobulin. 4′,6-Diamidino-2-phenylindole (DAPI) staining of DNA is shown for comparison. Myc-tagged cyclin B1 is cytoplasmic, whereas an nuclear localization site-tagged cyclin B1 enters the nucleus; therefore the Myc tag does not contain a nuclear localization site (personal communication from J. Pines).

activation domain into pT7TSHA-HA. pT7TS-VP16^{A}-HA-HA-Swift and -Smad2 were constructed by inserting full-length Swift and Smad2 cDNAs, respectively, into pT7TS-VP16^{A}-HA-HA, linearized by *Xba*I, and transcribed with T7. pT7TS-GAL-HA was constructed by inserting a DNA fragment encoding a GAL4 DNA-binding domain into pT7TSHA. pT7TS-GAL-HA-Swift was constructed by inserting a Swift cDNA into pT7TS-GAL-HA, linearized by *Xba*I, and transcribed with T7. pT7TS-GST-Swift B3-6 was constructed by inserting a DNA fragment encoding glutathione *S*-transferase (GST) into pT7TS-Swift B3-6, linearized by *Xba*I, and transcribed with T7. *HA-Smad2* and *activin* mRNAs were prepared as described elsewhere (33, 36). pSP64-Smad1, pSP64-Smad2, and pdn-BRII were gifts from D. A. Melton. The *Engrailed* repressor construct and pActRIB* (pALK4*) were gifts from J. Smith.

Oocyte synthesis of activin. The procedure for oocyte synthesis of activin was as described elsewhere (31).

In vitro binding assays. ^{35}S-labeled Swift was in vitro translated using the TNT coupled reticulocyte lysate system (Promega). GST, GST-Smad1, and GST-Smad2 were purified from overexpressing *Escherichia coli* (37). One microgram of GST, GST-Smad1, or GST-Smad2 was incubated with 0.5 μl of ^{35}S-labeled Swift in 0.1 ml of buffer A (20 mM Tris-Cl [pH 8.0], 0.5 mM EDTA, 1 mM dithiothreitol, 1% NP-40, 0.2 M NaCl) at 4°C for 1 h; then 10 μl of glutathione-Sepharose beads was added. After 1 h of incubation, the beads were washed with buffer A four times. Bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie brilliant blue staining and autoradiography.

In vivo coprecipitation. Each embryo was injected with various combinations of 2 ng of *GST-Swift B3-6*, 2 ng of *HA-Smad2*, and 1 ng of *activin* mRNAs into an animal pole of the one-cell stage embryo and cultured to stage 9. Forty embryos were homogenized in 0.4 ml of buffer B (50 mM Tris-Cl [pH 7.5], 50 mM KCl, 25% glycerol, 25 mM β-glycerophosphate, phosphatase inhibitors [Sigma], protease inhibitors [Roche], 2 mM Na_3VO_4, 0.2 mM NaF, 1 mM dithiothreitol) and cleared by centrifugation at 4°C; then 20 μl of glutathione-Sepharose beads was added. After 2 h of incubation, the beads were washed four times with buffer B containing 0.25% NP-40 and 0.2 M NaCl. Bound proteins were resolved by SDS-PAGE and analyzed by Western blotting.

Northern blot, in situ, and RNase protection assays. Northern blot, in situ, and RNase protection assays were performed as described elsewhere (33), and experiments were repeated at least three times. The RNase protection probe template for Swift was constructed as follows: pSwiftΔN(NcoI), which consisted of Swift nucleotides 1 to 2472, was linearized at the *Pst*I site (nucleotide 2231), and an antisense probe was synthesized using SP6 RNA polymerase.

Subcellular localization by immunofluorescence microscopy. U20S human osteosarcoma cells were maintained in Dulbecco Modified Eagle medium containing 10% fetal bovine serum under 5% CO_2, at 37°C. Cultured cells were transfected with pcDNAmyc-Swift (full length) using FuGENETM6 transfection reagent (Roche) and cultured for 24 h. Immunostaining was performed as described elsewhere (39). Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 5 min. After being washed with phosphate-buffered saline, cells were incubated with an anti-Myc antibody (9E10) (Roche) for 1 h. Fluorescein-conjugated rabbit anti-mouse immunoglobulin (Sigma) was used as the second antibody. Cells were observed and photographed under fluorescein or UV illumination using immunofluorescence microscopy.

Luciferase assays. Each embryo was coinjected in the animal pole at stage 1 with mRNA (0.5 ng) encoding a GAL fusion construct with 5xGAL4-luciferase reporter plasmid DNA (0.3 ng) (46) or 5xGAL4-TK-luciferase reporter plasmid DNA (0.1 ng) (12) with or without *ActRIB** (0.2 ng) or *VP16^{A}-Smad2* (3 ng) mRNA. Embryos were cultured to stage 10. Five embryos at stage 10 were homogenized in 0.1 ml of buffer C (50 mM Tris-Cl [pH 7.5], protease inhibitors [Roche]) and cleared by centrifugation at 4°C. One to five microliters of the supernatant was assayed for luciferase activity in a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

Nucleotide sequence accession number. The cDNA sequence has been deposited in GenBank under accession number AF172855.

RESULTS

Identification of Swift. Smad2 is a critical intracellular mediator of the TGFβ signaling pathway during early *Xenopus* development (14). To identify Smad2-binding proteins that participate in TGFβ signaling, we used a two-hybrid screen of a *Xenopus* egg cDNA library with pLexA-Smad2. This LexA

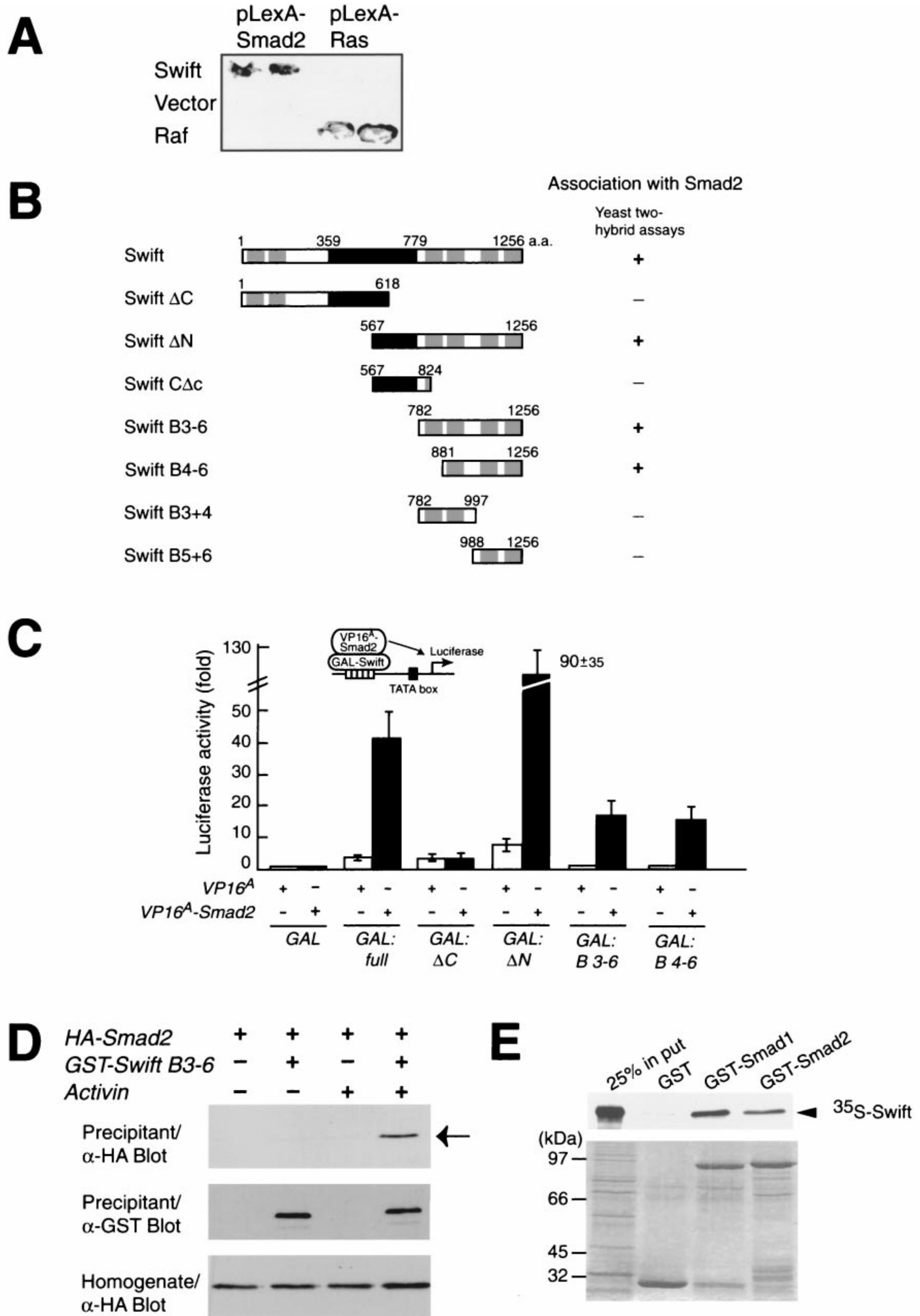

FIG. 3. Interaction of Swift with Smad2. (A) Interaction of Swift with Smad2 in yeast two-hybrid assays. Strain L40 was transformed with pLexA-Smad2 (amino acids 180 to 432) or pLexA-Ras (G12V) with pACTIIHK-Swift (full length), pACTIIHK, or pVP-Raf. Interaction of Swift with Smad2 was examined by qualitative assays for β-galactosidase activity, and pLexA-Ras and pVP-Raf were used as positive controls (40). (B) Determination of the Smad2-interacting region of Swift by yeast two-hybrid assays. The indicated fragments of Swift were tested for their

fusion construct encoded the linker region and a part of the MH2 domain of Smad2. Of 31 positive clones isolated, six were identical and encoded a novel amino acid sequence. Because all isolated cDNAs were partial clones, we performed a 5′ primer extension on a *Xenopus* egg cDNA library to obtain the entire open reading frames. The complete protein consists of 1,256 amino acids (Fig. 1A). Based on our subsequent functional analysis, we name this protein Swift, for *Xenopus* Smad wing for transcriptional activation.

Sequence analysis reveals that Swift has six BRCT domains, first identified in the C-terminal region of the breast cancer suppressor protein BRCA1 (24) (Fig. 1B and C). The BRCT domain is defined by distinct hydrophobic clusters of amino acids and is believed to occur as an autonomous folding unit of ~95 amino acids that is implicated in protein-protein interactions (3). Swift also contains a glutamine-rich region and a putative nuclear localization signal (amino acids 857 to 874). GenBank searches reveal a mouse homologue named PTIP (Pax transcription activation domain-interacting protein) (27) and a human homologue named CAGF28 (28) (GenBank accession numbers AF104261 and U80735, respectively). PTIP is a nuclear protein with five BRCT domains that interacts with the Pax2 DNA-binding protein (27). However, these two genes were not previously implicated in TGFβ signaling. Their C-terminal domains share about 80% amino acid sequence identity with that of Swift and contain BRCT domains (Fig. 1B). These results suggest that Swift is conserved at least from *Xenopus* to humans.

Expression pattern and nuclear localization of Swift. Northern blot analysis of the developmental expression pattern of *Swift* reveals that *Swift* mRNA is maternally expressed and that the level of its mRNA declines during gastrulation and continues at a lower level until late stages (Fig. 2A). In situ hybridization analysis reveals that *Swift* mRNA is ubiquitously expressed at the gastrula stage, and its expression pattern is similar to that of *Smad2* mRNA (14) (Fig. 2B, left). This result is confirmed by RNase protection assays (Fig. 2B, right). Later in development, *Swift* mRNA becomes enriched in the head and brain as does *Smad2* mRNA (Fig. 2C). The coincident expression pattern of *Swift* and *Smad2* mRNAs supports the possibility that they interact functionally during early development. A Myc-tagged Swift expressed in U20S human osteosarcoma cells is mostly localized in the nucleus (Fig. 2D), indicating that Swift is a nuclear protein.

Interaction of Swift with Smad2. To confirm that full-length Swift interacts with Smad2, we transformed pLexA-Smad2 with pACTIIHK-Swift in yeast, where Swift binds to Smad2 but not to the negative control bait (Fig. 3A). To define the domain of Swift required for Smad2 binding, various fragments of Swift were examined by yeast two-hybrid assays. We tested the fragments encoding full-length Swift, the N-terminal fragment (ΔC), the C-terminal fragment (ΔN), the C-terminal fragment lacking BRCT domains (CΔc), the last four BRCT domains (B3−6), the last three BRCT domains (B4−6), the two BRCT domains (B3+4), and the last two BRCT domains (B5+6). Smad2 binds to full-length Swift, ΔN, B3−6, and B4−6 but not to ΔC, CΔc, B3+4, or B5+6 (Fig. 3B). Then, using luciferase assays, we confirmed the Smad2-binding region of Swift in *Xenopus* embryos. We coinjected mRNA encoding Swift fused to a GAL4 DNA-binding domain and GAL4-luciferase reporter plasmid DNA with *VP16^A* or *VP16^A-Smad2* mRNA at stage 1 and measured the luciferase activity at stage 10. VP16^A Smad2 enhances the transcriptional activity of full-length, ΔN, B3−6, and B4−6 but not that of ΔC fused to a GAL4 DNA-binding domain (Fig. 3C). This result indicates that Smad2 binds to full-length Swift, ΔN, B3−6, and B4−6 but not ΔC in embryos. In summary, the last three BRCT domains, B4−6, are necessary and sufficient for Smad2 interaction in yeast and embryos.

We then asked if Swift interacts with Smad2 in an activin signal-dependent manner in embryos. We injected *HA-Smad2* (full-length) mRNA with or without *GST-Swift B3−6* and *activin* mRNAs at stage 1 and performed in vivo coprecipitation assays at stage 9. GST-Swift B3−6 interacts with HA-Smad2 in the presence but not in the absence of activin signaling (Fig. 3D). We conclude that Swift interacts with Smad2 in an activin signal-dependent manner in embryos.

To test if Swift directly binds to Smad2, we prepared ^{35}S-labeled Swift protein. ^{35}S-Swift directly binds to GST-Smad2 but not to GST alone (Fig. 3E). Swift also binds to Smad1 as well as Smad2 in in vitro binding assays. First, we focused on the analysis of Swift function for Smad2 in early embryos, because Smad2, but not Smad1, is a downstream component of TGFβ signaling. Then, we tested if Swift functioned with Smad1 in early embryos (see Fig. 7A). We conclude that Swift physically interacts with Smad2 via its last three BRCT domain-containing regions in an activin signal-dependent fashion.

Swift enhances activin-Smad2-dependent transcription. If Swift functionally interacts with Smad2, Swift should have some effect on activin-induced gene transcription in *Xenopus* blastula cells. Overexpressed Swift alone in animal caps does not activate gene expression (Fig. 4A). We therefore examined the effect of overexpressed Swift on gene expression induced by a constitutively active activin receptor IB (ActRIB*). ActRIB* transduces activin dose-dependent gene responses in the same way as activin (2). We injected the indicated amount of *ActRIB** mRNA with or without *Swift* mRNA in the animal

interaction with Smad2 in yeast two-hybrid assays. Interaction of Swift with Smad2 in yeast was examined by qualitative assays for β-galactosidase activity. (C) Determination of the Smad2-interacting region of Swift in embryos by luciferase assays. Each embryo was coinjected in the animal pole of the one-cell-stage embryo with mRNA encoding a GAL fusion construct and 5xGAL4-luciferase reporter plasmid DNA with *VP16^A* or *VP16^A-Smad2* mRNA. Interaction of Swift with Smad2 in embryos was examined by luciferase assays for luciferase activity. The values are means ± standard error of three independent experiments. Closed and open bars show luciferase activities with *VP16^A-Smad2* and *VP16^A* mRNAs, respectively. (D) In vivo coprecipitation of Smad2 with Swift in embryos. Embryos were coinjected with various combinations of the indicated mRNAs at stage 1; in vivo coprecipitation assays were performed at stage 9, followed by Western blotting using the indicated antibodies. The arrow indicates HA-Smad2 coprecipitated with GST-Swift B3-6. (E) Direct interaction of Swift with Smads using in vitro binding assays. GST, GST-Smad1, or GST-Smad2 was incubated with ^{35}S-labeled full-length Swift, and glutathione-Sepharose beads were then added. Bound proteins were resolved on SDS-PAGE and visualized by autoradiography (top) and Coomassie brilliant blue staining (bottom).

FIG. 4. Swift is an activin-dependent transcriptional cofactor of Smad2. (A) Effects of Swift on activin-induced gene transcription. Each embryo was coinjected with the indicated amount of *ActRIB** mRNA with *Swift* or *lacZ* mRNA at stage 1, animal caps were dissected at stage 8.5, and then at stage 10.25 gene induction was analyzed by RNase protection assays. *lacZ* was used as a negative control. (B) Effects of coinjected *Swift* mRNA on expression levels of ActRIB* protein. *HA-ActRIB** mRNA (0.1 ng) was injected with or without *Swift* mRNA (1 ng) in the animal pole of the one-cell-stage embryo, and animal caps were dissected at stage 8.5. At stage 10.5, an animal cap was homogenized and proteins were resolved by SDS-PAGE and then analyzed by Western blotting using an anti-HA antibody (control from noninjected embryos in panel D). (C) Effects of wild-type Swift and VP16^{A}-Swift on activin-induced gene transcription. Each embryo was injected with the indicated amount of *Swift* or *VP16^{A}-Swift* mRNA with or without *ActRIB** mRNA at stage 1, animal caps were dissected at stage 8.5, and then at stage 10.25 *Mix.1* gene induction was analyzed by RNase protection assays. Left, quantitation of gel analysis; right, gel analyses. (D) Expression levels of Swift and VP16^{A}-Swift proteins. Each embryo was injected with *Swift* or *VP16^{A}-Swift* mRNA (1 ng) with *ActRIB** mRNA (25 pg) at stage 1. At stage 10.25, an embryo was homogenized; proteins were resolved by SDS-PAGE and then analyzed by Western blotting using an anti-HA antibody. The arrow and arrowhead indicate HA-tagged VP16^{A}-Swift and HA-tagged Swift proteins, respectively.

FIG. 5. Determination of the Swift intrinsic transactivation domain. Each embryo was coinjected with mRNA encoding various regions of Swift fused to a GAL4 DNA-binding domain and with 5xGAL4-luciferase promoter plasmid DNA with or without *ActRIB** mRNA at stage 1, and luciferase activity was measured at stage 10. The values are means ± standard errors of three independent experiments. Closed and open bars show luciferase activities with and without *ActRIB** mRNA, respectively.

pole of the one-cell-stage embryo, dissected animal caps at stage 8.5, and measured gene expression at stage 10.25 using RNase protection assays. ActRIB* activates *Xgsc, Chd, Mix.1*, and *Eomes* gene transcription in a dose-dependent manner (Fig. 4A). The coinjection of *Swift* mRNA with *ActRIB** mRNA results in a synergistic activation of the ActRIB*-induced transcription of all the tested genes (Fig. 4A), including *Xbra* (data not shown).

It is possible that the coinjected *Swift* mRNA increases the expression level of ActRIB* protein and indirectly enhances transcription. To test this possibility, we injected *HA-ActRIB** mRNA with or without *Swift* mRNA at stage 1 and dissected animal caps at stage 8.5. At stage 10.25, animal cap proteins were resolved by SDS-PAGE and analyzed by Western blotting using an anti-HA antibody. The coinjected *Swift* mRNA does not have any affect on the expression level of HA-ActRIB* protein (Fig. 4B). HA-ActRIB* had the same effect on mesendoderm gene expression as ActRIB* (data not shown). If the coinjected *Swift* mRNA affects the endogenous level of ActRIB protein and if the increased levels of endogenous ActRIB protein activate mesendoderm gene expression, overexpressed Swift alone should activate mesendoderm gene transcription. However, overexpressed Swift alone does not activate mesendoderm gene expression (Fig. 4A). These results indicate that the coinjected *Swift* mRNA exerts its effect directly on transcription but not on the expression levels of ActRIB* or endogenous ActRIB protein.

We also confirmed that this synergy occurred at the level of Smad2. When we coinjected *Swift* mRNA with *Smad2* mRNA instead of *ActRIB** mRNA, Swift had a similar, but somewhat weaker, activating effect on Smad2-induced gene transcription (data not shown). It is not surprising that Swift more effectively enhances gene transcription induced by ActRIB* than by Smad2, because overexpressed Smad2 may not be properly phosphorylated, while ActRIB* efficiently phosphorylates and activates endogenous Smad2 (9). Therefore, we used ActRIB* instead of Smad2 in our subsequent experiments. We conclude

FIG. 6. Swift is involved in TGFβ signaling in embryos. (A) Suppression of activin-induced gene expression by En^R-Swift ΔN. Each embryo was coinjected with various combinations of *En^R-Swift ΔN, ActRIB**, and wild-type *Swift* mRNAs at stage 1; and at stage 10.25, gene induction in animal caps was analyzed by RNase protection assays. (B) Quantitation of RNase protection assays of *Mix.1* gene induction. (C) In situ hybridization of the *Mix.1* gene. *En^R-Swift ΔN* (0.4 ng) mRNA was injected into the equatorial region of a blastomere of the four-cell-stage embryo, and expression of *Mix.1* was assayed by in situ hybridization. Left, coinjection of *En^R-Swift ΔN* mRNA (0.4 ng) and *lacZ* mRNA (0.5 ng); right, injection of *lacZ* mRNA (0.5 ng) alone. En^R-Swift ΔN (n = 47), 100% *Mix.1* suppression; LacZ alone (n = 22), 0%. Arrows indicate sites of injection. (D) Phenotypic effects of En^R-Swift ΔN. A low (0.1 ng/embryo) or high (1 ng/embryo) dose of *En^R-Swift ΔN* mRNA was injected radially in all blastomeres of the four-cell-stage embryo, and the phenotypes were observed at stage 36. Left, control (no injection); center, *En^R-Swift ΔN* (0.1 ng) (n = 25, 72% trunk defect); right, *En^R-Swift ΔN* (1 ng) (n = 40, 100% severe head defect).

that Swift is involved in directly stimulating activin-Smad2-induced gene transcription. Furthermore, the fact that Swift has similar effects on all of the response genes which we tested suggests that Swift may be a general component of TGFβ/activin signaling in *Xenopus* embryos.

Swift is a transcriptional cofactor of Smad2. We show that Swift interacts with Smad2 in an activin signal-dependent manner (Fig. 3D). Therefore, if Swift does indeed behave as a cofactor of Smad2 at the promoter level, Swift fused to a VP16 transactivation domain from herpes simplex virus ($VP16^A$-Swift) (34) should potentiate an activin response more strongly than wild-type Swift. In contrast, if Swift acts at a level upstream of the promoter, then $VP16^A$-Swift should not be more effective than wild-type Swift. We injected the indicated amount of wild-type *Swift* or *$VP16^A$-Swift* mRNA with or without a small amount of *ActRIB** mRNA (25 pg) that only weakly induced transcription of *Mix.1* at stage 1 and assayed *Mix.1* gene expression in animal caps at stage 10.25. Indeed, $VP16^A$-Swift synergistically activates *Mix.1* gene transcription with ActRIB* more strongly than wild-type Swift (Fig. 4C). In addition, like wild-type Swift, $VP16^A$-Swift alone does not activate *Mix.1* gene transcription (Fig. 4A and C). $VP16^A$-Swift had a similar activating effect on ActRIB*-induced gene transcription of *Xgsc, Chd, Eomes*, and *Xbra* (data not shown).

To confirm that the effect of $VP16^A$-Swift on ActRIB*-induced gene transcription is not due to the higher expression levels of $VP16^A$-Swift protein than wild-type Swift protein, we coinjected *$VP16^A$-Swift* or *Swift* mRNA with *ActRIB** mRNA at stage 1. At stage 10.25, embryos were homogenized and proteins were resolved by SDS-PAGE and then analyzed by Western blotting using an anti-HA antibody. The expression level of $VP16^A$-Swift protein is somewhat less than that of Swift protein (Fig. 4D). This result indicates that the synergistic activation of mesendoderm genes by $VP16^A$-Swift with ActRIB* more strongly than by wild-type Swift is not due to the higher expression levels of $VP16^A$-Swift protein than wild-type Swift. We conclude that Swift is an activin signal-dependent transcriptional cofactor of Smad2.

Swift has intrinsic transactivation activity. How does Swift synergize with Smad2 at the promoter level? We found that Swift has a glutamine-rich region (Fig. 1); in some transcription factors such as Sp1 and Oct1, glutamine-rich regions mediate transcriptional activation (8). We examined whether Swift has a transactivation activity by fusing full-length Swift to a GAL4 DNA-binding domain. The ability of the fusion, termed GAL:Swift, to activate a GAL4 reporter construct was tested in embryos. We coinjected *GAL:Swift* mRNA and GAL4-luciferase reporter plasmid DNA with or without *ActRIB** mRNA at stage 1 and measured the luciferase activity at stage 10. GAL:Swift has intrinsic transactivation activity that is enhanced by ActRIB* (Fig. 5). We next defined the transactivation domain by testing various regions of Swift in this GAL4 fusion assay. GAL:Swift ΔC, which contains a part of the glutamine-rich region, has intrinsic transactivation activity that is not enhanced by ActRIB*. GAL:Swift ΔN, which contains a part of the glutamine-rich region and four BRCT domains, has intrinsic transactivation activity that is enhanced by ActRIB*. GAL:Swift B3−6, which contains only the last four BRCT domains, does not have any intrinsic transactivation activity, but some transcriptional activity is observed in the presence of ActRIB*. GAL:Swift B4−6 has an effect similar to that of GAL:Swift B3−6. These results indicate that the Swift intrinsic transactivation activity is located between amino acids 567 and 782 in the glutamine-rich region and that its activity is enhanced by activin signaling. In addition, the last three BRCT domains are required for activin signaling-dependent stimulation, suggesting that in the presence of activin signaling, other coactivators may be recruited via Smad2 and the BRCT domains interaction. Therefore, both the glutamine-rich region and the last three BRCT domains of Swift are necessary for the Swift transactivation activity in response to an activin signal.

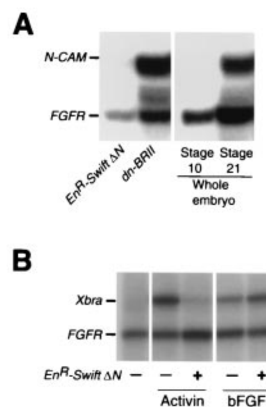

FIG. 7. (A) Effects of En^R-Swift ΔN on the BMP pathway. *En^R-Swift ΔN* (2 ng) or *dn-BRII* (0.5 ng) mRNA was injected into the ventral part of the animal region at the two-cell stage. Animal caps were dissected at stage 8.5; at stage 21, *N-CAM* gene induction was analyzed by RNase protection assays. (B) Effects of En^R-Swift ΔN on the FGF pathway. *En^R-Swift ΔN* mRNA (0.6 ng) was injected into the animal pole of the two-cell-stage embryo. At stage 8.5, animal caps were explanted, treated with a low dose of activin (0.2 ng/ml) or bFGF (0.2 μg/ml), and analyzed for expression of *Xbra* at stage 10.25 by RNase protection assays. bFGF was purchased from R&D Systems Inc.

Swift is involved in TGFβ signaling in embryos. To test if Swift is involved in TGFβ signaling in embryos, we used the *Drosophila Engrailed* repressor (22) attached to Swift. If Swift is involved in TGFβ signaling in embryos, Swift fused to an *Engrailed* repressor should suppress TGFβ-induced gene expression. Expression of full-length Swift protein fused to the *Engrailed* repressor was not detectable in mRNA-injected embryos by Western blotting (data not shown). The transactivation activity of Swift ΔN is similar to that of full-length Swift (Fig. 5), and Swift ΔN also binds to Smad2 (Fig. 3B and C). Therefore, we used Swift ΔN fused to the *Engrailed* repressor (En^R-Swift ΔN), and expression of this protein was detectable (data not shown). We coinjected *En^R-Swift ΔN* mRNA with *ActRIB** mRNA in the one-cell-stage embryo and assayed gene expression in animal caps at stage 10.25. The coinjection of *En^R-Swift ΔN* mRNA with *ActRIB** mRNA results in the suppression of ActRIB*-induced transcription of *Xgsc, Chd, Mix.1, Eomes*, and *Xbra* in a dose-dependent manner (Fig. 6A). To establish whether the suppression of ActRIB*-induced gene transcription is specific for the En^R-Swift ΔN activity, we coinjected wild-type *Swift* mRNA with *En^R-Swift ΔN* and *ActRIB** mRNAs. Wild-type Swift rescues the suppression of ActRIB*-induced gene transcription by En^R-Swift ΔN in a dose-depen-

dent manner (Fig. 6A). We confirmed that wild-type Swift completely rescues the suppression of ActRIB*-induced transcription of *Mix.1* by En^R-Swift ΔN (Fig. 6B). To test if endogenous *Mix.1* expression is suppressed by En^R-Swift ΔN, we injected *En^R-Swift ΔN* mRNA into the equatorial region of a blastomere of the four-cell-stage embryo and assayed expression of endogenous *Mix.1* by in situ hybridization at stage 10.25. The injection of *En^R-Swift ΔN* mRNA results in the complete suppression of endogenous *Mix.1* gene expression (Fig. 6C).

Since En^R-Swift ΔN blocks expression of activin-induced mesendoderm genes in animal cap explants, we expect that overexpressed En^R-Swift ΔN would cause loss of mesoderm-derived tissues in embryos and that its phenotype would be same as those observed by blocking activin, Smad2, and FAST functions. To test this possibility, we injected *En^R-Swift ΔN* mRNA radially in all blastomeres of the four-cell-stage embryo and observed the phenotypes at stage 36. The injection of a low concentration of mRNA (0.1 ng/embryo) results in defective trunk development (Fig. 6D, center). At a high dose of injected mRNA (1 ng/embryo), embryos show a complete loss of axial structure (Fig. 6D, right). These phenotypes are reminiscent of those observed using a dominant negative activin receptor, a dominant negative Smad2, or En^R-FAST in embryos (18, 20, 41), but not those observed using a dominant negative fibroblast growth factor (FGF) receptor (1) or by blocking bone morphogenic protein (BMP) signaling (11, 38). Moreover, this dose-responsive severity of developmental defects is observed in another TGFβ signaling component, FAST. The injection of a low dose of En^R-FAST results in a trunk defect, while high doses lead to both head and trunk defects (41). Taken together, these observations lead us to conclude that Swift is involved in embryonic TGFβ signaling.

Swift functions specifically in TGFβ signaling during early development. Using in vitro binding assays, we have shown that Swift binds to Smad1 as well as Smad2 (Fig. 3E). Although the phenotypes induced by En^R-Swift ΔN suggest that Swift is interacting with TGFβ signaling but not with BMP or FGF signaling (Fig. 6D), it is important to directly verify that Swift does not function with Smad1 in early embryos. Smad1 mediates signaling of BMP2/4 (other TGFβ family members) in early *Xenopus* development and regulates epidermal/neural as well as ventral mesoderm gene transcription (14, 43). Inhibition of BMP signals in *Xenopus* causes neuralization of an epidermal cell (reviewed in reference 42). If Swift interacts functionally with Smad1 in the BMP pathway in the gastrula, overexpressed En^R-Swift ΔN should suppress BMP signaling and induce expression of the *N-CAM* gene, a pan-neural marker. We injected the ventral side of two-cell-stage embryos with mRNA encoding En^R-Swift ΔN or a dominant negative BMP receptor II (dn-BRII) and dissected animal caps at stage 8.5. Animal caps were cultured until stage 21 and analyzed for expression of *N-CAM* by RNase protection assays. En^R-Swift ΔN in ventral animal caps does not induce *N-CAM* gene transcription, while dn-BRII does (Fig. 7A). We conclude that Swift does not function in the BMP pathway in early embryos.

Next we asked if Swift functions in FGF signaling. It is reported that mesoderm induction by activin requires FGF-mediated intracellular signals and that FGF induces some mesoderm genes, including *Xbra* (26). If Swift functions in FGF signaling as well as TGFβ signaling, overexpression of En^R-Swift ΔN should result in the suppression of FGF-induced *Xbra* gene expression. To examine this possibility, we injected *En^R-Swift ΔN* mRNA (0.6 ng) into the animal pole of the two-cell-stage stage embryo and explanted animal caps at stage 8.5. Animal caps were treated with a low concentration of activin (0.2 ng/ml) or basic FGF (bFGF)(0.2 μg/ml) and analyzed for expression of *Xbra* by RNase protection assays. Activin-induced *Xbra* gene expression is suppressed by En^R-Swift ΔN, while bFGF-induced *Xbra* gene transcription is not (Fig. 7B). Taken together, these findings lead us to conclude that Swift functions specifically in the TGFβ pathway but not in the BMP/Smad1 or FGF pathways in early embryos.

Swift is required for early development. Our finding that Swift is involved in TGFβ signaling in embryos does not necessarily mean that Swift is required for early *Xenopus* development. To test if Swift is required for early development, we designed a very subtle dominant negative form of Swift (dn-Swift) to interfere with endogenous Swift function. We prepared dn-Swift lacking the transactivation domain located between amino acids 567 and 782. We confirmed using luciferase assays that dn-Swift has neither intrinsic transactivation activity nor transcriptional repression activity (Fig. 8A and B), suggesting that dn-Swift is a weaker dominant negative than En^R-Swift ΔN. To confirm that dn-Swift interacts with Smad2, we transformed pLexA-Smad2 with pACTIIHK-dn-Swift or pACTIIHK-Swift in yeast, where dn-Swift and Swift bind to Smad2 but not to the negative control (Fig. 8C). If dn-Swift functions as a dominant negative, overexpressed dn-Swift should suppress Swift-enhanced mesendoderm gene expression. To test this possibility, we coinjected *dn-Swift* mRNA with *Swift* and/or *ActRIB** mRNAs at stage 1 and measured mesendoderm gene expression in animal caps at stage 10.25 by RNase protection assays. Swift enhances ActRIB*-induced gene expression of *Xgsc, Mix.1*, and *Xbra* (Fig. 8D). dn-Swift suppresses the effects of wild-type Swift (Fig. 8D). dn-Swift had the same effects on *Chd* and *Eomes* gene expression (data not shown). We conclude that dn-Swift functions as a dominant negative of wild-type Swift.

If endogenous Swift transactivation activity is required for TGFβ-induced gene expression in embryos, overexpressed dn-Swift should suppress expression of the target genes. To test this possibility, we coinjected *dn-Swift* mRNA (4 ng) with *ActRIB** mRNA (50 pg) at stage 1 and measured mesendoderm gene expression in animal caps. dn-Swift suppresses ActRIB*-induced *Xbra* gene expression, while wild-type Swift enhances ActRIB*-induced *Xbra* gene expression (Fig. 8E). dn-Swift did not suppress ActRIB*-induced gene transcription of *Xgsc, Chd, Mix.1*, or *Eomes* (data not shown). Because dn-Swift is a more subtle dominant negative than En^R-Swift ΔN and *Xbra* expression may be very sensitive to Swift function, the effects of dn-Swift might be obvious on *Xbra*. To test if endogenous Swift is required for endogenous *Xbra* transcription, we injected *dn-Swift* mRNA radially at the four-cell stage and characterized *Xbra* expression by in situ hybridization at stage 10.25. The injection of *dn-Swift* mRNA into embryos results in down-regulation of *Xbra* (Fig. 8F). The embryos injected with *dn-Swift* mRNA into dorsal blastomeres of the four-cell-stage embryo were allowed to develop until tailbud stages and show defective trunk development (Fig. 8G). The

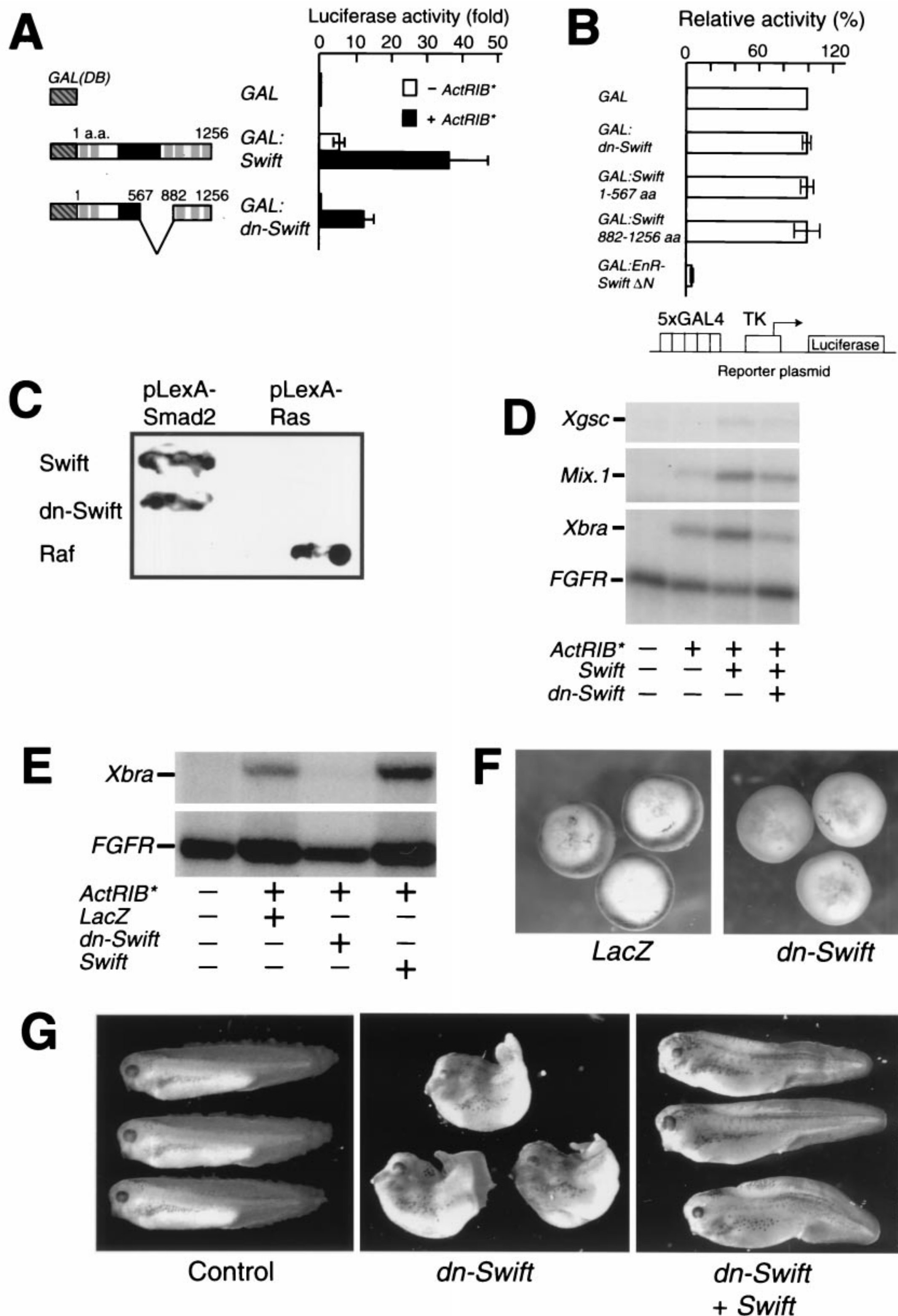

FIG. 8. Swift is required for early development. (A) dn-Swift lacks intrinsic transactivation activity. dn-Swift consists of nucleotides 1 to 1700 and 2649 to 3765. Each embryo was coinjected with mRNA encoding dn-Swift or wild-type Swift fused to a GAL4 DNA-binding domain and 5xGAL4-luciferase reporter plasmid DNA with or without *ActRIB** mRNA at stage 1, and luciferase activity was measured at stage 10. The values are means ± standard errors of three independent experiments. Closed and open bars show luciferase activities with and without *ActRIB** mRNA, respectively. (B) dn-Swift does not have transcriptional repression activity. Each embryo was coinjected with mRNA encoding a GAL fusion construct and 5xGAL4-TK-luciferase reporter plasmid DNA at stage 1, and luciferase activity was measured at stage 10. The activity of the reporter in the absence of GAL fusion was normalized to a value of 100. The values are means ± standard errors of three independent experiments. (C)

same phenotypes were observed when *dn-Swift* mRNA was injected radially (data not shown). These phenotypes are similar to those observed using a low dose of *En^R-Swift ΔN* mRNA (0.1 ng/embryo) (Fig. 6D, center), a result consistent with dn-Swift being a weaker dominant negative than En^R-Swift ΔN. The phenotype looks similar to that observed when Xbra function is inhibited (6). The dn-Swift-induced defect of trunk development is rescued by coinjection of wild-type *Swift* mRNA, indicating that the effects are due to specific inhibition of endogenous Swift. These results indicate that Swift is required at least for embryonic TGFβ-induced *Xbra* gene expression and normal mesoderm development.

DISCUSSION

In this study we show that Swift is a novel coactivator of Smad2 in *Xenopus*. Smad2 binds to the Swift C-terminal region via the last three BRCT domains in an activin signaling-dependent manner. The glutamine-rich region of Swift has latent transactivation capacity that is potentiated by activin signaling. We show that Swift enhances activin-induced gene expression at the promoter level by interacting with Smad2. Our data suggest that Swift itself is unlikely to directly bind to DNA in the promoters of responding genes in the absence of an activin signal, because VP16^A-Swift alone does not induce gene expression. We favor a model in which, upon TGFβ signaling, phosphorylated Smad2 enters the nucleus and binds to Swift along with DNA-binding proteins to form a TGFβ-responsive transcriptional complex assembled on mesendoderm gene promoters. Thus, Swift exerts its function as a ligand-dependent transcriptional coactivator of the complex.

The specificity of Swift. We have shown that Swift functions in the TGFβ pathway, but not in the BMP or FGF pathway, during early development (Fig. 7). These results indicate that Swift interacts functionally with Smad2 but not with Smad1, another receptor-regulated Smad, in early embryos. Does Swift functionally interact with other Smads during early *Xenopus* development? Smad4 is a common Smad and forms a complex with Smad1 as well as with Smad2 (30). Swift is not likely to interact with Smad4, because Swift does not function in the BMP pathway. *Xenopus* Smad3 has been neither identified nor characterized. Smad3 null mice are viable and survive to adulthood, suggesting that Smad3 is not required during gastrulation (45). Thus, Swift is not likely to function with the putative *Xenopus* Smad3 during gastrulation. In summary, Swift may interact functionally with Smad2 but not Smad1/3/4 in early embryos. Since we show that Smad1 binds to Swift in vitro (Fig. 3E), it is possible that Swift and Smad1 interact in some other contexts but not in early embryos.

En^R-Swift ΔN and dn-Swift. In general, En^R-Swift ΔN and dn-Swift have the same effects on development (Fig. 6 and 8), but the effects of En^R-Swift ΔN are stronger, because En^R-Swift ΔN has a strong transcriptional repressor activity whereas dn-Swift does not (Fig. 8B). We show that low levels of En^R-Swift ΔN block trunk development in a way that is indistinguishable from the effects of high levels of dn-Swift (Fig. 6D and 8G), while high levels of En^R-Swift ΔN block both head and trunk development (Fig. 6D). This dose-responsive severity of developmental defects has been observed in another TGFβ signaling component, FAST (41). Because dn-Swift is less potent than En^R-Swift ΔN, we would have to inject very large amounts of *dn-Swift* mRNA to see a severe head deficiency. However, the injection of such large levels of mRNA results in nonspecific and toxic effects.

Smad coactivators. p300 and CBP are reported as coactivators of Smad2 and Smad3 in mammalian cells (10, 21, 32). p300 and CBP function to regulate transcription and chromatin structure as general transcriptional activators (35). In *Xenopus*, p300 and CBP regulate neurogenesis, and inhibition of their activity blocks mesendoderm induction (23), suggesting that like Swift, p300 and CBP function as coactivators of Smads during early *Xenopus* development. How do both Smad2 coactivators, Swift and p300/CBP, cooperate in TGFβ signaling? We consistently find that Xbra expression and trunk development are very sensitive to Swift function (Fig. 6A and D and 8D to G) and this may be why the effects of En^R-Swift ΔN and dn-Swift are very obvious on Xbra and/or trunk development. This may reflect some aspect of specificity of Swift function between different TGFβ responses. It is possible that Swift functions together or in parallel with p300 and CBP in embryonic TGFβ signaling to efficiently activate gene expression.

The generality of Swift. Swift may define a new class of BRCT domain proteins that function as transcriptional cofactors in TGFβ signaling. GenBank searches during this study revealed two mammalian Swift homologues, mouse PTIP and human CAGF28 (Fig. 1B). Their C-terminal regions share about 80% amino acid sequence identity with that of Swift and contain BRCT domains. The C-terminal BRCT domains of Swift are required for Smad2 binding (Fig. 3B and C), suggesting that PTIP and CAGF28 may also bind to Smad2 and may regulate TGFβ signaling. Interestingly, mouse PTIP binds to the Pax2 DNA-binding protein (27), suggesting that Swift may interact with other DNA-binding proteins as well as with Smad2. FASTs, the other Smad2-binding proteins, identified

Interaction of dn-Swift with Smad2 in yeast. Interaction of dn-Swift with Smad2 using yeast two-hybrid assays was examined by qualitative assays for β-galactosidase activity. (D) dn-Swift functions as a dominant negative. Each embryo was coinjected with various combinations of *ActRIB** (25 pg), *Swift* (0.5 ng), and *dn-Swift* (8 ng) mRNAs at stage 1. Animal caps were explanted at stage 8.5 and analyzed for expression of mesendoderm genes by RNase protection assays at stage 10.25. (E) dn-Swift suppresses activin-induced *Xbra* gene transcription. Each embryo was coinjected with *lacZ, dn-Swift*, or wild-type *Swift* mRNA (4 ng) with *ActRIB** mRNA (50 pg) at stage 1; at stage 10.25, *Xbra* gene induction in animal caps was analyzed by RNase protection assays. (F) In situ hybridization of *Xbra*. Embryos were injected radially in all blastomeres of the four-cell-stage embryo with 2 ng of *dn-Swift* or *lacZ* mRNA per blastomere. Expression of *Xbra* gene was assayed by in situ hybridization. dn-Swift (n = 35), 89% *Xbra* suppression; LacZ (n = 24), 0%. (G) dn-Swift inhibits trunk development. *dn-Swift* (4 ng/embryo) and/or wild-type *Swift* (0.2 ng/embryo) mRNAs with *lacZ* mRNA (0.5 ng/embryo) were injected into dorsal blastomeres of the four-cell-stage embryo, and phenotypes were observed at stage 36. dn-Swift (n = 36), 67% defective trunk development; dn-Swift+Swift (n = 25), 0%. The lineage tracing with β-galactosidase was detected by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining (grey).

first in *Xenopus* (4) and then in mice and humans (25, 44), were found to have a general role in TGFβ signaling in vertebrate development. Thus, like FAST, Swift may be a general component of TGFβ signaling during vertebrate development.

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