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The Role of Heterodimeric AP-1 Protein Comprised of JunD and c-Fos Proteins in Hematopoiesis*[S]

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Sung-Young Lee[‡], Jaeho Yoon[§], Mee-Hyun Lee[‡], Sung Keun Jung[‡], Dong Joon Kim[‡¶], Ann M. Bode[‡], Jaebong Kim[§1], and Zigang Dong[‡2]

From the [‡]Hormel Institute, University of Minnesota, Austin, Minnesota 55912 and [§]Department of Biochemistry, College of Medicine, Hallym University, ChunCheon, Kangwon-Do, 200–702, Korea and the [¶]Medical Genomics Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea

Background: AP-1 (activator protein-1) is a transcription factor comprised of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) family members.
Results: AP-1 comprised of JunD and c-Fos induces hematopoietic gene expression and is regulated by BMP-4.
Conclusion: AP-1$^{JunD/c\text{-}Fos}$ has a crucial role in hematopoiesis and is required for BMP-4-induced hematopoiesis.
Significance: This work provides new insights regarding the role of AP-1 in hematopoiesis.

Activator protein-1 (AP-1) regulates a wide range of cellular processes including proliferation, differentiation, and apoptosis. As a transcription factor, AP-1 is commonly found as a heterodimer comprised of c-Jun and c-Fos proteins. However, other heterodimers may also be formed. The function of these dimers, specifically the heterodimeric AP-1 comprised of JunD and c-Fos (AP-1$^{JunD/c\text{-}Fos}$), has not been elucidated. Here, we identified a function of AP-1$^{JunD/c\text{-}Fos}$ in *Xenopus* hematopoiesis. A gain-of-function study performed by overexpressing *junD* and c-*fos* and a loss-of-function study using morpholino *junD* demonstrate a critical role for AP-1$^{JunD/c\text{-}Fos}$ in hematopoiesis during *Xenopus* embryogenesis. Additionally, we confirmed that JunD of AP-1$^{JunD/c\text{-}Fos}$ is required for BMP-4-induced hematopoiesis. We also demonstrated that BMP-4 regulated JunD activity at the transcriptional regulation and post-translational modification levels. Collectively, our findings identify AP-1$^{JunD/c\text{-}Fos}$ as a novel hematopoietic transcription factor and the requirement of AP-1$^{JunD/c\text{-}Fos}$ in BMP-4-induced hematopoiesis during *Xenopus* hematopoiesis.

Activator protein-1 (AP-1)[3] is an evolutionarily conserved bZip family transcription factor composed of Jun family members (*e.g.* c-Jun, JunB, and JunD) and Fos family members (*e.g.* c-Fos, FosB, Fra-1, and Fra-2). Whereas the Fos proteins can only heterodimerize with members of the Jun family, the Jun proteins can both homodimerize and heterodimerize with Fos members to form transcriptionally active complexes (1). Each of the AP-1 components is differentially expressed and regulated to perform subtly different functions (2). Moreover, different combinations of AP-1 components have been implicated in a large variety of biological processes, including proliferation, differentiation, apoptosis, and development (2–5). However, despite increasing knowledge regarding the physiological functions of AP-1, a specific role for distinct AP-1 components in early embryogenesis has only been partially elucidated (5–8).

In the *Xenopus* embryo, two sites of hematopoiesis are present and include the ventral blood island (VBI), which is analogous to the yolk sac blood islands of higher vertebrates, and the dorsal lateral plate region that is analogous to the aorta, gonads and mesonephros region (AGM) of vertebrates (9). Specifically, the VBI is located on the ventral side of the embryo, and the hematopoietic stem cells of the VBI differentiate primarily into embryonic erythroid cells (primitive hematopoiesis) (9, 10).

BMP-4 (bone morphogenetic protein-4) and transforming growth factor-β (TGF-β) are known to be required during gastrulation for specifying the ventral character of the embryonic mesoderm and are therefore candidates for controlling the development of VBI-derived blood progenitors (11, 12). Ectopic expression of BMP-4 in animal cap explants induces expression of the hematopoietic-specific transcription factor, *SCL* (stem cell leukemia), *GATA-1*, *GATA-2*, and *GATA-3*, *LMO2*, *Neptune*, and globin blood marker (11, 13, 14).

Here, we characterize the novel function of heterodimeric AP-1 comprised of *junD* and c-*fos* (AP-1$^{JunD/c\text{-}Fos}$) in hematopoiesis during *Xenopus* development. Furthermore, we provide evidence showing that AP-1$^{JunD/c\text{-}Fos}$ function is required for BMP-4-induced hematopoiesis. In addition, we demonstrate that BMP-4 controls AP-1$^{JunD/c\text{-}Fos}$ activity through transcriptional and post-translational regulation of *junD* during hematopoiesis.

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[1] To whom correspondence may be addressed: Dept. of Biochemistry, College of Medicine, Hallym University, 1 Okchon-Dong, ChunCheon, Kangwon-Do 200-702, Korea. Tel.: 82-33-248-2544; Fax: 82-33-244-8425; E-mail: jbkim@hallym.ac.kr.
[2] To whom correspondence may be addressed: The Hormel Institute, University of Minnesota, 801 16th Ave. NE, Austin, MN 55912. Tel.: 507-437-9600; Fax: 507-437-9606; E-mail: zgdong@hi.umn.edu.
[3] The abbreviations used are: AP-1, activator protein-1; VBI, ventral blood island; MO, morpholino oligonucleotide; qRT-PCR, quantitative RT-PCR.

EXPERIMENTAL PROCEDURES

Xenopus Embryo Manipulations and Animal Cap Assay—All procedures were followed as described previously (15).

Plasmid Constructs and in Vitro Transcription—*XjunD* (Genbank™ accession no. BC079782) and *Xc-fos* (Genbank™ accession no. BC079689) cDNAs were isolated from *Xenopus* and were inserted into the EcoRI/XbaI site of the pCS2 (+) vector and the FLAG- or HA-tagged pCS2 (+) vector by PCR. For examining *XjunD*-MO specificity, the *XjunD* cDNA was cloned into the pCS2-HA vector at the BamHI/EcoRV site. For rescue experiments, mouse *junD* cDNA was cloned into the pcDNA3.1 (+) vector. Capped mRNAs were synthesized from linearized vectors using with Ambion mMESSAGE Machine kit (Ambion, Austin, TX). The *SCL-mAP1*-luciferase reporter gene, which is mutated at the consensus AP-1 binding site, was generated by site-directed mutagenesis kit (Stratagene, LA Jolla, CA).

Morpholino—The translation-blocking morpholino oligonucleotides (MOs) for *Xenopus junD*, MO1 (5′-CAGGTTAAGGTTCAGATCCTTTTTC-3′) and MO2 (5′-TGATAGAAGGGTATTTCCATC-ATCC-3′), were generated by GeneTools. MO1 and MO2 were designed to block translation of endogenous *XjunD*. For loss of function of *XjunD*, a mixture of two MOs was used and control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′), provided by GeneTools, was used as a toxicity control. Oligonucleotides were resuspended in sterile water and injected at doses of 20 ng per embryo.

qRT-PCR—Total RNA was prepared using the TRIzol reagent (Tel-Test, Inc., Friendswood, TX), and cDNA was synthesized using Superscript pre-amplification system (Invitrogen). The PCR primers and cycling conditions are described at the *Xenopus* Molecular Marker Resource (University of Texas).[4] Additional primers are described in supplemental data. PCR reactions were carried out with SYBR Premix (Qiagen, Valencia, CA) and a thermal cycler real time system (Qiagen Rotor-Gene-Q, Valencia, CA).

Luciferase Assays—All procedures were followed as described previously (15).

Benzidine Staining of Blood—Animal caps were dissected from the injected embryos at stages 8 and 9 and cultured until stage 30. Animal caps were fixed for 5 min in 12% glacial acetic acid containing 0.4% benzidine (Sigma B-3503). The reaction was initiated by the addition of hydrogen peroxide to a final concentration of 0.3% and incubated at room temperature. The reaction was monitored for color development and photographed immediately.

Cell Lineage Tracing—GFP mRNA (200–300 pg) alone or together with mRNA of *junD* and c-*fos* was injected into dorsal animal blastomeres (D1) or ventral animal blastomeres (V1) of 8-cell stage embryos. Injected embryos were cultured to stages 27–30. The embryos were rinsed several times with 1× PBS and fixed for 1 h at room temperature in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) and stored at 4 °C. Fixed embryos were observed with a fluorescent microscope (15).

Whole Mount in Situ Hybridization—Embryos were injected with the indicated mRNAs and then processed for whole-mount *in situ* hybridization by using standard methods (16) with α-globin probes.

Western Blot Analysis—All procedures were followed as described previously (15). For Western blotting, anti-HA-peroxidase conjugate (Roche Applied Science), anti-phospho-Jun (α-73) (Cell Signaling, Danvers, MA), and monoclonal anti-actin (Sigma) were used. Proteins were visualized using ECL Western blotting detection reagents (Amersham Biosciences).

RESULTS

AP-1$^{JunD/c\text{-}Fos}$ Has a Role in Hematopoiesis—To define the function of AP-1$^{JunD/c\text{-}Fos}$ in hematopoiesis, we analyzed the gene expression profile induced by AP-1$^{JunD/c\text{-}Fos}$ in embryonic stem cells of *Xenopus* animal caps explants. Co-expression of *junD* and c-*fos* induced hematopoietic transcription factors, *GATA-1*, *SCL*, *LMO-2*, and *Neptune*, which are involved in the initial blood program (Fig. 1*A*), and α-globin, the erythrocyte specific marker, in a dose-dependent manner (Fig. 1*B*). Otherwise, *junD* or c-*fos* mRNA alone could not induce globin (supplemental Fig. 1). Hematopoietic transcription factors such as *GATA-1*, *SCL*, *LMO-2*, and *Neptune* alone do not induce hematopoietic cells from the embryonic ectoderm. Interestingly, AP-1$^{JunD/c\text{-}Fos}$ alone is sufficient to induce blood cell formation from the ectoderm similar to BMP-4 signaling.

Additionally, we examined whether AP-1$^{JunD/c\text{-}Fos}$ could regulate the transcription of *SCL* using an *SCL* promoter-luciferase reporter gene. AP-1$^{JunD/c\text{-}Fos}$ enhanced the promoter activity of this gene (Fig. 1*C*) but not the activity of *SCL*-mAP-1-luciferase, which is mutated at the consensus AP-1 binding site, suggesting the involvement of AP-1$^{JunD/c\text{-}Fos}$ in the transcriptional regulation of the *SCL* gene.

To further determine whether AP-1$^{JunD/c\text{-}Fos}$ is indeed necessary for hematopoiesis in *Xenopus laevis* development, we performed a loss-of-function study using morpholino antisense directed against *junD* (MO-junD). We generated two types of antisense morpholino oligonucleotides (MO1 and MO2) capable of depleting the XJunD protein. The MO-junDs were effective in specifically reducing the level of the 3′-terminal end HA-tagged XJunD protein (XJunD-HA) (Fig. 1*D*), whereas the control *MO* had no effect on the translation of *XjunD* mRNA (supplemental Fig. 2*A*). qRT-PCR analysis showed that the *junD*-depleted embryos repressed the expression of *SCL* and globin compared with control embryos without affecting the dorsal mesoderm marker, actin. Additionally, co-injection of mRNA of mouse *junD* (*mjunD*) rescued these markers that had been repressed by MO-junDs (Fig. 1*E*). The control *MO*-injected embryos had no effect on the expression of these markers (supplemental Fig. 2*B*). Furthermore, *XjunD*- or control *MO* were injected into one blastomere of two-cell embryos, together with the lineage tracer β-galactosidase (200 pg; Fig. 1*F*). Embryos were cultured until stage 32 and stained for β-galactosidase activity to confirm the accuracy of injections (*blue* staining, Fig. 1, *G* and *H*), followed by *in situ* hybridization to detect expression of globin, a marker of differentiated red blood cells (RBCs) (*purple* staining, Fig. 1, *G* and *H*). Embryos injected with MO-*junD* appeared normal but showed a severe repres-

[4] E. M. De Robertis, personal communication.

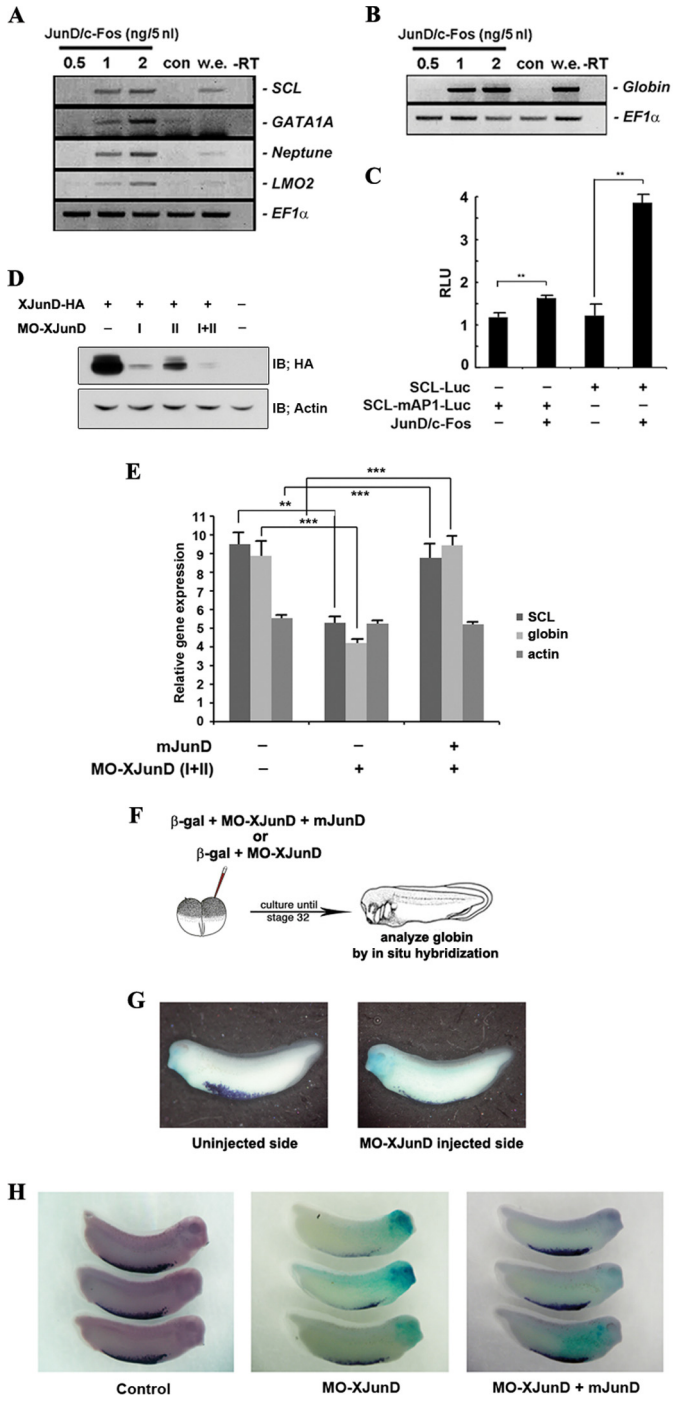

FIGURE 1. **AP-1$^{JunD/c\text{-}Fos}$ is required for hematopoiesis.** *A* and *B*, co-expression of *junD* and c-*fos* induces hematopoietic makers (*SCL*, *GATA1A*, *Neptune*, and *LMO2*) and globin. Animal caps, explanted from embryos injected with the indicated concentration of mRNAs encoding *junD* or/and c-*fos*, were incubated until stages 18–20 (*A*) or 24–28 (*B*) and used for qRT-PCR analysis. *C*, co-expression of *junD* and c-*fos* enhances the promoter activities of the *SCL* but not the mutant *SCL*-mAP1. Embryos injected with the *SCL*- or *SCL*-mAP1-luciferase reporter gene alone or together with 2 ng of *junD* and c-*fos* were incubated until stages 18–20. Luciferase activity was measured. Values are shown as means ± S.D. from at least three independent experiments. *RLU*, relative luciferase activity. *D*, MO *junD* (20 ng) specifically knocks down the translation of the overexpressed C-terminal HA-tagged XJunD protein at stage 18. Actin served as a specificity control. *E*, mouse *junD* rescues *SCL* and globin, which are repressed by MO *junD* expression without changing the dorsal mesoderm marker, actin. qRT-PCR analysis of whole embryos expressing MO *junD* (20 ng) alone or in combination with 1 ng of mouse *junD* at stages 20–24. *F*, illustration of the scheme of the experiment. One blastomere of two-cell embryos was injected with mRNA encoding β-galactosidase together with MO *junD* (20 ng) or mouse *junD* (1 ng) (*mjunD*) as illustrated. *G* and *H*, embryos were stained for β-galactosidase (*β-gal*) activity at stage 30 (blue stain) followed by *in situ* hybridization analysis of globin expression (*purple* stain). *G*, the expression of globin is repressed in the MO *junD*-injected side. *H*, mouse *junD* mRNA (*mjunD*) rescues globin expression that is repressed by expression of MO *junD*. EF1α, loading control; *w.e.*, whole embryo was used as a positive control for PCR; *−RT*, control reaction without reverse transcriptase; *con*, animal cap samples obtained from non-injected embryos. **, p value < 0.01; ***, p value < 0.001. *IB*, immunoblot.

sion of globin expression in the MO-*junD*-injected side relative to those of non-injected side or control *MO*-injected side (Fig. 1*G*). Expression of globin was effectively rescued by co-injection of mouse *junD* (Fig. 1*H*). Taken together, these results indicate that AP-1$^{JunD/c\text{-}Fos}$ is required for normal primitive erythropoiesis of *X. laevis*.

AP-1$^{JunD/c\text{-}Fos}$ Converts Dorsal-fated Tissue into Ventral-fated tissue, Specifically VBI—To elucidate the *in vivo* function of AP-1$^{JunD/c\text{-}Fos}$ during *Xenopus* embryogenesis, we performed a cell lineage tracing experiment. Embryos at the eight-cell stage were injected with GFP mRNA alone or together with *AP-1* mRNA (*junD* and c-*fos*) into animal blastomeres at the dorsal portion of the embryos (D1) (Fig. 2*A*). Two of the dorsal animal blastomeres at the eight-cell stage embryo (D1) differentiated into dorsal and anterior neural tissues. Thus, when GFP mRNA alone was injected into the D1 region, fluorescent cells were detected in dorsal and anterior structures (Fig. 2*B*). However, when GFP and a low dose of *AP-1$^{JunD/c\text{-}Fos}$* mRNAs (500 pg) were co-injected into the D1 regions, fluorescent cells were detected in the VBI region but also remained in the dorsal region (Fig. 2*C*). The VBI is indicated by *in situ* hybridization of globin (Fig. 2, *B* and *C*, *lower panels*). When *AP-1$^{JunD/c\text{-}Fos}$* mRNAs were injected into animal blastomeres of the ventral part of the embryos (V1) at the eight-cell stage as a control experiment (Fig. 2*D*), GFP was expressed at the ventroposterior epidermis (Fig. 2*E*) (17, 18). A similar pattern was seen with control embryos injected with GFP mRNA into the ventral part of animal blastomeres (Fig. 2*F*). These results indicate that *junD* and c-*fos* not only induce hematopoiesis but also have the ability to change a part of dorsal fated tissue into VBI tissue in whole embryos.

AP-1$^{JunD/c\text{-}Fos}$ Is Required for the BMP-4-induced Hematopoiesis—BMP-4 is a TGF-β family member that has been shown to be an essential factor in the differentiation of primitive blood cells in the *X. laevis* embryo.

To further verify the role of AP-1$^{JunD/c\text{-}Fos}$ in hematopoiesis, we investigated whether the role of AP-1$^{JunD/c\text{-}Fos}$ in blood formation is associated with BMP-4. First, we determined whether AP-1$^{JunD/c\text{-}Fos}$ and BMP-4 have synergistic effects in hematopoiesis. For analyzing the synergistic effect between AP-1 and BMP-4, we used a low dose of mRNA. A low dose of *AP-1$^{JunD/c\text{-}Fos}$* or *BMP-4* alone had no apparent effect on the expression of hematopoietic markers (Fig. 3*A*). As indicated earlier, for hematopoietic markers and globin expression, a high dose of *BMP-4* mRNA is required in animal cap explants. A combination of *AP-1$^{JunD/c\text{-}Fos}$* and *BMP-4* resulted in a marked increase in the level of hematopoietic markers, including globin (Fig. 3*A*). The expression of hematopoietic markers and globin induced by the combination of AP-1$^{JunD/c\text{-}Fos}$ and BMP-4 is higher than the additive value of each alone. To fur-

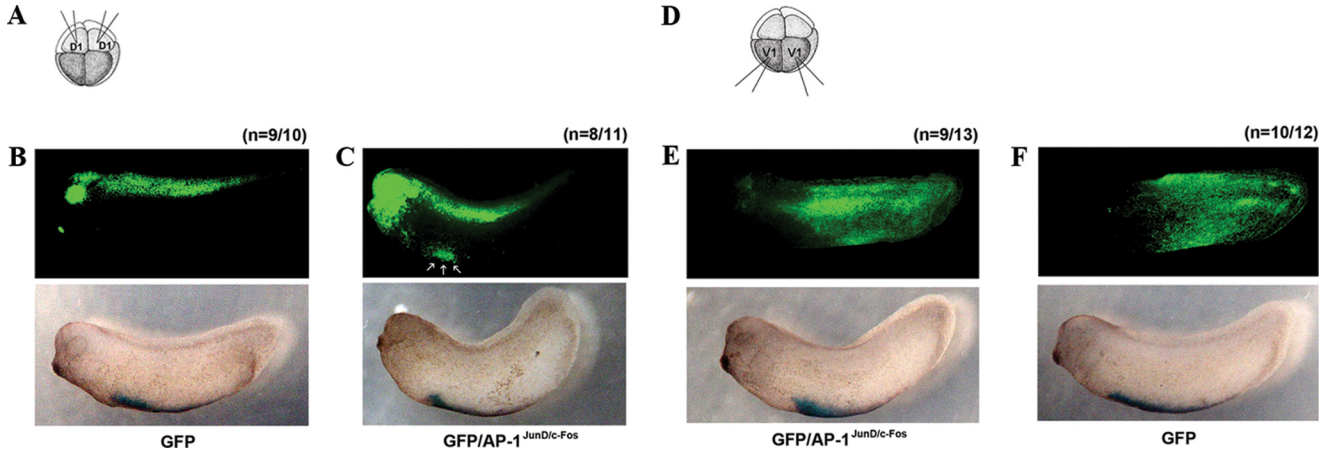

FIGURE 2. **AP-1$^{JunD/c\text{-}Fos}$ converts dorsal-fated tissue into ventral-fated tissue (ventral blood island).** *A* and *D*, illustration of the scheme of the experiment. GFP mRNA (200 pg, *B*) only (*B* and *F*) or GFP together with *AP-1* (*junD* and *c-fos*, 500 pg) mRNA (*C* and *E*) were injected into dorsal animal blastomeres (D1) or ventral animal blastomeres (V1) of eight-cell stage embryos and then cultured until stages 28–30. Embryos were fixed, and GFP expression was observed by green fluorescent microscopy. *B* and *C*, dorsally expressed GFP (*B*, *upper panel*) is partially transferred into the ventral blood island region (*C*, *three arrows* in *upper panel*). *E* and *F*, embryos injected with either GFP alone (*F*, *upper panel*) or together with AP-1$^{JunD/c\text{-}Fos}$ (*E*, *upper panel*) show a similar expression pattern of GFP, which is expressed at the ventroposterior epidermis. The ventral blood island is indicated by *in situ* hybridization of globin (*B–F*, *lower panel*). The number (*n*) of phenotypes for each group is presented.

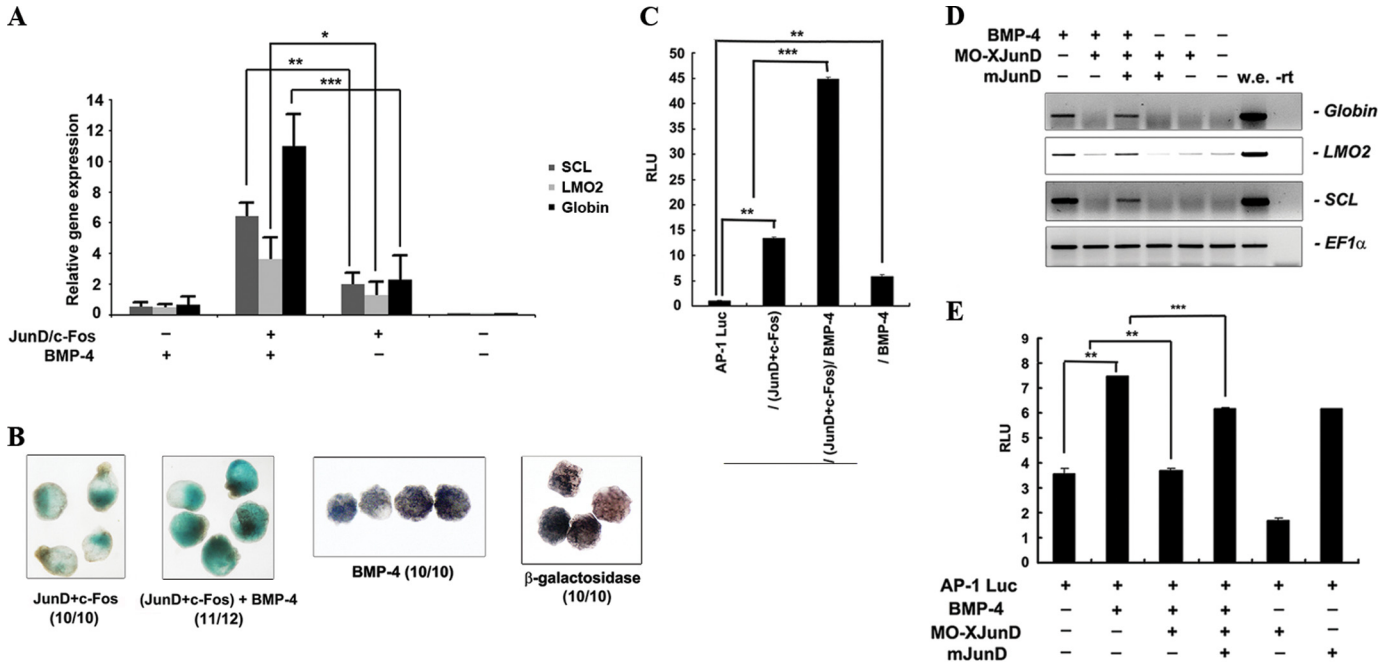

FIGURE 3. **AP-1$^{JunD/c\text{-}Fos}$ is required for hematopoiesis induced by BMP-4.** *A* and *B*, animal caps, explanted from embryos injected with the indicated mRNAs were incubated until stage 20–24 and used for qRT-PCR analysis (*A*) or benzidine staining (*B*). *A*, AP-1$^{JunD/c\text{-}Fos}$ and BMP-4 synergistically induce hematopoietic markers and globin. *B*, blood formation stained by benzidine is synergistically enhanced by AP-1$^{JunD/c\text{-}Fos}$ and BMP-4. *C*, the activity of the $(AP\text{-}1)_4$-luciferase reporter gene is synergistically activated by co-injection of *AP-1*$^{JunD/c\text{-}Fos}$ and *BMP-4*. An $(AP\text{-}1)_4$-luciferase assay using animal cap explants derived from embryos injected with the $(AP\text{-}1)_4$-luciferase reporter gene alone or in combination with the indicated mRNA was performed. Luciferase activity was measured at stage 18. Values are averages from at least three independent experiments. *RLU*, relative luciferase activity. *D*, animal caps, explanted from embryos injected with the indicated mRNAs or MO *junD* (20 ng), were incubated until stage 20–24 and used for qRT-PCR analysis. MO JunD selectively blocks BMP-4-induced expression of globin, *LMO2*, and *SCL*. Injection of mouse *junD* mRNA rescues the BMP-4 induction of globin, *LMO2*, and *SCL* as well as the activity of the $(AP\text{-}1)_4$-luciferase reporter gene in MO *junD*-injected animal caps (*E*). EF1α, loading control; *w.e.*, whole embryo was used as a positive control for PCR; $-rt$, control reaction without reverse transcriptase. **, p value < 0.01; ***, p value < 0.001.

ther confirm this finding, we performed benzidine staining, a specific histochemical stain for differentiated red blood cells. Animal caps derived from embryos injected with β-galactosidase as a control (Fig. 3*B*, *fourth panel*) were not stained by benzidine. The low dose of BMP-4-injected animal caps also did not exhibit detectable benzidine staining (Fig. 3*B*, *third panel*). However, consistent with the qRT-PCR data, a combination of AP-1$^{JunD/c\text{-}Fos}$ and BMP-4 enhanced benzidine staining (Fig. 3*B*, *second panel*) compared with each alone (Fig. 3*B*, *first panel*). These results indicate a synergistic effect of AP-1$^{JunD/c\text{-}Fos}$ and BMP-4 on blood formation.

On the basis of these results, we examined whether BMP signaling acts upstream of AP-1$^{JunD/c\text{-}Fos}$ during blood formation. We first determined whether BMP-4 could regulate AP-1 activity in an animal cap assay. The activity of the $(AP\text{-}1)_4$-luciferase reporter gene, which contains four AP-1 binding

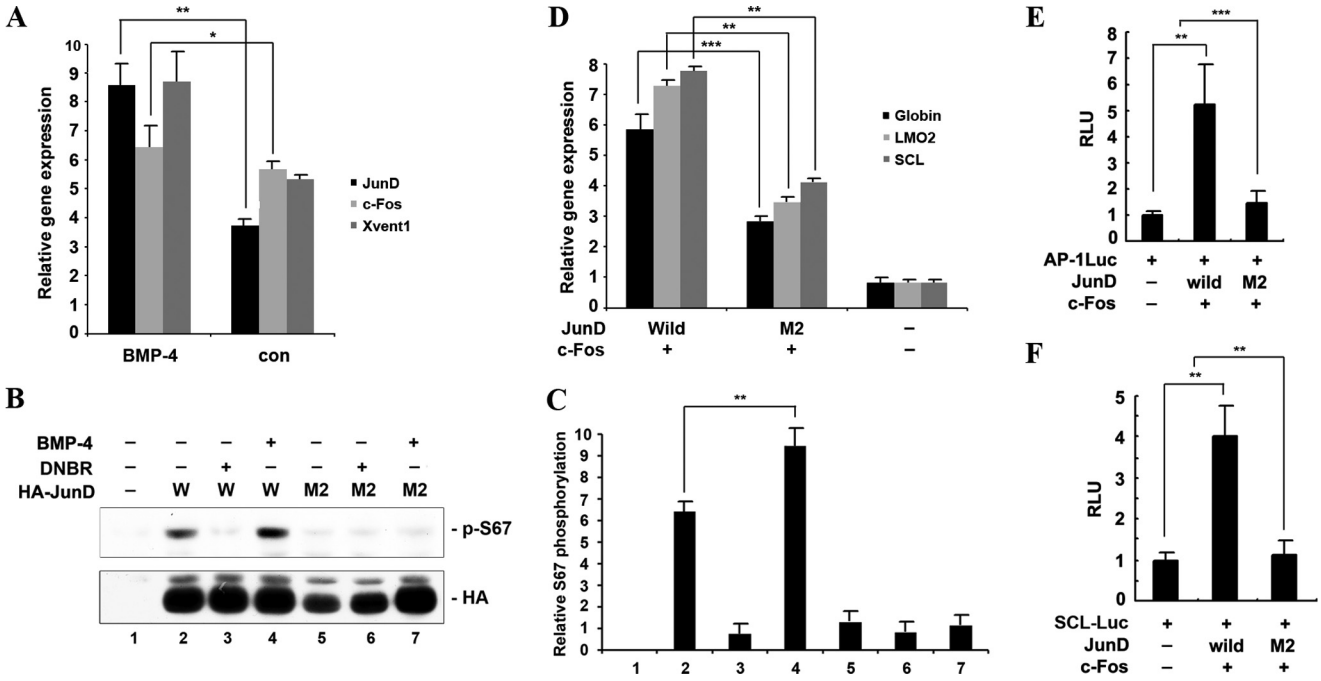

FIGURE 4. **Transcriptional and post-translational modification of AP-1$^{JunD/c\text{-}Fos}$ by BMP-4 is required for hematopoiesis.** *A*, BMP-4 activates the transcription of *junD*, but not c-*fos*. *Xvent1* (ventral marker) was used as a positive control for BMP-4. Animal caps derived from embryos injected with the indicated mRNA were excised and cultured until stage 13 and used for qRT-PCR analysis. *con*, animal cap samples obtained from non-injected embryos. *B*, BMP-4 enhances phosphorylation of XJunD at serine 67; in contrast, dominant-negative BMP receptor inhibits the phosphorylation of XJunD. Embryos injected with wild-type HA-*junD* (*W*) or mutant HA-*junD* (M2) alone or in combination with 1 ng of *BMP-4* or dominant-negative BMP receptor mRNA were used for Western blotting. Phosphorylation of JunD was analyzed by Western blot using anti-phospho-Jun (α-73). Western blotting with anti-HA shows that equal amounts of expressed JunD were loaded. *C*, band density was measured using the NIH ImageJ program. *D–F*, animal caps, explanted from embryos injected with the indicated mRNAs or in combination with the $(AP\text{-}1)_4$-luciferase reporter gene or *SCL*-luciferase reporter gene, were used for qRT-PCR analysis (*D*) and the luciferase assay (*E* and *F*). The concentration of each mRNA injected into embryos was 1 ng. *D*, AP-1$^{M2JunD/c\text{-}Fos}$ shows a lower induction of hematopoietic markers and globin, compared with AP-1$^{JunD/c\text{-}Fos}$. *E* and *F*, the AP-1- and *SCL*-luciferase activities are consistent with *D*. Data are shown as means ± S.D. of values from at least three independent experiments. *RLU*, relative luciferase activity. **, p value < 0.01; ***, p value < 0.001.

sequences (TGAC/GTCA), was enhanced by AP-1 (*junD* and c-*fos*) or *BMP-4* alone. These results suggest that BMP-4 regulates AP-1$^{JunD/c\text{-}Fos}$ activity.

Additionally, co-injection of *AP-1* and *BMP-4* caused a synergistic effect on the activation of AP-1 (Fig. 3*C*). This result is consistent with the synergistic effect of AP-1$^{JunD/c\text{-}Fos}$ and BMP-4 on blood formation of *Xenopus* embryos.

To confirm whether AP-1$^{JunD/c\text{-}Fos}$ is indeed necessary for BMP-4-induced blood formation, we investigated whether depletion of *junD* could inhibit the expression of BMP-4-induced hematopoietic markers. *MO-junD* (Fig. 3*D*) but not control *MO* (supplemental Fig. 3) effectively inhibited the BMP-4-induced expression of hematopoietic transcription markers (*SCL* and *LMO2*), including globin. Consistent with this result, the activity of the *AP-1 reporter* gene that was enhanced by BMP-4 was suppressed by depletion of *junD* (Fig. 3*E*, *lanes 1–3*). Additionally, the expression of hematopoietic markers and the activity of the *AP-1* reporter gene, which were suppressed by MO-*junD* in the presence of BMP-4, were effectively rescued by co-overexpression of wild-type *mjunD* mRNA (Fig. 4, *D* and *E*). Taken together, these data support the idea that AP-1$^{JunD/c\text{-}Fos}$ is required for BMP-4-mediated hematopoiesis during *X. laevis* development.

Phosphorylation and Transcriptional Regulation of JunD by BMP-4 Is Important for the Function of AP-1$^{JunD/c\text{-}Fos}$ in Hematopoiesis—As demonstrated above, the biological ability of AP-1$^{JunD/c\text{-}Fos}$ is required for BMP-4-induced hematopoiesis. To study whether BMP-4 regulates AP-1$^{JunD/c\text{-}Fos}$ in the embryo, we investigated transcriptional regulation of AP-1$^{JunD/c\text{-}Fos}$ by BMP-4. Interestingly, BMP-4 increased the transcription of *junD*, but not c-*fos* (Fig. 4*A*). *Xvent1* was used for positive control of BMP-4 (19, 20). Additionally, we investigated whether BMP-4 could phosphorylate JunD in embryos. Serine phosphorylation is a mechanism for regulating AP-1-dependent gene transcription (21, 22). A phospho-Jun antibody (α-73) was used for recognizing *junD* only if it is phosphorylated at serine 100 (23, 24). To examine phosphorylation of *junD* by BMP-4, we generated HA-tagged wild-type *junD* and mutant (M2) *junD* in which Ser-66, a sequence conserved with Ser-100 of *mouse junD*, was replaced with alanine. Phosphorylation of Ser-66 of *JunD* was detected using a phospho-Jun antibody (α-73). BMP-4 stimulation enhanced JunD phosphorylation at Ser-66, whereas dominant-negative BMP receptor efficiently inhibited phosphorylation of JunD (Ser-66) (Fig. 4*B*). The M2 mutant was not affected by BMP-4 or dominant-negative BMP receptor (Fig. 4*B*). The band density was measured using the NIH ImageJ program (Fig. 4*C*). This result indicates that BMP-4 is sufficient to regulate Ser-66 phosphorylation of JunD.

To study the biological role of Ser-66 phosphorylation in hematopoiesis of the *X. laevis* embryo, we compared the activity of mutant M2 *junD* and wild-type *JunD* in hematopoiesis of *Xenopus* embryos. Compared with wild-type *junD* and c-*fos* (AP-1$^{JunD/c\text{-}Fos}$), the mutant *junD* (M2) and c-*fos*

(AP-1$^{M2JunD/c\text{-}Fos}$) induced hematopoietic markers at a lower level as determined by an animal cap assay (Fig. 4*D*). Consistently, both *AP-1*- and *SCL*-luciferase activities were less activated by AP-1$^{M2JunD/c\text{-}Fos}$ compared with AP-1$^{JunD/c\text{-}Fos}$ (Fig. 4, *E* and *F*). Taken together, the results suggest that transcriptional regulation and phosphorylation by BMP-4 is important for the biological role of AP-1$^{JunD/c\text{-}Fos}$ in hematopoiesis of *Xenopus* development.

DISCUSSION

In the current study, we provide evidence for a novel function of the transcription factor AP-1$^{JunD/c\text{-}Fos}$ in hematopoiesis during *Xenopus* development. Furthermore, we suggest that transcriptional and post-translational regulation of *junD*, which is induced by BMP-4, is important for the biological function of AP-1$^{JunD/c\text{-}Fos}$ in hematopoiesis (supplemental Fig. 4).

The transcription factor AP-1 is composed of different possible dimer combinations formed between the Jun and Fos family of "bZip" transcription factors. These different AP-1 dimers exhibit similar DNA binding specificities (TGAC/GTCA) but have differences in their transactivation efficiencies (5–8), indicating that distinct AP-1 dimers differentially regulate AP-1 target genes. Thus, we hypothesized that the composition of AP-1 would determine the expression of specific target genes and allow prediction of the nature of the dimeric combinations, which are physiologically relevant under various conditions.

Previously, we have shown that AP-1 is involved in mesoderm induction as a major downstream mediator of FGF signaling (25, 26). Moreover, we demonstrated that heterodimeric AP-1, comprised of c-Jun and c-Fos, mediated activin-induced Spemann organizer gene expression (15). We also reported the involvement of AP-1 in BMP-4 signaling and in BMP-4 expression (26, 27). Although the role of other AP-1 components has been reported in *X. laevis* development, the physiological function of heterodimeric AP-1 comprised of JunD and c-Fos has not yet been elucidated in *X. laevis* development. In the present study, we elucidated the function of heterodimeric AP-1 comprised of JunD and c-Fos in hematopoiesis of *X. laevis* development.

To determine the specificity of *junD* in BMP-4-induced hematopoesis, we examined whether c-*jun* had an effect in BMP-4-induced hematopoiesis. Depletion of c-*jun* using MO c-*jun* had no effect on BMP-4-induced hematopoiesis; in contrast, MO c-*jun* effectively inhibited activin-induced gene expression (15), suggesting a specific biological function of AP-1$^{JunD/c\text{-}Fos}$ downstream of BMP-4 (supplemental Fig. 5). Additionally, depletion of *junD* using MO *junD* had no effect on activin-induced dorsal mesoderm and endoderm formation (supplemental Fig. 6). Taken together, these results indicated that c-Jun and JunD have distinct roles downstream of activin and BMP-4, suggesting that specific AP-1 composition regulated by diverse signaling determines the expression of specific target genes.

Transcription factors are tightly regulated at the transcriptional, post-transcriptional, and post-translational levels. In the current studies, we discovered that AP-1$^{JunD/c\text{-}Fos}$ is regulated downstream of BMP-4 signaling at the transcriptional and post-translational levels, and the regulation is important for AP-1$^{JunD/c\text{-}Fos}$-induced hematopoiesis. Serine phosphorylation is a representative mechanism for regulating AP-1-dependent gene transcription (21–22). Although numerous studies on the phosphorylation of c-Jun have been reported, JunD phosphorylation and its possible regulatory function have not been well characterized. Previously, JunD has been shown to be phosphorylated *in vivo* and *in vitro* (23–24, 28–31). The N terminus of *junD* contains three sites for MAP kinase phosphorylation (serines 90 and 100 and threonine 117), which are essentially identical to the well characterized regulatory phosphorylation sites of c-Jun (serines 63 and 73 and threonine 91). These sites share a conserved sequence with serines 56 and 66 and threonine 83 of *Xenopus* JunD. Generally, phosphorylation of serine 100 of JunD was studied to examine JunD activity because the phospho-Jun antibody (α-73) recognizes phosphorylated serine 100 of JunD. Thus, in the present study, we confirmed that phosphorylation of serine 66 of *Xenopus* JunD, which is identical to serine 100 of mouse JunD, was also detected with the phospho-Jun antibody (α-73). Results indicated that BMP-4 signaling could regulate phosphorylation of serine 66 of *Xenopus* JunD. However, the kinase activated by BMP-4 that is responsible for phosphorylating JunD at serine 66 remains to be identified in future studies (supplemental Fig. 4).

Additionally, we characterized the biological role of serine 66 of XJunD in hematopoiesis. Overexpression of the phosphorylation mutant form of AP-1$^{M2JunD/c\text{-}Fos}$ partially retained activity for its biological role in hematopoiesis, suggesting that other phosphorylation sites might also contribute to the complete biological function of AP-1$^{JunD/c\text{-}Fos}$ in hematopoiesis.

In the present study, we suggested a new function for AP-1 in hematopoiesis during *Xenopus* development providing additional evidence of a specific role for individual AP-1 members in distinct cellular process. Furthermore, we suggest that AP-1$^{JunD/c\text{-}Fos}$ functions downstream of BMP-4 signaling and BMP-4 alters AP-1$^{JunD/c\text{-}Fos}$ function at the post-translational level by phosphorylation of JunD at serine 66.

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REFERENCES

1. Angel, P., and Karin, M. (1991) The role of Jun, Fos, and the AP-1 complex in cell proliferation and transformation. *Biochim. Biophys. Acta* **1072,** 129–157
2. Jochum, W., Passegué, E., and Wagner, E. F. (2001) AP-1 in mouse development and tumorigenesis. *Oncogene* **20,** 2401–2412
3. Ameyar, M., Wisniewska, M., and Weitzman, J. B. (2003) A role for AP-1 in apoptosis: The case for and against. *Biochimie* **85,** 747–752
4. Shaulian, E., and Karin, M. (2002) AP-1 as a regulator of cell life and death. *Nat. Cell Biol.* **4,** E131–136
5. Shaulian, E., and Karin, M. (2001) AP-1 in cell proliferation and survival. *Oncogene* **20,** 2390–2400
6. Chinenov, Y., and Kerppola, T. K. (2001) Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene* **20,** 2438–2452
7. Tulchinsky, E. (2000) Fos family members: Regulation, structure, and role in oncogenic transformation. *Histol. Histopathol.* **15,** 921–928
8. Vogt, P. K. (2001) Jun, the oncoprotein. *Oncogene* **20,** 2365–2377
9. Mangia, F., Procicchiami, G., and Manelli, H. (1970) On the development of the blood island in Xenopus laevis embryos: light and electron micro-

scope study. *Acta Embryol. Exp. (Palermo)* **2,** 163–184
10. Kau, C. L., and Turpen, J. B. (1983) Dual contribution of embryonic ventral blood island and dorsal lateral plate mesoderm during ontogeny of hemopoietic cells in *Xenopus laevis*. *J. Immunol.* **131,** 2262–2266
11. Maéno, M. (2003) Regulatory signals and tissue interactions in the early hematopoietic cell differentiation in *Xenopus laevis* embryo. *Zoolog. Sci.* **20,** 939–946
12. Maeno, M., Mead, P. E., Kelley, C., Xu, R. H., Kung, H. F., Suzuki, A., Ueno, N., and Zon, L. I. (1996) The role of BMP-4 and GATA-2 in the induction and differentiation of hematopoietic mesoderm in *Xenopus laevis*. *Blood* **88,** 1965–1972
13. Huber, T. L., and Zon, L. I. (1998) Transcriptional regulation of blood formation during *Xenopus* development. *Semin. Immunol.* **10,** 103–109
14. Perry, C., and Soreq, H. (2002) Transcriptional regulation of erythropoiesis. Fine tuning of combinatorial multidomain elements. *Eur. J. Biochem.* **269,** 3607–3618
15. Lee, S. Y., Yoon, J., Lee, H. S., Hwang, Y. S., Cha, S. W., Jeong, C. H., Kim, J. I., Park, J. B., Lee, J. Y., Kim, S., Park, M. J., Dong, Z., and Kim, J. (2011) The function of heterodimeric AP-1 comprised of c-Jun and c-Fos in activin mediated Spemann organizer gene expression. *PloS one* **6,** e21796
16. Moore, K. B., Mood, K., Daar, I. O., and Moody, S. A. (2004) Morphogenetic movements underlying eye field formation require interactions between the FGF and ephrinB1 signaling pathways. *Dev. Cell* **6,** 55–67
17. Itoh, K., Tang, T. L., Neel, B. G., and Sokol, S. Y. (1995) Specific modulation of ectodermal cell fates in *Xenopus* embryos by glycogen synthase kinase. *Development* **121,** 3979–3988
18. Huang, S., Johnson, K. E., and Wang, H. Z. (1998) Blastomeres show differential fate changes in 8-cell *Xenopus laevis* embryos that are rotated 90 degrees before first cleavage. *Dev. Growth Differ.* **40,** 189–198
19. Friedle, H., Rastegar, S., Paul, H., Kaufmann, E., and Knöchel, W. (1998) Xvent-1 mediates BMP-4-induced suppression of the dorsal-lip-specific early response gene XFD-1′ in Xenopus embryos. *EMBO J.* **17,** 2298–2307
20. Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C., and Niehrs, C. (1995) Antagonizing the Spemann organizer: Role of the homeobox gene Xvent-1. *EMBO J.* **14,** 6268–6279
21. Weston, C. R., and Davis, R. J. (2002) The JNK signal transduction pathway. *Curr. Opin. Genet. Dev.* **12,** 14–21
22. Yazgan, O., and Pfarr, C. M. (2002) Regulation of two JunD isoforms by Jun N-terminal kinases. *J. Biol. Chem.* **277,** 29710–29718
23. Gallo, A., Cuozzo, C., Esposito, I., Maggiolini, M., Bonofiglio, D., Vivacqua, A., Garramone, M., Weiss, C., Bohmann, D., and Musti, A. M. (2002) Menin uncouples Elk-1, JunD, and c-Jun phosphorylation from MAP kinase activation. *Oncogene* **21,** 6434–6445
24. Vinciguerra, M., Vivacqua, A., Fasanella, G., Gallo, A., Cuozzo, C., Morano, A., Maggiolini, M., and Musti, A. M. (2004) Differential phosphorylation of c-Jun and JunD in response to the epidermal growth factor is determined by the structure of MAPK targeting sequences. *J. Biol. Chem.* **279,** 9634–9641
25. Dong, Z., Xu, R. H., Kim, J., Zhan, S. N., Ma, W. Y., Colburn, N. H., and Kung, H. (1996) AP-1/jun is required for early *Xenopus* development and mediates mesoderm induction by fibroblast growth factor but not by activin. *J. Biol. Chem.* **271,** 9942–9946
26. Kim, J., Lin, J. J., Xu, R. H., and Kung, H. F. (1998) Mesoderm induction by heterodimeric AP-1 (c-Jun and c-Fos) and its involvement in mesoderm formation through the embryonic fibroblast growth factor/Xbra autocatalytic loop during the early development of *Xenopus* embryos. *J. Biol. Chem.* **273,** 1542–1550
27. Xu, R. H., Dong, Z., Maeno, M., Kim, J., Suzuki, A., Ueno, N., Sredni, D., Colburn, N. H., and Kung, H. F. (1996) Involvement of Ras/Raf/AP-1 in BMP-4 signaling during *Xenopus* embryonic development. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 834–838
28. Stocco, C. O., Lau, L. F., and Gibori, G. (2002) A calcium/calmodulin-dependent activation of ERK1/2 mediates JunD phosphorylation and induction of nur77 and 20α-hsd genes by prostaglandin F2α in ovarian cells. *J. Biol. Chem.* **277,** 3293–3302
29. Rosenberger, S. F., Finch, J. S., Gupta, A., and Bowden, G. T. (1999) Extracellular signal-regulated kinase 1/2-mediated phosphorylation of JunD and FosB is required for okadaic acid-induced activator protein 1 activation. *J. Biol. Chem.* **274,** 1124–1130
30. Smart, D. E., Green, K., Oakley, F., Weitzman, J. B., Yaniv, M., Reynolds, G., Mann, J., Millward-Sadler, H., and Mann, D. A. (2006) JunD is a profibrogenic transcription factor regulated by Jun N-terminal kinase-independent phosphorylation. *Hepatology* **44,** 1432–1440
31. Wang, H., Xie, Z., and Scott, R. E. (1996) JunD phosphorylation and expression of AP-1 DNA binding activity modulated by serum growth factors in quiescent murine 3T3T cells. *Oncogene* **13,** 2639–2647