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The Proto-oncogene Transcription Factor Ets1 Regulates Neural Crest Development through Histone Deacetylase 1 to Mediate Output of Bone Morphogenetic Protein Signaling*

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Background: A proper level of BMP signal is essential for the maintenance of neural crest cells.
Results: Ets1 cooperates with HDAC1 to down-regulate BMP signaling output and reduce *id3* expression.
Conclusion: Ets1 regulates neural crest development through epigenetic control of BMP signaling.
Significance: This is the first study linking Ets1 to BMP signaling.

The neural crest (NC) is a transient, migratory cell population that differentiates into a large variety of tissues including craniofacial cartilage, melanocytes, and peripheral nervous system. NC is initially induced at the border of neural plate and non-neural ectoderm by balanced regulation of multiple signaling pathways among which an intermediate bone morphogenetic protein (BMP) signaling is essential for NC formation. *ets1*, a proto-oncogene playing important roles in tumor invasion, has also been implicated in delamination of NC cells. In this study, we investigated Ets1 function in NC formation using *Xenopus*. Overexpression of *ets1* repressed NC formation through down-regulation of BMP signaling. Moreover, *ets1* repressed the BMP-responsive gene *id3* that is essential for NC formation. Conversely, overexpression of *id3* can partially rescue the phenotype of NC inhibition induced by ectopic *ets1*. Mechanistically, we found that Ets1 binds to *id3* promoter as well as histone deacetylase 1, suggesting that Ets1 recruits histone deacetylase 1 to the promoter of *id3*, thereby inducing histone deacetylation of the *id3* promoter. Thus, our studies indicate that Ets1 regulates NC formation through attenuating BMP signaling epigenetically.

The neural crest (NC)[2] is a transient population of cells unique to vertebrates during embryonic development that is pluripotent and highly migratory. Arising at the border between neural plate and non-neural ectoderm, NC cells migrate extensively and differentiate into various tissues (1). The initial induction of NC occurs during gastrulation in response to the combined effects of bone morphogenetic protein (BMP), Wnt, FGF, retinoic acid, and Notch signals (2–6). These signaling pathways orchestrate and firstly activate neural plate border (NPB) specifiers including Pax3, Msx1 (7), and Zic1 (8), which establish a broad competence domain at the NPB. The cooperation of NPB specifiers, especially Pax3 and Zic1 (8, 9), induces more restrictedly localized NC specifiers including c-Myc (10), Foxd3 (11, 12), Sox9 (13), Snail1 (14, 15), Snail2 (16), Tfap2 (17), and Id3 (18, 19), which coordinate each other to regulate NC delamination and migration. The gene regulatory network of NC formation is conserved across vertebrate including zebrafish, frog, and mouse (20).

Among the mentioned signaling pathways, BMPs are postulated to pattern NC formation of zebrafish and *Xenopus* in a gradient fashion. A modest attenuation of endogenous BMP signal is required for the initial induction of NC (21–23), whereas the activated BMP signal is required for the maintenance of NC cells at neurula stages (24). In both *Xenopus* and zebrafish neurulae, a conserved intermediate level of BMP signaling has been detected in the NPB region (25). However, the molecular mechanisms as to how the intermediate BMP activity is defined are not well understood. It has been reported that among the target genes of BMP signaling pathway, *id3*, a member of the Id gene family, is expressed in NC where it plays an

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[2] The abbreviations used are: NC, neural crest; BMP, bone morphogenetic protein; co-IP, co-immunoprecipitation; DEX, dexamethasone; ETS, E26 transformation-specific; GR, glucocorticoid receptor; HDAC, histone deacetylase; MO, morpholino antisense oligonucleotide; NPB, neural plate border; TSA, trichostatin A; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; *sncg*, γ-*synuclein*; *dnets1*, dominant negative *ets1*.

essential role in mediating NC cell proliferation and cell survival (19, 26).

In addition to genetic regulation, the NC development is also subject to epigenetic regulation such as histone acetylation and deacetylation catalyzed by histone acetyltransferases and histone deacetylases (HDACs), respectively. Histone deacetylation is in general associated with gene silence. In mice, knockout of *Hdac8* in the NC caused defects in cranial NC cells and losses of cranial skeletal elements (27). Studies with *Xenopus* embryos have also shown an obvious NC inhibition after treatment with trichostatin A (TSA), a common HDAC inhibitor (28).

The proto-oncogene Ets1 belongs to the E26 transformation-specific (ETS) family of transcription factors. Previous reports showed that Ets1 is involved in hematopoietic development, angiogenesis, and tumor invasion (29). In *Xenopus* embryos, it was found that *ets1* is strongly expressed in premigratory and migratory NC cells (30, 31). A recent study with chicken embryos suggested that *Ets1* is regulated by a group of NC specifiers including Sox10, Pax7, Msx1/2, Foxd3, and Tfap2 (31). Other studies also suggested that Ets1 is required for cranial NC delamination as well as the migration and differentiation of cardiac NC (32, 33). In chicken embryos, *Ets1* cooperates with *Sox9* and c-*Myb* to activate *Sox10* expression (34). In this study, we investigated the functions of *ets1* during NC development using *Xenopus* embryos. We found that overexpression of *ets1* represses NC formation, which is at least partially due to the attenuation of BMP signaling by down-regulation of *id3*, a BMP target gene. In addition, Ets1 physically interacts with HDAC1 and recruits HDAC1 to the *id3* promoter, resulting in histone deacetylation of this region. Collectively, our study adds important insights into the epigenetic regulation of NC development.

Experimental Procedures

DNA Constructs—The open reading frames of *Xenopus laevis ets1a*, *id3*, and *hdac1* were amplified using PCR and cloned into pCS2+ or into pCS2GR, pCS2FLAG, pCS2HA, and pCS2Myc. Deletion mutants of *ets1* were generated by PCR and subcloned into pCS2Myc. All constructs were confirmed by DNA sequencing.

Injection, Whole-mount in Situ Hybridization, LacZ Staining, Cartilage Staining, and Vibratome Sectioning—X. laevis embryos were obtained by *in vitro* fertilization and cultured using methods described previously (35). Embryos were staged according to Nieuwkoop and Faber (36). Capped mRNAs were synthesized *in vitro* using the mMessage mMachine kit (Invitrogen). Translation-blocking MOs targeting *ets1* mRNA were purchased from Gene Tools, Inc. (ets1MO1, 5′-TCCTTCCAAATAGAGAAATGTGTGT-3′; ets1MO2a, 5′-TGAGATCTAGCGCAGCTTTCATGGC-3′; ets1MO2b, 5′-TAAGGTCTAGTGCAGCTTTCATGGC-3′). Embryos were injected with mRNAs or MO at the two-cell or four-cell stage and raised to the indicated stages. LacZ staining was performed using X-Gal or red X-Gal as described previously (37). Whole-mount *in situ* hybridization was performed using a digoxigenin-labeled antisense RNA probe, and signals were developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science) (38). Alcian blue cartilage staining was performed as described previously (39).

TABLE 1
Primers used for RT-PCR
Re, reverse; Fw, forward; *odc*, ornithine decarboxylase.

Gene	Sequence (5′–3′)	Ref.
ets1a Fw	TTGAACAGGCAATAGCAG	This study
ets1a Re	ACATCTGTAGTCCCTCCC	
id3 Fw	GGGAAACTTTGAGAGAGAGAGAG	This study
id3 Re	CGGGTTGCAAAGGGTTAAAG	
snail2 Fw	TCCCGCACTGAAAATGCCACGATC	22
snail2 Re	CCGTCCTAAAGATGAAGGGTATCCTG	
sox9 Fw	AAGCAGAATGTCCTCTGTGA	35
sox9 Re	AAGGCCAGATTCAGTTCTTC	
foxd3 Fw	GGAGGGAGGGGGCAATGCAC	12
foxd3 Re	CCCCGAGCTCGCCTACT	
odc Fw	CAGCTAGCTGTGGTGTGG	E. M. De Robertis
odc Re	CAACATGGAAACTCACACC	
pax3 Fw	CTACCTCGGTTTCTTGACTG	35
pax3 Re	TGGTCAATCCTTCTTAATGG	
zic1 Fw	ATGAAGGTCCACGAAGCATC	8
zic1 Re	CGTGCTGTGATTGGACGTGT	
sox2 Fw	GAGGATGGACACTTATGCCCAC	E. M. De Robertis
sox2 Re	GGACATGCTGTAGGTAGGCGA	
sox3 Fw	TGATGCAGGACCAGTTGGGC	35
sox3 Re	TGAAGTGAAGGGTCGCTGGC	
msx1 Fw	GCTAAAAATGGCTGCTAA	E. M. De Robertis
msx1 Re	AGGTGGGCTGTGTAAAGT	

Animal Cap Assay and RT-PCR—The animal cap assay was performed as described previously (40). Total RNA was extracted using the TRIzol reagent (Invitrogen), and cDNA was synthesized using Superscript III (Invitrogen) following the manufacturer's manual. Primers for RT-PCR are listed in Table 1.

Protein Co-immunoprecipitation (Co-IP) and Chromatin Immunoprecipitation (ChIP)—For co-IPs, embryos or HEK293T cells were lysed in lysis buffer (137 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5, 0.5% Triton X-100) containing protease inhibitors. The cell lysates were incubated with the indicated antibody for 2 h at 4 °C followed by incubation with protein G-Sepharose beads (GE Healthcare) for 1 h and washing in lysis buffer five times. Precipitates were separated by SDS-PAGE and transferred to nitrocellulose membrane for blotting. Protein bands were quantified using ImageJ. ChIP was performed according to the published protocols using *Xenopus* embryos or HEK293T cells (41, 42). The immunoprecipitated DNA fragments were purified and analyzed by PCR using primers listed in Table 2. Two independent experiments were performed for each ChIP assay, and one representative result is depicted. The antibodies used in this study are listed in Table 3.

Luciferase Assay—HEK293T cells were plated into 48-well plates and transfected with BRE-Id1 reporter plasmid, *Renilla* luciferase pRL-CMV, and the indicated plasmids. BMP4 was added 1 day after the transfection, and luciferase activity was measured using the Dual-Luciferase system (Promega) 24 h later.

TUNEL Assay—One dorsal blastomere of four-cell stage embryos was injected with *ets1* and *lacZ* mRNAs and collected at the indicated stages. LacZ staining was performed to identify the injection side. The fixed embryos were rehydrated in 1× SSC. After bleaching, the embryos were transferred to 1× PBS and incubated in terminal deoxynucleotidyltransferase buffer for 1 h. End labeling was carried out in terminal deoxynucleotidyltransferase buffer containing 1 μM digoxigenin-11-dUTP (Roche Applied Science) and 200 units/ml terminal deoxy-

TABLE 2
Primers used for ChIP assay in this study
Fw, forward; Re, reverse.

	Sequence (5′–3′)	Locus
Primer pair 1		
Fw	GCATTTCAGCCTGGAAGCAT	NT_004610.19
Re	ATGCCTCTTCTCTGCAAAGCAC	
Primer pair 2		
Fw	TTTCCCTCCTCCTTCTGCAATC	NT_004610.19
Re	CCCACCCCCCGTTTTTTTTA	
Primer pair 3		
Fw	CGGTATGTGCAATTATCTA	JGI Xenla 6.0
Re	AAAAGCCATTATTCTGTATC	Scaffold159155
Primer pair 4		
Fw	GCATTAATGACACAGTCC	JGI Xenla 6.0
Re	CTCTTTTATAGTCACACTC	Scaffold159155
GAPDH		
Fw	CGCTTTCTTTCCTTTCGC	AC_000144
Re	TGCCCATTCATTTCCTTCC	
Primer control		
Fw	TCCACATGCACAACCCTTTA	JGI Xenla 6.0,
Re	TCTTTGCCCACAAATCTGGT	Scaffold28661 (57)

TABLE 3
Primary antibodies used in this study
p-Smad1, phosphorylated Smad1.

Antibody	Company	Catalog no.
Ets1	Abcam	ab26096
Smad1	Invitrogen	38-5400
p-Smad1	Cell Signaling Technology	9511
FLAG	Sigma-Aldrich	F1804
HA	Sigma-Aldrich	H3663
Myc	Cell Signaling Technology	2276, 2278

nucleotidyltransferase (Roche Applied Science) overnight at room temperature. The reaction was terminated by washing in 1× PBS containing 1 mM EDTA at 65 °C for 1 h. The digoxigenin epitope was detected using antibody conjugated with alkaline phosphatase (Roche Applied Science) as in *in situ* hybridization.

Results

Expression of ets1 in NC Is Regulated by Lrig3 and FGF Signaling—Our previous study revealed that Lrig3 regulates NC formation downstream of Pax3 and Zic1 in *Xenopus* (40). Using microarray analysis, we further identified *ets1*, an ETS transcription factor, as a downstream effector of Lrig3 during NC formation. The microarray data were deposited into NCBI Gene Expression Omnibus under accession number GSE50487. The representatives of NC-related genes with -fold change and *p* value after knockdown of *lrig3* are listed in Table 4. We validated the microarray results with RT-PCR and found that, along with NC marker genes including *sox9*, *snail2*, and *foxd3*, *ets1* was repressed by knockdown of *lrig3*; all of these genes were otherwise induced by co-expression of *wnt3a* and *chordin* (Fig. 1*A*). This result further indicated that Lrig3 regulates *ets1* during NC formation.

The spatial expression pattern of *ets1* showed that *ets1* expression was not detected in stage 11 embryos (Fig. 1*B*), and weak signals began to appear from stage 13 (Fig. 1*C*) after which *ets1* was strongly expressed in the premigratory and migratory NC cells (Fig. 1, *D* and *E*). Apart from the NC and its derivatives, *ets1* expression was also detected in ventral blood island and developing blood vessels (Fig. 1, *D*′, *F*, and *G*). The temporal expression of *ets1* indicated that it is a maternal factor with the expression reduced until gastrula stages but recovered and maintained at a relative constant level at neurula and tail bud stages (Fig. 1*H*). Thus, at early developmental stages, the expression pattern of *ets1* suggests that its functions are associated with NC development.

TABLE 4
-Fold changes of representative genes that are involved in NC formation in comparison of Wnt3a + chordin + Lrig3MO versus Wnt3a + chordin

Gene	Accession number	-Fold change	*p* value
sox10	Xl.1588	−12.2514	0.0142723
pcns	Xl.15089	−10.5758	0.0023312
twist1b	Xl.56708	−10.3569	8.67E − 07
twist1a	Xl.879	−7.07247	0.0044942
snail2b	Xl.11972	−6.89003	0.0059053
ets1b	Xl.142	−6.61179	0.0017931
incab	Xl.4117	−5.00622	0.0022
sox8	Xl.29789	−4.34594	0.0015472
snail2a	Xl.3818	−4.20333	7.46E − 06
foxd3a	Xl.525	−4.07228	0.0165975
ets1a	Xl.1148	−4.01187	0.0028018
foxd3b	Xl.523	−4.01002	0.0585129
id3	Xl.8060	−1.17389	0.0348254
tubulin1b	Xl.13561	−1.16048	0.0594225
msx1b	Xl.45216	−1.12427	0.026907
pax3a	Xl.49495	1.89796	0.0333841
zic1	Xl.1796	1.91721	0.0834522
sox2	Xl.188	9.19403	0.0020139
sox3	Xl.22	15.1422	0.0015098

Because Lrig3 acts as an FGF signaling modulator (40), we reasoned that *ets1* expression could be regulated by FGF signaling. When embryos were treated with increasing doses of the FGF signaling inhibitor SU5402, the expression of *ets1* was reduced in a dose-dependent manner (Fig. 1, *I–K*) as that of the FGF target gene *brachyury* (Fig. 1, *L–N*; see legend for the percentage of affected embryos). Moreover, the animal cap assay indicated that overexpression of *efgf* induced expression of *ets1* but not *snail2* at this injection dose (Fig. 1*O*), and overexpression of *wnt3a* could induce *ets1* as well although to a lesser extent than did *efgf* overexpression. As the positive controls, *brachyury* and *nr3* were induced by *efgf* and *wnt3a*, respectively (Fig. 1*O*). Taken together, the above data suggested that *ets1* is regulated by FGF signaling during early embryonic development.

Overexpression and Knockdown of ets1 Interfere with the Development of NC Derivatives—As a first step to analyzing Ets1 function, we examined embryonic morphology upon overexpression or knockdown of *ets1*. We found that overexpression of *ets1* resulted in apparent defects of embryonic development including repression of head and anterior axis and loss of pigment in cranial and trunk regions in the embryos at stage 39 (Fig. 2, *A* and *B*). *X. laevis* is a pseudotetraploid, and like many other genes that have two nonidentical copies in its genome, *ets1* also has two copies, *ets1a* and *ets1b*. Therefore, for knockdown studies, an MO (ets1MO1) was designed to target the consensus sequence in 5′-UTRs of both copies of *ets1* (*ets1a* and *ets1b*) to knock down both simultaneously. The endogenous Ets1 protein level was reduced by approximately 50% after injection of ets1MO1 (60 ng/embryo; Fig. 2, *H* and *I*). Interestingly, *ets1* morphants also exhibited inhibition of head with pigment loss in the cranial and trunk regions when examined at

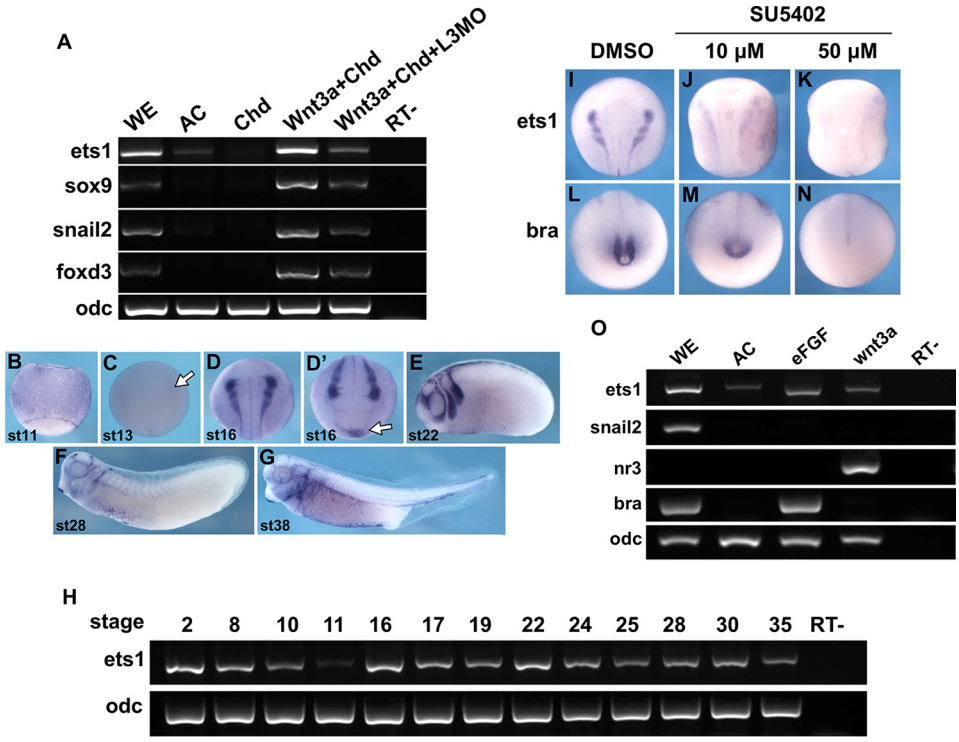


FIGURE 1. **Expression of *ets1* in NC is regulated by Lrig3 and FGF signaling.** *A*, embryos were injected with *chordin* (*Chd*), *chordin* + *wnt3a*, or *chordin* + *wnt3a* + lrig3MO (*L3MO*). Animal caps were dissected at stage 9 and cultured to stage 17. Expression of the indicated genes in animal caps was assayed by RT-PCR. Ornithine decarboxylase (*odc*) was used as an internal standard. *WE*, uninjected whole embryo; *AC*, uninjected animal caps; *RT*−, without reverse transcriptase. *B*–*G*, spatial expression pattern of *ets1* as detected by *in situ* hybridization. *B*, *C*, and *D*, dorsal view; *D*′, anterior view; *E*, *F*, and *G*, lateral view. The *white arrow* in *C* indicates the weak *ets1* signal starting at stage 13. The ventral blood island is indicated by a *white arrow* in *D*′. *H*, temporal expression of *Xenopus ets1a* at the indicated stages. *I*–*N*, embryos treated with the indicated concentrations of SU5402 from stage 12. *ets1* and *brachyury* (*bra*) expression was examined at stage 16 by whole-mount *in situ* hybridization. The expression of the indicated genes was affected in the following percentage of embryos: *M*, 73% (8 of 11); *N*, 100% (20 of 20); *J*, 33% (7 of 21); *K*, 61% (11 of 18). *O*, embryos were injected with 10 pg of *efgf* or 300 pg of *wnt3a* mRNA, respectively. The caps were dissected from embryos at stage 9 and then cultured to stage 15. Expression of the indicated genes in animal caps was assayed by RT-PCR.

stage 35 (Fig. 2, *C* and *D*). Moreover, the defects induced by ets1MO1 could be partially rescued by co-expression of *ets1* mRNA (Fig. 2*G*), which supported the specificity of ets1MO1. To further confirm the phenotypes induced by ets1MO1, we designed another pair of MOs, ets1MO2a and ets1MO2b, that target start codons of *Xenopus ets1a* and *ets1b*, respectively. Co-injection of ets1MO2a and ets1MO2b (hereafter collectively named as ets1MO2; 60 ng/embryos) reduced the endogenous Ets1 by 60% (Fig. 2, *H* and *I*) and caused a phenotype similar to that of ets1MO1 (Fig. 2, *E* and *F*). In addition, we also examined the expression of γ-*synuclein* (*sncg*), which is a gene expressed in the cranial nerves and dorsal root ganglions (35), and found that either overexpression or knockdown of *ets1* disrupted the expression of *sncg* in cranial nerves, particularly in the trigeminal nerve (Fig. 2, *J*–*L*). To clarify whether these abnormalities are direct effects of manipulating *ets1* expression, we generated two inducible constructs, *ets1-GR* and dominant negative *ets1-GR* (*dnets1-GR*) (43) by fusing a glucocorticoid receptor domain 3′ to the full *ets1* open reading frame or to the ETS domain, respectively. Both can be activated by dexamethasone (DEX) as reported previously (44). After injection with *ets1-GR* or *dnets1-GR*, the embryos treated with DEX exhibited inhibition of anterior axis formation and abnormalities in pigment formation (Fig. 2, *M*–*P*). Moreover, the *sncg* expression was much reduced (Fig. 2, *R* and *T*), which is similar to the embryos injected with *ets1* mRNA or ets1MOs. The embryos without DEX treatment did not show obvious defects (Fig. 2, *M*, *O*, *Q*, and *S*), suggesting that the phenotypes induced by *ets1-GR* or *dnets1-GR* are specific. Likewise, in both *ets1*-overexpressing embryos and *ets1* morphants, cranial cartilage formation was impaired on the injected side (Fig. 2, *U*–*W*). However, it is notable that the inhibition of cranial cartilage induction by overexpression of *ets1* was much more profound than that caused by knockdown of *ets1*. These results indicate that an optimal level of Ets1 is essential for normal NC development.

Knockdown of ets1 Does Not Obviously Affect NC Formation—We then compared NC induction at stage 17 in *ets1* morphants with that in the control embryos by examining the expression of NC marker genes such as *foxd3* and *snail2*. ets1MO1 (60 ng/embryo) or ets1MO2 (60 ng/embryo) was injected into two blastomeres of *Xenopus* embryos at the two-cell stage. At this injection dose, neither ets1MO1 nor ets1MO2 apparently affected *foxd3* (Fig. 3, *A*, *B*, *E*, and *F*) or *snail2* expression (Fig. 3, *C*, *D*, *G*, and *H*). Likewise, ets1MO2 (30 ng/embryo) injected into one blastomere of embryos at the two-cell stage did not obviously affect the expression of *foxd3* (Fig. 3*I*) or *snail2* (Fig. 3*J*). However, in animal caps co-injected with *chordin* and *wnt3a*, knockdown of *ets1* repressed the NPB marker *pax3* and the NC markers *sox9* and *snail2* (Fig. 3*K*). This discrepancy might be due to redundant factors that exist in embryos but not in *in vitro* induced NC tissues. In fact, *ets2*, the

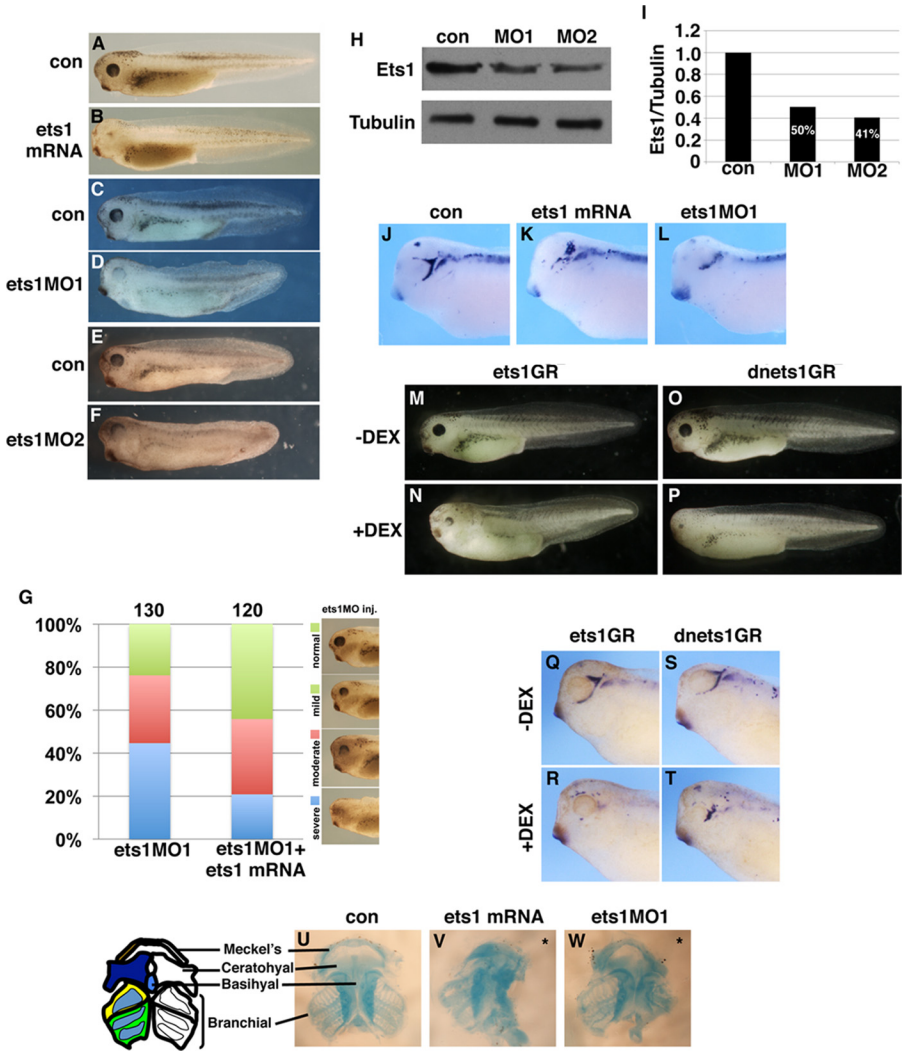


FIGURE 2. **Overexpression or knockdown of *ets1* causes defects in NC derivatives.** *A* and *B*, *ets1* overexpression (500 pg/embryo; 85%; 23 of 27) caused loss of pigment throughout the body and inhibition of head structures. *C–F*, knockdown of *ets1* using ets1MO1 (94%; 30 of 32) or ets1MO2 (94%; 33 of 35) showed similar pigment loss and repression of head. *G*, categories of defects induced by either 30 ng of ets1MO1 separately or by co-injection (*inj.*) with 250 pg of *ets1* mRNA. *Numbers* at the *top* indicate total embryos scored at stage 35 from three independent experiments. *H* and *I*, either 60 ng of ets1MO1 or ets1MO2 was injected into two-cell stage embryos, the embryos were collected at stage 11, and Ets1 protein was detected by α-Ets1 antibody. Tubulin was used as an internal control. The bands on Western blots (*H*) were quantified in *I*. *J–L*, the expression of cranial nerve marker gene *sncg* is disrupted in embryos injected with 250 pg of *ets1* mRNA (*K*; 85%, 28 of 33) or 30 ng of ets1MO1 (*L*; 72%, 18 of 25). *con*, control. *M–P*, either *ets1-GR* or *dnets1-GR* mRNA (500 pg/embryo) was injected into two-cell stage embryos. The injected embryos were treated with DEX starting at stage 13. Loss of pigment and inhibition of anterior axis were observed in DEX-treated embryos (*N*, 81%, 26 of 32; *P*, 74%, 17 of 23) but not in untreated embryos (*M*, 4%, 1 of 26; *O*, 0%, 0 of 11). Likewise, the expression of *sncg* was much reduced in the embryos injected with either *ets1-GR* or *dnets1-GR* and sequentially treated with DEX (*R*, 84%, 26 of 31; *T*, 84%, 21 of 25) but remained normal in embryos without DEX treatment (*Q*, 0%, 0 of 29; *S*, 0%, 0 of 18). *U–W*, cranial cartilage formation in control (*U*) and embryos injected with *ets1* mRNA (*V*; 85%, 51 of 60) or ets1MO (*W*; 83%, 33 of 40) was examined by Alcian blue staining. An *asterisk* indicates the injected side. *con*, control.

closest member to *ets1* in the Ets family, is expressed in NC cells (30) but was not induced in animal caps by co-injection of *wnt3a* and *chordin* (Fig. 3*K*). We examined *ets2* expression in *Xenopus* embryos. *ets2* was expressed strongly in the animal pole in two-cell stage embryos (Fig. 3*L*). We detected its expression in the mesoderm surrounding the yolk plug at stage 11 (gastrula; Fig. 3*M*). Although its expression at neurula stages is low (Fig. 3, *N* and *O*), strong signals were clearly detected in the migrating NC at tail bud and tadpole stage embryos (Fig. 3, *P* and *Q*). Thus, it is likely that Ets2 can compensate some of the functions of Ets1 in *ets1* morphants.

To investigate the migration of NC cells, ets1MO1 was injected into one dorsal blastomere of *Xenopus* embryos at the four-cell stage. The injected embryos were collected at late neurula stages around stage 20 for examining the expression of *foxd3*, *snail2*, and *twist1*. NC cells started to extend laterally from neural tube in the uninjected side, whereas they were restricted to a narrow region along the neural tube in the injected side (Fig. 3, *R*, *S*, and *U*). In line with this observation, the expression of migratory NC marker gene *twist1* was also reduced in the injected side at tail bud stages (Fig. 3, *V* and *W*), although the reduction was not apparent at late neurula stages (Fig. 3, *T* and *U*). The *twist1* signal stripes marking cranial NC did not extend laterally as far as those in the uninjected side either (Fig. 3, *V* and *W*). Sections from stage 20 embryos suggested that the NC cells labeled by *twist1* staining at the

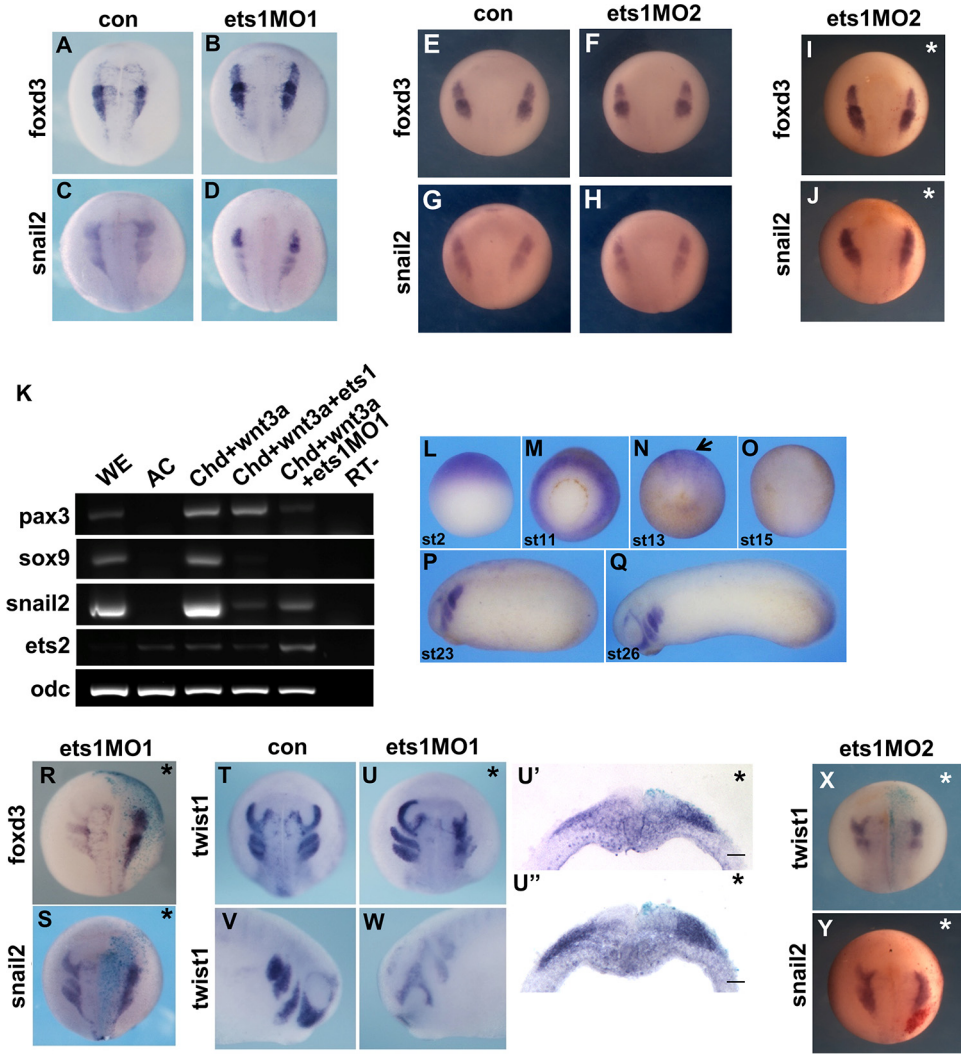


FIGURE 3. **Knockdown of *ets1* does not obviously affect NC formation but blocks NC migration.** *A–J*, the expression of *foxd3* or *snail2* was not inhibited by ets1MOs. ets1MO1 or ets1MO2 was injected either separately into both blastomeres (*A–D*, 60 ng of ets1MO1/embryo; *E–H*, 60 ng of ets1MO2/embryo) or together with 100 pg of *lacZ* mRNA into one blastomere (*I* and *J*, 30 ng/embryo) at the two-cell stage. The injected embryos were collected at stage 17 and examined for *foxd3* (*B*, 90%, 18 of 20; *F*, 96%, 24 of 25; *I*, 95%, 21 of 22) and *snail2* (*D*, 95%, 22 of 23; *H*, 92%, 23 of 25; *J*, 90%, 18 of 20) by whole-mount *in situ* hybridization. The injected side was traced by red X-Gal staining and is marked with an *asterisk* (*I* and *J*). *K*, overexpression and knockdown of *ets1* repressed NC formation in an animal cap assay. Expression of the indicated genes in animal caps injected with either *chordin* (*Chd*) + *wnt3a*, *chordin* + *wnt3a* + *ets1*, or *chordin* + *wnt3a* + ets1MO1 was examined by RT-PCR. *L–Q*, spatial expression pattern of *ets2* as detected by *in situ* hybridization. *st*, stage. *L*, *P*, and *Q*, lateral view; *M* and *N*, vegetal view; *O*, dorsal view. The *black arrow* in *N* indicates weak expression of *ets2*. *R* and *S*, ets1MO1 (30 ng) and *lacZ* mRNA (100 pg) were co-injected into one dorsal blastomere of four-cell stage embryos, and the embryos were collected at stage 20. Segmentation and extension of cranial NC were blocked at the ets1MO1-injected side (*R*, 85%, 17 of 20; *S*, 83%, 20 of 24). *T–W*, knockdown of *ets1* decreased the expression of *twist1* in both neurula (*T* and *U*) and tail bud embryos (*V* and *W*). *T*, control embryo at stage 20. *U*, embryos injected with ets1MO1 at one side. *V* and *W*, embryos at stage 25 injected with ets1MO1 at one side. At the injected side (*U*, 85%, 11 of 13; *W*, 90%, 9 of 10), the *twist1* signal stripes were weaker and did not extend laterally as far as those at the uninjected side (*T* and *V*). An *asterisk* indicates the injected side. *U′* and *U″*, transverse sections of embryos shown in *U* (5 of 5 embryos). ets1MO1 and *lacZ* mRNA were co-injected at one side of the embryos. In the injected side, NC cells marked by *twist1* staining were concentrated laterally to the neural tube and seemed not to detach from the neural plate. In the uninjected side, NC cells extended out of the neural plate, and signal spots were scattered underneath the mesoderm region, suggesting that NC cells migrated into the arches. An *asterisk* indicates the injected side. *Scale bars* in *U′* and *U″* indicate 100 μm. *X* and *Y*, Ets1MO2 (30 ng/embryo) was co-injected with *lacZ* mRNA (100 pg; used as a lineage tracer) into one blastomere of two-cell stage embryos, and embryos were stained for the expression of *twist1* (81%, 17 of 21) and *snail2* (79%, 11 of 14). The extension of cranial NC was decreased in the ets1MO2-injected side. *WE*, uninjected whole embryo; *AC*, uninjected animal caps; *RT−*, without reverse transcriptase; *con*, control.

ets1MO1-injected side cannot detach and migrate from the neural plate, forming a concentrated signal zone when compared with those at the uninjected side (Fig. 3, *U′* and *U″*). Injection of ets1MO2 into one blastomere of embryos at the two-cell stage also attenuated lateral extension of NC expressing *twist1* and *snail2* (Fig. 3, *X* and *Y*).

Taken together, these results suggested that, although the initial induction of NC was not apparently affected by *ets1* depletion, Ets1 was required for the migration of NC cells. This is consistent with a previous study in chicken embryos that showed that knockdown of *ets1* resulted in NC delamination defects, thereby affecting NC migration (33).

Overexpression of ets1 Represses NC Formation—We also investigated effects of overexpression of *est1* on NC formation. Increasing doses of *ets1* mRNA were injected into two blastomeres of *Xenopus* embryos at the two-cell stage, and the expression of *foxd3*, *snail2*, and *twist1* at stage 17 was examined. Low injection doses of *ets1* (100 or 200 pg/embryo) did not obviously

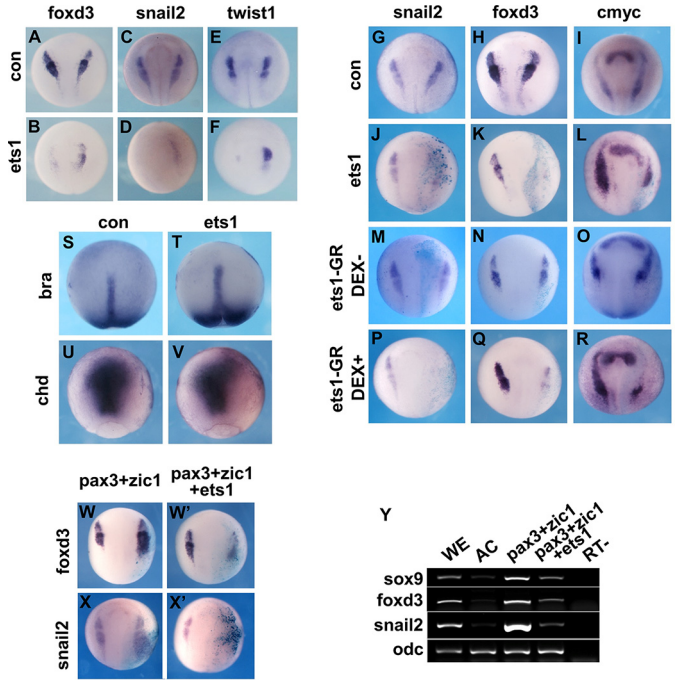


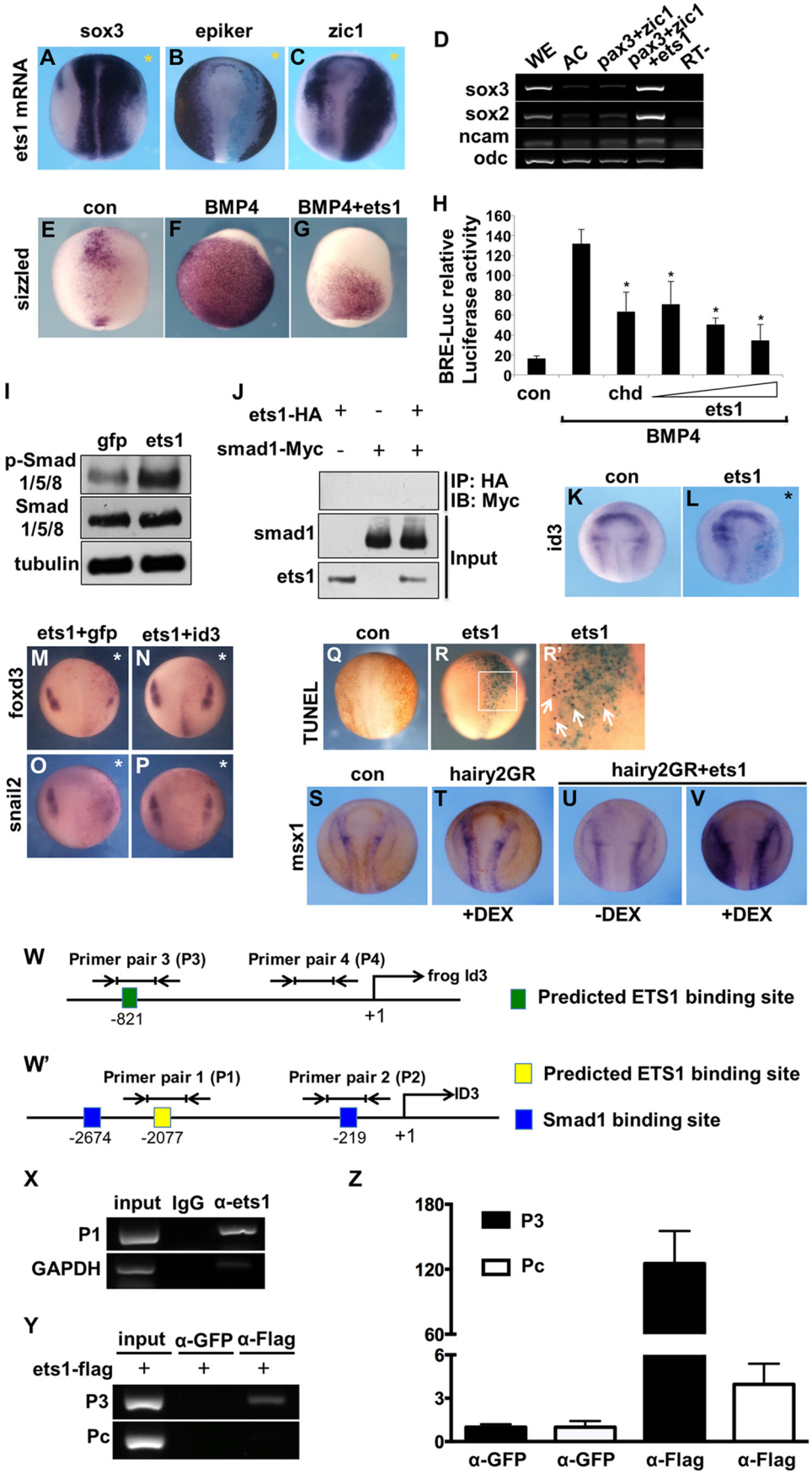
FIGURE 4. **Overexpression of *ets1* represses formation of NC in embryos and in animal caps injected with *pax3* and *zic1* mRNAs.** *A–F*, the NC markers *foxd3* (100%, 10 of 10), *snail2* (78%, 7 of 9), and *twist1* (80%, 12 of 15) were repressed by overexpression of *ets1*. *G–R*, either *ets1* or *ets1-GR* mRNA was co-injected with *lacZ* mRNA into one dorsal blastomere of four-cell embryos, and embryos were collected at around stage 17. Embryos injected with *ets1-GR* were treated with dimethyl sulfoxide or DEX from stage 12 to stage 17. Whole-mount *in situ* hybridization was used to examine the NC marker genes *snail2* (*J*, 71%, 17 of 24; *M*, 100%, 19 of 19; *P*, 65%, 13 of 20), *foxd3* (*K*, 63%, 17 of 27; *N*, 95%, 18 of 19; *Q*, 62%, 8 of 13), and c-*myc* (*L*, 62%, 13 of 21; *O*, 100%, 20 of 20; *R*, 60%, 9 of 15). The injected side was traced by LacZ staining. *S–V*, mesoderm marker genes *brachyury* (*bra*; *S* and *T*) and *chordin* (*chd*; *U* and *V*) were examined in embryos injected with *ets1*. *S* and *U*, control embryo; *T* and *V*, *ets1*-injected embryos. *W–X′*, the mRNA mixtures of *pax3* + *zic1* or *pax3* + *zic1* + *ets1* were injected into one dorsal blastomere at the four-cell stage, and the expression of *foxd3* (*W*, 90%, 17 of 19; *W′*, 74%, 29 of 39) and *snail2* (*X*, 94%, 15 of 16; *X′*, 75%, 24 of 32) was examined by whole-mount *in situ* hybridization at stage 16. *Y*, animal cap assays indicated that overexpression of *ets1* suppressed the expression of NC maker genes induced by *pax3* and *zic1* and enhanced neural marker genes. *WE*, uninjected whole embryo; *AC*, uninjected animal caps; *RT−*, without reverse transcriptase; *con*, control.

interfere with NC formation (data not shown), whereas the high injection dose of *ets1* (500 pg/embryo) severely repressed *foxd3* (Fig. 4, *A* and *B*), *snail2* (Fig. 4, *C* and *D*), and *twist1* (Fig. 4, *E* and *F*). We then compared NC formation in the injected side of embryo with that in the uninjected side of the same embryo. *ets1* mRNA (250 pg/embryo) was injected into one dorsal blastomere of four-cell stage embryos, which resulted in strong inhibition of the expression of *snail2*, *foxd3*, and *c-myc* in the injected side (Fig. 4, *G–L*). To further confirm this result, the *ets1-GR* mRNA was injected into one dorsal blastomere of embryos at the four-cell stage, and Ets1-GR was then activated by adding DEX at stage 12 when the mesoderm was formed and the induction of NC was initiated. We found that the expression of *snail2*, *foxd3*, and c-*myc* was repressed upon activation of Ets1-GR by DEX (Fig. 4, *P–R*), whereas the injected embryos without DEX treatment were unaffected (Fig. 4, *M–O*). To exclude the possibility that the repression of NC was due to defective mesoderm formation (45, 46), we examined the expression of two mesoderm marker genes, *brachyury* and *chordin*, in *ets1*-injected embryos. The expression of *brachyury* and *chordin* was not obviously altered by ectopic *ets1* (Fig. 4, *S–V*), suggesting that the initial development of mesoderm was not affected by *ets1* overexpression and that inhibition of NC formation was due to disturbed differentiation of ectoderm rather than mesoderm. Moreover, *ets1* overexpression also repressed the NC marker genes including *sox9* and *snail2* but not the NPB marker *pax3* in animal caps injected with *chordin* and *wnt3a* (Fig. 3*K*). This was further confirmed in ectopic NC induced by *pax3* and *zic1* (8, 9) (Fig. 4, *W* and *X*) where *foxd3* (Fig. 4*W′*) and *snail2* (Fig. 4*X′*) were repressed by ectopic *ets1* in whole embryos. It is notable that in animal caps the up-regulation of *sox9*, *foxd3*, and *snail2* induced by co-injection of *pax3* and *zic1* was also repressed by *ets1* overexpression (Fig. 4*Y*). Taken together, overexpression of *ets1* repressed NC formation in whole embryos as well as in animal cap assays.

Ets1 Attenuates BMP Signaling Downstream of Phosphorylation of Smad1/5/8—Because overexpression of *ets1* did not seem to affect mesoderm formation and NC is an ectodermal derivative, we examined genes involved in ectoderm differentiation in *ets1*-injected embryos. *ets1* mRNA was injected into one dorsal blastomere of four-cell stage embryos, and the expression of *sox3*, *zic1*, and *epi-keratin* was examined. Overexpression of *ets1* expanded the pan-neural marker *sox3* (Fig. 5*A*) but suppressed the epidermis marker *epi-keratin* (Fig. 5*B*) in the injected side. These observations strongly suggested that BMP signaling was inhibited as attenuation of BMP signaling is essential for neural induction (21, 47). Consistent with these findings, *zic1* (Fig. 5*C*) was strongly induced by ectopic *ets1*. *zic1* is regarded as a gene directly induced by the inhibition of BMP signaling (48). The strong induction of *zic1* represents additional evidence supporting the view that BMP signaling was attenuated by *ets1* overexpression. Induction of NC markers such as *foxd3* and *snail2* in animal caps by injection of *pax3* and *zic1* was suppressed by co-injection of *ets1*, whereas expression of the neural markers *sox2*, *sox3*, and *ncam* was increased (Fig. 5*D*), again supporting the view that overexpression of *ets1* inhibits BMP signaling.

We next overexpressed *bmp4* to induce expansion of *sizzled*, a direct target of BMP signaling in embryos. The induced *sizzled* expression was repressed by co-expression of *ets1* (Fig. 5, *E–G*), confirming again the inhibition of BMP signaling by ectopic *ets1*. In the luciferase assay performed in HEK293T cells, the recombinant BMP4-activated luciferase reporter signals were also decreased by *ets1* overexpression in a dose-dependent way with *chordin* used as a positive control (Fig. 5*H*). Collectively, these data strongly indicated that overexpression of *ets1* attenuates BMP signaling. We next wanted to investigate how Ets1 regulates BMP signaling. Phosphorylation of Smad1/5/8, which respond to the activation of BMP signaling, was not reduced but rather moderately enhanced by overexpression of *ets1* (Fig. 5*I*). Moreover, Ets1 does not appear to physically interact with Smad1 because co-immunoprecipitation did not show binding between them (Fig. 5*J*). These results suggested that Ets1 functioned downstream of Smad1/5/8 phosphorylation to regulate the output of BMP signaling.

Ets1 Represses NC Formation Partially through Down-regulation of Id3—The BMP target gene *id3* plays essential roles in maintaining NC progenitors (18, 19, 26, 49). Knockdown of *id3*


sox3
epiker
zic1
ets1 mRNA
A
B
C
D
WE
AC
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pax3+zic1 +ets1
RT-
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ncam
odc
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BMP4
BMP4+ets1
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BRE-Luc relative Luciferase activity
160
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Smad 1/5/8
tubulin
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IB: Myc
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ets1
id3
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ets1+gfp
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foxd3
M
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snail2
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ets1
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TUNEL
Q
R
R'
con
hairy2GR
hairy2GR+ets1
msx1
S
T
U
V
+DEX
-DEX
+DEX
W
Primer pair 3 (P3)
Primer pair 4 (P4)
frog Id3
-821
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Predicted ETS1 binding site
W'
Primer pair 1 (P1)
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ID3
-2674
-2077
-219
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α-ets1
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GAPDH
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caused the absence of NC precursors and losses of NC derivatives (19). These findings prompted us to investigate whether Id3 is a downstream target of Ets1 in regulating NC formation. To test this hypothesis, we injected *ets1* mRNA into one dorsal blastomere of four-cell stage embryos and examined the expression of *id3* at stage 17 by whole-mount *in situ* hybridization, which showed that *id3* was strongly repressed by overexpression of *ets1* in the injected side (Fig. 5, *K* and *L*). Moreover, we found that *ets1*-induced repression of *foxd3* and *snail2* (Fig. 5, *M* and *O*) was partially rescued by co-expression of *id3* (Fig. 5, *N* and *P*). In addition, a TUNEL assay showed that *ets1* overexpression induced a profound increase of apoptotic cells in the injected region (Fig. 5, *Q*, *R*, and *R′*), suggesting furthermore that repression of NC induced by *ets1* overexpression at least in part is due to the decreased expression of *id3*. In a previous study, Id3 was found to interact with Hairy2 and negatively regulated Hairy2 activity, which plays an essential role in NC specification (50). We co-injected *hairy2-GR* and *ets1*, and the injected embryos were treated with DEX. We observed a strong increase of *msx1*, which is similar to the effect of co-injection of *hairy2* and id3MO (Fig. 5, *S–V*) (50). This result suggested that Hairy2 is related to the Ets1-Id3 axis in the NC regulatory network.

Because a previous study revealed that Ets binding motifs are enriched in Smad1/5 binding regions of the human genome (51), we examined the promoter region of *ID3* and found potential Ets1 binding sites in the promoters of both human and *Xenopus id3* (Fig. 5, *W* and *W′*). This was further confirmed by ChIP assay using antibody against endogenous ETS1 in HEK293T cells that showed that ETS1 bound to the predicted site in human *ID3* promoter (Fig. 5*X*). In *Xenopus* embryos, FLAG-tagged Ets1 also bound to the predicted Ets1 binding site in the *id3* promoter (Fig. 5, *Y* and *Z*).

Ets1 Physically Interacts with HDAC1 and Regulates BMP Signaling and NC Formation through Regulating Histone Acetylation of id3 Promoter—A previous study has reported that Ets1 interacts with HDAC1 to repress target genes in Th1 cells (52). To explore the possibility that Ets1 represses BMP target genes through epigenetic control during NC development, we first assessed the interaction between Ets1 and HDAC1 in both *Xenopus* embryos and cultured mammalian cells. *Xenopus ets1-Myc* and human *HDAC1-HA* were co-transfected into

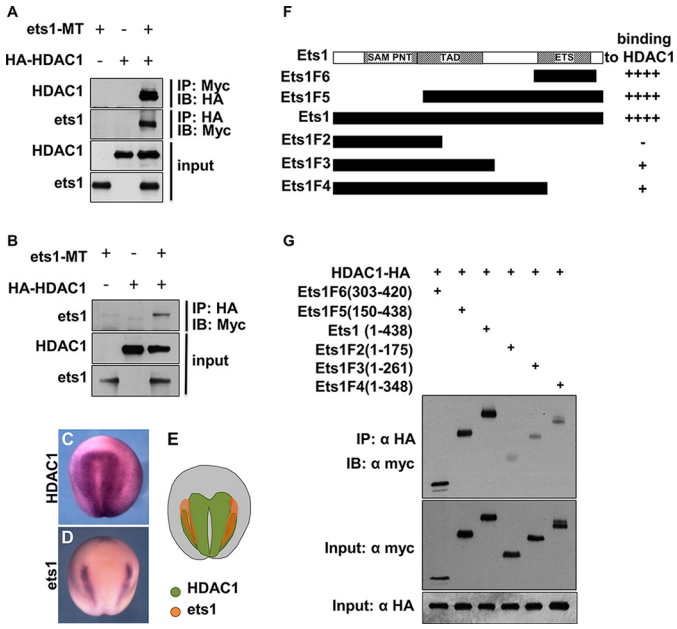

FIGURE 6. **Ets1 physically interacts with HDAC1 through the ETS domain.** *A*, co-IP was carried out with extracts of HEK293T cells transfected with either Ets1-Myc, HDAC1-HA separately, or both using antibodies against Myc or HA. *B*, co-IP indicates that Ets1-Myc binds to HDAC1-HA in *Xenopus* embryos. *C–E*, expression patterns of *Xenopus hdac1* (*C*) and *ets1* (*D*) in *Xenopus* embryo at stage 17. A schematic diagram of *Xenopus hdac1* and *ets1* expression (*E*) indicates overlap between them. *F*, Ets1 deletion mutants and their binding ability to HDAC1. *G*, co-IP was carried out to examine the binding of Myc-tagged Ets1 deletion mutants and HDAC1-HA in HEK293T cells. *IB*, immunoblot; *TAD*, transactivation domain; *SAM*, sterile α motif; *PNT*, pointed.

HEK293T cells, and a co-IP assay was performed. Strong interaction between Ets1 and HDAC1 was demonstrated (Fig. 6*A*). Alternatively, in *Xenopus* embryos injected with *Xenopus ets1-Myc* and human *HDAC1-HA* mRNAs, Ets1 was also co-precipitated with HDAC1 in the injected embryos (Fig. 6*B*). We also carefully compared the expression of *Xenopus hdac1* and *ets1*. Apart from the strong expression in neural plate, *hdac1* was also detected in NC, largely overlapping with the *ets1* expression region (Fig. 6, *C–E*), suggesting the interaction of *Xenopus* Hdac1 and Ets1 during NC development.

We then constructed a series of C-terminal and N-terminal deletion mutants of Ets1 to map the domains that interact with HDAC1. The co-IP results revealed that Ets1F5 (amino acids

FIGURE 5. **Overexpression of *ets1* attenuates BMP signal downstream of Smad1/5/8 phosphorylation and represses *id3* through binding to its promoter.** *A–C*, *in situ* hybridization analysis of the expression of the indicated markers in embryos injected with *ets1* mRNA (*sox3*, 87%, 13 of 15; *epi-keratin* (*epiker*), 75%, 12 of 16; *zic1*, 100%, 22 of 22). An *asterisk* indicates the injected side. *D*, neural markers *sox3*, *sox2*, and *ncam* were induced by *ets1* overexpression in an animal cap assay. *E–G*, expression of *sizzled* was examined in embryos injected with *bmp4* mRNA (89%, 32 of 36) alone or together with *ets1* mRNA (68%, 27 of 40). *H*, luciferase assay was performed to assay BMP signaling. *chordin* (*chd*) was used as a control. *, $p < 0.05$ between indicated group and the group only treated with BMP4. *I*, Western blot showing the level of phosphorylated Smad1/5/8 (*p-Smad1/5/8*) and total Smad1/5/8 in stage 16 embryos overexpressing *ets1*. Endogenous tubulin was used as a loading control. *J*, co-immunoprecipitation was performed using lysates from embryos injected with either *ets1-HA*, *smad1-Myc* separately, or both. *K* and *L*, expression of *id3* in control (*K*) and *ets1*-injected embryos (*L*; 65%, 22 of 34) was examined by *in situ* hybridization. *M–P*, expression of *foxd3* and *snail2* in embryos injected with either the mixture of *ets1* and *gfp* (*M*, 69%, 24 of 35; *O*, 61%, 22 of 36) or the mixture of *ets1* and *id3* (*N*, 69%, 31 of 45; *P*, 60%, 29 of 48). *Q–R′*, cell apoptosis was detected by TUNEL assay (*black spots*) in embryos with one side injection of *ets1* mRNA (100%, 5 of 5). LacZ staining (*light blue*) was used to indicate the injected side. *Q*, control embryos; *R*, *ets1*-injected embryo; *R′*, high magnification of framed region in *R*. *S–V*, *hairy2-GR* mRNA (500 pg/embryos) was injected into embryos at the two-cell stage alone or together with *ets1* (500 pg/embryos), and DEX was used to treat embryos from stage 13 to stage17. The expression of *msx1* was examined using *in situ* hybridization. Although *hairy2* slightly promoted *msx1* expression (*T*, 88%, 23 of 26), co-expression with *ets1* enhanced *msx1* expression (*U*, 95%, 19 of 20; *V*, 96%, 25 of 26). *W*, the predicated Ets1 binding site in the *id3* promoter of *X. laevis* and the primers used in ChIP. *W′*, schematic diagram illustrating the predicted SMAD1 and ETS1 binding sites in the human *ID3* promoter and the primers used in the ChIP assay. *X*, ChIP was performed using antibodies against ETS1 in HEK293T cells, and the precipitated DNA fragments were amplified using primer pair 1 (*P1*) or the GAPDH primer pair, respectively. Endogenous ETS1 can bind to the predicted binding site. The GAPDH primer pair was used as the control. *Y* and *Z*, ChIP was done in *X. laevis* embryos injected with 500 pg of *ets1-FLAG*. Semiquantitative (*Y*) and real time PCR (*Z*) were performed using primer pair 3 (*P3*) or primer pair control (*Pc*) (Table 2). *WE*, uninjected whole embryo; *AC*, uninjected animal caps; *RT−*, without reverse transcriptase; *con*, control; *IB*, immunoblot. *Error bars* represent S.D.

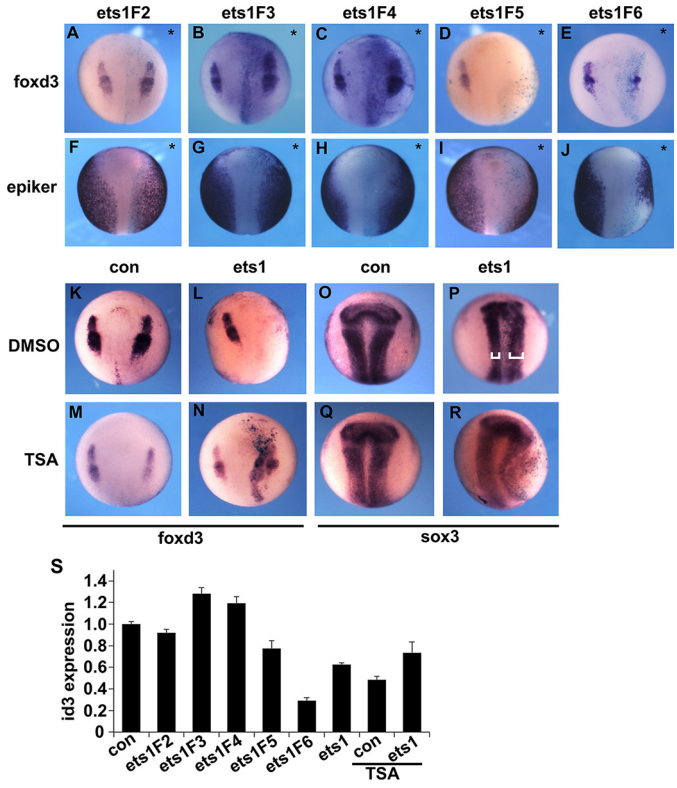

FIGURE 7. **The interaction with HDAC1 is required for Ets1 to regulate NC and epidermis formation as well as *id3* expression.** *A–J*, embryos were injected with mRNAs of different *ets1* deletion mutants (250 pg/embryo) in one side and stained for the expression of *foxd3*. Injection of *ets1F2*, *ets1F3*, and *ets1F4* did not apparently affect the expression of *foxd3* (*A–C*) and *epi-keratin* (*epiker*; *F–H*), whereas overexpression of *ets1F5* and *ets1F6* suppressed these two marker genes at the injected side (*foxd3*: *D*, 65%, 13 of 20; *E*, 94%, 15 of 16; *epi-keratin*: *I*, 90%, 9 of 10; *J*, 93%, 26 of 28). An *asterisk* indicates the injected side in embryos. *K–R*, embryos injected with 250 pg of *ets1* mRNA were treated with 50 nM TSA from stage 13 to stage 17. *foxd3* was repressed by overexpression of *ets1* alone (67%, 16 of 24), whereas ectopic *foxd3* in *ets1*-injected embryos was induced after TSA treatment (57%, 8 of 14). The expression of *sox3* was expanded by overexpressing *ets1* (83%, 15 of 18), whereas TSA treatment reduced the expression of *sox3* in the *ets1*-injected side (93%, 14 of 15). LacZ staining was used to trace the injected side. *S*, real time PCR analysis of *id3* expression in embryos injected with the different mRNAs as indicated or treated with TSA. Results are presented as -fold changes after normalization to ornithine decarboxylase expression. *con*, control. *Error bars* represent S.D.

150–438) and Ets1F6 (amino acids 303–420), which both contain the ETS domain, interacted with HDAC1 to the same degree as did wild-type Ets1, whereas other deletion mutants that do not contain the ETS domain showed only weak association with HDAC1 (Fig. 6, *F* and *G*). These results suggest that the ETS domain is essential for Ets1 interaction with HDAC1.

We further assessed whether the interaction is required for Ets1-mediated regulation of BMP signal transduction and NC formation. The mRNAs encoding deletion mutants of *ets1* were injected into *Xenopus* embryos, and the effects on NC and epidermis development were compared with those induced by wild-type *ets1*. We found that Ets1F5 and Ets1F6, which showed strong interaction with HDAC1, repressed NC marker gene *foxd3* (Fig. 7, *D* and *E*) and *epi-keratin* (Fig. 7, *I* and *J*) as did wild-type *ets1* (Figs. 4 and 5*B*). In contrast, *ets1F2*, *ets1F3*, and *ets1F4* that only weakly associated with HDAC1 failed to repress the examined markers (Fig. 7, *A–C* and *F–H*). In fact, we observed that *ets1F3* and *ets1F4* moderately expanded *foxd3* expression (Fig. 7, *B* and *C*).Thus, the interaction with HDAC1 seems to be required for Ets1 to repress NC and epidermis markers. To determine whether the activity of HDAC is required for the effects of ectopic *ets1*, we used the HDAC inhibitor TSA to inhibit HDAC1 activity in the *ets1*-injected embryos. As shown above, overexpression of *ets1* resulted in repression of *foxd3* (Fig. 7, *K* and *L*) and expansion of *sox3* (Fig. 7, *O* and *P*), whereas TSA treatment reversed the inhibitory effects of *ets1* as ectopic *foxd3* was induced (Fig. 7, *M* and *N*) and *sox3* was repressed (Fig. 7, *Q* and *R*). These results suggested that in the presence of HDAC overexpression of *ets1* inhibits NC formation, whereas when HDAC is inhibited, overexpression of *ets1* promotes NC formation. We next used real time PCR to examine *id3* expression in the embryos injected with *ets1* deletion mutants or treated with TSA. Overexpression of *ets1F3* and *ets1F4* enhanced *id3* expression (Fig. 7*S*). In contrast, *ets1F5*, *ets1F6*, and *ets1* repressed *id3* expression (Fig. 7*S*). With TSA treatment, *ets1* moderately enhanced *id3* expression compared with that in control embryos (Fig. 7*S*). These observations are in line with the change of *foxd3* revealed by whole-mount *in situ* hybridization (Fig. 7, *A–R*). Because these results are in good agreements with the aforementioned results that Ets1 represses NC formation through down-regulation of BMP signaling target gene *Id3*, it prompted us to investigate the role of Ets1 in epigenetic control of BMP signaling and NC formation. ChIP assay showed that co-transfection of *ets1* and *hdac1* enhanced the binding of HDAC1 to the *ID3* promoter compared with that in the cells transfected with HDAC1 alone (Fig. 8*A*). In *Xenopus* embryos, overexpression of Ets1, Ets1F5, or Ets1F6 that strongly bound to HDAC1 also resulted in reduced acetylated histone 4 on *id3* promoter (Fig. 8, *B–D*). In contrast, overexpression of *ets1F3* that weakly interacted with HDAC1 promoted histone 4 acetylation on *id3* promoter (Fig. 8*E*).

Taken all together, these results suggested that *ets1* cooperates with HDAC1 to repress gene transcription of *id3* through reducing histone acetylation. Based on our findings and previous reports, we therefore propose that the interaction between Ets1 and HDAC1 allows HDAC1 to bind to *id3* promoter, resulting in the reduced histone acetylation, decreased *id3* transcription, and ultimately inhibition of NC formation, whereas overexpression of Ets1F3 or Ets1F4 that lack the ETS domain increased histone acetylation and *id3* expression and expanded NC formation (Fig. 9).

Discussion

We have demonstrated previously that Lrig3 regulates NC formation downstream of Pax3 and Zic1 in *Xenopus* (40). In this study, we identified *ets1* as a Lrig3 effector in microarray analysis and revealed that Ets1 plays an important role in NC development through attenuation of the BMP signaling pathway.

We observed that the zygotic activation of *ets1* occurs during neurulation, which is later than that of NPB specifiers during gastrulation. Expression of NPB specifiers *ap2a*, *hairy2*, *msx1*, *pax3*, and *zic1* was initiated at stage 11–12 (53), whereas weak *ets1* expression was first detected at stage 13 (Fig. 1*C*), suggesting that *ets1* functions downstream of NPB specification to regulate NC formation. In addition, expression of NC marker genes induced by *pax3* and *zic1* were repressed by *ets1* overex-

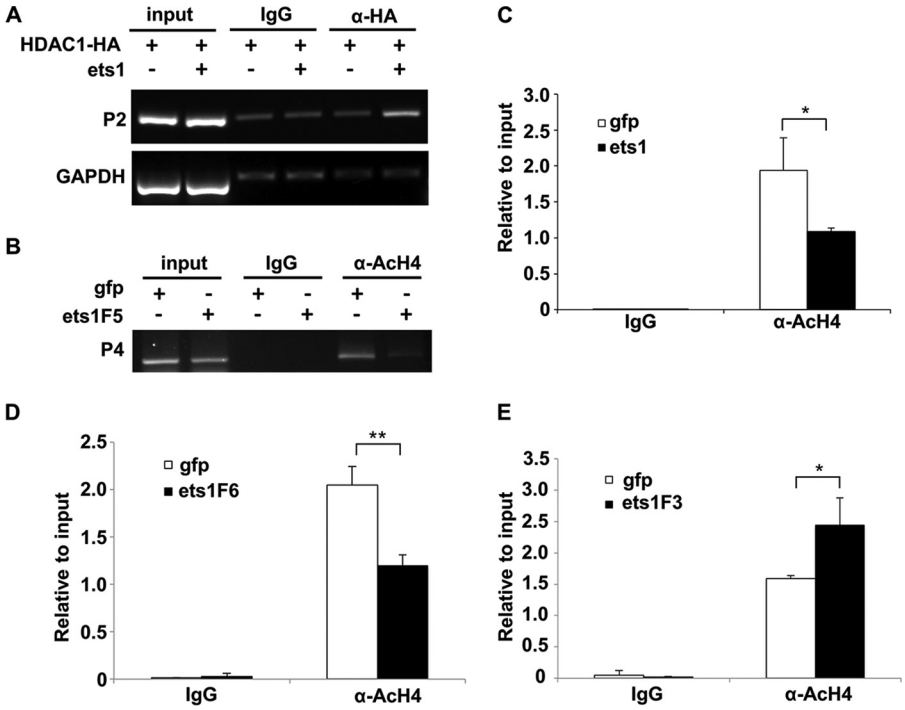

FIGURE 8. **Ets1 recruits HDAC1 to the *id3* promoter and reduces the level of acetylated histone 4.** *A*, ChIP was done using HEK293T cells transfected with either HDAC1-HA alone or *Xenopus* Ets1. The precipitated DNA fragments were amplified using primer pair 2 (*P2*) or the GAPDH primer pair. The co-expression of Ets1 promoted the binding of HDAC1 to the *ID3* promoter. *B–E*, ChIP was performed in the embryos injected with the indicated mRNAs. The precipitated DNA fragments were analyzed by semiquantitative PCR (*B*) or real time PCR (*C–E*). *Error bars* represent S.D. Asterisk represents p value <0.05 and double-asterisk represents p value <0.01.

pression both in whole embryos and in animal caps (Fig. 4, *W*, *W′*, *X*, *X′*, and *Y*). Moreover, activation of Ets1-GR from stage 14 when NPB specification has been completed could still repress *foxd3* (data not shown). Thus, in the normal NC formation process, Ets1 appears to function downstream of NPB specification. This is in agreement with the results obtained in chicken embryos where *ets1* is expressed in NC just before the onset of emigration, suggesting that Ets1 is not essential in neural plate border specification (54). Interestingly, knockdown of *ets1* by ets1MOs in *Xenopus* embryos did not affect NC formation (Fig. 3, *A–J*) but inhibited NC migration (Fig. 3, *R–Y*). However, in animal cap assays, knockdown of *ets1* blocked the NC formation induced by co-overexpression of *wnt3a* and *chordin*. This discrepancy may be due to the functional redundancy of other factors such as *ets2*, which is expressed in whole *Xenopus* embryos (Fig. 3, *L–Q*) but not in the induced animal caps (Fig. 3*K*). The NC development is tightly and precisely regulated by a signaling network (20). Our finding that the timing and strength of Ets1 expression are essential for NC formation and subsequent migration prompted us to postulate that Ets1 is induced by NPB specifiers during the NC formation, and the Ets1 might establish a negative feedback to attenuate NPB specifiers, which may be required for initiation of NC migration during embryonic development.

Our study provides further evidence that Ets1 regulates NC formation through attenuating BMP signaling in *Xenopus* embryos. It has been proposed that a modest attenuation of endogenous BMP signal is required for the initial induction of NC (21–23), whereas the activated BMP signal is required for maintaining NC cells (24). This is conserved across vertebrate including zebrafish (24, 25), *Xenopus* (24, 25), chicken (55), and mouse (56, 57). In this study, we found that overexpression of *ets1* up-regulated the expression of neural marker genes *sox3* and *zic1* (Fig. 5, *A*, *C*, and *D*) but down-regulated *epi-keratin* in *Xenopus* embryos (Fig. 5*B*), suggesting that BMP signaling was attenuated by *ets1* overexpression. In addition, overexpression of *ets1* suppressed the *sizzled* expression induced by BMP4 (Fig. 5, *E–G*). This was further supported by an *in vitro* luciferase assay in which Ets1 decreased the luciferase activity of the BMP reporter pGL-Id1 induced by BMP4 (Fig. 5*H*). Although the BMP signal was attenuated by overexpression of *ets1*, the phosphorylation of Smad1/5/8 was not inhibited (Fig. 5*I*) but instead moderately increased, suggesting that Ets1 functions downstream of the phosphorylated Smad1/5/8 in the BMP signaling cascade. We notice that some BMP target genes such as *sizzled* and *bambi* also function as BMP signaling inhibitors, and knockdown of *sizzled* enhances Smad1 phosphorylation (58). Thus, the enhanced Smad1/5/8 phosphorylation might result from this negative feedback regulation in BMP signaling as overexpression of *ets1* repressed *sizzled*.

The role of BMP signal in maintaining NC is partially mediated by activation of *id3*, a BMP target gene required for the survival of NC cells (19, 26). Overexpression of *ets1* repressed *id3* (Fig. 5, *K* and *L*). Conversely, overexpression of *id3* partially rescued the repression of *foxd3* and *snail2* induced by ectopic *ets1* (Fig. 5, *M–P*). The ETS binding motif is enriched in Smad1/5 binding regions in the human genome (51). In this study, we found that endogenous human ETS1 directly bound to the promoter of *ID3* in HEK293T cells (Fig. 5*X*), and the binding was also observed in *Xenopus* embryos injected with

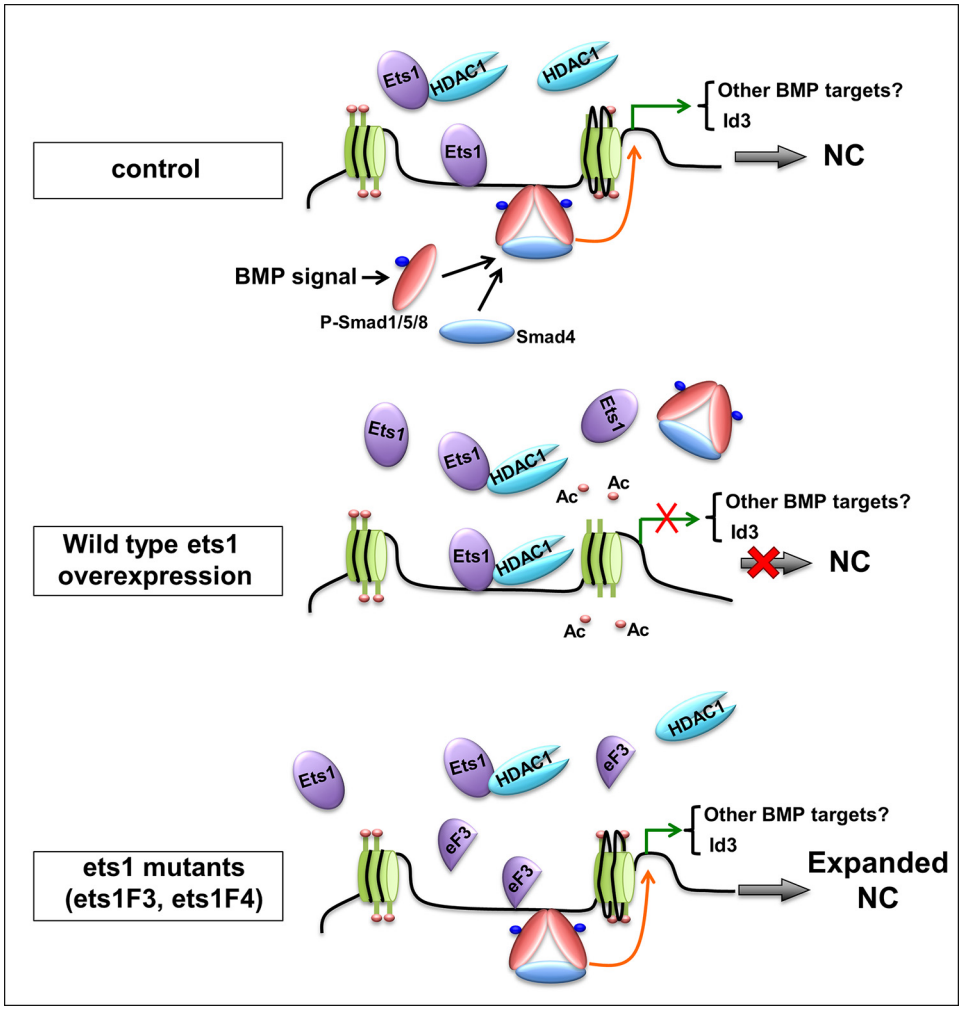

FIGURE 9. **Proposed model showing Ets1 regulation of BMP signal and NC formation through Hdac1.** When *ets1* is overexpressed in embryos, it binds to Hdac1, and recruits Hdac1 to the *id3* promoter, leading to deacetylation of histone and chromatin condensation, which prevents transcription of *id3* and causes inhibition of NC formation. Other BMP targets involved in NC development may also be affected by this mechanism. When *ets1F3* or *ets1F4* Ets1 mutant is overexpressed, it enhances histone acetylation of *id3* promoter and therefore promotes NC formation. *eF3*, Ets1F3; *Ac*, acetyl group; *P-Smad*, phosphorylated Smad.

ets1-FLAG mRNA (Fig. 5, *Y* and *Z*). In addition, we also found that Ets1 physically interacts with HDAC1 through the ETS domain, which was previously regarded as a DNA binding domain (Fig. 6). This was supported by overlapping expression of *ets1* and *hdac1* in *Xenopus* embryo as revealed by whole-mount *in situ* hybridization (Fig. 6, *C–E*). Several genes have been reported to regulate BMP signaling through HDACs. During osteoblast differentiation, Twist1 inhibits BMP signaling through forming a complex with Smad4 and HDAC1 (59). Moreover, it has been reported that the ETS domain of the Ets transcription factor Elk-1 is involved in recruiting HDAC to the promoters of its target genes (60).

During the early stage of NC formation, high level of Id3 and other NC specifiers cooperate to create conditions that promote NC specification. Ets1 is induced during this stage, and this stage is independent of Ets1 and hence unaffected by loss of Ets1. With the process of NC development, Ets1 protein is accumulated to a high level, which may result in a decrease of *id3* and *foxd3*, and thereby promotes NC migration. Ets1 may serve a negative fine-tuning modulator to maintain expression of *id3* or other BMP target genes at a proper level. Indeed, overexpression of ETS domain-containing Ets1 mutants that interact with HDAC1 strongly (Ets1F5 and Ets1F6) effectively repressed NC formation or *id3* expression and attenuated BMP signaling (Fig. 7, *D*, *E*, *I*, *J*, and *S*), whereas the mutants without the ETS domain (EtsF2, Ets1F3, and Ets1F4) lost this ability (Fig. 7, *A–C* and *F–H*). These results suggest that the interaction between Ets1 and HDAC1 is required for the repression of NC formation and that the C-terminal regions of Ets1 that contain the ETS domain work as a transcriptional repression domain. In line with these finding, overexpression of Ets1 mutants (Ets1F3 and Ets1F4) that lack the ETS domain promoted NC formation and *id3* expression (Fig. 7, *B*, *C*, and *S*). Overexpression of *ets1F3* promoted histone acetylation in *id3* promoter (Fig. 8*E*). These results suggest that the N-terminal region of Ets1 without the ETS domain may serve as a transcriptional activation domain. Furthermore, when HDAC activity was inhibited by TSA, overexpression of *ets1* induced rather than repressed *foxd3* expression (Fig. 7*N*), whereas TSA treatment alone decreased *foxd3* expression (Fig. 7*M*) as reported in a previous study (28), suggesting that HDAC activity is also required for the inhibitory role of Ets1. Taken together, our

findings suggest that different protein domains of Ets1 have distinct effects on NC formation. A number of proteins were reported to interact with different domains in Ets1 (29). For example, Ets1 was shown to interact with cAMP-response element-binding protein (CREB)-binding protein/p300, an acetyltransferase (61, 62), with its *transactivation* domain. It is possible that Ets1 can activate or repress gene expression by interacting with p300 or HDACs, respectively. Further studies are required to address the effects of dynamic interactions between Ets1 and its partners on the regulation of transcriptional activity during embryonic development. It should be mentioned that we focused on *id3* expression to illustrate the regulatory mechanism of Ets1 for mediating the output of BMP signaling. The NC development is a rather sophisticated process, and many factors are involved in this multistep development event. In addition to *id3*, other genes responding to the BMP signal could also be regulated by interaction of Ets1 and HDAC1, serving one of the mechanisms during NC development.

In summary, our data indicated that optimal levels of Ets1 are essential for NC formation and migration and that Ets1 regulates NC formation at least in part through mediating *id3* expression. We further demonstrated for the first time that using a multivalent interaction mechanism Ets1 can attenuate outputs of BMP signaling epigenetically through interaction with HDAC1.

Author Contributions—C. W., R. K. T. K., W. S., and Y. X. performed experiments. X. C., Y. C., J. S., G. L., Y. D., and Z. C. analyzed the data. S. O. C., W. Y. C., and Y. D. provided analytical tools. H. Z. conceived and coordinated the study. C. W., Y. D., and H. Z. wrote the paper.

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