EENA PROMOTES MYELOID PROLIFERATION THROUGH STIMULATING ERK1/2 PHOSPHORYLATION IN ZEBRAFISH

Huang-Ying Le1, 2, Yong Zhang1, 2, Han Liu1, Li-Heng Ma1, Yi Jin1, Qiu-Hua Huang1, Yi Chen1, Min Deng1, 2, Zhu Chen1, 3, Sai-Juan Chen1, 3 and Ting Xi Liu1, 2

From 1State Key Laboratory of Medical Genomics & Laboratory of Development and Diseases, Institute of Health Sciences (IHS), Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences, 225 Chong Qing South Road, Shanghai 200025 & Shanghai Institute of Hematology, RuiJin Hospital, Shanghai Jiao Tong University (SJTU), 197 RuiJin Road II, Shanghai 200025, 2Model Organism Division, E-institutes of Shanghai Universities, SJTU, Shanghai 200025, 3Shanghai Center for Systems Biomedicine, SJTU, Shanghai 200240

Address correspondence to: TingXi Liu, Laboratory of Development and Diseases, IHS, Room 408, Building 1, 225 South Chong Qing Road, Shanghai 200025. Tel: 86-21-63857025; Fax: 86-21-63857029; E-mail: txliu@sibs.ac.cn; Sai-Juan Chen, State Key Laboratory for Medical Genomics and Shanghai Institute of Hematology, RuiJin Hospital, 197 RuiJin Road II, Shanghai 200025, China; E-mail: sjchen@stn.sh.cn.

The EEN (extra eleven nineteen) gene is one of fusion partners of MLL (mixed-lineage leukemia), located on chromosome 19p13. Here, we cloned two een genes (designated as eena and eenb) in zebrafish, which are assigned to linkage groups 8 and 2, respectively. Whole-mount in situ hybridization assay showed that eena and eenb have overlapping, but distinct expression patterns during embryogenesis. Ubiquitious or targeted overexpression of eena, but not eenb, into wild-type or transgenic embryos (green-fluorescent protein-labeled myeloid progenitors) induced a significant proliferation and ectopic distribution of myeloid progenitors in the yolk sac. Using a morpholino-antisense gene knockdown approach, we showed that the number of myeloid progenitors and their downstream mature myelomonocytic cells was significantly decreased in the eena-deficient embryos. Mechanistically, overexpression of eena selectively stimulated ERK phosphorylation and increased the level of transcription factor c-Fos in vitro and in vivo, while eena lacking the SH3 domain completely abolished these effects. Furthermore, a MAPK/ERK kinase (MEK) inhibitor, PD98059, blocked the eena-induced cell proliferation and activation of ERK signaling. The results suggest that eena plays an important role in the development of myeloid cell through activation of the ERK pathway and may provide a valuable reference for future studies of the role of EEN in leukemogenesis.

INTRODUCTION

The EEN (extra eleven nineteen), was originally cloned from a case of infant acute myeloid leukemia M5 subtype with a chromosomal translocation at the breakpoint of t(11; 19) (q23; p13), in which EEN is fused with MLL (myeloid-lymphoid leukemia) (1). EEN is the founding member of the endophilin SH3 domain-containing protein family, which comprises two subfamilies—endophilins A and B. In mammals, the A subfamily consists of three members, endophilin A1 (EA1), A2 (EEN/EA2) and A3 (EA3), whereas the recently identified B subfamily comprises B1 (also called SH3GLB1) and B2 (also called Bif1/SH3GLB2) (2-4). They all have an identical domain structure, consisting of the following domains: a relatively conserved N-terminal BAR (BIN/amphiphysin/Rvsp) domain; a conserved C-terminal region containing the SH3 domain which shared 70% homology with the adaptor protein GRB2; and a variable region in between, which, despite its lack of extensive sequence homology, containing clusters of proline and other turn-inducing amino acids, suggesting that it might act as a flexible joint linking the BAR domain and SH3 domain (5,6).

Most of the studies of the endophilin family...
have been focused on their functions in endocytosis in neuronal cells. The members of this family have been shown to form complexes with several proteins, such as amphiphysin, synaptotagmin and dynamin, all of which are implicated in presynaptic vesicle trafficking (7-9). They are also binding partners of the G protein–coupled β1-adrenergic receptor (10). In addition, endophilin-Ca$^{2+}$ channel complex is required for synaptic vesicle endocytosis (11). Recent studies demonstrated that EEN could bind BPGAP1 and is involved in the activation of EGF receptor endocytosis and ERK1/2 signaling (12). EEN is also engaged in ras signaling and cellular transformation through binding EBP (13). EEN is the only member in the family expressed in hematopoietic tissues, including bone marrow and fetal liver (1). However, as a myeloid leukemia-associated gene, the role of EEN in myeloid development and leukemogenesis has not been fully explored. In mammals, it is relatively difficult to access early developmental processes such as primitive myelopoiesis because they occur within the mother's body.

Zebrafish has become an ideal organism for the study of normal and abnormal hematopoiesis. Its hematopoiesis has been shown to be very similar to that of higher vertebrates, and homologues of a large number of genes involved in mammalian hematopoiesis have been identified in the zebrafish (14,15). As in mammals, zebrafish primitive and definitive hematopoiesis arise successively in the intermediate cell mass (ICM) and the ventral wall of the dorsal aorta in the developing embryos (16).

In this report, we have cloned two zebrafish orthologues of the mammalian EEN gene, termed eena and eenb, and investigated the evolutionary relationship of these genes and expression patterns during early embryonic development. Our results suggest that zebrafish eena and eenb result from a gene duplication event at the een locus and that this may lead to their functional divergence. We also showed that eena and eenb were expressed maternally and ubiquitously, implying a role in early embryonic development. However, at the later stages, eenb was only observed in hatching gland, whereas eena expression was still ubiquitously and richly detected in intermediate cell mass (ICM) at 22 hour post-fertilization (hpf). Further, we focused on the function of eena and eenb in myeloid development in vivo because EEN has been found fused to MLL in acute myeloid leukemia. Here, we showed that knockdown of eena caused a significant suppression in the myeloid cells development, while overexpression of eena, but not eenb, led to an increased number of myeloid progenitors through stimulating extracellular signal-regulated kinase (ERK) phosphorylation and the subsequent up-regulation of zebrafish c-fos mRNA. In addition, the N-terminal part of eena, which lacked the SH3 domain, acted as a potential dominant negative mutant completely abolished these effects. Our data indicate that eena regulates myeloid development through activation of the ERK signal pathway, which provides a novel insight into the role of EEN in leukemogenesis caused by MLL-EEN.

**EXPERIMENTAL PROCEDURES**

Zebrafish strains- Zebrafish strains were maintained at 28.5°C as described by Westfield (17). Transgenic line TG(zpu.1:EGFP) was kindly provided by Drs. John Kanki and A. Thomas Look at Dana-Farber Cancer Institute (18). Embryos were staged as described by Kimmel (19). Developmental stages refer to hours (hpf) or days (dpf) post-fertilization.

**een cloning and plasmids construction-** Zebrafish eena and eenb genes were identified based on homology to human EEN. The specific primers were designed according to genomic sequence in the UCSC database (primer sequences available at supplemental Table 1). The amplified PCR product was purified and subcloned into pCS2$^+$ for in vitro synthesis of capped mRNA for microinjection. Then eena and eenaΔSH3 fragment were digested with BamHI and XhoI sites from pCS2$^+$ vector and cloned into an I-sccl-containing plasmid vector between a 9.0 kb zebrafish pu.1 (zpu.1) promoter sequence (18) and a SV40 polyadenylation site, which resulted in the constructs of pu.1-eena and pu.1-eenaΔSH3 for microinjection, respectively. The plasmid of pEGFP-EEN was constructed by Liu et al. (20).

**Multiple sequence alignment, phylogenetic trees and gene structure analysis-** Protein sequences for zebrafish, and other species endophilins were obtained from GenBank to determine the phylogenetic relationships. Multiple
alignments of amino acid sequences for eena genes were obtained by using the ClustalX 1.83 alignment program and subsequently analyzed with the Neighbor Joining method to construct a phylogenetic tree by using a Mega3.1 software (21,22). A bootstrap test was performed with 1,000 repetitions.

Genomic sequences of human EEN and zebrafish eena and eenb were obtained by searching the corresponding genome sequence databases (http://genome.ucsc.edu, and http://www.ensembl.org/Danio_rerio, respectively), which have complete information on eena genes loci and putative exon arrangement, as well as on transcript and protein sequences. The exon and intron arrangement of zebrafish eena and eeenb genes were defined based on the information deposited in the DNA database, and checked by amino acid sequence alignment of the corresponding cDNAs.

Identification of syntenic relationship- The Sanger Zv6 zebrafish genome assembly (http://www.ensembl.org) was searched for sequences with similarity to human EEN protein using tBLASTn. We applied the reciprocal best “hit” (RBH) method (23) for syntenic analysis and compared genes neighboring the zebrafish eena to the genes neighboring the human EEN. The map positions of the corresponding human genes were obtained at http://genome.ucsc.edu.

Whole-mount in situ hybridization- To localize eena and eenb, as well as c-fos mRNAs in zebrafish embryo, whole-mount in situ hybridization was carried out. pCS2* containing eena or eenb 3′UTR (352 and 150 bp, respectively) was utilized to generate antisense RNA probes for eena and eenb using digoxigenin-11-uridine 5′-triphosphate (Roche, Switzerland). Embryos were fixed in 4% paraformaldehyde at the stages indicated, and whole-mount in situ hybridization was performed as described (24). These probes were detected with alkaline-phosphatase-conjugated antibodies (1:5,000) (Roche, Switzerland) using the substrates BCIP/NBT (purple colour) (Vector Laboratories, Burlingame, CA). At least 10 embryos were used at each stage and all showed similar expression patterns. After staining, embryos were mounted in 3% methylcellulose and captured under the Nikon SMZ1500 microscope equipped with a Nikon DXM1200F digital camera and ACT-1 software.

Microinjection of zebrafish embryos- The expression vector pCS2*-eena and pCS2*-eennb were linearized with NotI and transcribed in vitro with SP6 RNA polymerase in the presence of m7G (59)ppp(59)G (Ambion, USA) to produce capped transcripts and were microinjected into one-cell stage embryos. Embryos were microinjected with 100 pg of EGFP mRNA or DEPC water as controls, using an air pressure injector and glass capillaries. To specifically express eena in myeloid progenitors, the pu.1-eena and pu.1-eenaASH3 plasmids were prepared with endotoxin-free midiprep kit (Promega, USA) and injected into TG(zpu.1:EGFP) embryos with 100 pg of DNA, 0.5 x 1-scel buffer and 0.5 units/μl 1-scel meganuclease (New England Biolabs, USA). Injection experiments were performed three times. EGFP expression was directly observed under a fluorescent microscope (Zeiss Lumar V12 stremomicroscope equipped with an AxioCam MRC5digital camera and AxioVision Rel.4.5 software).

The morpholino antisense oligonucleotide (MO) targeted against eena was synthesized by Gene-Tools LLC (Philomath, OR, USA). The sequence was as follows:5′-CTTCTTTAAGCCCGCAGGGACAT C-3′. The 5-base mismatch MO sequence was 5′-CTTgTTTAacCCcCGAGAATC-3′. MOs were diluted to obtain 1 mM stock solution in 1×Danieau buffer and were microinjected at a volume of 2 nl into one-cell stage embryos.

Cell culture and transfection- NIH3T3 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD), 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C in a 5% CO2 humidified incubator. For transfection, NIH3T3 cells were seeded on sterile glass coverslips (BDH) in six-well plates (BD Biosciences) and grown for 24 h before transfection. Transfections were performed using 2-6 μg of plasmid cDNA and SuperFect transfection reagent (Qiagen, Germany) according to the manufacturer’s instructions. Cells were harvested for RT-PCR at 24 h after transfection.

RT-PCR and immunofluorescence- After injection or transfection, embryos or cells were harvested at 22 hpf or 24 h, respectively, and lysed.
in Trizol (Invitrogen, USA), followed by standard total RNA extraction. The expression of nuclear transcription factors was detected by serial dilution semi-quantitative RT-PCR assay using indicated primers (Supplemental Table 1).

Embryos were fixed after 22 hpf with 4% paraformaldehyde at 4°C overnight and perforated with 0.3% Triton X-100 for 10 min, then blocked with 10% FBS for 1h, followed by incubation with anti-EGFP antibody (SantaCruz Biotech, Santa Cruz, CA, USA) and anti-phosphohistone H3 antibody or anti-c-Fos antibody (Cell Signaling Technology, Beverly, MA) at 4°C overnight. The embryos were incubated with fluorochrome-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies for 1 h (Molecular Probes, Invitrogen, USA).

Western blot- Deyolking embryos and protein immunoblotting were performed as previously described (25). Anti-ERK1/2, anti-phospho-ERK1/2 (p-ERK1/2), anti-JNK, anti-p-JNK, anti-p38, anti-p-p38 and anti-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-een antibody was purchase from SantaCruz Biotech (Santa Cruz, CA, USA). MEK1/2 inhibitor, PD98059, was purchased from Calbiochem (San Diego, CA).

Statistical analysis- Embryos were mounted in 95% glycerol and the number of cells in the anterior yolk sac was counted under the zeiss microscope (80×). Data was reported as mean values ± SEM, and statistically compared using one-way ANOVA followed with LSD or Student’s t test. Statistical significance was accepted when p < 0.05.

RESULTS

Characterization, phylogenetic and syntenic analyses of the zebrafish een orthologues.

After searching the zebrafish genome database, we identified two een genes which we refer to as een and eenb, respectively. The zebrafish een cDNA (NM_201497) consisted of 1,662-bp and encoded a predicted protein of 351 amino acids (~38 kDa). And eenb cDNA (NM_200301) was 1,460-bp and encoded a predicted protein of 365 amino acids (~39 kDa) (Fig. 1A). At the nucleic acid level, within the coding region, the cDNAs of zebrafish een and eenb were 72% identical, whereas within the 3’UTR, the similarity was dropped to 8%.

We then aligned the complete cDNA coding sequence of een and eenb with the genomic sequence to reveal the exon-intron structure. eenb spans 30.89 kb in the genome and contains ten exons, each aligning with the ten human exons. In contrast, een spans 37.49 kb in the genome and has retained only nine exons, with exons 1 through 8 aligning with human exons 1 through 8 and exon 9 aligning with human exon 10, suggesting that een is missing a sequence homologous to human exon 9, which encodes a variable region acting as a joint linking the BAR and SH3 domain. Comparisons of exon lengths of the two zebrafish een genes with the human EEN indicated a high degree of cross-species conservation (Supplemental Table 2).

We next examined the relationship of een and eenb amino acid sequences in zebrafish to mammalian endophilin family members in more detail. Results suggested that the highest BLASTP bit scores were with EEN (Supplemental Table 3). In the BAR and SH3 domains, zebrafish een and eenb were showed a very good conservation in comparison to mammalian EENs (Supplemental Fig. 1). Using the neighbor-joining method, we constructed a phylogenetic tree with several known tetrapod sequences, including human, mouse, chick and xenopus (Fig. 1B). We were also able to identify two candidate copies of predicted een in the fugu, medaka, and stickleback genomes.

To more precisely confirm the evolutionary conservation of EEN, we investigated the distribution of genes located adjacent to the EEN locus on chromosomes in zebrafish and human genomes. As shown in Fig. 1C, four genes, HLRC1, CREB3L3, STAP2 and SEMA6B, were located close to each other on human chromosome 19 near EEN, and their zebrafish orthologues can be found in the vicinity of een on linkage group (LG) 8, indicating that zebrafish een was an orthologue of human EEN. Human genes MBDA3, SF3A2, CHICO, CCDC94 and STAP2 close to EEN also have their zebrafish orthologues on LG2 near eenb, supporting the idea that eenb was also an orthologue of human EEN.

Taken together, it shows that the two zebrafish copies arose from a duplication event in the teleost lineage after the divergence from the tetrapod lineage but before the teleost radiation. This observation was consistent with the notion of a whole genome duplication in the teleost lineage
een and eenb expression during embryogenesis

The temporal and spatial expression patterns of een and eenb were investigated in zebrafish embryos from 0.75 to 120 hpf by in situ hybridization using digoxigenin-labeled antisense RNA probe. As shown in Fig. 2, strong signals were detectable in the blastomeres of the two-cell stage and the sphere period (4 hpf), indicating that both een and eenb transcripts were maternally derived and suggesting a role in early embryonic development. Although the een and eenb genes exhibited similar patterns of expression during the early stage, differential expression of the een genes was observed after the eighteen-somite stage (18 hpf). At that stage, high levels of een transcripts were still strongly detected throughout the entire embryo, predominantly expressed in neural tissues (brain, spinal cord and eye) and skeletal muscle, identical to that of EEN in mice described previously (27) (Fig. 2A). At 22 hpf, hematopoietic expression of een was observed in the posterior ICM (Fig. 2A, red arrow in inset), a region analogous to the blood island of the yolk sac in mammals (28). By 28 hpf, very strong expression was also seen in the brain, and weaker staining in the tail and the trunk. After 2 dpf, expression decreased and became mostly restricted to the notochord and optic capsule (Fig. 2A, black arrow), then became undetectable by 5 dpf.

By comparison, we found that eenb was restricted to the hatching gland during 18 hpf and 22 hpf (Fig. 2B, black arrow), which has not been previously reported, then was gradually disappeared after 30 hpf (data not shown). By 3 to 5 dpf, eenb expression was no longer observed. The eenb mRNA was not detected in ICM.

To control for specificity, embryos of each stage were also probed in parallel with a sense probe, which gave no signal at any stage (data not shown). Our data provides the first insight into the embryonic expression profile of een genes in zebrafish.

een mRNA overexpression in wild-type or transgenic TG(zpu.1:EGFP) zebrafish embryos

It has been shown that een was expressed in the ICM region, suggesting its possible role in zebrafish hematopoiesis. It was, therefore, interesting to explore the possible function of een on hematopoietic cells. We first microinjected synthetic zebrafish een mRNA into wild-type embryos at the one-cell stage, and examined the hematopoietic marker expression, including scl (hematopoietic progenitors), gata1 (primitive erythroid lineage) and pu.1 (primitive myeloid precursors) (29,30).

As shown in supplemental Figs. 2A & 2B, no obvious morphological changes were detected and the expression of scl and gata1 was similar in 100 pg of either EGFP-, een- or eenb- injected embryos. However, compared to the EGFP-injected or eenb-injected embryos, injection with een mRNA induced ectopic pu.1 expression (Figs. 3A & 3B, red arrows and insets) and caused an increase in the number of pu.1-expressing myeloid cells at 14 hpf and 22 hpf embryos (Figs. 3A & 3B). To quantify this effect, we measured the number of pu.1-positive cells in the yolk sac. At 22 hpf, the mean number of control EGFP-injected or eenb-injected was 57.1 ± 2.92 (n=83, pooled from 3 independent experiments) and 53.6 ± 2.36 (n=91), respectively, whereas 40% eenb-injected embryos reached to 80.1 ± 3.54 (n=103). This value was significantly higher than the other two (p < 0.001) (Fig. 3C).

The above results showed that een overexpression affected early myeloid cells but not erythroid cells. To investigate whether een function is limited to the myeloid cells, we also examined the expressions of two non-myeloid markers krox-20 (ectoderm hindbrain-specific marker) (31) and myoD (mesodermal muscle-expressing marker) (32), after overexpression of een or eenb mRNA. Like scl and gata1 expression, expression of krox-20 and myoD was not affected in een overexpression embryos (supplemental Figs. 2C & 2D).

In order to further verify that the increased cells were indeed pu.1-positive cells but not from trans-differentiation of other cell types, we employed the well-characterized transgenic line TG(zpu.1:EGFP), in which EGFP was expressed in the myeloid progenitors under the zpu.1 promoter (18), and injected capped mRNAs encoding een or eenb into embryos. As expected, about 43% een-overexpression embryos (n=108) significantly increased the number of pu.1+/EGFP+ cells to about 1.5-fold higher than that of the control (n=101) (Figs. 3D & 3E, the
mean number of pu.1+/EGFP+ cells was 68.7 ± 4.35 and 40.5 ± 2.53, respectively) and the cells were also exhibited abnormal distribution in the yolk sac (Fig. 3D, white arrowheads).

In addition, to test if mammalian EEN and zebrafish een proteins are functionally conserved, we injected human EEN mRNA into TG(zpu.1:EGFP) embryos. Overexpression of hEEN resulted in the significant expansion of pu.1+/EGFP+ cells, similar to the effect of zebrafish eena overexpression (supplemental Fig. 3, white arrowheads). These results suggest that een and EEN proteins are functional orthologues.

**Morpholino knockdown of een results in significant suppression of myeloid cells**

To determine whether eena is required for zebrafish myeloid development, we first determined whether the een morpholino oligonucleotide (MO) was able to inhibit the synthesis of endogenous eena protein by Western blot analysis. Microinjection of een MO at the dose of 8 and 16 ng per embryo resulted in a dose-dependent reduction of eena expression (Fig. 4A, lanes 1 and 2). Microinjection of een MO at the dose of 8 and 16 ng per embryo resulted in a dose-dependent reduction of eena expression (Fig. 4A, lanes 1 and 2). Nearly 90% of eenaknockdown embryos at 22 hpf (n=100). Consequently, the number of cells expressing l-plastin (primitive macrophage marker) was also dramatically reduced in 85% of eenaknockdown embryos at 22 hpf (n=92, Fig. 4C). The mean number of l-plastin-expressing cells was about 83.2 ± 3.35 v.s. 136.6 ± 2.96 in eenaknockdown and control embryos, respectively (Fig. 4C & 4F). Consistently, mpo-expressing granulocytes were nearly completely absent in 80% of eenaknockdown embryos within anterior yolk sac and posterior ICM (Fig. 4D). The number of mpo-expressing cells in the anterior yolk sac was 46.2 ± 3.57 v.s. 16.6 ± 4.13 in control and eenaknockdown embryos, respectively (Fig. 4F, n=60). The result is consistent with previous observation that the l-plastin and mpo-expressing cells were absent or dramatically decreased in the pu.1-deficient embryos (33). In contrast, expression of scl was unaffected by een deficiency (Fig. 4E).

**Eena enhances pu.1-expressing myeloid cells expansion via its SH3 domain**

The I-scl approach was originally utilized in Medaka to induce stronger promoter activity in F0 founder, and to decrease non-specific expression and increase the stable integration of transgene into the genome (34). For example, it has been used to study the BMP signaling under the control of zlmo2 promoter in zebrafish hemato-vascular development, which decreased the mosaicism substantially caused by transient transgenic expression and acquired a specific expression pattern in ~30% of the injected embryos (35). Taking advantage of the I-scl approach, we constructed een expression vector driven by pu.1 myeloid promoter flanked by I-scel meganuclease recognition sites, named as pu.1-eena, to further determine if een expression vector driven by pu.1 myeloid promoter flanked by I-scl meganuclease recognition sites, named as pu.1-eena, to further determine if een indeed act as activator of myeloid cells expansion. As a control, the SH3 domain-deleted een was also made as described in Fig. 5A, and referred to as pu.1-eenaΔSH3, to determine the function of SH3 domain in myeloid development.

The pu.1-eena or pu.1-eenaΔSH3 was injected into the TG(zpu.1:EGFP) embryos. The pu.1-eena-injected embryos (45%, n=114) showed a 2-fold increase in the number of pu.1+/EGFP+ cells (77.3±3.02) with ectopic distribution observed in the ventral side of yolk sac (Fig. 5B & 5C, red arrows and white arrowheads), compared to control or pu.1-eenaΔSH3 injected siblings (38.6±1.26 v.s. 41.7±3.67, respectively). To determine the underlying mechanism responsible for increased number of pu.1+/EGFP+ cells, we employed whole-mount immunofluorescence using the anti-EGFP antibody and the anti-phosphohistone H3 antibody. Phosphohistone H3 is a specific marker for cells undergoing mitosis (36). EGFP- and pu.1-expressing cells derived from TG(zpu.1:EGFP) were immunostained for EGFP by green fluorescence and phosphohistone H3-positive proliferating cells by red fluorescence. The result shows that the phosphohistone H3-positive pu.1+/EGFP+ cells were significantly increased in the yolk sac of embryos overexpressing een, compared with...
control or pu.1-eenASH3-injected embryos (Fig. 5D). Taken together, the data strongly suggested that the eena stimulated proliferation through enhancing the mitosis of pu.1-positive myeloid cells and its SH3 domain likely mediated this effect.

**eena enhances myeloid proliferation through stimulating ERK1/2 phosphorylation.**

A number of studies have shown that activation of MAPK is involved in a diverse set of responses affecting cell proliferation and differentiation, adaptation to environmental stress, and apoptosis (37,38). To investigate whether the regulation of myeloid cell proliferation by eena during embryonic development is through the cross-talk with MAPK pathway, western analysis was first performed. As shown in Fig. 5E, at 22 hpf, the level of phospho-ERK was significantly up-regulated by about 5~6-fold in pu.1-eeana-injected embryos compared with controls, while overexpression of eenaΔSH3 showed normal level of ERK phosphorylation. In contrast, eena had no effects on the activation of phospho-JNK and phospho-p38 (Fig. 5E). These data suggested that overexpression of eena might induce proliferation and migration of pu.1-positive cells via selective activation of the ERK signal pathway.

**Effects of eena on activation of nuclear transcription factors**

It has been reported that, in the nucleus, c-Fos, c-Myc, and c-Jun are probably major downstream targets in MAPK pathway (39). To determine whether these transcription factors were influenced, we used a combination of in vitro and in vivo experiments. In vitro, NIH3T3 cells were transfected with the vector control or expression plasmid encoding human wild-type EEN. Cells overexpressing the EEN showed normal level of ERK phosphorylation. In contrast, eena had no effects on the activation of phospho-JNK and phospho-p38 (Fig. 5E). These data suggested that overexpression of eena might induce proliferation and migration of pu.1-positive cells via selective activation of the ERK signal pathway.

To further confirm that phosphorylation of ERK-c-Fos signaling is involved in eena induced proliferation of pu.1-positive myeloid cells, embryos injected with pu.1-eeana were incubated with or without PD98059 (100mM), a specific MEK1/2 inhibitor (40) at 16 hpf for 6 h, followed by analyzing the level of phospho-ERK and c-Fos, and quantitating the number of pu.1+/EGFP+ cells in the yolk sac. The results showed that eena-induced phosphorylation of ERK and up-regulation of ze-fos transcripts were significantly blocked by PD98059. Western blot analysis using a specific antibody against ze-fos protein showed that the levels of eena protein were approximately 2-fold higher in eena-injected embryos than that of control or eenaΔSH3-injected embryos. Conversely, the expressions of ze-jun and ze-myc mRNAs were not changed (Fig. 6C).

The above result implied c-Fos as an important factor for myeloid cells proliferation and led us to further examine its expression in these cells of 22 hpf embryos after injected with pu.1-eeana, using ze-fos as probe of whole-mount **in situ** hybridization. In the control embryos, only a diffuse staining was observed in the head and the trunk. After injected with pu.1-eeana, however, high level of ze-fos mRNA expression was detected in the individual pu.1-positive myeloid cells (Fig. 6D, left panel, black arrowheads). To verify these hybridization results, immunofluorescence assay was used to determine the EGFP and c-Fos protein in pu.1-positive cells with specific anti-EGFP (green channel) and anti-c-Fos (red channel) antibodies **in vivo**. In agreement with observations, c-Fos was also up-regulated in the pu.1+/EGFP+ cells (Fig. 6D, middle and right panels) after injected with pu.1-eeana in Tg(zpu.1:EGFP).

**Blocking the ERK signaling inhibits proliferation of pu.1-positive myeloid cells induced by eena.**

To further confirm that phosphorylation of ERK-c-Fos signaling is involved in eena induced proliferation of pu.1-positive myeloid cells, embryos injected with pu.1-eeana were incubated with or without PD98059 (100mM), a specific MEK1/2 inhibitor (40) at 16 hpf for 6 h, followed by analyzing the level of phospho-ERK and c-Fos, and quantitating the number of pu.1+/EGFP+ cells in the yolk sac. The results showed that eena-induced phosphorylation of ERK and up-regulation of ze-fos transcripts were significantly blocked by PD98059. Western blot analysis using a specific antibody against ze-fos protein showed that the levels of eena protein was similar in pu.1-eeana-injected embryos treated with and without PD98059 and was about 1.5~2-fold higher than endogenous expression level (Fig. 7A). Quantification assay confirmed that the number of pu.1+/EGFP+ cells was strongly increased in ~45% of the eena-overexpressed embryos, but reduced after
AML1-ETO associated leukemogenesis. Mechanistic insights into MLL-EEN mediated or EEN will undoubtedly contribute to further understanding the function of the fusion protein AML1-ETO could aberrantly transactivate the fusion gene, higher levels of EEN transcripts were observed in primary M2 leukemia cells carrying the AML1-ETO fusion gene (44). Therefore, fully understanding the function of EEN will undoubtedly contribute to further mechanistic insights into MLL-EEN mediated or AML1-ETO associated leukemogenesis.

It has been well documented that there is not a one-to-one correspondence between zebrafish and human chromosomes, and several genes unique in mammals have two or more copies in teleost fish (45,46). In this study, we have identified two highly similar zebrafish een genes and investigated their spatio-temporal expression and the possible functions of these proteins in hematopoiesis. Several lines of evidence suggest that the two een genes are co-orthologues of the mammalian EEN. The conserved synteny suggests that portions of these two zebrafish chromosomes are derived by genome duplication or by segmental duplication of a chromosome sharing a common ancestor with human chromosome 19. Changes in gene order within this conserved synteny support the frequent occurrence of inversions and other intrachromosomal rearrangements in these regions since the divergence of teleost and tetrapod ancestors (47).

After a gene is duplicated, one of the copies usually accumulates nonsense mutations and becomes a pseudogene, although sometimes both paralogue copies are retained in the genome (48). Zebrafish with partial genome duplication had become a critical model system to study the functional divergence of duplicated genes (49). The finding that both of the duplicated een genes are preserved in zebrafish also raised an important question regarding their functional relationship. In this study, we first explored the possible difference in the spatio-temporal expression patterns of the zebrafish eena and eenb genes. At 18 hpf, expression of eenb was restricted to the hatching gland, while expression of eena was still detected ubiquitously and expressed in hematopoietic cells. Therefore, the differential expression patterns of the zebrafish eena and eenb genes imply a hierarchical subfunctionalization that may account for the retention of both the duplicated een genes in the zebrafish genome and also indicate that eena may have more general and/or different functions from eenb in hematopoietic cells. Previous data suggests that EEN is the only member in the endophilin family expressed in hematopoietic cells capable of transforming NIH3T3 and enhancing the self-renewal capacity and proliferative potential of clonogenic hematopoietic progenitors in vitro (1,44). Our previous study also showed that the HL-60 cells transfected with pEGFP-EEN grew much faster than the control cells (44). Consistently, in this report, we found that knockdown of zebrafish eena resulted in the significantly decreased number of myeloid cells. Furthermore, targeted expression of eena led to proliferation of pu.1-positive myeloid cells in vitro. Evidences suggested that overexpression of cortactin in NIH3T3 fibroblasts and endothelial cells resulted in an enhanced cell migration and invasive potential (50,51). It has been shown that cortactin is found to interact with dynamin (52), which is also a target for EEN (6,9). Likewise, we found that targeted overexpression of zebrafish eena led to abnormal migration of pu.1-positive myeloid cells in the yolk sac. Therefore, it raises the possibility that eena may couple dynamin to cortactin to regulate myeloid cell migration in zebrafish.

As previously described, endophilin family members encode proteins with highly conserved SH3 domains that share the highest homology to the SH3 domain found in Grb-2/Sem-5, a member of the family of adaptor proteins conserved from fruitfly to mammals (53-55). These adaptor
proteins couple receptor tyrosine kinases to the Ras signalling pathway and participate in both normal and pathogenic pathways through the interaction of their SH3 domains with proline-rich peptide sequences of the target proteins (56). Mutations in the SH3 domain of the Grb2/Sem-5 protein abolish its binding to downstream effectors, and result in a loss of function (54,55). Based on the high sequence homology between the SH3 domains of EEN and Grb-2 proteins, we speculated that eeNA might play a role in mitogenic signaling via its SH3 domain. Coincident with the hypothesis, our results showed that eeNA overexpression specifically increased the phosphorylation of ERK1/2 and promoted the transcription of its downstream target zc-fos. However, eeNA lacking the SH3 domain completely abolished these effects. MEKs are the only known upstream activators of ERKs, and PD98059, a specific MEK1/2 inhibitor partially blocked the phosphorylation of ERK1/2 and subsequent down-regulation of c-Fos, and also inhibited the myeloid cell proliferation induced by eeNA (Fig. 7). Therefore, our results clearly demonstrated that eeNA overexpression led to the proliferation of myeloid cells mainly via the ERK-c-Fos signal pathway and that the SH3 domain plays an important role. The activator protein-1 (AP-1) complex of transcription factors, which is composed of the Jun and Fos proteins, is related to many oncogenic signal transduction pathways (57). Our previous results indicated that overexpression of EEN transactivates AP-1 in the NIH3T3 and HL60 cell lines, as well as in COS7, K562 and U937 cells by luciferase reporter assay (44). In this report, we showed that EEN selectively induced the up-regulation of c-Fos in NIH3T3 cells again. In addition, we provided further evidence that overexpression of eeNA did stimulate up-regulation of zc-fos mRNA but not zc-jun in vivo, suggesting that the evolutionarily conserved EEN-ERK-c-Fos signal pathway played a significant role in the proliferation of myeloid cells.

Accumulating evidence suggests that endocytosis of certain RTKs and G protein-coupled receptors is obligatory for MAP kinase activation (58-62). For example, EGF receptor mediated MAPK activation is inhibited by a mutant version of dynamin that blocks endocytosis (60,61). Given the involvement of EEN in endocytosis (8,9,11,12), along with the findings that overexpression of eeNA activate the MAPK pathway and induce myeloid proliferation, our studies raise a intriguing question as to whether eeNA may serve as a potential link between the endocytic machinery and the MAPK signal transduction machinery that need to be addressed.

REFERENCES
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FOOTNOTES

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Protein sequences with Entrez protein accession numbers from human EEN (NP_003016), mouse EEN (NP_038692), chick EEN (NP_989860) and xenopus EEN (AAH61395) can be found at http://www.ncbi.nlm.nih.gov. Protein sequences for Danio rerio (zebrafish), Fugu rubripes (fugu), Gasterosteus aculeatus (stickleback), and Oryzias latipes (medaka) were found by searching the predicted protein database of each species and can be found on Ensembl (http://www.ensembl.org). Zebrafish eena (NP_958905), zebrafish eenb (NP_956595), fugu eena (SINFRUT00000144757), fugu eenb (SINFRUP00000151916), stickleback eena (ENSGACP00000016230), stickleback eenb (ENSGACP00000020231), medaka eena (ENSORLP00000004783), and medaka eenb (ENSORLP00000018713).

Abbreviations used: EEN, extra eleven nineteen; MLL, mixed-lineage leukemia; MEK, MAPK/ERK kinase; SH3, Src homology 3; BAR, BIN/amphiphysin/Rvsp; ICM, intermediate cell mass; hpf, hours post-fertilization; dpf, days post-fertilization; LG, linkeage group; AML, acute myeloid leukemia; AP-1, activator protein-1.

FIGURE LEGENDS

Figure 1. Structural, phylogenetic and syntenic analyses of zebrafish eena and eenb. A, A schematic diagram comparing the genomic structures of the zebrafish eena and eenb genes. Closed boxes represent exons, thin lines introns. Areas shaded in black represent UTRs and the numbers of amino acids encoded by each exon are shown above the boxes. UTR sizes are indicated in base pairs. ATG initiation codon and stop codons are represented by an arrow and a bar, respectively. B, Phylogenetic analysis of zebrafish eena and eenb. The numbers on the branches of the phylogenetic tree are bootstrap values. The topology of the tree suggests that the eena locus duplicated before the teleost radiation. Hs, Homo sapiens (human), Mm, Mus musculus (mouse), Gg, Gallus gallus (chick), Xl, Xenopus laevis (Xenopus), Dr, Danio rerio (zebrafish), Ga, Gasterosteus aculeatus (stickleback), Fr, Fugu rubripes (fugu), Ol, Oryzias latipes (medaka). A scale of ‘0.1’ means 0.1 nucleotide substitution per site. C, Comparison of the syntenic relationship of the zebrafish eena genes with the human orthologue. Orthologous eena gene symbols are in bold. Another pair of duplicated genes (stap2a and stap2b) on zebrafish and the human STAP2 orthologue are underlined. Mb, megabase.

Figure 2. Expression of zebrafish eena and eenb during embryonic development. A, Zebrafish eena expression in wild-type AB strain embryo. B, Zebrafish eenb expression pattern. HG, hatching gland; ICM, intermediate cell mass; NC, notochord; OC, optic capsule.

Figure 3. Effects of eena mRNA overexpression in wild-type or TG(zpu.1:EGFP) transgenic embryo. A, Expression of pu.1 mRNA at 14 and 22 hpf wild-type embryos injected with control EGFP, eena, or eenb mRNA. Red arrows and insets indicate that proliferation and abnormal distribution of pu.1-positive cells after eena overexpression. Left panels, lateral views with anterior to the up; right panels, lateral views with anterior to the left. B, Morphologies of eena-injected embryos with outgrowth and abnormal distribution of pu.1 cells at 22 hpf. (a) ventral views; (b) higher magnification of the inset in a, lateral views; (c-e) lateral views. C, pu.1 cells in the yolk sac were counted under the microscope (x 80). The bar
The graph shows *pu.1*-positive cell number presented by means ± SEM. *, *p < 0.05. *D, een mRNA overexpression induces ectopic *pu.1*-expressing myeloid cell formation in *TG(zpu.1:EGFP)* embryos. Note the increase in the number of *pu.1+/EGFP*+ cells, some of which are located ectopically (red arrows). Left panels, lateral views with anterior to the left and dorsal up; middle panels, dorsal views; right panels, ventral views. *E, pu.1+/EGFP*+ cells were counted in the same way as shown in Fig. 3C. Similar results were obtained in three independent experiments. Data are presented as means ± SEM. *, *p < 0.05.

**Figure 4.** *Eena* is required for the development of myeloid cells. *A, Western analysis of embryos injected with *eena* or control morpholinos at the indicated doses. The proteins extracted from twenty embryos at 22 hpf were loaded onto each lane. WT, uninjected; C, control morpholino. *B-E, Whole-mount mRNA in situ analyses of *pu.1, l-plastin, mpo* and *scl* expression at 22 hpf. The black arrow in *D* indicates *mpo*-expressing cells in the posterior ICM. *F, Quantitative analysis of myeloid cell number in anterior yolk sac of control embryos or injected with *eena* MO. *, *p < 0.05.*

**Figure 5.** Zebrafish *eena* enhances *pu.1*-positive myeloid proliferation via the ERK signaling. *A, eena* expression vectors used in studies, which are driven by *zpu.1* promotor, and flanked by 1-sceI sites. *B, Fluorescent image of 22 hpf living embryos injected with *pu.1-eena* or *pu.1-eenaASH3* at one-cell stage. Left panels, lateral views are shown with anterior to the left and dorsal up; middle panels, dorsal views; right panels, ventral views. *C, Quantification of *pu.1+/EGFP*+ cells. Data are presented as means ± SEM. *, *p < 0.05. *D, Anti-phosphohistone H3 immunostaining was performed on 22 hpf embryos to detect proliferating cells at mitotic phase of cell cycle. All *pu.1+/EGFP*+ cells in the yolk sac are stained with green (left panels) and nuclei of proliferating, phosphohistone H3-positive cells are stained with red (middle panels), and merged in right panels. *E, Embryos injected with *pu.1-eena* or *pu.1-eenaASH3* were analyzed by western blot with anti-total and anti-p-MAPK antibodies. Comparable results were obtained in three independent experiments.

**Figure 6.** The effects of *eena* on the activation of nuclear transcription factors by semi-quantitative RT-PCR analysis *in vitro* and *in vivo*. *A, Analysis of murine *c-Fos* and *c-Jun* mRNA levels in NIH3T3 cells transfected with control or human pEGFP-EEN plasmid by serial dilution semi-quantitative RT-PCR *in vitro*. Expression of *Gapdh* was used as an internal control. And the extent of serial dilution of cDNA samples was listed in the bottom. *B, Analysis of *c-Fos* protein expression levels in NIH3T3 cells transfected with pEGFP, pEGFP-*eena* or pEGFP-*eenaSH3* plasmid, respectively. *C, Study of *zc-fos, zc-jun* and *zc-myc* mRNA expression in *eena* or *eenaASH3*-injected *TG(zpu.1:EGFP)* embryo by single-embryo RT-PCR *in vivo*. Expression of *β-actin* was used as an internal control. And the serial dilution of cDNA samples was listed in the bottom. *D, Detection of *zc-fos* mRNA or protein in *pu.1-eena*-injected *TG(zpu.1:EGFP)* embryos. Left panel, expression of *zc-fos* mRNA using whole-mount *in situ* hybridization; middle panels, EGFP and c-Fos immunofluorescent images were recorded with appropriate filters, respectively; right panels, the merge of EGFP and c-Fos images indicates that c-Fos was upregulated in *pu.1+/EGFP*+ cells (yellow).

**Figure 7.** The effects of kinase inhibitor on *eena*-induced ERK1/2 activation and cell proliferation in *TG(zpu.1:EGFP)* embryos. *A, eena, c-Fos, phosphorylated and total ERK1/2 proteins were assessed by western blot. *B, Quantification of *pu.1+/EGFP*+ myeloid cells with or without PD98059 administration after *eena* overexpression. *C, Proposed model for *eena*-mediated myeloid cells proliferation in zebrafish embryo.*
Figure 1

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{W}

{X}

{Y}

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Figure 2

A
Figure 3

A lateral lateral pu.1

Control 14 hpf pu.1

eena mRNA

22 hpf

eenb mRNA

B

eena mRNA

a b c
d e

D lateral dorsal ventral

Control

22 hpf

eena mRNA

22 hpf

eenb mRNA

E

C

Control (n = 83)
eena mRNA (n = 103)
eenb mRNA (n = 91)

pu.1 cell number

90 80 70 60 50 40 30 20 10

22 hpf

Control (n = 108)
eena mRNA (n = 101)
eenb mRNA (n = 93)

pu.1/EGFP+ cell number

80 70 60 50 40 30 20 10

22 hpf
Figure 4

A

eena morpholino injection (ng)

WT C(16.0) 8.0 16.0

een Actin

B

Control
eena MO

22 hpf

C

Control
eena MO

22 hpf

D

Control
eena MO

22 hpf

E

Control
eena MO

22 hpf

F

L-plastin

Control eena MO

pu.1

mpo

myeloid cell number

*p*
Figure 5A-C

A

pu.1-eena
I-sceI—pu.1-promoter
pu.1-eena ΔSH3
I-sceI—pu.1-promoter

BAR SH3
sv40—I-sceI

B

lateral
dorsal
ventral

Control (n = 112)
pu.1-eena (n = 114)
pu.1-eena ΔSH3 (n = 105)

C

pu.1+/GFP+ cell number

*
Figure 5D and E

D

Control

22 hpf

pu.1-eena

22 hpf

pu.1-eena ΔSH3

22 hpf

E

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Fold induction

- p-ERK/ERK
- p-JNK/JNK
- p-p38/p38
Figure 6

A  In vitro NIH3T3 cells

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B  In vitro NIH3T3 cells

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C  In vivo zebrafish embryos

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D  In vivo zebrafish embryos

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Figure 7

A

Control  pu.1-eena  PD98059

[Images of protein blots showing eena, p-ERK, ERK, c-Fos, Actin]

B

- Control (n = 102)
- pu.1-eena (n = 97)
- pu.1-eena+PD98059 (n = 93)

[Bar graph showing pu.1+EGFP+ cell number at 22 hpf]

C

[Diagram showing the interaction of eena, ERK (p), PD98059, c-fos, AP-1, nucleoplasma, and proliferation]

* indicates statistical significance.
EENA promotes myeloid proliferation through stimulating ERK1/2 phosphorylation in zebrafish
Huang-Ying Le, Yong Zhang, Han Liu, Li-Heng Ma, Yi Jin, Qiu-Hua Huang, Yi Chen, Min Deng, Zhu Chen, Sai-Juan Chen and TingXi Liu

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