A novel myeloid-restricted zebrafish CCAAT/enhancer-binding protein with a potent transcriptional activation domain

Susan E. Lyons, Bixiong C. Shue, Andrew C. Oates, Leonard I. Zon, and P. Paul Liu

The CCAAT/enhancer-binding protein (C/EBP) family consists of transcription factors essential for hematopoiesis. The defining feature of the C/EBPs is a highly conserved carboxy-terminal bZIP domain that is necessary and sufficient for dimerization and DNA binding, whereas their amino-terminal domains are unique. This study reports a novel c/ebp gene (c/ebp1) from zebrafish that encodes a protein homologous to mammalian C/EBPs within the bZIP domain, but with an amino terminus lacking homology to any C/EBP or to any known sequence. In zebrafish embryos, c/ebp1 expression was initially observed in cells within the yolk sac circulation valley at approximately the 16- to 18-somite stage, and at 24 hours postfertilization (hpf), also in circulating cells. Most c/ebp1 cells also expressed a known early macrophage marker, leukocyte-specific plastin (lplastin). Expression of both markers was retained in m683 and spadetail, mutants affecting erythropoiesis, but not myelopoiesis. Further, c/ebp1 expression was lost in a mutant with defective myelopoiesis, but intact erythropoiesis. These data suggest that c/ebp1 is expressed exclusively in myeloid cells. In electrophoretic mobility shift assays, c/ebp1 was able to bind a C/EBP consensus DNA site. Further, a chimeric protein containing the amino-terminal domain of c/ebp1 fused to the DNA-binding domain of GAL4 induced a GAL4 reporter 4000-fold in NIH3T3 cells. These results suggest that c/ebp1 is a novel member of the C/EBP family that may function as a potent transcriptional activator in myeloid cells.

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affect later steps along the erythroid pathway. However, data on mutants that specifically affect the myeloid lineage have not yet been published.

To facilitate analysis of myelopoiesis in *D. rerio*, a zebrafish kidney complementary DNA (cDNA) library was screened for c/ebp cDNAs using a probe encoding the conserved bZIP region of human C/EBPβ. A gene encoding a c/ebp family member was identified that has no known ortholog in any other organism and was named *c/ebp1*. The *c/ebp1* gene was mapped within the zebrafish genome and its expression was analyzed by RNA in situ hybridization both in wild-type and hematopoietic mutant embryos. The *c/ebp1* protein was shown to bind DNA containing a canonical mammalian C/EBP binding site motif. Further, *c/ebp1* contains a novel amino-terminal domain that, although apparently unrelated to any known activation domain, acted as a potent activator of transcription in a mammalian system.

Materials and methods

Library screening and sequence analysis of cDNAs

Random-primed and oligo-dT primed zebrafish adult kidney cDNA libraries in the phagemid pBKCMV 37 were screened using a probe encoding the bZIP domain of human C/EBPβ generated via polymerase chain reaction (PCR). The probe was amplified from a plasmid containing human C/EBPβ (courtesy of K. G. Xanthopoulos) 14 using the forward primer, 5'-AAGGCCAAAGAAGGCTAGGAC-3' (nucleotides [nt] 583-603), and the reverse primer 5'-TCAGCTGCCAACCCTCCCCGCAGC-3' (nt 846-826). The amplified fragment (264 bp) was labeled using 32P-dCTP with the Rediprime kit (Amersham-Pharmacia, Piscataway, NJ), according to the manufacturer's instructions. Positive clones were isolated and sequenced in one direction with a T7 primer (performed by ACGT, Northbrook, IL). Sequences were placed into a contig map using the Sequencing Project Manager program within DNASTAR. A single clone encompassing the open reading frame was sequenced in both directions. The sequence has been submitted to GenBank and the accession number is AF306857.

Radiation hybrid mapping

The *c/ebp1* gene was mapped within the zebrafish genome using a 94-clone radiation hybrid panel generated from a zebrafish primary fibroblast cell line, *A. ceylanicus*, and a hamster fibroblast cell line, WGHI. The primers used to amplify a portion of *c/ebp1* were 5'-CACTTTAACTCGAAAGTT-TCAGG-3' (nt 364-383) and 5'-ATCCAGATGGACCCGAGCAGG-3' (nt 476-457). PCR results on the panel of 94 radiation hybrid DNAs and 2 control DNAs from the zebrafish and hamster parental lines were submitted to http://www.eb.tuebingen.mpg.de/abt.3 for analysis and placement on the zebrafish map. 27

Zebrafish maintenance and breeding

Zebrafish were maintained and bred essentially as described 28 under an approved animal use protocol of the National Institutes of Health. After breeding, embryos were maintained in egg water (0.006% Instant Ocean in distilled water) with 2 parts per million methylene blue to prevent fungal growth.

Whole-mount in situ hybridization

Embryos used for in situ hybridization were obtained from breedings of the wild-type EK strain (Ekkwill) 29 and 3 hematopoietic mutants, cloche (clo 39), spadetail (sp 8090), 30 and mshh 31. Embryos were staged as described. 28 When growing embryos for harvest at more than 24 hours postfertilization (hpf), embryos were grown in 0.003% 1-phenyl-2-thiourea (Sigma, St Louis, MO) to prevent melanization. Embryos were dechorionated in 2 mg/mL pronase followed by extensive washing in 30% Danieau solution (58 mM NaCl, 0.67 mM KCl, 0.4 mM MgSO 4, 0.6 mM Ca(NO 3 ) 2, and 4.5 mM HEPES, pH 7.5) and killed in 0.2% 3-amino benzoic acid ethyl ester (Tricaine). Whole-mount in situ hybridization was performed essentially as described with the following modifications. Embryos less than 24 hpf were not treated with proteinase K. Embryos between 24 and 36 hpf were treated for 5 minutes in 10 μg/mL proteinase K and embryos 36 hpf to 2 days postfertilization (dpf) were treated for 10 to 20 minutes. Hybridization and washing were performed at 55°C for all probes. RNA antisense probes were synthesized according to the manufacturer's instructions with either UTP-digoxigenin (c/ebp1) or UTP-fluorescein (l-plastin) (Boehringer Mannheim, Indianapolis, IN). Probes labeled with digoxigenin were visualized with BM-purple (Boehringer Mannheim) and probes with fluorescein with fast red (Boehringer Mannheim). The *c/ebp1* cDNA used for in situ hybridization started at nt +3 and included over 600 bp of 3' untranslated sequence. The plasmid was digested with EcoRI and T7 RNA polymerase was used to synthesize the antisense probe. The plasmid containing *l-plastin* (kindly provided by B. Thisse) 31 was linearized with NotI and transcribed with T7 RNA polymerase. Embryos were examined with an Olympus dissecting microscope or a Nikon Microphot-FXA compound microscope and photographed with a Spot (Diagnostic Instruments, Sterling Heights, MI) or Quantix (Photometrics, Tucson, AZ) CCD camera. In some cases, images focused in 2 different planes were merged using Adobe Photoshop to allow both yolk sac and tail regions to be visualized in the same image.

Electrophoretic mobility shift assays

For DNA-binding assays, a double-stranded probe containing an optimal C/EBP binding site was prepared by annealing a self-complementary oligonucleotide with a GATC overhang (shown in bold), 5'-GATCTGGAATTCGGAGATGCAGAGACTAGTCTCTGCA-3' and labeling using Klenow polymerase and 32P-dCTP. The probe was amplified from a plasmid containing human C/EBPα and used as a negative control. DNA-binding assay samples were separated on a poly[dI-dC], 1 ng labeled probe (1 × 10 6 cpm), and varied amounts of unlabeled probe as indicated. A probe with a mutated C/EBP binding site, 5'-GATCTGGAATTCGGAGATGCAGAGACTAGTCTGCAGCA-3' (the GATC overhang is shown in bold and changes from the wild-type probe are underlined) was used in vitro to transcribe and translate proteins (TNT reticulocyte lysate system, Promega, Madison, WI) according to a manufacturer's directions. Products from 40 ng of each plasmid were combined with 1 μg poly[dI-dC], 1 ng labeled probe (1 × 10 6 cpm), and various amounts of unlabeled probe as indicated. A probe with a mutated C/EBP binding site, 5'-GATCTGGAATTCGGAGATGCAGAGACTAGTCTGCA-3', was used to test for protein-DNA interactions. DNA-binding assay samples were separated on a 4% polyacrylamide/0.25 × TBE (tris borate EDTA) gel. 35S-methionine-labeled proteins were analyzed on a 4% to 12% bis-tris gradient gel (Invitrogen, Carlsbad, CA), fixed, treated with Amplify (Amersham-Pharmacia), and dried followed by autoradiography.

Transfection and activation assays

The cDNAs encoding putative activation domains were placed in pcDNA3-based vector containing the region encoding the DNA-binding domain (DBD) of GAL4 (amino acids [aa] 1-147) (plasmid kindly provided by N. Perkins, University of Dundee). A PCR fragment encoding the region of the DBD of c/ebp1 was ligated downstream of the GAL4 DBD using EcoRI and BamHI. The primers used to amplify a portion of the DBD of *c/ebp1* were 5'-CCGGAATTCCTAGATCGGTGTCTGCAAAATCATC-3' (nt 1-21, EcoRI site in bold letters) and 5'-CCGGAATTCCTAGATCGGTGTCTGCAAAATCATC-3' (nt 1-21, EcoRI site in bold letters) and 5'-CCGGAATTCCTAGATCGGTGTCTGCAAAATCATC-3' (nt 1-21, EcoRI site in bold letters). The product encoding aa 1-194 was amplified with the reverse primer: 5'-CCGGAATTCCTAGATCGGTGTCTGCAAAATCATC-3', and the primers used to amplify a portion of the DBD of *c/ebp1* were 5'-GGCCATCCGGCGCCCCGGCCGCGC-3' (nt 262-241, BamHI site in bold letters). PCR fragments encoding the first 194 aa and the first 102 aa of human C/EBPα were amplified from a human C/EBPα plasmid and placed downstream of the GAL4 DBD with EcoRI and BamHI. Both PCR products were obtained using the forward primer: 5'-CCGGAATTCCTAGATCGGTGTCTGCAAAATCATC-3'. The product encoding aa 1-194 was amplified with the reverse primer: 5'-GGCCATCCGGCGCCCCGGCCGCGC-3' (nt 856-565, BamHI site in bold letters) and the product encoding aa 1-102 was amplified with the reverse primer: 5'-CCGGAATTCCTAGATCGGTGTCTGCAAAATCATC-3'. The amplified regions and the restriction site junctions were sequenced in both directions for all

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constructs. NIH3T3 cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum (Life Technologies, Rockville, MD) at 37°C under 5% CO2. Approximately 5 x 10⁶ cells were cotransfected with 125 to 250 ng of a cytomegalovirus promoter-driven β-galactosidase expression vector and the indicated quantities of plasmids in 6-well dishes using Superfect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Transfected cells were harvested 24 to 48 hours after transfection for luciferase and β-galactosidase assays. Cells were lysed by the addition of 500 μL reporter lysis buffer (Promega) into each well. Cells were scraped from each well and lysates were incubated at room temperature with gentle shaking for 10 minutes followed by centrifugation at 14,000 rpm for 5 minutes to pellet debris. Twenty microliters of lysate was used to measure luminescence from luciferase or from a chemiluminescent β-galactosidase assay in a Tropix TR717 microplate reader (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol (Promega). Luminescence units were normalized for transfection efficiency using β-galactosidase activity.

Results

Isolation and mapping of c/ebp1 in zebrafish

A zebrafish kidney cDNA library was screened with a 264-bp PCR product encoding the conserved bZIP domain of human C/EBPκ to identify C/EBP family members from zebrafish. One c/ebp cDNA, represented by 25% of the clones, was not orthologous to any mammalian C/EBP genes and was named c/ebp1. The start of the open reading frame for c/ebp1 was determined based on the rules of Kozak.22 c/ebp1 encodes a 170-aa protein including a bZIP domain containing 4 leucines in a heptad periodicity. The bZIP domain of c/ebp1 was between 26% and 50% identical and 66% and 79% conserved when compared to the bZIP domains of the human C/EBP family members. However, the amino-terminal region of c/ebp1 showed no significant homology with any of the human C/EBPs (Figure 1A). Further, BLAST analysis did not reveal homology between the amino-terminal region of c/ebp1 and any open reading frame in GenBank. To clarify the evolutionary relationship of c/ebp1 to other C/EBP family members, a phylogenetic tree was generated using an alignment of the conserved bZIP region of c/ebp1 with those of all the human C/EBP family members and the recently cloned zebrafish c/ebpa, c/ebpb, c/ebpc, and c/ebpd genes (S.E.L. et al., manuscript in preparation) (Figure 1B). In this analysis, c/ebp1 was excluded from the branches containing the human C/EBP family members and thus does not appear to have a human ortholog.

The c/ebp1 gene was mapped to the zebrafish genome to aid future mutant screening analyses. PCR primers within the coding sequence of c/ebp1 were used to type the Goodfellow radiation hybrid panel.20 The PCR primers used did not amplify other zebrafish c/ebps or sequences in hamster genomic DNA (data not shown). The c/ebp1 gene was mapped to linkage group 24 (LG24) between 71 and 72.1 cM from the top of LG24 with a LOD score of 10.51 (Figure 2).

Expression of c/ebp1 in normal embryos

Expression of c/ebp1 throughout embryonic development was analyzed by RNA in situ hybridizations using digoxigenin-labeled antisense RNA. At approximately the 16- to 18-somite (17-18 hpf) stage of development, expression was first detected in a few cells overlying the yolk sac in the embryo (data not shown). By the 20- to 21-somite stage (19.5 hpf), approximately 25 cells located between the epithelial layer and the yolk sac mass were stained (Figure 3A). At approximately 24 hpf, 25 to 50 stained cells could be seen overlying the yolk sac (Figure 3B,D) and stained cells could be seen within the caudal portion of the axial vein. The exact locations of the c/ebp1+ cells over yolk sac and in the axial vein are not fixed, because they are cells in circulation. The stained cells appeared small and round, differing from the yolk syncytial layer underneath, consistent with circulating hematopoietic cells (Figure 3D). The majority of cells appeared to have a scant amount of cytoplasm with a large, circular nucleus (Figure 3E). At 2 dpf, stained cells were still visible within the circulation and the surrounding mesenchyme around the caudal part of the axial vein (Figure 3C). Further, cells were seen in the mesenchyme of the head. There is minimal staining at 3.5 days, with only a few circulating cells visible (data not shown).

The expression pattern appeared similar to that seen with a known myeloid marker, l-plastin, which is expressed in circulating...
cells along the yolk sac initially at the 16- to 18-somite stage (17-18 hpf) (data not shown) and subsequently in the axial vein and the head.17 It is difficult to compare expression patterns between 2 embryos hybridized separately with l-plastin and c/ebp1 probes, due to the fact that cells expressing either of these 2 genes are in circulation, and therefore, likely to change their locations from embryo to embryo. However, when embryos at 24 hpf were double-labeled with the c/ebp1 and l-plastin probes, the majority of cells visualized expressed both c/ebp1 and l-plastin (Figure 4). Individual cells appeared to express different ratios of l-plastin and c/ebp1, as shown by arrows in Figure 4. These data suggest that c/ebp1 is expressed specifically in myeloid cells from the 16- to 18-somite stage through 2 dpf stages of development.

Expression of c/ebp1 in hematopoietic mutant embryos

To determine if c/ebp1 gene expression is altered in hematopoietic mutants, RNA in situ hybridization was performed with mutant embryos. The bloodless mutant, cloche, has been shown to be affected at a very early stage in both hematopoietic and vascular development.22 The mutation occurs upstream of scl, a gene encoding a transcription factor required for hematopoietic stem cell differentiation.24 Expression of c/ebp1 was absent in cloche embryos as was l-plastin expression (Figure 5).

In a bloodless mutant, m683, scl expression was intact, but gata-1 expression was absent, consistent with a defect beyond the stem cell level.25 In this mutant, expression of both c/ebp1 and l-plastin was normal, suggesting a defect in the erythroid pathway with normal myeloid development. Therefore, c/ebp1 expression parallels l-plastin expression in hematopoietic mutants, consistent with a common lineage for the cells marked by c/ebp1 and l-plastin. Another mutant, spadetail (spu104), which affects somitic mesoderm specification,30,33 has defects in erythropoiesis with normal vascular development24 and normal expression of pu.1, another early myeloid marker.35 The c/ebp1 gene was expressed later than pu.1 and was maintained in the spadetail mutant (spu104).
myeloid lineage. 

in-crosses. 

using wild-type, cloche, and m683 embryos at 24 hpf. Stained cells are seen over the observed in 25% of the cloche embryos from at least 3 independent heterozygote staining patterns with a single probe (either c/ebp1 or l-plastin) because the stained cells were freely flowing over the yolk sac and in the circulation before sacrifice and fixation of the embryos. Lack of staining using c/ebp1 or l-plastin probes was 

cells were freely flowing over the yolk sac and in the circulation before sacrifice and fixation of the embryos. Lack of staining using c/ebp1 or l-plastin probes was observed in 25% of the cloche embryos from at least 3 independent heterozygote in-crosses.

(data not shown), again consistent with c/ebp1 as a marker of the myeloid lineage.

Finally, a mutant was recently isolated from N-nitroso-N-ethyleurea (ENU) mutagenesis screening that has lost expression of l-plastin (S.E.L. et al. results to be described elsewhere). In this mutant, c/ebp1 expression was also lost while expression of the stem cell marker, cbf1,25 and the erythroid marker, gata1, was maintained. Characterization of this mutant suggests a specific defect in the myeloid pathway with intact hematopoietic progenitor and erythroid pathways. Therefore, the loss of c/ebp1 expression in this mutant points strongly toward expression of c/ebp1 specifically in cells of myeloid lineage.

Analysis of c/ebp1 as a transcriptional activator

Sequence comparison between c/ebp1 and mammalian C/EBP family members revealed significant homology within the bZIP domain, but no homology at the amino terminus. Consistent with the high homology within the bZIP domain, full-length c/ebp1 was able to bind a palindromic C/EBP binding site by an electrophoretic mobility shift assay (Figure 6). This binding was specific because it was competed away with addition of an excess amount of unlabeled self DNA, but not with a mutated site probe. In the same experiment, human C/EBPγ also bound the C/EBP site specifically (data not shown).

To assess whether the amino terminus of c/ebp1 could function as a transcriptional activator, a chimera (GAL4-c/ebp1 [1-87]) containing the DBD of GAL4 fused to the region of c/ebp1 amino terminal to the bZIP domain (aa 1-87) was used to activate a GAL4 DNA recognition site-driven luciferase reporter gene (GAL4-luc) (Figure 7A,B). The GAL4-c/ebp1 (1-87) expression construct induced the GAL4-luc reporter up to 4000-fold in NIH3T3 cells relative to induction of the GAL4-luc reporter by the GAL4 DBD without an activation domain (Figure 7B). A dose response was seen when increasing amounts of GAL4-c/ebp1 (1-87) were used to induce the GAL4-luc reporter (Figure 7B). To compare the transcriptional activation activity of c/ebp1 to human C/EBPγ, the amino terminus of human C/EBPγ was fused to the GAL4-DBD and tested for transcriptional activation ability. The chimera including the GAL4-DBD and the amino-terminus of human C/EBPγ resulted in only a 2-fold induction compared to a 900-fold induction of GAL4-luc by the GAL4-c/ebp1 (1-87) protein under the same conditions (Figure 7C). The human C/EBPγ protein has been shown to contain a repression region between aa 115 and 162, whereas its activation domain resides in aa 1-70.36 Therefore, a chimera containing the GAL4 DBD and the first 70 aa of C/EBPγ was also compared to c/ebp1. GAL4-C/EBPγ (1-70) activated approximately 170-fold, suggesting that the amino-terminus of c/ebp1 is a more potent activator than the activation domain of C/EBPγ. Taken together, these data indicate that c/ebp1 may act as a potent transcriptional activator of genes with a C/EBP DNA binding site motif.

Discussion

In this report, we have identified a novel myeloid-restricted member of the C/EBP family in zebrafish containing a highly conserved bZIP domain and a unique amino-terminal transactivation domain. The existence of 2 copies of many mammalian genes in zebrafish has led to the belief that an additional genome-wide duplication occurred during evolution of teleost fish compared to other vertebrates.37 For example, each HOX-bearing chromosome in humans has 2 paralogous chromosomes in zebrafish.38 However, c/ebp1 is not likely to be the result of this tetraploidization event or a tandem duplication based on its sequence divergence from the other c/ebp family members. No c/ebp1 ortholog has been found in the nearly completed human genome, and therefore, the mammalian ortholog of c/ebp1 may have been lost in humans while being maintained in the zebrafish.

The myeloid specificity of c/ebp1 expression in zebrafish
embryos is supported by the following observations: (1) c/ebp1 is only expressed in a fraction of circulating cells over the yolk sac and the axial vein; (2) most of the c/ebp1+ cells also express the myeloid marker, l-plastin, another myeloid marker; (3) its expression is abolished in cloche, a mutant affecting hematopoiesis at the level of stem cells; and (4) its expression is retained in mutants affecting specifically the erythroid lineage, whereas it is lost in a mutant affecting specifically the myeloid lineage. It is possible that this gene is also expressed in lymphoid and megakaryocytic lineages, although the location and the timing of the expression make such possibilities unlikely.

Most of the cells expressing c/ebp1 also express the macrophage marker, l-plastin. However, there are cells that express varying ratios of c/ebp1 and l-plastin. The cells with these different expression patterns may represent myeloid cells at slightly different stages of development or may represent separate subsets of myeloid cells. Further studies with additional hematopoietic markers, as they become available, will help to elucidate the identity of the cells observed in this analysis.

In our transcripational activation assay, the amino-terminal 87 aa of c/ebp1 act as a strong transcripational activation domain, far more potent than that of human C/EBPβ.36 In mammals, C/EBPα, β, δ, and ε contain amino-terminal activation domains and act as transcripational activators. In contrast, C/EBPγ and χ lack these domains and usually act as trans-dominant repressors.41 Deletion and mutation analysis of C/EBPs has shown that it contains multiple activation regions that act cooperatively.42 One of these regions is proline rich, but the prolines do not appear to be required for activity.43 In C/EBPβ an acidic, hydrophobic region has been demonstrated to have transactivation ability.44 Many transactivating domains are acidic, proline rich, glutamine rich, or in some cases, serine/threonine rich. The activation domain of c/ebp1 contains 26% serines/threonines, but does not appear to be proline or glutamine rich. Further, although the activation domain carries a net charge of −5, the region is not significantly acidic in comparison to described activation domains such as VP16 with a charge of −18. Much is still not understood about the sequence and structural requirements of transactivation domains, and activation domains defined by deletion and mutation analysis do not always fall into the classic categories. Further studies on the amino-terminal region of c/ebp1 will be required to assess the structure of the c/ebp1 activation domain and the elements of the region (aa 1-87) that are essential for transactivation.

c/ebp1 binds a consensus C/EBP site with high affinity and its amino terminus acts as a potent transcripational activation domain in a mammalian system. Therefore, c/ebp1 likely acts as a transcripational activator in vivo. Like the mammalian C/EBPαs, c/ebp1 may heterodimerize with other c/ebps, such as c/ebpα and c/ebpβ, or other bZIP proteins to act on different DNA targets. Further, as for other c/ebps, additional cofactors such as c-myb might be required for optimal transactivation.45,46 Only a few myeloid-specific genes have been isolated in the zebrafish to date, and none of their promoters have been characterized, but it is likely that many will contain c/ebp sites. Differential affinity of these sites for the c/ebps and their partners would be expected to determine gene expression. However, analysis of the c/ebp1 target genes will have to await further characterization of zebrafish promoters.

The myeloid-specific expression pattern of c/ebp1 is similar to the expression of mammalian C/EBPα, which is seen primarily in myeloid and lymphoid cells. To date, an ortholog of mammalian C/EBPα has not been discovered in zebrafish (S.E.L. et al, unpublished results). However, c/ebp1 may perform some or all of the functions of the mammalian C/EBPα. Alternatively, if a zebrafish ortholog to C/EBPα does exist, c/ebp1 and the zebrafish C/EBPα ortholog may share the functions performed by the mammalian C/EBPα. Given the myeloid expression pattern of c/ebp1, it may play a role in cell-type specification or maturation of myeloid cells.
In this study, we have identified a novel member of the c/ebp family with myeloid-specific expression. This factor has a previously undescribed amino-terminal domain with potent transcriptional activation properties. Our findings suggest that c/ebp1 may play a role in myelopoiesis in the zebrafish. In the near future, the use of c/ebp1 as a marker in directed mutation screens should lead to the identification and characterization of myeloid mutants and thus to the identification of genes crucial to myeloid development.

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