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UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Zebrafish Expression Screen Identifies a Novel Myeloid Specific Gene

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

Jennifer Marie Tamai

Committee in charge:

Professor David Traver, Chair
Professor Amy Kiger
Professor Cornelis Murre

2008

The Thesis of Jennifer Marie Tamai is approved and it is acceptable in
quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2008

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ABSTRACT OF THE THESIS

A Zebrafish Expression Screen Identifies a Novel Myeloid Specific Gene

by

Jennifer Marie Tamai

Master of Science in Biology

University of California, San Diego, 2008

David Traver, Chair

In vertebrates, conserved genetic pathways tightly regulate the production and function of myeloid cells, such as granulocytes, macrophages, monocytes, and dendritic cells. Myeloid cells perform key functions in the immune response including clearance of pathogens and antigen presentation. Although myeloid cell regulation

and function in vertebrates is best understood in mammals, the zebrafish presents a new model to better understand myeloid cell biology due to embryonic transparency, which allows direct observation of cell populations using transgenic reporter lines and gene expression patterns using whole-mount in situ hybridization (WISH). Because the genetic regulation of the hematopoietic hierarchy and blood cell function in zebrafish is well conserved with mammals, the insights obtained studying zebrafish myeloid cell function can be applied to our overall understanding of vertebrate myeloid cell biology.

In this study, we took advantage of the optical transparency and rapid development of zebrafish embryos to identify new genes involved in myeloid cell function. We generated a cDNA library from adult kidney myelomonocytes isolated by flow cytometry to identify novel genes. We performed WISH to identify genes expressed in known sites of embryonic myelopoiesis and identified a novel gene, clone 2B4. Using quantitative PCR, we found high levels of expression of clone 2B4 in other purified myeloid cell populations. Bioinformatics analysis revealed that clone 2B4 belongs to the PLAC8 family. Here, we present the initial characterization of the novel myeloid gene, clone 2B4.

INTRODUCTION

Mature leukocytes are traditionally categorized into two lineages, lymphoid and myeloid. The lymphoid lineage includes T cells, B cells and natural killer (NK) cells (Laiosa et al., 2006). The myeloid lineage consists of a number of morphologically, phenotypically and functionally distinct cell types, including different subsets of granulocytes (neutrophils, eosinophils and basophils), monocytes, macrophages, and mast cells (Laiosa et al., 2006). Dendritic cells (DCs) have a unique developmental program that can be activated from either the lymphoid or myeloid pathway (Laiosa et al., 2006; Traver et al., 2000). These mature myeloid cells play a key role in both the innate and the adaptive immunological response. Figure 1 displays the first or innate immune response, where local resident macrophages first detect an invading pathogen and secrete various signaling molecules that recruit neutrophils and other effector cells to the infected area (Ley et al., 2007). Together, neutrophils and macrophages aid in eradicating the foreign pathogen by phagocytosis (Kantari et al., 2008). Recruited monocytes enter the affected area and differentiate into mature macrophages and DCs to contribute to the innate immune response. Generally, the following inflammatory response efficiently eradicates a pathogen. Evasive or chronic infections however, may require a further adaptive immune response, involving T cells, B cells and DCs. The regulation of the immune response is dependant upon a delicately controlled expression of target genes that effect cell functions (Alper et al., 2008). Slight disturbances of this tight regulation can lead to irregular cell function and eventually to the emergence of many diseases,

such as cancers, autoimmune diseases and inflammatory diseases (Dinauer, 2005; Pham, 2006). Because many aspects of immunological diseases are not well understood, there is a need to investigate genes involved in the regulation of the immune response. By searching for new target genes and investigating their functions, we can improve our understanding of the gene regulatory pathways involved in the immune system.

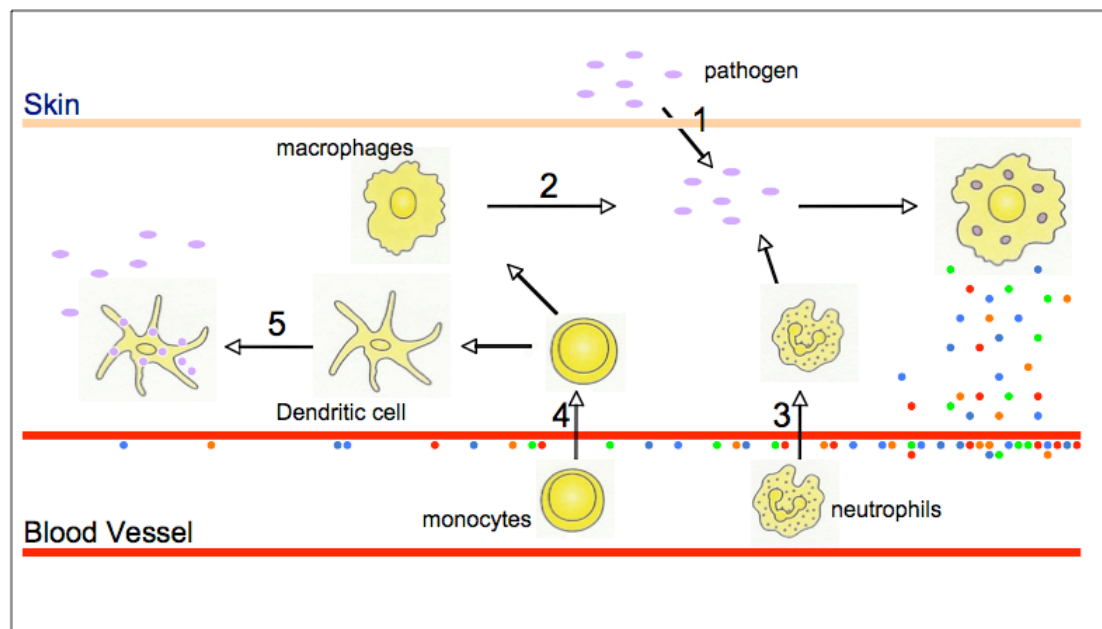


Figure 1. The innate immune response. Once an invading pathogen (1) is detected by resident macrophages (2), cytokines are released into the blood stream in order to recruit neutrophils (3) and various effector cells to the infected area. Neutrophils and macrophages phagocytose foreign particles while recruited monocytes (4) differentiate into macrophages for further elimination of the pathogen. If the innate immune response is not sufficient in eradicating the pathogen, the adaptive immune response (5) is triggered, involving T cells, B cells and DC cells.

All mature blood cells of the immune system develop from multipotent, self-renewing hematopoietic stem cells (HSCs) (Cumano & Godin, 2007; Orkin & Zon, 2008). HSCs produce differentiated blood cell types through fate decisions regulated by transcriptional regulation of lineage-specific genes, as shown in Figure 2a. One of

these early decisions is the commitment to the myeloid and erythroid or to the lymphoid fate. HSCs produce common myeloid progenitors (CMPs) that have the capacity to further differentiate into all the mature myeloid and erythroid cell types (Akashi & Traver et al., 1997). Differentiation of CMPs to successively fate-restricted intermediates is regulated by important transcription factors such as *pu.1*, *gata1*, *gfi1*, and *c/epb* (Crowhurst et al., 2002; Miyamoto et al., 2002; Orkin & Zon, 2008; Su, et al., 2007). Regulation by these transcription factors directs the path of these fate-restricted progenitors, shown in Figure 2b, such as the antagonistic interplay between *pu.1* and *gata1*. This interplay determines cell fate along the myeloid lineages, where *gata1* triggers the differentiation of CMPs to the megakaryocyte-erythrocyte progenitors (MEPs) while expression of *pu.1* commits CMPs down the granulocyte-macrophage progenitor (GMPs) lineage (Laiosa et al., 2006; Orkin & Zon, 2008). Through the regulation by other transcription factors, MEPs generate erythrocytes and megakaryocytes, while GMPs generate granulocytes (neutrophils, eosinophils, and basophils), monocytes and macrophages (Akashi & Traver et al., 1997; reviewed in Orkin & Zon, 2008).

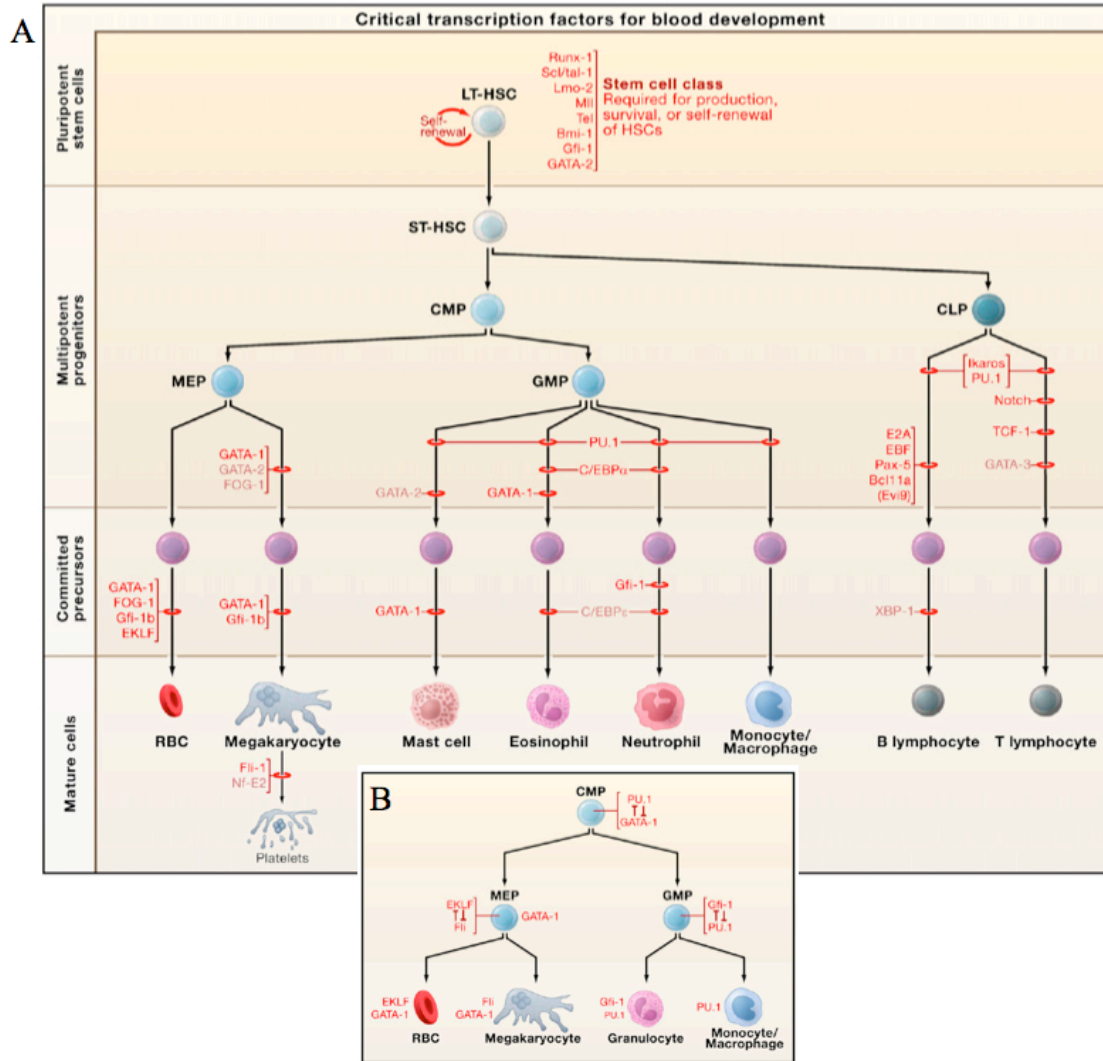


Figure 2. The hematopoietic cellular pathway. (A) All blood cells mature from HSCs and differentiate into the lymphoid lineage or the myeloid lineage. Transcription factors that influence cellular differentiation are shown (red). (B) Gene regulation of the myelopoiesis cellular pathway. The interplay between genes regulates the differentiation from a CMP into the megakaryocyte-erythrocyte lineage or the granulocyte-macrophage lineage. (Images provided by Orkin & Zon, 2008.)

The zebrafish (*Danio rerio*) has emerged as a promising vertebrate model for examining aspects of immunity and disease. It is uniquely suited for developmental and functional studies because of its small size, rapid external development and prolific fecundity (Lieschke & Currie, 2007; Trede et al., 2004; Yoder et al., 2002). A

key feature is the optical clarity that allows for the direct observation of all systems during development. Also, recent advances in fluorescent transgenesis allows for visualizing and monitoring of the behavior of various cell types in living animals (Lieschke & Currie, 2007). With fluorescent transgenic lines marking specific blood lineages, such as transgenes driven by *mpo*, *lck* and *gatal* promoters, we can independently visualize myeloid, lymphoid and erythroid populations within live zebrafish embryos (Langenau et al., 2004; Mathias et al., 2006; Renshaw et al., 2006; Traver et al., 2003). Furthermore, understanding the transcriptional regulation of the immune system is facilitated by access to established mutant lines with defects in hematopoiesis (Trede et al., 2004; Weber, et al., 2005). Though conventional vertebrate immune models allow for forward genetic screens, the investigation of gene function in a high throughput manner is generally not feasible. In zebrafish, it is possible to assay gene expression on a large scale using whole-mount in situ hybridization (WISH) and examine functionality using antisense morpholino oligonucleotides to study knockdown of gene function or RNA injection for gene overexpression studies (Trede et al., 2004). Importantly, both hematopoiesis and the immune system are highly conserved from zebrafish to mammals (Rombout et al., 2005; Traver, 2004). The zebrafish possess analogous blood cell lineages to mammals, including erythroid, myeloid and lymphoid lineages (Traver, 2004). In addition, homologues of transcription factors that regulate myeloid cell development, such as *pu.1*, *gatal*, *gf1l*, and *c/epbl*, are required for specification of mesodermal cells to the myeloid lineages in both fish and mammals (Berman et al., 2005;

Crowhurst et al., 2002; Orkin & Zon, 2008; Su, et al., 2007). The antagonistic regulation of *pu.1* and *gata1* is also observed in zebrafish, where the inhibition of *pu.1* shifts progenitor cell fate to a myeloid fate, while the knockdown of *gata1* expression directs an erythroid fate (Galloway et al., 2005; Rhodes, et al., 2005). Taken together, these advantages make the zebrafish model appealing for immunological studies, as well as for cell biological and functional investigations.

Because most fish species hatch during embryonic stages of life, they are exposed early on to a myriad of pathogens in an aquatic environment (Carradice & Lieschke, 2008). Consequently, the zebrafish must depend on its immune system early in development. The ontogeny of the zebrafish immune system is shown in Figure 3. The first immune cells observed in the zebrafish embryo are macrophages generated during the primitive wave of hematopoiesis (Herbomel et al., 1999). They appear at approximately 18 hours post fertilization (hpf) from the cephalic mesoderm/rostral blood island (RBI) (Herbomel et al., 1999; Herbomel et al., 2001). Around 48hpf, granulocytes and monocytes are produced from committed erythromyeloid progenitors (EMPs) in the posterior blood island (PBI), a region of the embryonic trunk that exists transiently (Bertrand & Kim et al., 2007). These granulocytes and monocytes are functionally active and have been shown to accumulate at sites of inflammation (Mathias et al., 2006; Renshaw et al., 2006). A second definitive wave produces all mature blood cell types from HSCs. HSCs emerge along the ventral aspect of the dorsal aorta and their lymphoid progeny can be observed to directly colonize the thymic anlage by 48hpf (Bertrand & Kim et al.,

2007). Once HSCs emerge, they transit to in the kidney marrow, which is the teleost equivalent of mammalian bone marrow, and can generate all hematopoietic lineages including all mature myeloid lineages throughout adulthood (Traver, 2004).

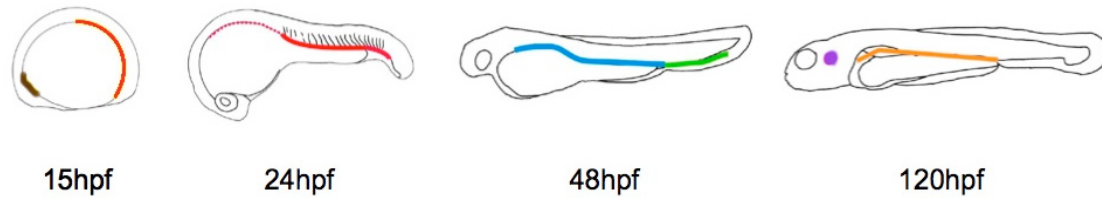


Figure 3. The hematopoietic sites of zebrafish ontogeny. The RBI (brown) produces primitive macrophages, while the intermediate cell mass (ICM, red) produces primitive erythrocytes. The PBI (green) gives rise to EMPs. HSCs are suggested to arise in the ventral wall of the dorsal aorta (blue). The thymus (purple) is seeded by immigrating precursors while the kidney marrow (orange) produces HSCs for the rest of adult life. (Images from Rombout et al., 2005).

In the mouse, specific subsets of blood cells can be purified by combinations of antibody staining and fluorescence activated cell sorting (FACS) analysis. Although as a system the zebrafish lack suitable antibodies for doing similar isolation, it has been shown that zebrafish blood cells can be purified by light scatter characteristics into 4 groups: precursor, myeloid, erythroid and lymphoid (Traver, et al., 2003). With this prospective isolation of hematopoietic subgroups, it is possible to purify cells and to generate cDNA libraries that can be used to uncover novel target genes required within the immune response and, specifically, genes required for myeloid cell function. By taking advantage of this method, we developed an expression screen that combines the power of genomics and early zebrafish development. We generated a cDNA library from myeloid cells highly purified by FACS from adult zebrafish kidney. We screened clones chosen at random for patterns of myeloid expression using WISH and identified three genes. Here we present the

identification, validation, and initial investigation of clone 2B4, a gene not precisely characterized. Preliminary experiments revealed a WISH expression profile similar to the expression of a known myeloid gene, *pu.1*. We validated clone 2B4 as a myeloid specific gene by examining the WISH expression profile in *vlad tepes* mutants. The protein encoded by clone 2B4 was discovered to be homologous to the relatively uncharacterized PLAC8 domain. We have also discovered six uncharacterized genes, which are all homologous to clone 2B4, in the zebrafish that belong to the PLAC8 protein family. We also showed that clone 2B4 is expressed in embryonic and adult *mpo*⁺ populations, which are believed to be comprised of monocytes, macrophages, and neutrophils. Our results suggest that there are many target genes yet to be characterized that may regulate cells of the immune response. Further investigation of these target genes may lead to a more precise understanding of the immune response, and the function and biology of the cells involved.

MATERIALS AND METHODS

Zebrafish maintenance

AB* zebrafish and embryos were maintained, collected and staged as described (Westerfield, 1993). Hematopoietic mutants *vlad tepes* were obtained from crosses of heterozygous parents and were scored for hematopoietic defects at 24hpf (Lyons et al., 2002). Embryos were dechorionated at 24hpf by pronase (Sigma, St. Louis, MO) treatment.

Cell suspension preparation

Wild-type adult zebrafish were anesthetized with 0.02% tricane (Sigma Aldrich, St. Louis, MO) before kidney removal. Following a ventral, midline incision, the kidney was dissected, washed and placed into ice-cold 0.9x PBS containing 5% FCS. Single-cell suspensions were generated by aspiration followed by gentle teasing of the organ on a 40 µm nylon mesh filter with a plunger from a 1 ml syringe.

Flow cytometry

Single cell suspensions were processed as stated above. Propidium iodide (PI, Sigma Aldrich, St. Louis, MO) was added at 1 µg/ml to exclude dead cells and debris. Flow cytometric analysis and sorting was based on PI exclusion, forward scatter, side scatter, GFP, and/or DsRed fluorescence using a FACS Aria flow cytometer (Becton Dickinson, La Jolla, CA). Cells were sorted twice to optimize purity. FACS data was analyzed using FlowJo software (TreeStar, Ashland, OR).

Generation of myeloid cDNA library

Adult zebrafish whole kidney marrow sorted cells were obtained by flow cytometry as previously described (Traver et al., 2003). Cells from the myelomonocyte (FSC^{hi}SSC^{hi}) fraction were collected in TRIzol and total mRNA was purified according to manufacturer protocol (Life Technologies, Inc., Gaithersburg, MD). cDNA was synthesized by Clontech using SMART cDNA synthesis [Oligo(dT) primed] and directionally cloned into pTrip1Ex2 at the *Sfi*I cloning site (Clontech, Palo Alto, CA). Clones were sequenced using the following primers: 5' pTrip1Ex2 sequencing primer (5' – TCCGAGATCTGGACGAGC – 3') and 3' pTrip1Ex2 sequencing primer (5' – TAATACGACTCACTATAGGG – 3').

Bioinformatics Analysis

BLAST searches were done using NCBI (<http://blast.ncbi.nlm.nih.gov>) and Ensembl databases (http://www.ensembl.org/danio_rerio) with cDNA sequences. Alignments were analyzed using EMBL-EBI ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Protein domains were assed by searches against the Pfam database (<http://pfam.wustl.edu/hmmmsearch.shtml>).

Q-PCR analysis

Total mRNA was isolated from whole embryos or dissected adult organs. Organs were dissociated with Liberase Blendzyme II for 2 hours at 33°C. Embryos at indicated time points and the dissociated organ tissues were treated with TRIZOL

(Invitrogen, Philadelphia, PA) and mRNA purification was performed using RNeasy kit (Qiagen, Valencia, CA) according to manufacturer protocol. Synthesis of cDNA was performed using First Strand cDNA Superscript III kit (Invitrogen, Philadelphia, PA). The following primers were used: 2B4-F (5' – GCTGCAGTGACCTACTTGT – 3'), 2B4-R (5' – CATATTGTGCCCTGTATTCC – 3'), cornifelin1-F (5' – ATCTGCTGTTGTGGTTTCTG – 3'), cornifelin1-R (5' – CTCATGGCGAGTGTAATAGG – 3'), cornifelin2-F (5' – AACATACAGGGTTCGATGT – 3'), cornifelin2-R (5' – CCTAAGCGCTTCCTAATTTTC – 3'), cornifelin3-F (5' – GCAGTCGCTATGGAATAAAG – 3'), cornifelin3-R (5' – AATGTCAAACCTTTGCTCTCG – 3'), cornifelin4-F (5' – TTTTCTGTCTCCCGTGTATG – 3'), cornifelin4-R (5' – ACATGGATCCCTCTATGTTG – 3'), cornifelin5-F (5' – TAAGGTGGCACAGGATAAT – 3'), cornifelin5-R (5' – ATCACCCAGTCATCACAGAC – 3'), cornifelin6-F (5' – AGTCTGGCTCATCAGTATGG – 3'), cornifelin6-R (5' – AACAGCAATACACCGTCATC – 3'). Q-PCR reactions were performed using Brilliant SYBER Green QPCR Master Mix (Stratagene, La Jolla, CA).

Cloning of zebrafish cornifelin genes

cDNA was synthesized as indicated above. Each cornifelin 1-6 gene was amplified by PCR using the following primers: cornifelin1-F (5' –

ATAGTCATGATGGCTCAGGCAGTAAG – 3'), cornifelin1-R (5' –
 CTGTCGGACCTTCAGCTCTCTG – 3'), cornifelin2-F (5' –
 ATGGAGGTGACATCTCAACCC – 3'), cornifelin2-R (5' –
 CAGAACTGTCCTAAGCGCTTCC – 3'), cornifelin3-F (5' –
 AGAATGACCACTCAGCAGCCC – 3'), cornifelin3-R (5' –
 GTGACAAATGTCAAACCTTTGCTCTCG – 3'), cornifelin4-F (5' –
 ATGGAGGTGACATCTCAACCGTC – 3'), cornifelin4-R (5' –
 CATAATTCAGCGTGCCGTTACTCTTTC – 3'), cornifelin5-F (5' –
 ATGTCATATCAGGCAGAATCAATGACG – 3'), cornifelin5-R (5' –
 GCCTCTCGCCTTCTGTTCTCG – 3'), cornifelin6-F (5' –
 CAACCTGGCCTTGGTGAAACC – 3'), cornifelin6-R (5' –
 CTCTCTGATCATTTGACAGAGGGC – 3'). PCR was performed with 0.5 µl Taq
 polymerase (Invitrogen, Philadelphia, PA) in 50 µl reactions containing 10 µM each
 dNTP, 2 µM MgCl₂, 0.2 µM each primer, 5 µl Taq buffer, 37.5 µl sterile water, and 2
 µl cDNA template (1 ng). The following conditions were used: initial denaturation
 step at 94°C for 3 mins, 35 cycles of amplification at 94°C for 1 min, primer annealing
 temperature for 1 min (cornifelin 1-3, 6: 62°C, cornifelin-4: 63.5°C, cornifelin-5:
 64°C), 72°C for 1 min, and a final extension at 72°C for 30 min.

The coding sequence of cornifelin-1 and cornifelin-6 were amplified by PCR
 using the following primers: cornifelin1 cds-F (5' – GA GAATTC
 ACCATGATGGCTCAGGCAGTAAGTGTC – 3'), cornifelin1 cds-R (5' – GA
CTCGAG CACATTCAGTAAACATATTGGCAAATGTAGC – 3'), cornifelin6 cds-

F (5' – GA GAATTC ACCATGGACTCACCTGGTCCAAACG – 3'), cornifelin6 cds-R (5' – GA CTCGAG TCAGGAGCTTTCCAGTGCAGTAGAC – 3') (adding 5' – *EcoRI* and 3' – *XhoI* sites, underlined sequences). PCR was performed with 0.5 µl Taq polymerase (Invitrogen, Philadelphia, PA) in 50 µl reactions containing 10 µM each dNTP, 2 µM MgCl₂, 0.2 µM each primer, 5 µl Taq buffer, 38.5 µl sterile water, and 1 µl cDNA template (1 ng). The following conditions were used: initial denaturation step at 94°C for 3 mins, 40 cycles of amplification at 94°C for 1 min, primer annealing temperature for 1 min (cornifelin-1: 62°C, cornifelin-6: 64°C), 72°C for 1 min, and a final extension at 72°C for 15 min.

Each sequence was cloned using TOPO TA Cloning Kit, Dual Promoter (Invitrogen, Philadelphia, PA) in accordance with manufacturer protocol. Clones were sequenced using the T7/SP6 promoter primers (Eton Bioscience, Inc., San Diego, CA).

Whole-mount in situ hybridization

Dechorionated embryos were treated with 0.003% 1-phenyl-2thiourea (PTU, Sigma, St. Louis, MO) to block pigmentation, then fixed in 4% paraformaldehyde (PFA) at noted time points. cDNA clones were linearized and digoxigenin-labeled RNA probes were synthesized using a DIG RNA Labeling Kit (SP6/T7; Roche). Each probe was validated by gel electrophoresis to confirm a single digestion by restriction enzyme and to validate the integrity of the probe. Whole-mount in situ hybridization was performed as described (Thisse & Thisse, 2008). Embryos were mounted in

methylcellulose before photographs were taken with a Sony Cybershot camera attached to a microscope (Leica). Digital images were further processed with Adobe Photoshop CS software (Adobe Systems, San Jose, CA).

RESULTS

Development of a zebrafish myeloid cDNA library

With the high degree of conservation between zebrafish and mammalian immune cell types, the zebrafish provides a powerful system to find new genes involved in immune cell biology through forward genetic screens and large-scale expression screens. We previously demonstrated that the major hematopoietic lineages of the zebrafish can be identified from adult hematolymphoid tissues by FACS using light scatter characteristics, as shown in Figure 4 (Traver, et al., 2003). This simple technique allows for the separation of erythroid, myeloid, lymphoid and precursor populations to relative purity from the kidney, the spleen or the blood, with each of these populations being amenable to examination of gene expression and library construction.

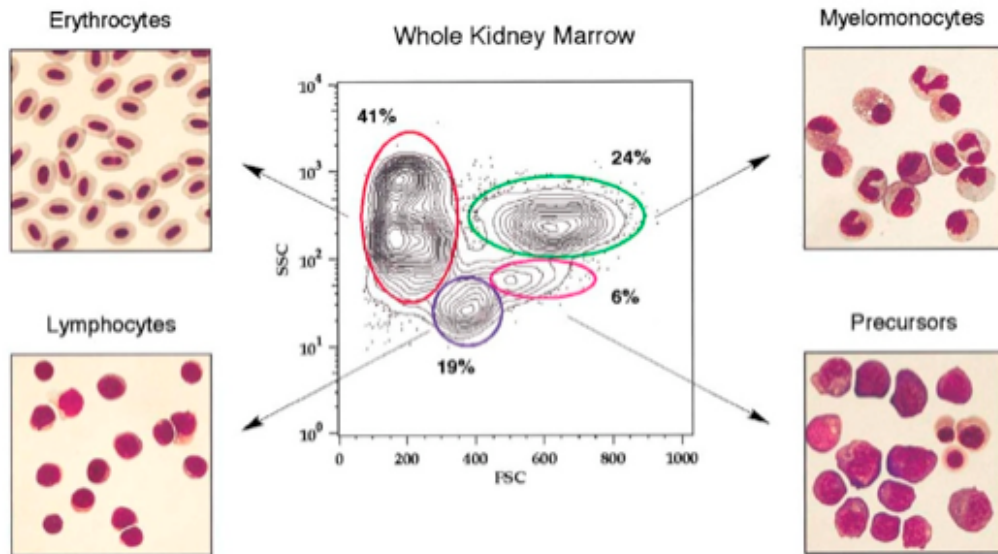


Figure 4. Separation of blood lineages by FACS. Adult kidney cells separate into hematopoietic cell lineages when analyzed by size (FSC) and granularity (SSC). Sorting of each population yields cells within four different gates, which are comprised of only mature erythrocytes (upper left panel), only lymphocytes (lower left panel), immature precursors (lower right panel), and only myelomonocytic cells (upper right panel). (Image provided by Traver et al., 2003).

In order to identify novel genes involved in myeloid cell biology, a zebrafish myeloid specific cDNA library was generated from highly purified myeloid cells, as shown in Figure 5. Morphological analyses showed that mature myelomonocytic cells, including neutrophils, basophils, eosinophils, monocytes and macrophages, all fall within the $FSC^{hi}SSC^{hi}$ myeloid gate. Therefore, this constituted as an ideal source for novel myeloid-related genes. Due to the limited number of cells obtained by FACS, PCR amplification of cDNA was necessary for the construction of a cDNA library. The Clontech SMART cDNA library system was chosen for the construction of this library (Clontech, Palo Alto, CA). This system was designed to amplify full-length cDNAs and preserve the representation of message diversity. In addition, directional cloning was performed by the use of adaptor sequences that was incorporated onto both ends of the cDNA. The amplified cDNAs were first cloned into a phage vector pTrip1Ex2 and then later excised *in vivo* to create a phagemid pTrip1Ex2 library used for further analysis. 10,000 clones were sequenced and comparative bioinformatics was carried out in order to removed redundancy and housekeeping genes.

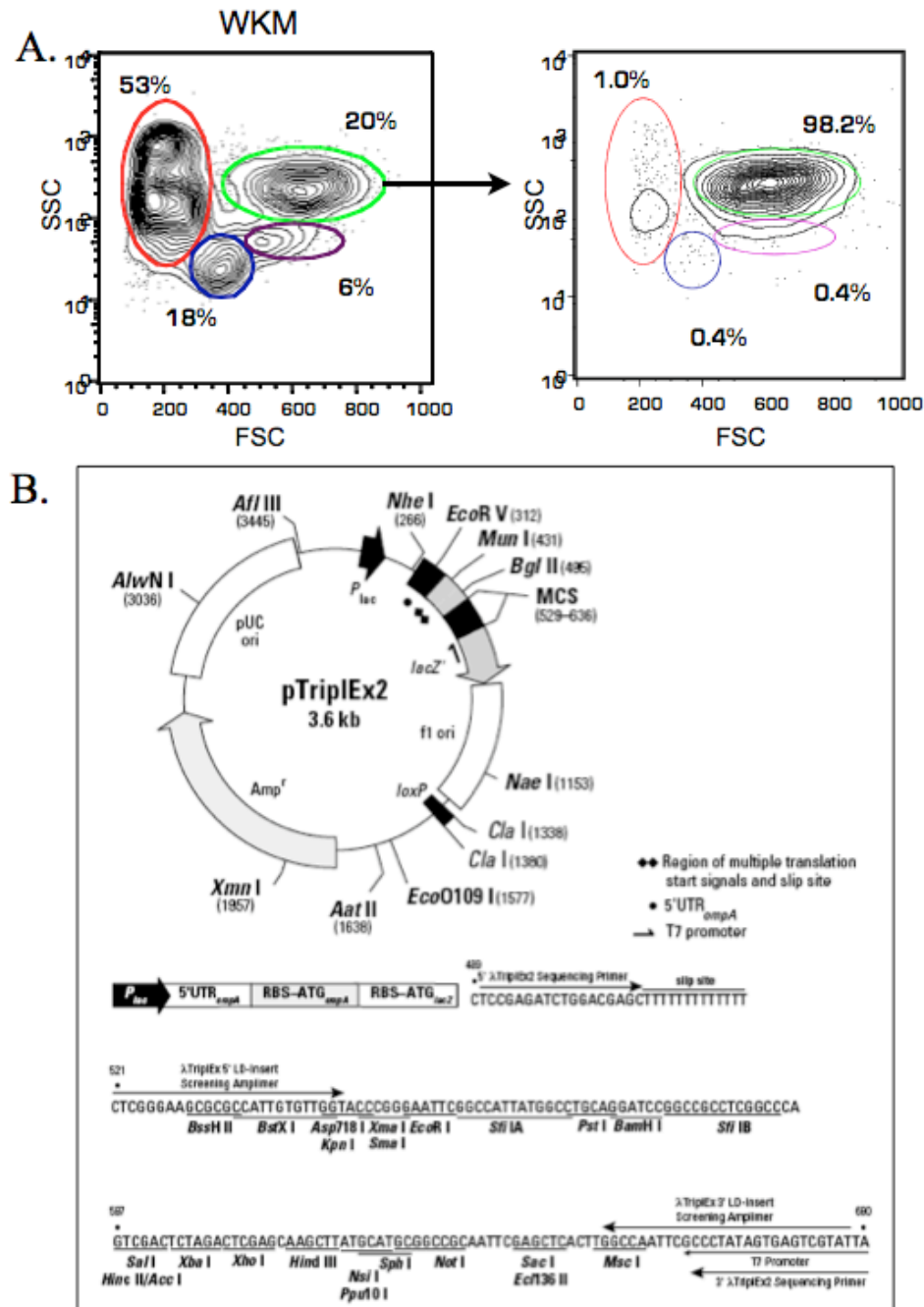


Figure 5. Generation of the myeloid cDNA library from zebrafish adult WKM. (A) FACS sort of the WKM from 42 adult wild-type zebrafish using light scatter characteristics (left panel). The myeloid gate (green) was resorted for purity (right panel). RNA extracted from the purified myeloid fraction was used for full-length cDNA amplification. (B) pTriplEx2 vector map with the multiple cloning sequence and restriction sites. Each cDNA sequence was directionally cloned into the vector at the *Sfi*I site. The average insert size is 1-2 kb. Digoxigenin-labeled RNA probes were generated by linearizing using *EcoR*I and transcribing using the T7 promoter (Image provided by CLONTECH Laboratories, Inc., 2000).

WISH-based screen and the identification of myeloid specific genes

Using the zebrafish myeloid cDNA library, we initiated a WISH-based screen to identify novel myeloid genes. WISH analysis exploits the optical transparency and rapid embryonic development of the zebrafish to provide a thorough spatial and temporal expression pattern of genes in their natural context. Digoxigenin-labeled RNA probes were generated from randomly selected cDNA clones following *EcoRI* linearization. To date, we have synthesized roughly 300 RNA probes from the myeloid cDNA library. Probes made from each cDNA clone were hybridized to zebrafish embryos staged at 24hpf, 48hpf, and 6 days post fertilization (dpf). Since maturing myeloid cells are found within the yolk (~ 1dpf), in the PBI (~1-4dpf) and in the kidney (>5dpf), these time points are ideal developmental stages for the initial selection of myeloid specific genes. So far, there have been about 200 clones screened. WISH analysis on several clones showed myeloid expression patterns similar to that seen for the myeloid specific gene, *pu.1*. Figure 6 shows select results from our ongoing study. The cDNA clones have been named after their plate locations. Of three clones discovered by WISH, clone 3A7 represents a zebrafish orthologue of a known mammalian myeloid protein (*lysozyme C*), and the other two clones have no sequence similarity to any published gene (clone 2B4 and clone 3A8).

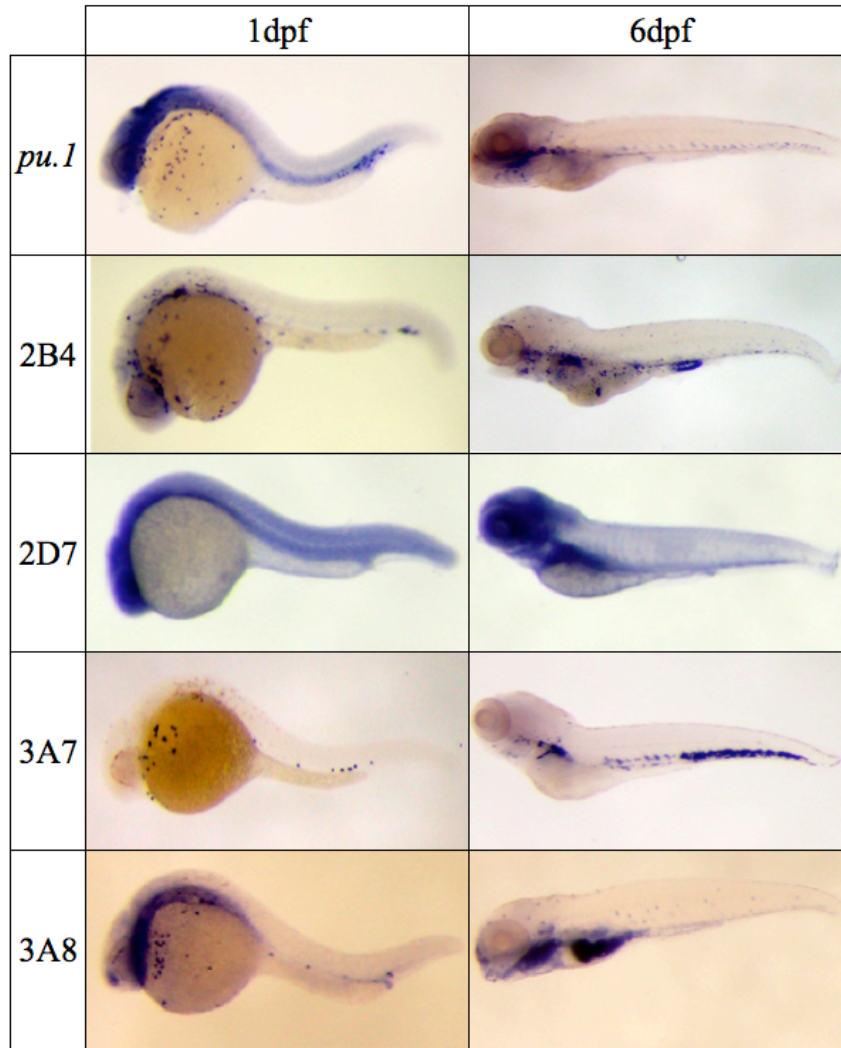


Figure 6. Selected results from the WISH-based screen on a myeloid cDNA library. The myeloid specific gene, *pu.1*, was used as a control for typical myeloid expression patterns. At 1dpf, sites of myeloid expression are in the yolk and in the PBI. At 6dpf, expression expands into the tissues of the embryo and in the kidney, the adult site of hematopoiesis. Clone 2D7 represents a non-specific expression pattern. Clones 2B4, 3A7 and 3A8 showed WISH expression patterns similar to *pu.1*.

We focused on the characterization of clone 2B4. First, we tested clone 2B4 at more extensive time points by WISH analysis to evaluate its role in the hematopoietic program. The expression profile for clone 2B4 presented in Figure 7 displays a typical myeloid WISH expression profile at various developmental stages. At 24hpf, clone 2B4 was expressed in the yolk and in the PBI. The expression of clone 2B4 increased

in the yolk and PBI in 30 to 36hpf. From 48hpf to 3dpf, the expression expanded into the tissues of the embryo and at 6dpf, we saw the expression of clone 2B4 emerge in the kidney. The expression profile is similar to the one seen for *pu.1*, suggesting that clone 2B4 is expressed in myeloid cells.

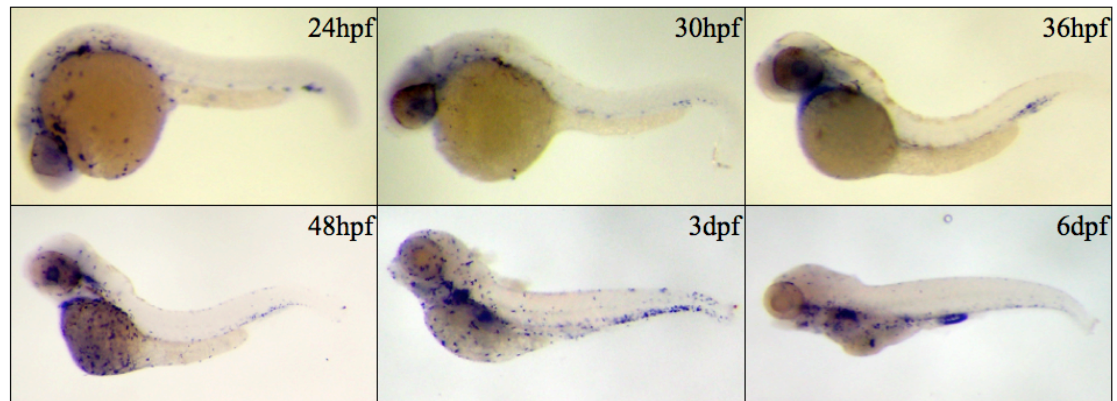


Figure 7. WISH expression profile of clone 2B4 at various developmental time points. From 24-36hpf, expression is localized in the RBI and the PBI, known sites of myelomonocyte production in the early embryo. From 48hpf to 6dpf, expression expands into the tissues of the embryo and into the kidney.

Validation of clone 2B4 as a myeloid specific gene

The initial WISH screen identified clone 2B4, a gene with typical myeloid expression patterns in the zebrafish embryo. To evaluate whether this gene may constitute an interesting candidate for further biological investigation, we examined its expression profile among WKM leukocyte populations by quantitative PCR. cDNA prepared from WKM and FACS purified lymphoid, myeloid and progenitor cell populations served as templates in this experiment. As the main hematopoietic site, the WKM was used as a reference to quantify the expression in isolated hematopoietic cell populations. As shown in Figure 8, expression of clone 2B4 is specifically confined within the WKM myeloid fraction. When compared to WKM, clone 2B4

showed a ~1.6 fold enrichment in the myeloid population, whereas there was no detection of gene expression in the lymphoid and precursor populations. These results demonstrated the feasibility of the expression-based screening, which should reveal an array of myeloid specific genes for functional testing.

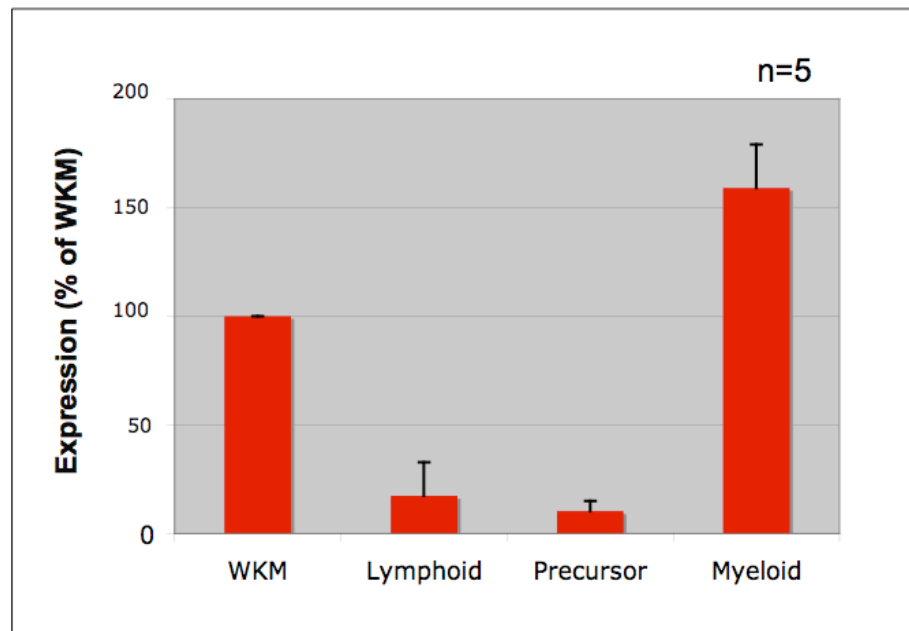


Figure 8. Quantitative PCR expression profile of clone 2B4 in adult hematopoietic cell populations. Each blood lineage of the WKM can be separated into distinct populations by FACS using light scatter characteristics. Clone 2B4 showed high enrichment in the myeloid population, with low expression in the lymphoid and precursor populations compared to the WKM control.

Genetic screens in zebrafish have identified mutants with defects in the development of the hematopoietic system, which have been instrumental in epistatic gene analyses. Blood mutants of particular interest are mutants that show an expansion of myelopoiesis. The *vlad tepes* mutant is characterized by the absence of the erythroid cell lineage caused by a defect in the erythroid specific transcription factor, *gata1* (Weber et al., 2005). There is an antagonistic interplay of *gata1* and *pu.1*, where the inhibition of *gata1* results in an expansion of *pu.1* expressing cells in

the ICM (Galloway et al., 2005; Rhodes, et al., 2005). To further investigate the requirement of clone 2B4 in hematopoiesis, we examined its WISH expression profile at different developmental stages in *vlad tepes* mutants. The expression profile of clone 2B4 was compared to the expression profile observed for *pu.1*. Figure 9 shows WISH expression patterns at 20, 24 and 48hpf in *vlad tepes* mutants, along with sibling wild-type embryos. As previously stated, *pu.1* showed ectopic expression in the ICM at 20 and 24hpf in the *vlad tepes* embryos compared to their wild-type siblings. At 48hpf, *pu.1* displayed an increased expression in the caudal hematopoietic tissue (CHT) in *vlad tepes* mutants compared to their wild-type siblings. Similarly, at 24 and 48hpf, we saw an ectopic expression of clone 2B4 in the ICM/CHT regions in *vlad tepes* mutants compared to their wild-type siblings. At 20hpf, clone 2B4 showed no expression in either the *vlad tepes* mutants or the wild-type siblings. Taken together, this data validated clone 2B4 as a myeloid specific gene. In addition, the early expression of *pu.1* suggests that clone 2B4 may act downstream of *pu.1* signaling in the zebrafish.

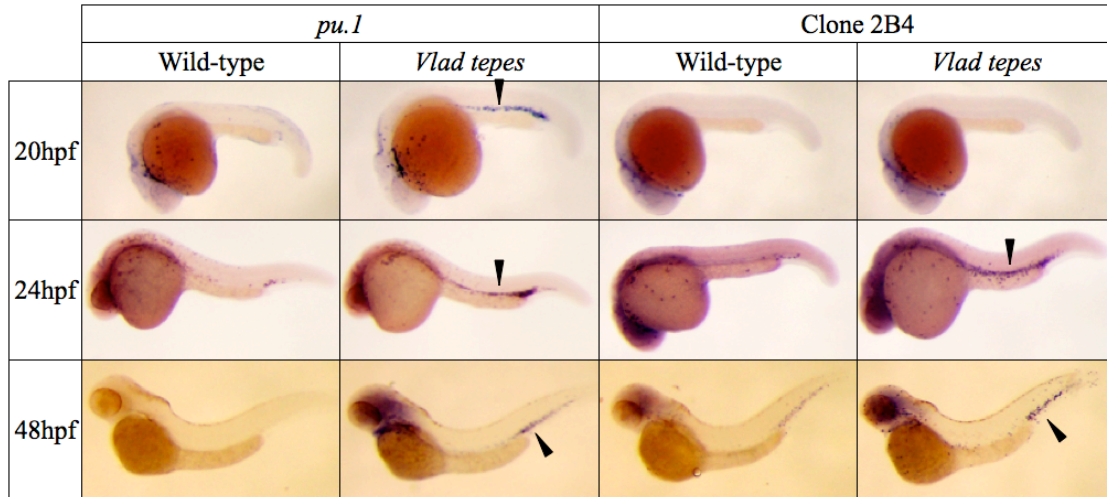


Figure 9. WISH expression patterns of clone 2B4 in *vlad tepes*. *Vlad tepes* mutants lack the erythroid lineage due to a defect in *gata1*. The inhibition of *gata1* results in the expansion of *pu.1* expressing cells. *Vlad tepes* mutants showed an ectopic expression of *pu.1* in the ICM/CHT region compared to wild-type siblings at 20, 24 and 48hpf. Clone 2B4 follows this expression pattern at 24 and 48hpf. At 20hpf, clone 2B4 is not yet expressed and suggests the gene acts downstream of *pu.1* signaling. The similar expression pattern of clone 2B4 and *pu.1*, a known myeloid gene, in the *vlad tepes* confirms clone 2B4 as myeloid specific gene.

Analysis of clone 2B4 using bioinformatics

Following the validation of clone 2B4 as a myeloid specific gene, we sought to examine the native protein encoded by clone 2B4. This protein, a polypeptide of 114 amino acids, was analyzed using BLAST and Pfam program. Analyses showed no indication of characterized motifs or signal peptide, but revealed that clone 2B4 encodes for a protein belonging to the PLAC8 family, as shown in Figure 10a. The PLAC8 domain contains a high cysteine residue content of roughly 10% and is conserved in all vertebrates and plants examined to date (Galaviz-Hernandez, et al., 2003; Ledford et al., 2007). In mammals, three members of the PLAC8 family have been identified: PLAC8/onzin, PLAC8-like and cornifelin. All mammalian PLAC8 members have variable N-terminal sequences in their protein structures, as shown in

Figure 10b. PLAC8/onzin has no homology with other protein families, which has made it difficult in obtaining clues as to its function. However, some studies suggest that it may have a role in regulation of apoptosis and cellular proliferation (Li et al., 2006; Rogulski, et al., 2005). Because PLAC8/onzin remains uncharacterized, the function of the protein encoded by clone 2B4 remains unknown. Therefore, to begin the characterization of the protein encoded by clone 2B4, we analyzed the sequence homology of clone 2B4 with the known mammalian PLAC8 members using BLAST. Figure 10c shows that of the three known mammalian PLAC8 members, clone 2B4 has the highest homology with the human and mouse PLAC8-like proteins. Of those two proteins, clone 2B4 has a 41% homology with human PLAC8-like protein and a 38% homology with mouse PLAC8-like protein.

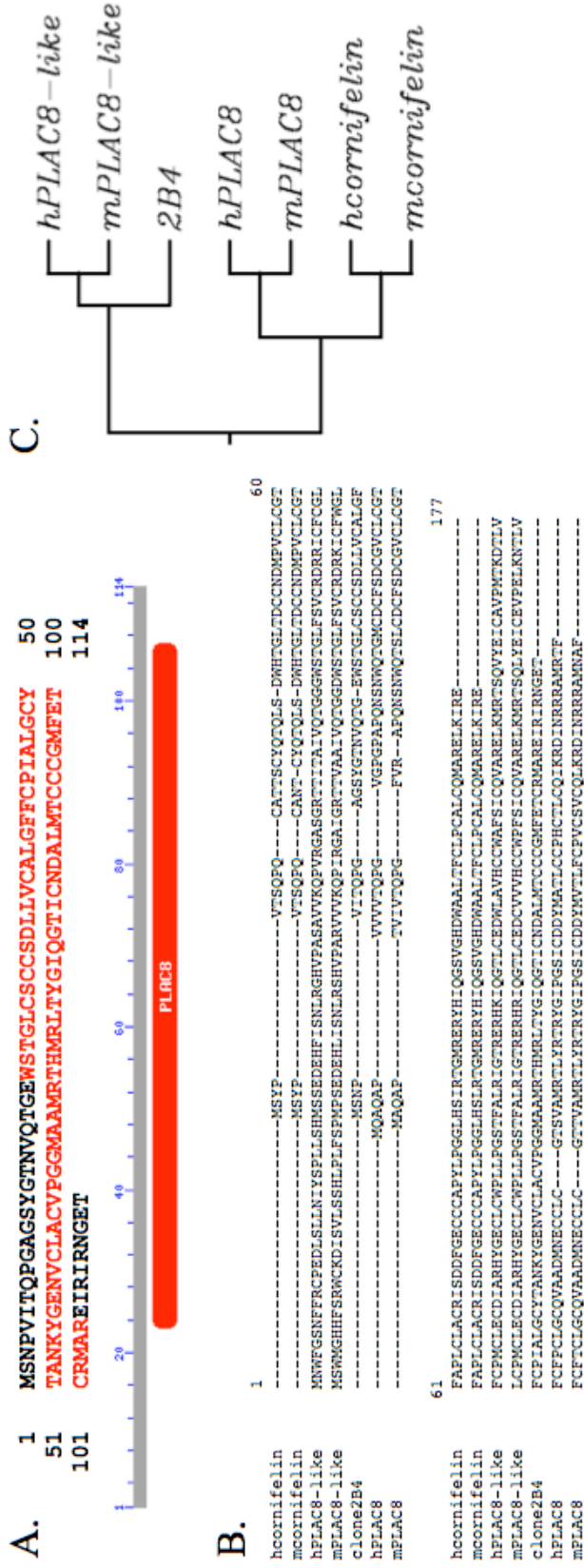


Figure 10. Clone 2B4 homology with the PLAC8 protein family in human and mouse. (A) Clone 2B4 protein sequence with the PLAC8 domain marked in red. (B) The alignment of the PLAC8 protein family members in human and in mouse with clone 2B4. (C) A dendrogram displaying the homology of clone 2B4 and the three mammalian PLAC8 family members. Clone 2B4 has the closest homology with the human and mouse PLAC8-like proteins.

Clone 2B4 homology with zebrafish cornifelin members

To further investigate the function of the protein encoded by clone 2B4, we searched for genes within the zebrafish genome that contain the PLAC8 domain. Using BLAST analysis, we identified six proteins belonging to the conserved PLAC8 protein family found in mammals. Further investigation of these zebrafish PLAC8 proteins using BLAST revealed that they have not been previously characterized and their functions remain hypothetical or unknown. These six genes, which have been termed cornifelin 1-6 (crn1-6), are displayed in Figure 11a. In order to obtain clues as to the functions of these zebrafish PLAC8 protein members, we compared cornifelin 1-6 to clone 2B4. Figure 11a shows that two pairs of the zebrafish cornifelin genes are located on the same chromosome; cornifelin-1 and cornifelin-3 are found on zebrafish chromosome 7, while cornifelin-2 and cornifelin-4 are found on chromosome 10. The two pairs of genes on similar chromosomes had corresponding protein descriptions, such that cornifelin-1 and cornifelin-3 are undescribed proteins, while cornifelin-2 and cornifelin-4 are hypothetical proteins. The cornifelin-5 protein is described as a cornifelin homologue and the cornifelin-6 protein is described as a PLAC8-like homologue. Using BLAST analysis, we examined the sequence homology of the six zebrafish cornifelin genes and clone 2B4, as shown in Figure 11b. Sequence analysis revealed that the zebrafish cornifelin genes and clone 2B4 do not have a highly conserved protein domain other than the PLAC8 domain. Figure 11c shows that clone 2B4 has the closest homology with cornifelin-6, with a 38% homology, compared to all six zebrafish cornifelin proteins. Since cornifelin-6 is described as a PLAC8-like

homologue, these results support the fact that clone 2B4 has the highest homology with the human and mouse PLAC8-like proteins. Then, we analyzed the sequence homology of the entire human, mouse and zebrafish PLAC8 protein family with clone 2B4 by BLAST in order to investigate the homology among all discovered PLAC8 proteins and clone 2B4. Clone 2B4 had a higher homology with zebrafish cornifelin-6 protein than human and mouse PLAC8-like proteins (data not shown). After BLAST analysis of the zebrafish cornifelin proteins and clone 2B4, the function of clone 2B4 remains vague.

A.

Gene Name	Source	Genome Location	Description
crn-1	LOC100002539	Chromosome 7: 13.39m	No Description
crn-2	zgc: 158845	Chromosome 10: 8.36m	hypothetical protein LOC799489
crn-3	LOC100002602	Chromosome 7: 13.28m	No Description
crn-4	zgc: 114201	Chromosome 10: 8.37m	hypothetical protein LOC606661
crn-5	zgc: 103671	Chromosome 16: 8.37m	Cornifelin homolog
crn-6	PLAC8L1	Chromosome 21: 32.70m	PLAC8-like 1
clone 2B4	Unnamed	Chromosome 21: 32.71m	No Description

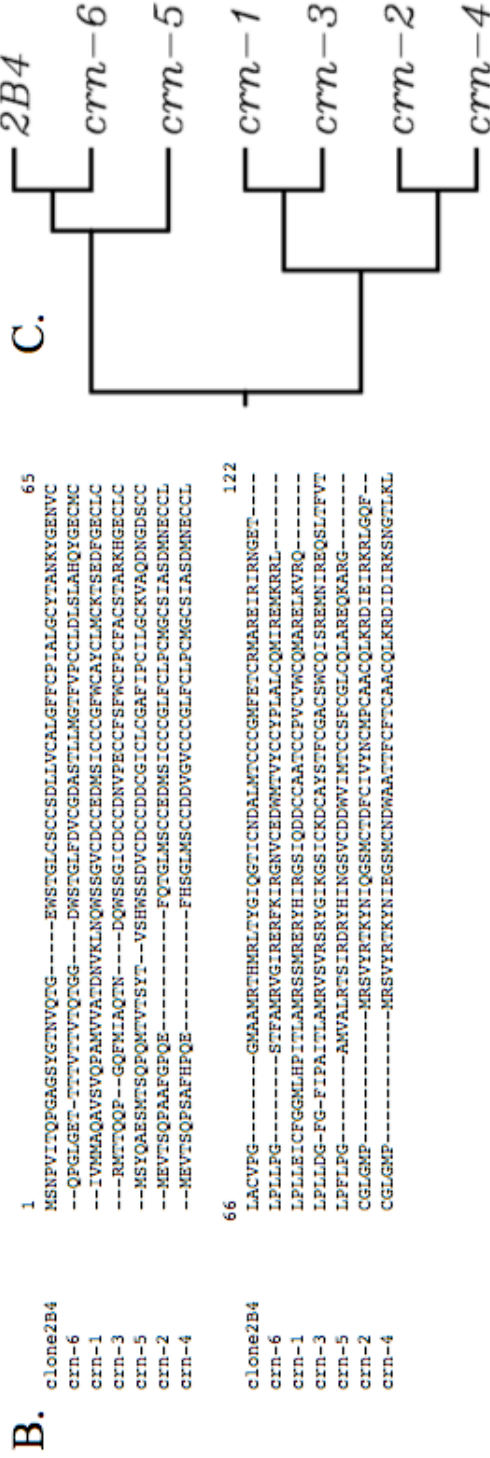


Figure 11. Clone 2B4 homology with PLAC8 protein family in zebrafish. (A) A table of the six zebrafish PLAC8 protein members and clone 2B4. Each gene has not been previously characterized. (B) Alignment of clone 2B4 with the zebrafish PLAC8 protein family. (C) A dendrogram of clone 2B4 and the zebrafish PLAC8 protein family. Clone 2B4 has the highest homology with zebrafish crn-6. (D) A dendrogram of clone 2B4 with all known PLAC8 proteins. Clone 2B4 has the highest homology with the zebrafish crn-6, then is homologous with the mammalian PLAC8-like proteins.

Characterization of clone 2B4 in myeloid cell populations

After the identification of clone 2B4 by the preliminary WISH-based screen, we validated its myeloid expression patterns in the embryo, but it remained to be functionally characterized. Clone 2B4 was specifically expressed in cells atop the yolk syncytial layer and within the PBI from 24hpf onward, an expression pattern reminiscent of embryonic macrophages. In order to investigate more broadly the expression of clone 2B4 within different hematopoietic cell lineages, we examined expression within different myeloid cell populations. We analyzed the expression of clone 2B4 in both adults and embryos to gain a comprehensive expression profile. By taking advantage of the established zebrafish transgenic lines that mark specific leukocyte populations, we can separate a variety of myeloid cell lineages by FACS. Figure 12 shows the established zebrafish transgenic lines that fluorescently label different hematopoietic cell populations.

Transgenic line:	Reporter gene expressed in:	Reference:
Mpo:eGFP	neutrophils, precursors of monocyte/macrophage lineage	Renshaw et al., 2006; Mathias et al., 2006
Lck:eGFP	mature T cells	Langenau et al., 2004
Rag1:eGFP	immature B and T cells	Jessen et al., 1999, 2001
Rag2:eGFP	immature B and T cells	Langenau et al., 2004
Lysozyme C:eGFP	macrophages, neutrophils	Hall et al., 2007
Pu.1:eGFP	myeloid cells	Ward et al., 2003; Hsu et al., 2004
Gata1:DsRed	erythrocytes	Traver et al., 2003
Gata2:eGFP	eosinophils	Traver et al., 2003
CD45:DsRed express	all leukocytes	Bertrand et al., unpublished

Figure 12. A table of available zebrafish transgenic lines. Each transgenic line was developed to identify different differentiated blood lineages.

In order to analyze the expression of clone 2B4 in myeloid cell populations of adult zebrafish, we first removed cellular debris by sorting WKM from double

transgenic *gata1*:DsRed; *mpo*:GFP adult zebrafish by FACS using light scatter characteristics. We choose *gata1*:DsRed; *mpo*:GFP double transgenic because *gata1*:DsRed marks erythrocytes and *mpo*:GFP marks neutrophils and precursors of the monocyte/macrophage lineage (Renshaw et al., 2006; Traver et al., 2003). By taking advantage of the fluorescently labeled cell populations, we were able to separate WKM into its different myeloid cell lineages by FACS using *mpo*:GFP and *gata1*:DsRed fluorescence expression levels. As shown in Figure 13, we isolated myeloid and erythroid cell populations, where the sort yielded a *gata1*⁺ population, a double negative (DN) populations, and two distinct populations of *mpo* expressing cells: *mpo*^{high} population and *mpo*^{low} population. After further sorting by FACS using light-scatter characteristics, these two *mpo* populations were confirmed to be distinct from one another. Based upon the location of the two *mpo* populations by FACS using light-scatter characteristics, we hypothesize that the *mpo*^{high} population consists of mostly macrophages and neutrophils, while the *mpo*^{low} population contains monocytes, though further experiments are necessary to confirm this. We next analyzed expression levels of clone 2B4 in the four populations isolated by FACS from *gata1*:DsRed; *mpo*:GFP adult zebrafish by quantitative PCR. The kidney was used as a hematopoietic reference to determine the degree of expression in the different *gata1*:DsRed; *mpo*:GFP subsets. Compared to WKM, the results showed a ~5.5 fold enrichment of clone 2B4 in the *mpo*^{high} fraction and an ~1.5 fold enrichment of clone 2B4 in the *mpo*^{low} fraction. There was no gene expression of clone 2B4 in the DN fraction or the *gata1*⁺ fraction. These results indicated that clone 2B4 is not

expressed in erythroid (*gata1*⁺) cells and is highly expressed in the myeloid (*mpo*⁺) cells. The five-fold enrichment of clone 2B4 in the *mpo*^{high} population suggests that clone 2B4 is highly expressed in macrophages and neutrophils. Enrichment in the *mpo*^{low} population suggests that clone 2B4 is expressed in monocytes, but to a lesser extent.

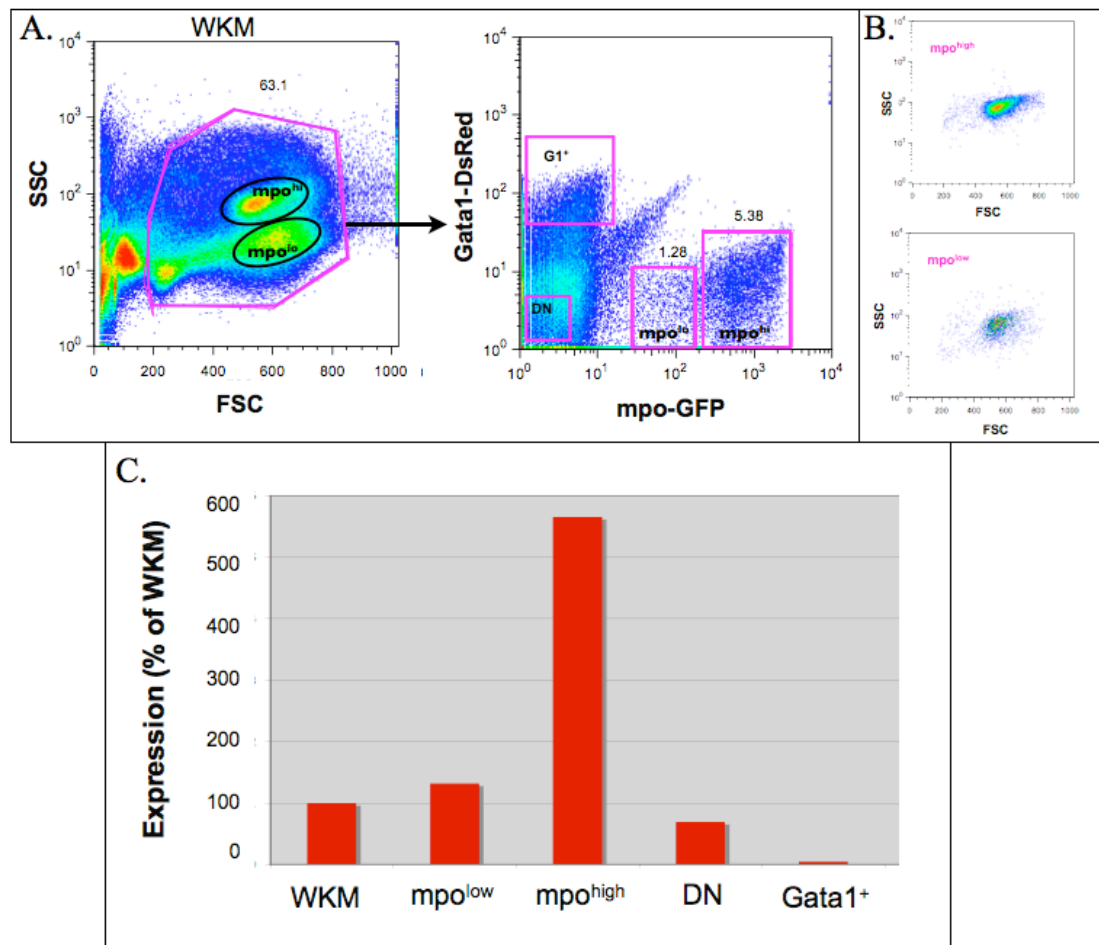


Figure 13. Quantitative PCR expression profile of clone 2B4 in cell populations isolated from double transgenic *gata1*:DsRed; *mpo*:GFP adult zebrafish. (A) FACS sort yielded four cell populations. The myeloid population is marked by *mpo*, where the *mpo*^{low} population consists of monocytes and the *mpo*^{high} population consists of macrophages and neutrophils. The *gata1*⁺ population consists of erythrocytes. The DN fraction consists of cells that lack *gata1* or *mpo* expression. (B) The two *mpo*⁺ populations prove to be distinct populations when sorted using light scatter characteristics by FACS. (C) Quantitative PCR analysis of clone 2B4 in the four hematopoietic cell populations. As the site of hematopoiesis in the adult zebrafish, WKM was used as a reference. Clone 2B4 showed enrichment in both *mpo*^{high} and *mpo*^{low} populations. There was no expression in *gata1*⁺ or DN populations.

Next, we analyzed the expression of clone 2B4 in embryonic myeloid cell populations. We sorted *gata1*:DsRed; *mpo*:GFP whole embryos using light scatter characteristics by FACS to remove cellular debris. Similar to *gata1*:DsRed; *mpo*:GFP adult zebrafish, we separated embryonic cell populations into different myeloid cell lineages using *gata1*:DsRed and *mpo*:GFP fluorescence activity. This sort yielded only three cell populations: a *mpo*⁺ population, a *gata1*⁺ population and a DN population. Then, we examined expression levels of clone 2B4 in each cell population using the WKM as a control, as shown in Figure 14. Compared to WKM, clone 2B4 had a ~1.5 fold enrichment in the *mpo*⁺ population, with no gene expression in the *gata1*⁺ or the DN population. Taken together, quantitative PCR analysis on embryonic myeloid cell populations correlate with the embryonic WISH expression profile of clone 2B4. We see that clone 2B4 is highly expressed in *mpo*⁺ populations, supporting our initial observation of the similarity between the embryonic WISH expression profile of clone 2B4 and primitive macrophage expression patterns. The expression of clone 2B4 in adult *mpo*⁺ cell lineages suggests that the expression of clone 2B4 is maintained throughout zebrafish adulthood within neutrophils and macrophages.

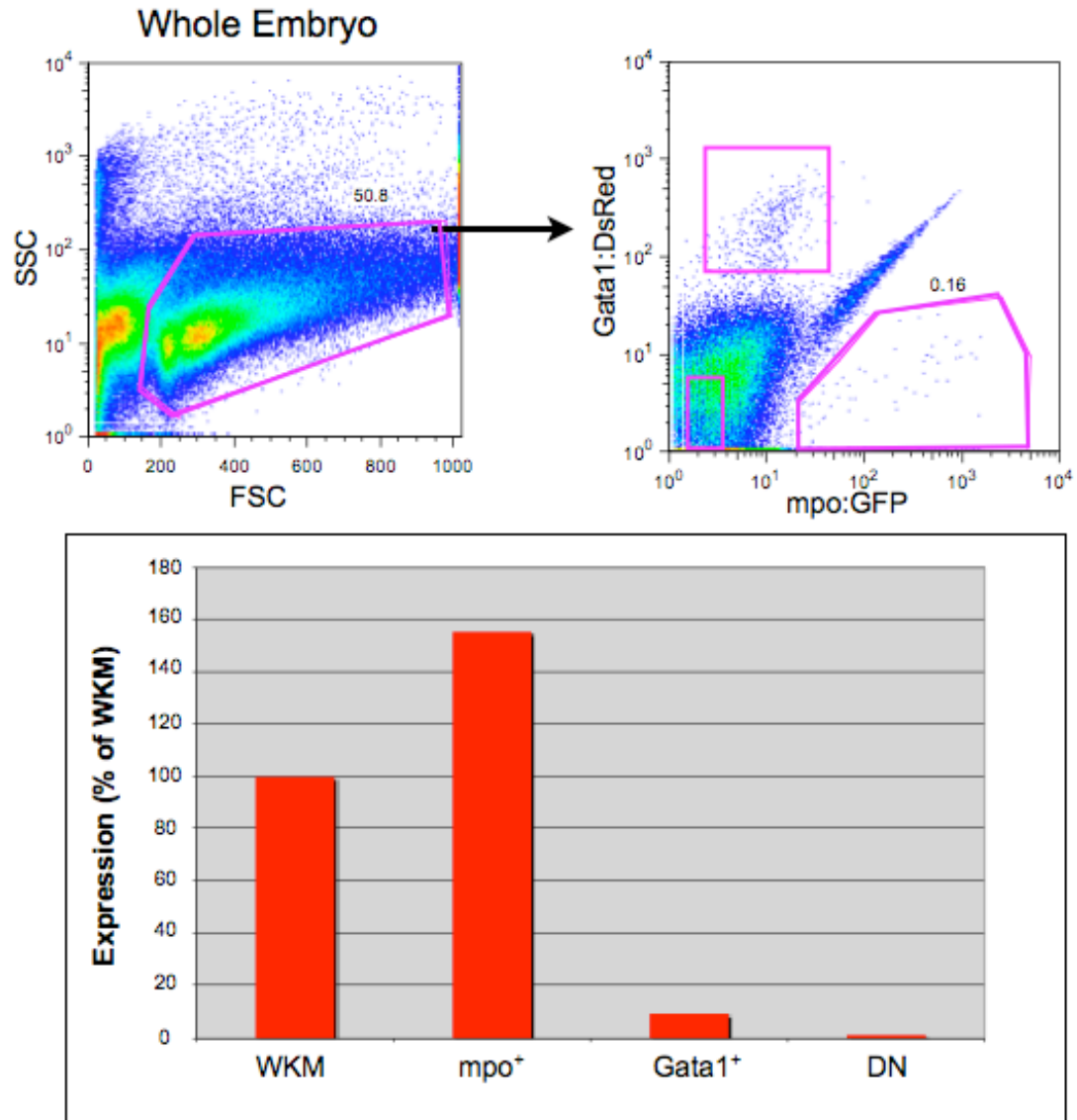


Figure 14. Quantitative PCR expression profile of clone 2B4 in cell populations isolated from double transgenic *gata1:DsRed; mpo:GFP* zebrafish embryos. A FACS sort of WKM yielded a myeloid population marked by *mpo*, an erythroid population marked by *gata1*⁺ a DN population, which lacks *gata1* or *mpo* expression. As the site of hematopoiesis in adult zebrafish, the WKM was used as a reference. Clone 2B4 showed an enrichment in *mpo*⁺ populations, with no gene expression in *gata1*⁺ or DN populations.

Investigation of the expression profile of the zebrafish cornifelin proteins

Bioinformatics analyses of clone 2B4 led to the discovery of six zebrafish genes that have not been previously characterized. By analyzing the expression

profiles of cornifelin 1-6, we sought to better understand clone 2B4 in myeloid cell populations. To begin, we analyzed the expression profiles of cornifelin 1-6 and clone 2B4 in different hematopoietic lineages by quantitative PCR. cDNA prepared from primitive macrophages of 5dpf embryos and from *mpo*⁺, *gata1*⁺, and DN cell populations separated from double transgenic *gata1*:DsRed; *mpo*:GFP 5dpf embryos by FACS served as templates in this experiment. cDNA templates of 30dpf embryos were used as a control to quantify expression levels in each cell population. With expression levels normalized to 30dpf embryo expression, Figure 15 displays clone 2B4 with a ~200 enrichment in the primitive macrophage population and a ~240 enrichment in the *mpo*⁺ population. Cornifelin-1 displayed a similar expression pattern to clone 2B4, with a ~37 enrichment in primitive macrophages and a ~30 enrichment in *mpo*⁺ cells. Cornifelin 2-4 displayed no enrichment in hematopoietic populations compared to the 30dpf embryo. Cornifelin-5 showed an expression in the *mpo*⁺ population, with a slight enrichment of ~12. Cornifelin-6 displayed a ~65 enrichment in *gata1*⁺ cells, which is unlike clone 2B4.

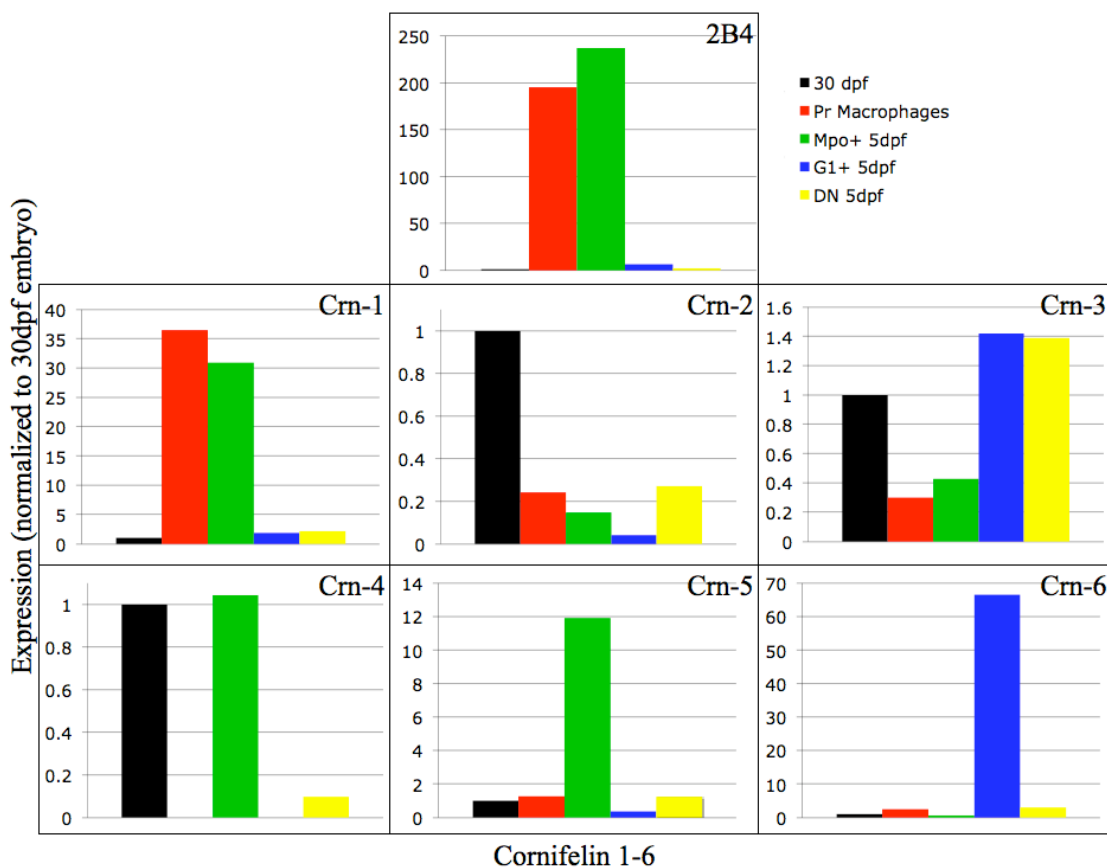


Figure 15. Quantitative PCR expression profiles of zebrafish cornifelin 1-6. Clone 2B4 showed high enrichment in primitive macrophages (~200) and *mpo*⁺ populations (~240). Cornifelin-1 had similar expression to clone 2B4, with a ~37 enrichment in primitive macrophages and a ~30 enrichment in *mpo*⁺ populations. Cornifelin 2-4 showed no enrichment in hematopoietic populations. Cornifelin-5 showed a slight enrichment in the *mpo*⁺ population (~12). Cornifelin-6 displayed a ~65 enrichment in the *gatal*⁺ population.

To further investigate the requirements of the zebrafish cornifelin family in the hematopoietic system, we analyzed their expression profiles by WISH. Each gene was PCR amplified using cDNA templates from cell populations of high expression from results obtained by quantitative PCR analysis in hematopoietic cell populations. Each PCR amplified gene was cloned into a TOPO-TA vector (Invitrogen, Philadelphia, PA) and a digoxigenin-labeled RNA probe was synthesized. Using these RNA probes, we performed WISH on zebrafish embryos at various developmental

stages. Of the six zebrafish cornifelin proteins, cornifelin 2-5 displayed non-specific hematopoietic WISH expression patterns, with expression in the endoderm, marking areas such as the stomach, gut, gills and pharynx. Cornifelin-6 showed WISH expression patterns similar to *gata1* at 24hpf and 30hpf, where expression was located in the ICM at 24hpf and in the AGM and PBI at 30hpf. This supports the enrichment of cornifelin-6 in *gata1* cell populations by quantitative PCR. We were, however, unable to obtain WISH expression patterns of cornifelin-1 after multiple WISH experiments. Figure 16 displays an expression pattern of cornifelin-6 alongside a *gata1* expression profile, as well as a representative expression pattern of cornifelin 2-5, with cornifelin-3 shown to mark the pharynx. Taken together, the WISH expression patterns of cornifelin 1-6 support the results obtained by quantitative PCR in hematopoietic cell populations.

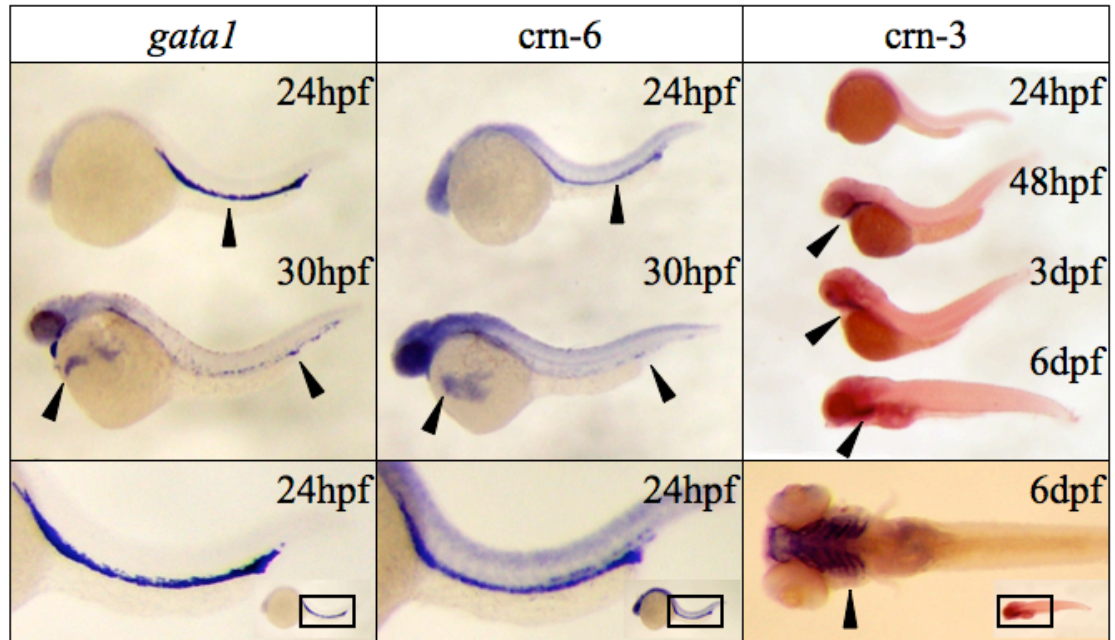


Figure 16. WISH expression profiles of zebrafish cornifelin 1-6. At 24hpf and 30hpf, *gatal* (left column) marks erythrocytes, where below is a magnified trunk section of a 24hpf embryo. Cornifelin-6 (middle column), expressed in *gatal* cell populations, showed a similar WISH expression profile to *gatal*, where below shows a magnified trunk section of the 24hpf embryo. WISH analysis on cornifelin-3 (right column) is a representative expression profile of the zebrafish cornifelin 2-5 family members, which showed expression patterns in the endoderm, such as in the pharynx, gills, and stomach, where below shows a magnified ventral view of a 6dpf embryo. WISH expression profile of cornifelin-1 is unavailable.

DISCUSSION

As a model for hematopoiesis, the zebrafish possesses a highly conserved hematopoietic system and genetic program with mammals (Amatruda & Zon, 1999; Trede et al., 2004). Though feasible in the mouse model, large-scale screens prove expensive, laborious and are often limited by the lethality of morphant phenotypes (Lieschke & Currie, 2007). The generation of a myeloid specific cDNA from adult WKM and the use of a WISH-based screen demonstrate the viability of the expression-based screen in the zebrafish model. Using this method as a tool, we have discovered novel genes that appear important in myeloid function. The initial WISH-based screen identified several genes with typical myeloid expression patterns in the zebrafish embryo that remain to be functionally characterized. Analysis by quantitative PCR on clone 2B4 displayed enrichment in the adult WKM myeloid population, which validates the generation of a myeloid specific cDNA library from the adult WKM myelomonocyte population. The specificity of this technique is also supported by the ectopic expression of clone 2B4 in *vlad tepes* mutant embryos by WISH analysis. As previously stated, *gata1* and *pu.1* have an antagonistic fate-regulating relationship (Rhodes, et al., 2005). Similar to *pu.1*, the ectopic expression of clone 2B4 in the *vlad tepes* mutants, which lacks expression of *gata1*, confirms clone 2B4 as a myeloid specific gene. Altogether, these results support the feasibility and ease of the large-scale expression screen in the zebrafish model and validate the generation of a myeloid specific cDNA library, while confirming our find, clone 2B4, as a myeloid specific gene.

Focusing on clone 2B4, our embryonic WISH expression profile shows a strikingly transient spatial and temporal expression pattern similar to that of developing myeloid cells throughout embryogenesis. With the high degree of conservation between zebrafish and mammalian hematopoiesis, it has been shown in both the zebrafish and mouse model that spatially distinct waves of hematopoiesis produce different myeloid cell types (Bertrand et al., 2005; Bertrand & Kim et al., 2007; Orkin & Zon, 2008; Palis et al., 1999). The first zebrafish myeloid cells, primitive macrophages, are shown to arise in the anterior lateral plate mesoderm (ALPM) and migrate to the yolk syncytial layer by 24hpf (Herbomel et al., 1999). These early macrophages precede the appearance of any other leukocyte in the embryo. Then, myeloid expression shifts to the PBI region, where it has been shown that a transient population of early definitive progenitors having both erythroid and myeloid potential emerges between 24-48hpf in the PBI (Bertrand & Kim et al., 2007). From this, definitive macrophages and granulocytes arise and are shown to colonize embryonic tissues after 48hpf, with detected expression of genes, such as *pu.1* and *mpo* (Crowhurst et al., 2002). The results displaying a high enrichment of clone 2B4 in *mpo*⁺ populations by quantitative PCR analyses in *gata1*:DsRed; *mpo*:GFP double transgenic embryonic and adult populations show that expression maybe confined within macrophage, monocytes and neutrophils. *Mpo* is a definitive myelopoietic gene marking mature granulocytes. It was shown to have the earliest zebrafish expression in cells of the ICM at 18hpf and by 4dpf, *mpo* expressing cells are in circulation and distributed throughout the embryo (Bennett, et al., 2001; Berman

et al., 2005). With high expression in the embryonic *mpo*⁺ population, clone 2B4 may be specifically expressed in developing granulocytes. In addition, our analysis of the variant expression levels within two distinct *mpo*⁺ populations of the adult zebrafish suggest that clone 2B4 may be specifically expressed in mature macrophages and neutrophils (*mpo*^{high}), while still expressed at low levels in less committed myeloid cells, such as monocytes (*mpo*^{low}). This hypothesis is based upon the location of the two *mpo* populations according to FACS using light scatter characteristics. The *mpo*^{high} population resides near the FSC^{hi}SSC^{hi} gate, which has been shown to include myelomonocytic cells (neutrophils, granulocytes, monocytes, macrophages and eosinophils), whereas the *mpo*^{low} population appears within a FSC^{hi}SSC^{int} gate, which has been shown to include immature precursors (Traver, et al., 2003). However, we did not perform morphological analyses of these two distinct *mpo* populations to confirm this hypothesis. Taken together, these results suggest that clone 2B4 may be specifically expressed in developing macrophages and/or granulocytes throughout development.

Clone 2B4 is predicted to encode a polypeptide of 114 amino acids and lacks a known signal peptide. The protein family database Pfam suggested similarity to the PLAC8 (for human placenta-specific gene 8) region, a cysteine-rich domain of unknown function found in many plant and animal proteins (Galaviz-Hernandez et al., 2003; Ledford et al., 2007; Marchler-Bauer et al., 2005). PLAC8 family proteins are described by the presence of a conserved PLAC8 domain that contains more than 10% cysteines and a highly divergent N-terminal region rich in low complexity sequences

(Galaviz-Hernandez et al., 2003; Ledford et al., 2007). There are three known mammalian PLAC8 proteins: PLAC8/onzin, PLAC8-like and cornifelin. However, PSI-BLAST reveals no clear similarity to any characterized proteins nor any obvious structural motifs to suggest a potential function.

In the mouse model, studies investigating the PLAC8 domain offer vague clues to its unknown function. The PLAC8/onzin protein was initially identified through a microarray analysis on placental and embryonic RNA (Galaviz-Hernandez et al., 2003). Mouse PLAC8/onzin is located on chromosome 5, where it is predicted to encode for a cysteine-rich protein of 112 amino acids with 5 exons (Galaviz-Hernandez et al., 2003). *In vitro* analyses suggest that the PLAC8 protein family may play a role in regulating proliferation and apoptosis. The protein is highly expressed in myeloid cells and promotes growth and survival by regulation of apoptotic responses through modulating the Akt1-Mdm2-p53 pathway (Rogulski et al., 2005). PLAC8/onzin was shown to be negatively regulated by Phospholipid Scramblase I (PLSCR1), a membrane protein that controls the movement of plasma membrane phospholipids during apoptosis (Li et al., 2006). In addition, cornifelin was identified in a study comparing mRNA populations from normal and psoriatic skin in humans, where it was shown to be highly expressed in psoriatic skin and located in the granular layer of the epidermis (Michibata et al., 2004). *In vivo* studies also suggested that PLAC8/onzin might play a role in innate immune function. It was found to be expressed at high levels in phagocytes, macrophages and neutrophils and was shown to be required for optimal killing of bacteria by phagocytes (Ledford et al., 2007).

Using onzin knockout mice (*onzin*^{-/-}), it was shown that there was no direct antimicrobial function for PLAC8/onzin, but suggested that the protein may contribute to the intracellular eradication of bacteria through a novel pathway (Ledford et al., 2007). These studies offer clues as to the function of the PLAC8 domain and suggest, due to the homology of clone 2B4 with the PLAC8 protein family, that clone 2B4 may function in the regulation of cell proliferation and/or apoptosis.

Through BLAST analysis, we identified six PLAC8 protein members in the zebrafish genome and termed them with the internal nomenclature: cornifelin 1-6. These six genes, like PLAC8 protein members, have no clear similarity with any characterized proteins nor any obvious structural motifs to suggest a potential function. However, our data show two pairs of the cornifelin genes each reside on the same chromosome (cornifelin-1 & cornifelin-3; cornifelin-2 & cornifelin-4), and each of these pairs have corresponding protein descriptions. Given that the zebrafish retains duplicated chromosome segments obtained through teleost ancestry, the proximity and description of these pairs suggest that they may be pairs of duplicated genes (Postlethwait et al., 2000). Yet, this hypothesis remains to be further tested. The analyses by quantitative PCR show that cornifelin-1 is highly enriched *mpo*⁺ and primitive macrophage populations, suggesting a specific expression in myeloid cells and possibly analogous to the expression of clone 2B4. However, we were unable to obtain a WISH expression profile for cornifelin-1 and lack a whole-embryo expression pattern. Cornifelin 2-5 were not highly expressed in hematopoietic cell populations by quantitative PCR analysis and WISH analysis did not display specific hematopoietic

cell expression patterns. However, cornifelin 2-5 were expressed in endoderm (pharynx, gills, gut and stomach). Interestingly, our results show that that clone 2B4 has the highest homology with cornifelin-6 of the six zebrafish cornifelin genes. Yet, cornifelin-6 was not expressed in myeloid cells, but was expressed in erythroid cells by quantitative PCR. In addition, cornifelin-6 displayed a WISH expression profile that mimics *gata1*, suggesting a specific expression in the erythroid population. In mouse studies, expression of *gata1* is found in erythrocytes, megakaryocytes, mast cells and eosinophils (Kadri et al., 2005; Orkin & Zon, 2008). It has been shown that GATA-1 is involved in the signal transduction pathway regulating erythroid-cell differentiation, where Epo activates GATA-1 transcriptional activity through the phosphatidylinositol 3-kinase (PI3K)/Akt signal cascade (Ghaffari et al., 2005; Kadri et al., 2005; Zhao et al., 2005). Activated GATA-1 then regulates the transcription of genes that coordinate proliferation during erythroid maturation (Gregory et al., 1999; Rylski et al., 2003; Zhao et al., 2005). Studies have also shown that the PI3K/Akt pathway is involved in hematopoietic cell signaling contributing to myeloid and erythroid differentiation by maintaining cell survival by activating Akt (Buitenhuis et al., 2007; Miranda & Johnson, 2007). The level of activated Akt has been shown to mediate myeloid lineage decisions between eosinophils and neutrophils, as well as play an important role in sustaining myeloid progenitor survival (Buitenhuis et al., 2007). Activation of Akt is also known to regulate cell survival, proliferation and differentiation by regulation of apoptosis pathways (Miranda & Johnson, 2007; Ghaffari et al., 2005. Zhao et al., 2005). Apoptosis plays an important role in cell development

because of the necessary rapid turnover rates among cell populations in a developing embryo (Elmore, 2007). The apoptosis pathway requires tight regulation, where failure of this process leads to can lead to diseases, such as autoimmunity or tumor growth (Ren & Savill, 1998). With the conservation of the PLAC8 domain in both clone 2B4 and cornifelin-6, it could suggest a link between the two genes, where their functions may involve regulation apoptotic or cell proliferation pathways in different cell types.

To continue to investigation of the expression of clone 2B4, we plan to isolate different hematopoietic cell populations (*mpo*⁺, *gata1*⁺, *gata2*⁺, etc.) from transgenic zebrafish and examine the expression profile of clone 2B4 by quantitative PCR. In doing so, we hope to gain an expression profile of clone 2B4 within all of the myeloid cell lineages throughout embryogenesis and adulthood. With this extensive expression profile, we can compare clone 2B4 expression levels to those of cornifelin-6 within the same hematopoietic cell populations in order to further understand the relationship between clone 2B4 and cornifelin-6.

Additionally, we can further examine clone 2B4 and its position in the hematopoietic gene tree by epistatic analysis using existing zebrafish blood mutants (Weber et al., 2005). WISH analysis in *vlad tepes* mutants suggested that clone 2B4 may act downstream of *pu.1* signaling. We can similarly use the mutants identified in previous forward genetic screens that exhibit defects in specific stages of hematopoiesis to serve as assay systems for understanding genetic heirarchies. For example, the *cloche* mutant lacks *scl* expression, an early gene necessary for normal

hematopoiesis, where the mutant lacks both vascular and hematopoietic stem cells (Laio et al., 1998). Forced expression of *scl* in *cloche* mutants demonstrated a partial rescue of both hematopoietic and vascular fates, showing that *scl* acts downstream of *cloche*. Similarly, we can use this simple model and other blood mutants to determine the hierarchical molecular network involved in zebrafish myelopoiesis for clone 2B4.

Also, to complement our initial studies, we will functionally characterize clone 2B4 *in vivo*. We can exploit the advantages of the zebrafish through the ability to easily test gene function using morpholino knockdown or mRNA over expression approaches. Studies show that antisense morpholino oligonucleotides can block blood-specific gene function with a transient nature (Ekker & Larson, 2001; Nasevicius & Ekker, 2000). Since primitive macrophages and definitive myeloid cells are formed within the first 5 days of development, morpholino knockdowns can be a highly effective in blocking gene functions within developing myeloid cells. We will perform morpholino knockdown by designing and injecting morpholino RNA sequences into single-cell stage wild-type embryos. Similarly, we can initiate gain of function studies by subcloning the full-length clone 2B4 into an mRNA expression vector and inject the mRNA into single-cell stage wild-type embryos. We will use WISH to assay the gain or loss of function of clone 2B4 along with an array of myeloid genes such as *pu.1*, *mpo*, *l-plastin*, and *c-myb* for comparison. We will also perform gain or loss of function experiments using fluorescent transgenic zebrafish lines that will enable the effects of gene misregulation to be monitored within living embryos. From this, we can further analyze isolated affected cell types by FACS,

quantitative PCR, transplantation, and *in vitro* cell culture assays. Altogether, the use of morpholino knockdown and mRNA overexpression approaches will provide us with rapid and efficient functional testing approaches for clone 2B4.

Additionally, we can further investigate the functionality of clone 2B4 by analyzing its expression within leukocyte populations during inflammatory conditions to examine its hypothetical role in apoptosis and cell proliferation. We plan to challenge zebrafish embryos at different developmental stages with bacterial infection or radiation and analyze the changes in expression in the leukocyte populations by quantitative PCR and WISH. By looking at the changes in expression patterns of clone 2B4, we will be able to investigate which cell types regulate clone 2B4 during an challenge response. This should allow us to further characterize the function of clone 2B4 in hematopoietic cell populations.

Overall, we have shown the feasibility of an expression-based screen used to discover novel genes from a myeloid specific cDNA library. The initial WISH-based screen discovered novel genes that have yet to be characterized. This study presents a preliminary investigation into one gene, clone 2B4, which may function in the differentiation and regulation of zebrafish hematopoiesis. Through further studies, we hope to characterize the function and regulatory pathways of clone 2B4, as well as continue to uncover more novel genes using the WISH-based screen on the myeloid cDNA library. In doing so, we hope to build upon the recognized hematopoiesis cellular pathway in the zebrafish and mammals by extending our knowledge of characterized genes that function in hematopoiesis.

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