HEMATOPOIETIC CELL LINEAGE SWITCHING MEDIATED BY ZEBRAFISH STAT1B

by

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Title: HEMATOPOIETIC CELL LINEAGE SWITCHING MEDIATED BY ZEBRAFISH STAT1B

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A critical question for developmental biology is the mechanism by which cells make fate decisions. In the hematopoietic system, stem cells differentiate into several different cell types, but the mechanisms that affect this process are incompletely known. Understanding these mechanisms is important because abnormal regulation of hematopoiesis can result in disease.

STAT1 protein plays crucial roles in mediating innate immunity by transducing interferon signals, but recent results have also related STAT1 to hematopoietic cell differentiation. Here we cloned a previously uncharacterized zebrafish co-ortholog of the human *STAT1* gene we call *stat1b* and investigated the functions of two zebrafish Stat1 proteins in hematopoiesis. The advantage of the zebrafish model is that, due to a whole genome duplication (WGD), some human genes have two co-orthologs in zebrafish. During evolution, co-orthologs have retained or acquired similar, complimentary, or new

functions.

Both *stat1a* and *stat1b* encode all four characteristic domains of the human STAT1 protein. Phylogenetic and conserved synteny analyses showed that *stat1b* and *stat1a* arose as duplicates in the teleost genome duplication event, and these analyses clarified the historical origin of the entire vertebrate STAT gene family. RT-PCR demonstrated maternal expression of both *stat1a* and *stat1b*. Expression of *stat1b*, but not *stat1a*, was detected in hematopoietic domains of embryos by in situ hybridization. Morpholino knockdown of *stat1b*, but not *stat1a*, mRNA expression resulted in a decrease in expression of the myeloid cell marker genes *spi* and *mpx* and an increase in expression of the hematopoietic progenitor marker gene *scl* and the erythrocyte marker gene *gata1*. These results show that in zebrafish, Stat1b protein functions in the commitment of hematopoietic cells to a myeloid cell fate.

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CHAPTER I

INTRODUCTION

1.1. Hematopoiesis in Zebrafish

The process of hematopoiesis consists of cell fate decision and lineage differentiation and maturation of all blood cells. SCL/TAL1 is a helix-loop-helix transcription factor that determines the commitment of hemangioblast, the common ancestor of endothelial cells and blood cells, to hematopoiesis stem cells (HSC) (Forrai and Robb, 2003; Xiong, 2008). Absence of *SCL/TAL1* was embryonic lethal in knockout mice due to complete and early block in embryonic erythropoiesis (Shivdasani et al., 1995). For each of the three main blood cell lineages, erythroid, myeloid, and lymphoid, several important transcription factors exist for regulating the development and are used as markers for specific cell lineages. Among them, GATA1 is a zinc finger protein essential for erythroid cell differentiation. GATA1 binds to its specific DNA sites and recruits co-factors to promote transcription of downstream genes, including hbbe1 for hemoglobin (Fujiwara et al., 1996; Crispino, 2005). PU.1/SPI1 is an Ets-family transcription factor and promotes expression of various myeloid specific genes including the M-CSF receptor (Oikawa et al., 1999; Kastner et

al., 2008). IKAROS, a hemopoietic-specific zinc finger transcription factors, is necessary for both embryonic lymphoid cell fate decision and adult T-/B- cell maturation, cooperating with many factors, especially AIOLOS (Georgopoulos, 1997; Schmitt, 2002). Nevertheless, the detailed mechanisms of their functions and the intricate networks of co-factors remain to be revealed. Besides, MPO/MPX (myeloperoxidase) is a heme-containing enzyme mainly synthesized during neutrophils maturation and executes the microbicidal activity of neutrophils by generating highly active free radical species (Hampton et al., 1998; Klebanoff, 2005) and thus is used as a maker for differentiated neutrophils.

Zebrafish is a good model in hematopoiesis research. Vertebrates have two waves of hematopoiesis, primitive (embryonic) hematopoiesis and definitive (adult) hematopoiesis. The transcriptional mechanisms of hematopoietic stem cell (HSC) differentiation are evolutionarily conserved among vertebrates in both hematopoietic waves, although the location varies among species (de Jong and Zon, 2005). In zebrafish, primitive hematopoiesis begins around 10 hours post fertilization (hpf) when embryonic HSC differentiate from the ventral lateral plate mesoderm (Davidson et al., 2003). Primitive HSC reside at two sites in zebrafish: the intermediate cell mass (ICM) and the rostral blood island (RBI). The ICM is located in the trunk ventral to the notochord and contains stem cells that differentiate mainly into pro-erythroblasts. The RBI develops from the cephalic mesoderm and produces mainly macrophages (Long et

al., 1997). Primitive hematopoiesis generates mainly erythrocytes. At about 24hpf, about 300 erythrocytes enter the blood stream from the ICM (Patterson et al., 2005). Primitive hematopoiesis lasts until about 32hpf, and about an hour earlier, definitive HSC begin to appear in the ventral wall of the dorsal aorta in a region known as the aorta-gonad-mesonephros (AGM) (Burns et al., 2005). Hematopoiesis continues in the AGM from 30hpf to 40hpf, when the kidney marrow takes over as the site of lifelong definitive hematopoiesis, analogous to the bone marrow in tetrapods. Cytospin preparations of kidney marrow reveal that all circulating hematopoietic blood cell types are present from 40hpf onwards (the embryo hatches on day 3) (Traver, 2004).

1.2. STAT1 in Hematopoiesis

Hematopoiesis may also involve STAT1 (signal transducer and activator of transcription-1). The lack of *FANCC* activity leads to Fanconi Anemia, a condition of hematopoietic bone marrow failure and leukemia (Joenje and Patel, 2001; Wang, 2007), and failure to activate optimally STAT1 in response to cytokines or growth factors (Pang et al., 2000; Pang et al., 2001). STAT1 mediates signaling of both type I and type II interferons (IFNs) in the innate immune response against viral infection through an IFN-JAK-STAT relay (Schindler et al., 1992). IFNs bind to IFN receptors, thereby activating JAKs, which then phosphorylate STAT1. Phosphorylated STAT1 translocates from the cytosol into the nucleus, and in minutes promotes changes in gene

expression (Stark et al., 1998). Now we know STAT1 can transduce signals in response to a variety of cytokines and growth factors (Najjar et al., 2010a) and regulate different cellular functions, mainly in immunity system. In addition to by type I and type II IFNs to fight against opportunistic virus infections (Meraz et al., 1996; Durbin et al., 1996), growth factors including EGF (Chin et al., 1996) and PDGF (Vignais et al., 1996) have been shown to activate STAT1. TNF has anti-viral function and can activate STAT1, although TNF is mainly released in response to bacterial lipopolysaccharide (LPS) rather than viruses (Singh K et al., 2010). Moreover, STAT1 is activated by IL-4 for cell growth inhibition and T cell maturation (Chang et al., 2000; Acacia et al., 2007); by IL-6 to inhibit cartilage growth (Legendre et al., 2003); by IL-7 to initiate T cell activity (Rose et al., 2010); by IL-9 for cell growth arrest and differentiation (Demoulin et al., 1999; Demoulin et al., 2001); by IL-10 to activate monocytic cells (Rahimi et al., 2005); by IL-11 to protect endothelial cells and trigger gastric tumorigenesis (Mahboubi et al., 2000; Ernst et al., 2008); by IL-13 to induce degranulation of mast cells leading to asthma (Chiba et al., 2009); by IL-21 to promote cell apoptosis (de Totero et al., 2008); by IL-22 to increase cultured cell line proliferation (Lejeune et al., 2002; Brand et al., 2006); and by IL-27 to regulation T cell differentiation and mediate inflammation through monocytes activation (Kalliolias and Ivashkiv, 2008; Ouaked et al., 2009). STAT1 also plays a role in the humoral immunity, promoting the expression of the B-cell receptor in the lymphoid lineage (Najjar et al., 2010b) and helping neutrophil

granulocytes from the myeloid lineage fight against mycobacterial infection (Al-Muhsen et al., 2008; Bussmeyer et al., 2010). In addition, STAT1 is involved in the activation of dendritic cells and macrophages in antigen presentation (Cantrell et al., 2009). STAT1 might even promote late-stage melanoma progression (Schultz et al., 2010). Many of these various functions of STAT1 could be related to its effect on cell growth retardation and apoptosis. For example, activation of STAT1 is involved in renal cells damage in hyperglycemia (Ortiz-Muñoz et al., 2010), pathophysiological change in the CNS (Hofer et al., 2010) and osteoblast differentiation inhibition in bone (Tajima et al., 2010). But several showed the opposite, especially in carcinogenesis and tumor metastasis.

Although a substantial amount of work on STAT1 has occurred in mature tissue culture cells, comparatively little study has been directed towards the roles of STAT1 in embryonic development. The zebrafish *Danio rerio* (Hamilton, 1822) has several advantages for an investigation of the roles of STAT1 in embryonic development. Due to a whole genome duplication event at the base of the teleost radiation, some single copy genes in human have two orthologs in zebrafish (Amores et al., 1998; Amores et al., 2004; Postlethwait et al., 2004). Such duplicates often share between them ancestral gene functions that are conserved with the mammalian genes (Force et al., 1999). Thus, the knock down of just one of the duplicates by morpholino (MO) antisense oligonucleotide while keep the other functional can reveal gene functions that might be

difficult to resolve in mammals due to pleiotropy. Our work on the two zebrafish *STAT1* co-orthologs provides new insights into the roles of STAT1 in hematopoietic development. The *stat1a* gene of zebrafish has been shown to rescue interferon-signaling mediated cell growth inhibition in a STAT1-deficient human cell line (Oates et al., 1999). Here we report the cloning of *stat1b*, an additional *stat1* co-ortholog in zebrafish. We show that *stat1b* arose in the teleost genome duplication event, that it is expressed in the embryonic hematopoietic domain, and that it promotes blood cell lineage switching in primitive hematopoiesis development.

CHAPTER II

MATERIALS AND METHODS

2.1. Primers for Cloning *stat1b* cDNA

Primers covering overlapping fragments of XR_029517 were designed as follows for RT-PCR, with two pairs of primers for each fragment. PCR products were sequenced with the TOPO TA cloning kit into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequences were assembled to obtain full-length cDNA sequence.

stat1bF10	CGTTGAAAGATGACGCTCTG
stat1bR10	ACGGATCTGCTGGAGACACT
stat1bF11	GCTTATCCCGAGATACACTCC
stat1bR11	TCTTCAGCTGCTGACGGATCT
stat1bF20	GCTGAAGGTGTGCAAATGAA
stat1bR20	AGGCACTGGGTAACTGGTTG
stat1bF21	GCTGAAGGTGTGCAAATGAA
stat1bR21	TTCACCGCTGAGCATGTTGTA
stat1bF30	GACGCCTCTCATCGTTACAGA

stat1bR30	TTTTGGGATGTTCGGGTAAA
stat1bF31	GCAGCTGAATTTCGTCATTTG
stat1bR31	GCGACCAAAAGCTTCATCTT
stat1bF40	TGGTGAACCCAAGATCCATT
stat1bR40	GTGAAATGGCCTGTTCATCC
stat1bF41	TGCGCTTTAGTGAAAGCTGT
stat1bR41	TGAAATGGCCTGTTCATCCA
stat1bF50	CAGCAGATGGCTTGTATTGG
stat1bR50	TGCCAGCTTATGACCTTTGA
stat1bF51	TATCAGAGGAGTTACCCGAGT
stat1bR51	TGCTGATTGAAGAAAACTGCC

2.2. Phylogenetic Analysis

Related STAT protein sequences were downloaded from NCBI and Ensembl and aligned by Muscle (MUltiple Sequence Comparison by Log-Expectation, http://www.ebi.ac.uk/Tools/muscle/index.html) using the JTT model with ProtTest (Abascal et al., 2005;). Finally, all information was submitted to PhyML (Guindon and Gascuel, 2003; http://www.atgc-montpellier.fr/phyml/) for construction of the phylogenetic tree.

2.3. Zebrafish, Morpholinos, and In Situ Hybridization

Zebrafish (ABC X TU strain) were maintained as described (Kimmel et al., 1995). The sequence of *stat1b* morpholino (MO) was AAAATGTAGCGGATGTTACTTCGAC (Gene Tools, LLC, Philomath, OR, USA), which targets the splice donor site of exon5-intron5, leading to the inclusion of intron 5 and a premature translation stop codon. The sequence of *stat1a* MO is TCATGTGGTCAACAGGCACCTGCAA, which targets the splice donor site of exon3-intron3, leading to the inclusion of intron 3 and a premature translation stop. The sequence of the control MO was CCTCTTACCTCAGTTACAATTTATA, whose sequence has no significant similarity found in the zebrafish reference genome sequence database. Embryos were collected at 16hpf, 24hpf, 32hpf, and 48hpf for in situ hybridization with zebrafish *stat1a* probe, *stat1b* probe, and a negative control (cuttlefish, GenBank accession number GU388435). In situ was carried out following methods previously described (Yan et al., 2002) except that probe incubation was done at 50 . MO (5 mg/ml for *stat1b* MO and 10 mg/ml for *stat1a* MO) was injected into one-cell stage embryos. In situ hybridization with blood markers was carried out at 16hpf and 32hpf following methods previously described (Yan et al., 2002).

2.4. RT-PCR and Quantitative RT-PCR

Fertilized eggs were collected at indicated time points. Total RNA was extracted

with TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and genomic DNA was removed with DNA-free™ DNase Treatment and Removal Reagents (ABI, Foster City, CA, USA). Further total RNA sample was quantified with spectrometry at 260nm and loading amount to RT-PCR was calibrated to be the same. Product from each pair of primers was sequenced and verified to be the target mRNA fragment before the actual RT-PCR. RT-PCR was run with OneStep RT-PCR kit (QIAGEN, Valencia, CA, USA). Primers used are listed below:

actinF GCCAACACAGTGCTGTCTGGAGG

actinR GGTCATGGACGCCCATTGTGAGG

stat1aMEF AGTCGCAGCAATGACTCAGTG

stat1aMER CTGCTGATGATCATCGCCATTG

stat1bMEF AGGTGACTCCATGCAGGGGAAC

stat1bMER GCGGGTCGTTGCTGTAGGTGA

cDNAs were prepared with the High Capacity RNA-to-cDNA kit (ABI, Foster City, CA, USA). Quantitative PCR was carried out using Power SYBR® Green PCR Master Mix (ABI, Foster City, CA, USA). Q-PCR requires biological repeats and sampling repeats. To make the level of RNA comparable among different samples, the expression level of a house-keeping gene zgc:136952 (NM_001040043), which encodes a ribosomal protein, was run as an internal control. Primers used are listed below:

gata1qF GAATGCAGCTTCAGAGGTTTATCC

gata1qR TGGGTTCAGAGAATACGCTCCTA

mpxqF CCAAACCTCAGGGATGTTCTTG

mpxqR CCCAAACTACGAGTCCCTATGC

spiqF GGGTAGCCATCACATCCCTCTAG

spiqR TGGACGTTGTGAGGGTAACACA

sclqF GACTAATTTCTCGGGCTGACAACTA

sclqR GGGCGTTAACAGAAAGTCTTACGTA

RPL32qF CCCTCACCAAACCTAAGATCGT

RPL32qR CTCCAGTTTGCCCTGATCTTG

CHAPTER III

RESULTS

3.1. Isolation of a cDNA for a Zebrafish Co-Ortholog of STAT1, and Gene and Protein Structure

To search for a possible duplicate of the described zebrafish *stat1a* gene, we used the *stat1a* gene sequence (RefSeq NM_131480) in a BLAST search against the zebrafish EST database and identified the gene model sequence XR_029517 as the most similar sequence. We designed primers for overlapping fragments of the EST sequence and used them to amplify cDNA from a total RNA sample of mixed 24hpf and 48hpf embryos from an ABC X TU cross, and then combined sequences of the cDNA fragments bioinformatically. We identified a 3,569bp nucleotide sequence that we called *stat1b* and submitted it to GenBank (accession number FJ986224). Comparing the structure of this cDNA with the genomic DNA at *Ensembl* shows 23 exons. The translated exons of human *STAT1* and zebrafish *stat1a* and *stat1b* are orthologs. The untranslated exon 1 in human *STAT1* was lost in both zebrafish *stat1* genes and exon 2 was partially kept. At 3' end, exon 24 to 26 in *stat1a* encodes the STAT domain (see below and Fig. 2) just like what exon 24 in human *STAT1* does (Fig. 1). The *stat1b* gene

should be translated into a 725-amino-acid peptide (accession number ACR83062). As predicted by NCBI BLASTP, the translated peptide Stat1b has four (Int, Alpha, Bind, and SH2 superfamily) of the five specific domains that are found in human STAT1 (Fig. 2). The two domains at the N-end, STAT-int and STAT-alpha, are responsible for the interaction between STAT proteins after they bind DNA sites, usually enhance their transcription activation effects; the STAT-bind domain recognizes and binds specific DNA fragments, determining the specificity of STAT proteins; SH2 domain, or Src Homology 2, near to the C-end, bind a phosphorylated tryosine residue in IFN receptors or other STAT proteins, thus recruiting STATs to IFN receptors or forming dimers with other STAT proteins, respectively. The human STAT1 gene has two splice forms, STAT1-α and STAT1-β. The difference between the two splicing isoforms is that STAT1-α has an extra domain at the C-end, which, interestingly, also appears in zebrafish Stat1a C-end. This domain is approximately 20 amino acids in length and binds selectively to the TAZ2 domain of CRB (CREB-binding protein) to act as a transcriptional activator. Taken together, we conclude the newly cloned gene is indeed a member of *STAT* family.

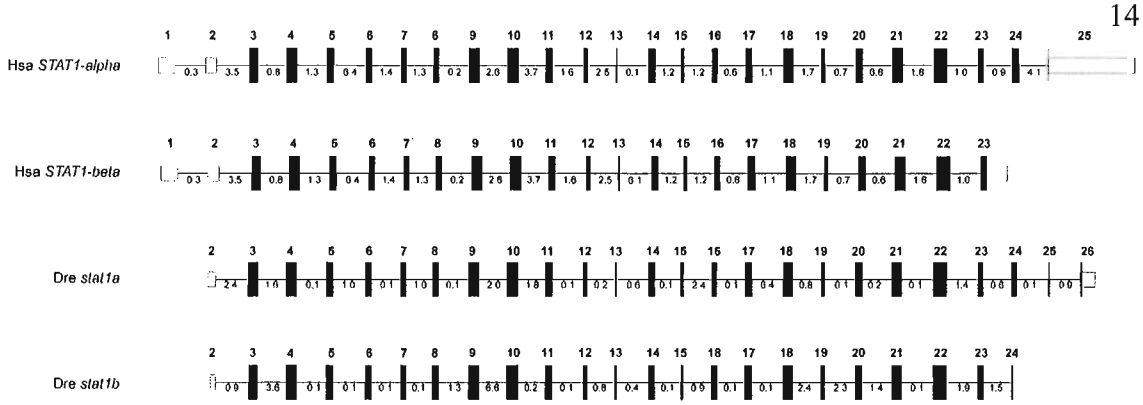

Fig. 1. Exon structures of human *STAT1 alpha* and *beta*, and zebrafish *stat1a* and *stat1b*. Exons are represented as labeled unfilled boxes (5' and 3' un-translated regions (UTRs)) or filled boxes (translated regions). The width of boxes is proportional to the actual length of each exon. Introns are represented with thin lines connecting exons and their length is indicated in kilobases but is not proportional to length.

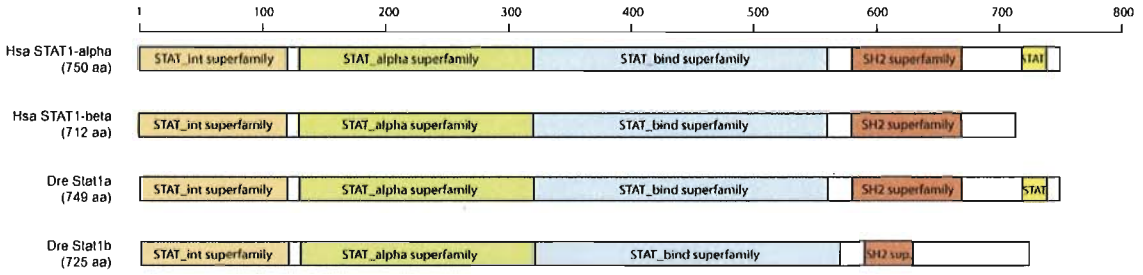

Fig. 2. STAT specific domains appear in proteins encoded by the two STAT1 splicing forms of the human gene and from zebrafish Stat1a and Stat1b. Five domains are represented as colored boxes. The length of each domain is indicated with a scale of numbers of amino acids on the top. Linker regions of domains in the proteins are represented as white boxes.

3.2. The Evolutionary Origin of *stat1b*

To help understand the origin of *stat1b*, we conducted a phylogenetic analysis of full-length STAT proteins using maximum likelihood (Guindon and Gascuel, 2003) rooted on the STATa protein of the urochordate *Ciona intestinalis* (Fig. 3). The STAT1, STAT2, and STAT3 clades are well supported with high bootstrap values (470/500 for

STAT1, 500/500 for STAT3, and 490/500 for STAT2). The newly cloned *stat1b* sequence clearly belongs to the STAT1 clade. Note that the teleost Stat1b and Stat1a clades do not branch as sisters basal to the tetrapod STAT1 clade. Because the bootstrap value for the zebrafish Stat1a branch is lower than 60%, the node was collapsed. The evidence from this tree topology neither strongly supports nor rules out the hypothesis that *stat1a* and *stat1b* arose in the teleost genome duplication event. We conducted BLAST searches with *stat1b* sequence against genome databases and found *stat1b* sequences for only two species, zebrafish and goldfish, both of which are Cypriniform fishes. In contrast, *stat1a* sequences were found not only in Cypriniforms, but also in Percomorph fish, including stickleback, pufferfish, and medaka. It appears that the *stat1b* gene was lost in the Percomorph lineage.

The phylogenetic analysis showed that the duplication event that produced *STAT1, STAT2,* and *STAT3* clades occurred before the divergence of tetrapod and teleost genomes. To determine the historical relationships of these genes, we used the Synteny Database (Catchen et al., 2009) to identify human paralogs of genes in a 20Mb interval surrounding *STAT1* on human chromosome 2 (Hsa2). The algorithm marches down Hsa2 gene by gene, identifies paralogs, and directly above or below the Hsa2 gene, plots a plus

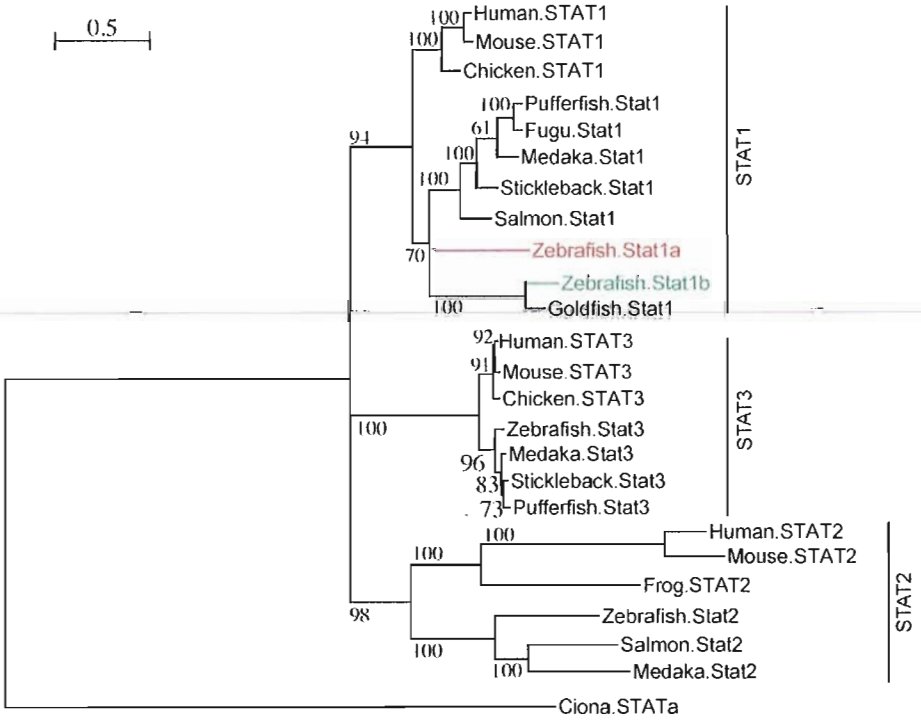

Fig. 3. Phylogenetic tree constructed by maximum likelihood for STAT1, STAT2, and STAT3 clades, using *Ciona intestinalis* STATa as outgroup. Bootstrap values are shown as the percentage of a data set of 500 trials. The newly cloned sequence clearly belongs to the STAT1 clade. Protein sequences used in tree construction: Chicken.STAT1: NP_001012932; Chicken.STAT3: NP_001026102; Ciona.STATa: BAE06716; Goldfish.Stat1: AAO88245; Human.STAT1: NP_009330; Human.STAT2: NP_005410; Human.STAT3: NP_644805; Medaka.Stat3: AAT64912; Mouse.STAT1: NP_033309; Mouse.STAT2: NP_064347; Mouse.STAT3: NP_035616; Pufferfish.Stat1: AAL09414; Pufferfish.Stat3: AAL09415; Stickleback.Stat1: GENSCAN00000019309; Stickleback.Stat3: GENSCAN00000017667; Fugu.Stat1: ENSTRUP00000000223; Zebrafish.Stat1a: NP_571555; Zebrafish.Stat1b: ACR83062; Zebrafish.Stat3: AAH68320;

sign on the human chromosome that has the paralog. Figure 4A shows that Hsa2 has numerous paralogs on Hsa3, 7, 12, and 17. The most parsimonious explanation is that these four chromosome segments (Hsa2, 3+7, 12, 17) arose in two rounds of whole genome duplication at the base of the vertebrate radiation (Dehal and Boore, 2005).

These events would have produced a fourth *STAT1*-related paralog that is now missing from all studied genomes. The *Ensembl* database shows that *STAT1* lies adjacent to *STAT4* and both genes are transcribed in the same orientation (Fig. 4B), suggesting that they arose by a tandem duplication event. *STAT3* lies adjacent to *STAT5A*, which is next to *STAT5B*, with *STAT3* and *STAT5B* transcribed in the same orientation. Finally, *STAT3* is very near *STAT6*, only about 750kb distant, on Hsa12. We conclude that the *STAT* gene family arose by a tandem duplication to give a *STAT123* gene and a *STAT456* gene in pre-vertebrate chordates. In the R1 and R2 rounds of genome duplication that preceded the vertebrate radiation, four copies of this tandem gene pair were produced, *STAT1 STAT4, STAT2 STAT6, STAT3 STAT5,* and a fourth pair that is now missing. A subsequent tandem duplication event produced *STAT5A* and *STAT5B* in the human lineage after it diverged from the chicken lineage.

Evidence from the phylogenetic analysis was not conclusive regarding the origin of *stat1a* and *stat1b* with respect to the teleost genome duplication event. To clarify the origin of the *stat1a* and *stat1b* gene pair, we used the Synteny Database (Catchen et al., 2009) to search for regions in the zebrafish genome with conserved synteny to the *STAT1* region on Hsa2. Results showed that *stat1b* lies next to *stat4* on Dre9 along with about 300 other genes with conserved synteny between the two chromosomes, only a few of which are shown in Fig. 3B. Except for two inversions, genes in human and zebrafish are in the same order in these regions, including *stat1b* and *stat4* and near

neighbors. Likewise, several orthologous pairs of genes with conserved synteny are shared between the human *STAT1* region and the region of Dre22 that contains *stat1a* (Fig. 4). An inversion breakpoint is in the location expected for a duplicate copy of *stat4*, suggesting that the inversion event may have destroyed the *stat4* copy on Dre22, which would have had no phenotypic penalty if its functions were fully redundant with the *stat4* copy on Dre9. Because the orientation of transcription is *stat4*> *stat1b*>, an inversion event between the genes could have broken *cis*-acting regulatory elements located in the intergenic region, which is 5' to *stat1b*. We conclude that *stat1a* and *stat1b* arose in the teleost genome duplication event.

3.3. Both *stat1a* and *stat1b* Are Expressed at Early Stage of Zebrafish Embryo Development

A gene's expression pattern provides important hints about its function. We followed the time course of *stat1a* and *stat1b* expression by RT-PCR using total RNA samples from zebrafish embryos of various ages. To calibrate this semi-quantitative technique, we made dilutions of the template and used just 21 cycles in PCRs (Fig. 5a). Results showed that the intensity of the amplified band depended on template concentration. When we used the same conditions on embryo RNAs, we found strong and steady expression of *stat1a* from 0.5hpf until 96hpf, about 1 day after hatching, as well as in unfertilized eggs. These results suggest a maternal expression pattern followed by

continued zygotic expression (Fig. 5b). In contrast, *stat1b* was not maternally expressed and started low expression around 0.5hpf but rose until about 9hpf and continued strong thereafter (Fig. 5b).

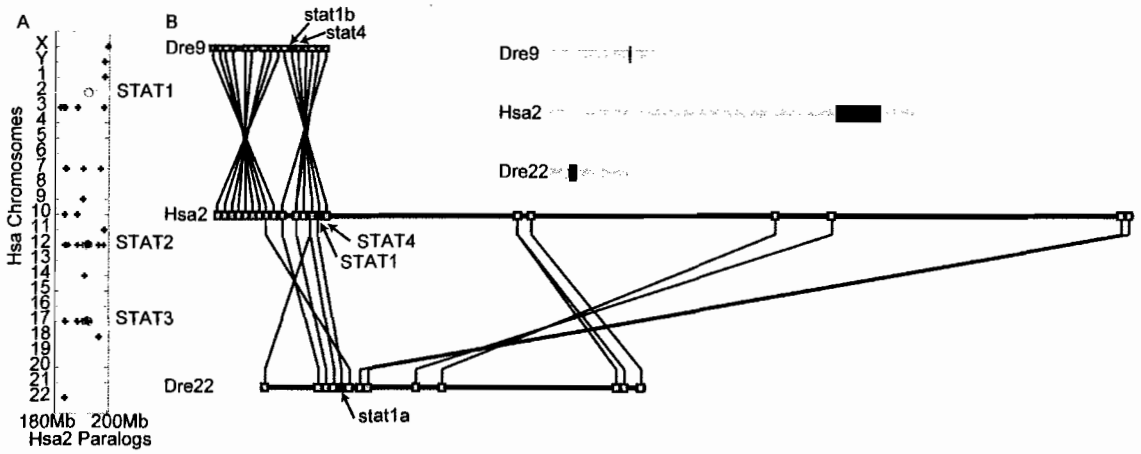

Fig. 4. Conserved syntenies verify the history of the *stat1* gene family. A. Paralogy analysis of 20Mb in the human genome surrounding *STAT1*. Human paralogs of genes residing in the region of Hsa2 between 180 and 200Mb were plotted on the rest of the human chromosomes with gene order according to the location on human chromosome 2 (Hsa2) using the Dotplot feature of the Synteny database (Catchen et al., 2009). Results showed that Hsa2 genes showed extensive paralogy with Hsa7, 12, and 17. *STAT1* and its paralogs *STAT2* and *STAT3* are circled. These results would be expected if these chromosome segments arose by two rounds of whole genome duplication. B. Conserved syntenies around human *STAT1* were searched in the zebrafish genome using the Synteny Database (Catchen et al., 2009). Extensive conserved syntenies were found around *stat1b* on zebrafish chromosome 9 (Dre9) and *stat1a* on Dre22. Lines connect orthologs. The positions shown in black rectangles on the chromosomes in the upper right are blown up in the main figure. Arrows indicate the positions of *stat* genes. Each box represents a gene. Genes: *HUMAN, dre9gene, dre22gene*: *STAT4, stat4; STAT1, stat1b, stat1a; GLS, gls, gls; NAB1, NP_001116745.1, si:dkeyp-84a8.1; TMEM194B, B0UYT4; MFSD6, zgc:92925, si:dkey-188p4.2; HIBCH, hibch; MSTN, mstn, gdf8; PMS1, pms1; ORMDL1, ormdl1; OSGEPL1, osgepl1; ANKAR, ankar; slc40a1, slc40a1; WDR75, wdr75.* The following genes are *HUMAN, dre22gene: RQCD1, rqcd1; PLCD4, plcd4a; PTH2R, si:dkeyp-4h4.1; FAM119A, zgc:110528; FAM119A; AOX1, aox1; BZW1, bzw1a.*

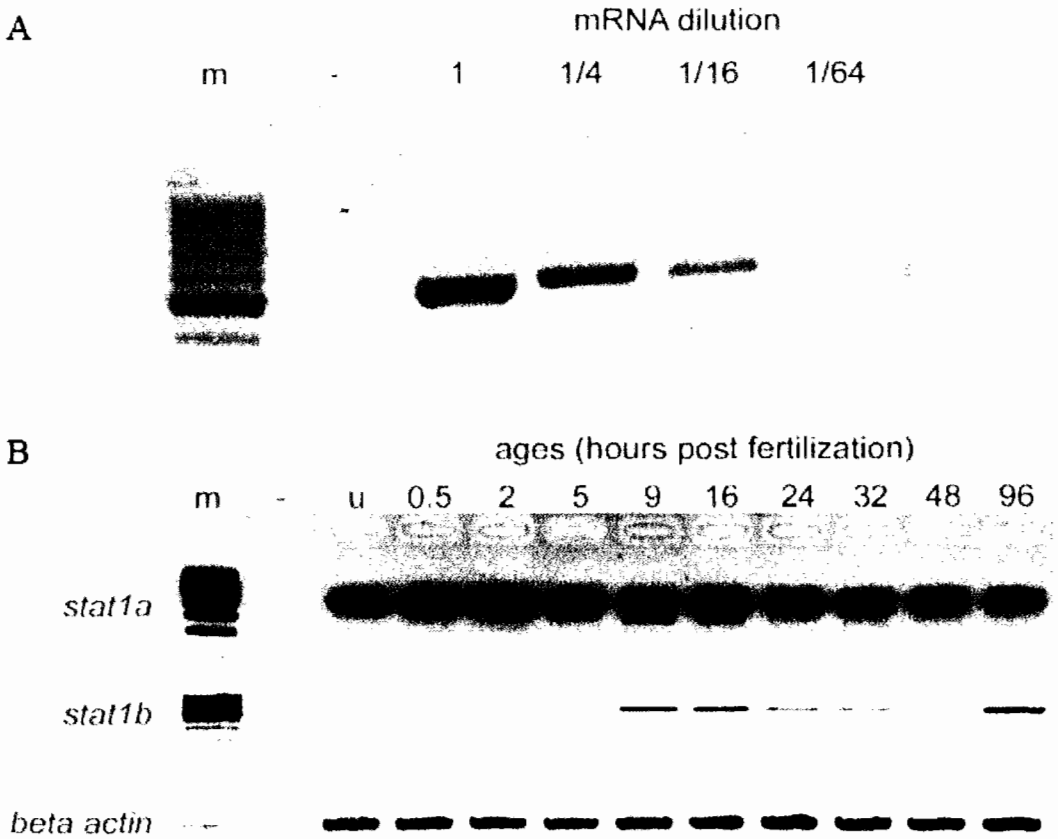

Fig. 5. A). Control experiment with a gradient of RNA concentrations and 21 amplification cycles. Results verified that these RT-PCR conditions can show the relative amount of target mRNA semi-quantitatively. B). RT-PCR with *stat1a* and *stat1b* primers were run to detect their expression levels along zebrafish development. The gene of *beta actin* was used as loading control. RNA samples from zebrafish embryos were extracted at different time points as indicated in the lanes. –, negative control; u, unfertilized eggs; 0.5, 2, et al., time points at hours post fertilization. While the expression level of *stat1a* mRNA is steady, that of *stat1b* varies. Especially, *stat1a* is clearly maternal expressed, while *stat1b* is turned on after fertilization.

3.4. The Expression of *stat1b* Is Specific in Hematopoiesis Region

To identify the spatial localization of *stat1* transcripts, we used whole mount in situ hybridization. Confirming a previous report (Oates et al., 1999), we did not find a

specific expression pattern for *stat1a* (data not shown), despite detecting it readily in RT-PCR studies. In contrast, *stat1b* RNA probes showed strong and restricted expression in the hematopoietic domain. Expression of *stat1b* appeared in the characteristic two stripes that flank the paraxial mesoderm of the posterior embryo starting at the 2-somite stage (about 11hpf) (Fig. 6CD) and meet anteriorly at the 18-somite stage (Fig. 6EF). Most cells in that region at 16hpf are erythroid progenitors (de Jong and Zon, 2005). At 24hpf, the expression pattern of *stat1b* is in the ICM (Fig. 6G), where most erythrocytes reside at this time. Later, at 32hpf and 48hpf, *stat1b* expression advanced to the heart and the ducts of Cuvier, which form after 25hpf and contain both erythrocytes and myeloid cells (Fig. 6HI). The expression patterns of *stat1b* are consistent with hematopoietic marker genes, including *gata1*, *gata2* and *hbbe* (Detrich et al., 1995).

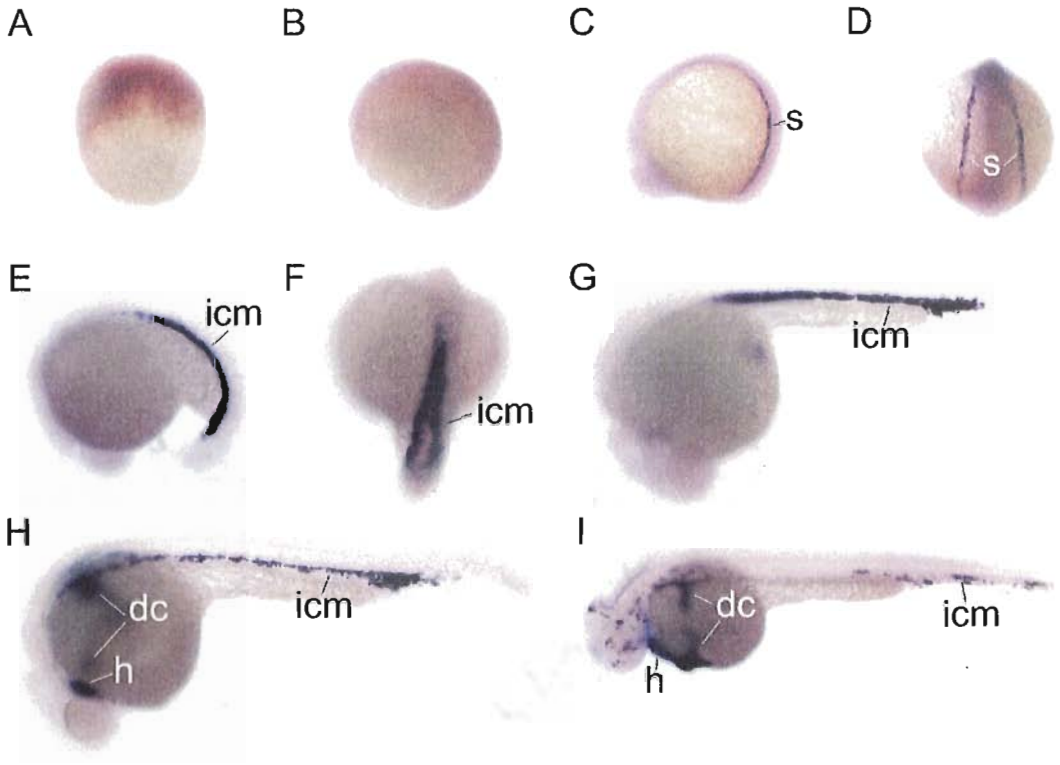

Fig. 6. Whole mount in situ hybridization shows specific expression pattern of *stat1b* in zebrafish hematopoietic regions. A. and B. No expression was detected at 3hpf and 9hpf. C. and D. First expression pattern of *stat1b* was detected at 2 somites and the expression was constrained to stripes that flank the paraxial mesoderm, shown with side and dorsal, respectively. E. and F. *stat1b* was expressed in internal cell mass at 18 somites, where hematopoietic progenitor cells reside, shown with side and dorsal, respectively. G. At 24hpf embryos, the expression region of *stat1b* is merging into one stripe. H. At 32hpf, in addition to internal cell mass, *stat1b* expression was found in the heart and ducts of Cuvier, a blood vessel network on yolk. I. At 48hpf, the expression of stat1b keeps on in the hematopoietic regions. Abbreviations: dc, ducts of Cuvier; h, heart; icm, intermediate cell mass; s, stripes flanking the paraxial mesoderm.

3.5. Loss of *stat1b* Function Results in Hematopoietic Cell Lineage Switching

The expression domain of *stat1b* in zebrafish ICM suggested the hypothesis that *stat1b* plays a role in primitive hematopoietic development. To knockdown activity of *stat1a* and *stat1b* in developing zebrafish embryos, we injected one-cell fertilized eggs with

either *stat1a* or *stat1b* MO, and then queried whether various lineages of blood cells developed differently by following the expression of lineage-specific gene markers. The *scl/tal1* gene is a marker for primitive hematopoietic progenitor cells. Results showed that the *scl/tal1* expression domain in 16hpf embryos injected with *stat1b* MO was broader than normal with more cells expressing this gene. In contrast, animals injected with the *stat1a* MO were normal (Fig. 7A-C). Quantitative PCR analysis confirmed the increased expression level of *scl/tal1* in *stat1b* knockdown animals (Fig. 7G). We conclude that *stat1b,* but not *stat1a,* normally narrows the stripe of *scl/tal1*-expressing hematopoietic progenitor cells. The *gata1* gene is a marker for cells in the primitive erythroid lineage. Knockdown of *stat1b* caused an elevation of *gata1* expression levels in both 16 and 32hpf embryos as detected by in situ hybridization experiments, and this result was confirmed by Q-PCR (Fig. 7H-N). The *pu.1/spi1* gene provides a marker for myeloid cells in general, and *mpo/mpx* is a marker of heterophil granulocytes. Both markers were depressed both in the in situ hybridization assays and in the Q-PCR assays (Fig. 7M-Y). We conclude that Stat1b normally promotes myeloid differentiation but depresses that of erythroid cells and those two cell lineages compete with each other for the same pluripotential progenitor cell group. Analysis of expression by in situ hybridization for *c-myb* (a marker of definitive HSC), *l-plastin* (a marker of macrophages), and *rag1* (a marker of lymphoid cells) were also performed, but no significant difference of those markers was found in *stat1b* or *stat1a* MO embryos and

controls (data not shown).

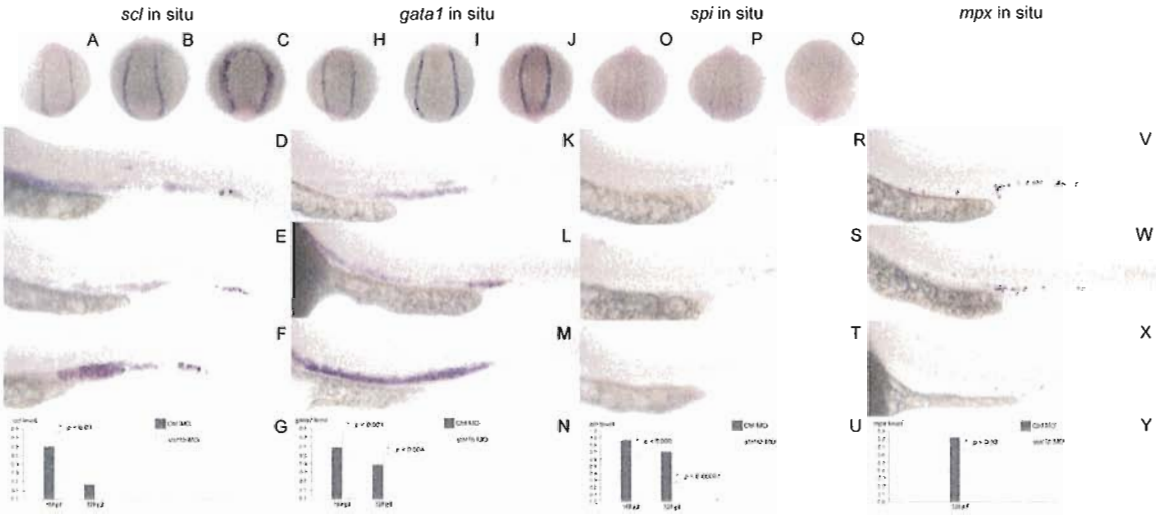

Fig. 7. In situ hybridization and quantitative PCR with blood gene markers after *stat1a* and *stat1b* MO injections. 16hpf embryos injected with control sequence MO (A), *stat1a* MO (B), and *stat1b* MO (C). The expression of *scl/tal1* in 16hpf embryos was elevated by *stat1b* MO and confirmed with quantitative PCR (G). Elevation of *scl/tal1* expression in 32hpf embryos by *stat1b* MO was found by in situ (D-F) but not consistent with q-PCR results (G). In situ with *gata1* probe showed that the expression was elevated by *stat1b* MO at both 16hpf (J) and 32hpf (M) and the results were confirmed by q-PCR (N). Whereas the *stat1a* MO had no effect (I and L) as those of control MO (H and K). On the contrary, the depression of *pu.1/spi1* expression by *stat1b* MO was found in both 16hpf (Q) and 32hpf (T) and confirmed with q-PCR (U). Again, the effects of *stat1b* MO (P and S) were similar to those of control MO (O and R). Finally *mpo/mpx* expression in *stat1b* knockdown embryos was suppressed dramatically at 32hpf (X) and the q-PCR suggested the same effect (Y). But the effect of *stat1b* MO (W) was similar to those of control MO (V). Taken together, those results suggested that *stat1b* is involved in promoting a lineage switch from erythroid cells to myeloid cells.

CHAPTER IV

DISCUSSION

We cloned and investigated the functions of *stat1b*, a previously unidentified zebrafish co-ortholog of the human *STAT1* gene. Phylogenetic analysis supports the conclusion that the newly cloned gene belongs to the STAT1 family with high bootstrap value (Fig. 3) and conserved synteny analysis shows that the human and zebrafish genomes have been conserved in the regions of both *STAT1* duplicates (Fig. 4). Only one other *stat1b* gene was identified in the NCBI database, a gene in gold fish, like zebrafish, a cyprinid fish. An explanation for this gene distribution could be a missing ohnolog (Postlethwait, 2007). Because most gene duplicates become pseudogenes, only about 20% genes in human genome have two co-orthologs in zebrafish (Postlethwait et al., 2000).

The alignment of translational exons of human *STAT1* and zebrafish *stat1a* and *stat1b* showed highly conserved exons structures (Fig. 1). The first non-coding exon in human *STAT1* was lost in both zebrafish *stat1*, which might be due to no selection pressure in that part. Four STAT specific domains appear in both Stat1a and Stat1b, with an extra domain at the C-end of Stat1a. The same scenario happens in two human

STAT1 splicing forms, of which STAT1-alpha has the extra domain (Fig. 2). It is interesting to postulate that some evolution pressure selected the splicing variants in human STAT1 while zebrafish may have evolved two different genes for a similar purpose after the WGD. While it is difficult to investigate the functional difference between the two human STAT1 splicing variants in vivo, zebrafish Stat1a and Stat1b provide us with an alternative way to investigate gene functions.

The *stat1a* and *stat1b* genes differed in several key respects. First, the in situ expression pattern of *stat1b* in the zebrafish hematopoietic region is a sharp contrast to that of *stat1a:* we and others (Oates et al., 1999) could not detect a specific expression pattern for *stat1a* by whole mount in situ hybridization. Because RT-PCR revealed substantial transcript for both *stat1a* and *stat1b* (Fig. 5), it may be that *stat1a* is expressed at low levels ubiquitously and that we detect *stat1b* expression by in situ hybridizatin due to its concentration in the hematopoietic system. That difference supports subfunction partitioning of two co-orthologs due to WGD. By conventional model of anti viral infection process, interferons were secreted by viral infected cells and then affected any surrounding cell through IFN-JAK-STAT1 pathway to invoke anti viral reaction. Stat1a in zebrafish might be the one against viral infection like revealed by Oates et al. in 1999 and Stat1b is involved in zebrafish hematopoiesis.

Second, our knockdown studies with anti-sense Morpholino (MO) did not suggest a role of Stat1a in blood cell lineage switching, even though RT-PCR experiments

showed high levels of maternal *stat1a* expression. Either of two possibilities might explain this result. First, *stat1a* may not play a role in blood development, or second, the splice-targeting MO for *stat1a*, which will not act on maternal message RNA, may not block the effect of maternal *stat1a*. A translation start site targeting MO for *stat1a* might resolve this question, but without a suitable antibody for zebrafish Stat1a, we could not verify the *stat1a* efficacy of the translation MO.

The specific expression pattern of *stat1b* in hematopoietic regions suggests that it plays a role in zebrafish hematopoiesis. Loss-of-function of *stat1b* by MO injection resulted in the increased expression of *scl* and *gata1* in primitive hematopoiesis but decreased expression of *spi* and mpx in both experiments of in situ and quantitative PCR. Because in situ expression analyses showed broadened expression regions, but not condensed expression in cells, we conclude that the increased expression of *scl* and *gata1* is due to increased cell numbers of corresponding blood lineage. The *scl* gene marks primitive hematopoietic stem cells (HSC) and *gata1* marks erythroid cells, whereas *spi* and *mpx* mark myeloid cell lineages. Along zebrafish development, erythrocytes are predominant initially, but later several myeloid cell lineages appear. Thus, our results suggested that 1) both RBC and neutrophils compete for the same pluripotential progenitor cell group; 2) although a marker for progenitor cells, *scl* biased to RBC differentiation; several other markers exist for primitive HSCs and they might be responsible for other cell lineage differentiation; and 3) *stat1b* promotes lineage

switching from erythroid (*gata1*-expressing) cells to myeloid (*spi*- and *mpx*-expressing) cells. Note that the result from knocking down *stat1b* is complementary to the result from knocking down *gata1*, which leads to an increase of *spi* and *mpx* expression in zebrafish but did not affect other cell lineages (Galloway et al., 2005) (Rhodes et al., 2005).

In addition, that result is consistent with recent experiments on mice that IFN-γ produced by Th1 cells stimulates a significant expansion of LSK, a specific HSC lineage, which tends to differentiate into myeloid lineages, through a STAT1 dependent pathway (Zhao et al., 2010). They showed that IFN-γ induced depletion of total progenitor cells, but promoted LSK cells proliferation capacity into myeloid cells, which compensate for the loss of total progenitors. IFN-γ has been known for long to be capable for regulating hematopoietic cell proliferation (Raefsky et al., 1985) but its effects are complicated. Depending on the stages and lineages of hematopoiesis, IFN-γ either inhibits colony formation through inducing over apoptosis (Young et al., 1997) (Shimozato et al., 2002) or promotes certain type of stem cell expansion and differentiation into myeloid cells (Caux et al., 1992) (Brugger et al., 1993). In all of those experiments, STAT1 is the important mediator for IFN-γ function. In our experiments, Stat1b could act in any of a number of ways. It might1) promote the apoptosis of erythroid cells; 2) inhibit the differentiation of progenitor cells into erythrocytes; or 3) promote the differentiation of progenitor cells into myeloid cells.

Taking into account the results mentioned above and the function of STAT1 in inducing apoptosis, we prefer the first hypothesis but cannot rule out the other two possibilities. Understanding the different regulatory effects of IFN and STAT1 in different hematopoietic cell lineages and stages would help to shed light on the mechanism of those two interesting proteins and to better manipulate them in clinical practice for the welfare of human patients. Meanwhile zebrafish represents a new model to dissect the intricate functions of STAT1 with function divergence between zebrafish Stat1a and Stat1b.

REFERENCES

Abascal, F., Zardoya, R., Posada, D. (2005). ProtTest: Selection of best-fit models of protein evolution. *Bioinformatics*: 21(9):2104-2105.

Acacia de Sa Pinheiro, A., Morrot, A., Chakravarty, S., Overstreet, M., Bream, J.H., Irusta, P.M., Zavala, F. (2007). IL-4 induces a wide-spectrum intracellular signaling cascade in CD8+ T cells. *J Leukoc Biol.* Apr;81(4):1102-10.

Al-Muhsen, S. and Casanova, J.L. (2008). The genetic heterogeneity of mendelian susceptibility to mycobacterial diseases. *J Allergy Clin Immunol.* 122(6):1043-51.

Amores, A., Force, A., Yan, Y.L., Joly, L., Amemiya, C., Fritz, A., Ho, R.K., Langeland, J., Prince, V., Wang, Y.L., Westerfield, M., Ekker, M., Postlethwait, J.H. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science.* 27;282(5394):1711-1714.

Amores, A., Suzuki, T., Yan, Y.L., Pomeroy, J., Singer, A., Amemiya, C., Postlethwait, J.H. (2004). Developmental roles of pufferfish Hox clusters and genome evolution in ray-fin fish. *Genome Res.* Jan;14(1):1-10.

Brand, S., Beigel, F., Olszak, T., Zitzmann, K., Eichhorst, S.T., Otte, J.M., Diepolder, H., Marquardt, A., Jagla, W., Popp, A., et al. (2006). IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol.* Apr;290(4):G827-38.

Brugger, W., Möcklin, W., Heimfeld, S., Berenson, R.J., Mertelsmann, R., Kanz, L. (1993) Ex vivo expansion of enriched peripheral blood CD34+ progenitor cells by stem cell factor, interleukin-1 beta (IL-1 beta), IL-6, IL-3, interferon-gamma, and erythropoietin. *Blood.* May 15;81(10):2579-84.

Burns, C.E., Traver, D., Mayhall, E., Shepard, J.L., Zon, L.I. (2005). Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev.* Oct 1; 19(19): 2331-2342.

Bussmeyer, U., Sarkar, A., Broszat, K., Lüdemann, T., Möller, S., van Zandbergen, G., Bogdan, C., Behnen, M., Dumler, J.S., von Loewenich, F.D., Solbach, W., Laskay, T. (2010). Impairment of gamma interferon signaling in human neutrophils infected with Anaplasma phagocytophilum. *Infect Immun.* 78(1):358-63.

Cantrell, J., Larmonier, C., Janikashvili, N., Bustamante, S., Fraszczak, J., Herrell, A., Lundeen, T.J., Lacasse, C., Situ, E., Larmonier, N., Katsanis, E. (2009). Signaling pathways induced by a tumor-derived vaccine in antigen presenting cells. *Immunobiology*. [Epub ahead of print]

Catchen, J.M., Conery, J.S., Postlethwait, J.H. 2009 Automated identification of conserved synteny after whole-genome duplication. *Genome Res.* Aug;19(8):1497-505.

Caux, C., Moreau, I., Saeland, S., Banchereau, J. (1992) Interferon-gamma enhances factor-dependent myeloid proliferation of human CD34+ hematopoietic progenitor cells. *Blood.* May 15;79(10):2628-35.

Chang, T.L., Peng, X., Fu, X.Y. (2000). Interleukin-4 mediates cell growth inhibition through activation of Stat1. *J Biol Chem.* Apr 7;275(14):10212-7.

Chiba, Y., Todoroki, M., Misawa, M. (2009). Activation of signal transducer and activator of transcription factor 1 by interleukins-13 and -4 in cultured human bronchial smooth muscle cells. *J Smooth Muscle Res.* 45(6):279-88.

Chin, Y.E., Kitagawa, M., Su, W.C., You, Z.H., Iwamoto, Y., Fu, X.Y. (1996). Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. *Science*. 3;272(5262):719-722.

Crispino, J.D. (2005). GATA1 in normal and malignant hematopoiesis. *Semin Cell Dev Biol.* Feb;16(1):137-47.

Davidson, A.J., Ernst, P., Wang, Y., Dekens, M.P., Kingsley, P.D., Palis, J., Korsmeyer, S.J., Daley, G.Q., Zon, L.I. (2003). cdx4 mutants fail to specify blood progenitors and can be rescued by multiple hox genes. *Nature*. Sep 18; 425(6955): 300-306.

de Jong, J.L. and Zon, L.I. (2005). Use of the zebrafish system to study primitive and definitive hematopoiesis. *Annu Rev Genet.* 39:481-501.

de Totero, D., Meazza, R., Capaia, M., Fabbi, M., Azzarone, B., Balleari, E., Gobbi, M., Cutrona, G., Ferrarini M., Ferrini, S. (2008). The opposite effects of IL-15 and IL-21 on CLL B cells correlate with differential activation of the JAK/STAT and ERK1/2 pathways. *Blood.* Jan 15;111(2):517-24.

Dehal, P., Boore, J.L. (2005). Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol.* Oct;3(10):e314.

Demoulin, J.B., Van Roost, E., Stevens, M., Groner, B., Renauld, J.C. (1999). Distinct roles for STAT1, STAT3, and STAT5 in differentiation gene induction and apoptosis inhibition by interleukin-9. *J Biol Chem.* Sep 3;274(36):25855-61.

Demoulin, J.B., Van Snick, J., Renauld, J.C. (2001). Interleukin-9 (IL-9) induces cell growth arrest associated with sustained signal transducer and activator of transcription activation in lymphoma cells overexpressing the IL-9 receptor. *Cell Growth Differ.* Mar;12(3):169-74.

Detrich, H.W. 3rd., Kieran, M.W., Chan, F.Y., Barone, L.M., Yee, K., Rundstadler, J.A., Pratt, S., Ransom, D., Zon, L.I. (1995) Intraembryonic hematopoietic cell migration during vertebrate development. *Proc Natl Acad Sci U S A.* Nov 7;92(23):10713-7.

Durbin, J.E., Hackenmiller, R., Simon, M.C., Levy, D.E. (1996). Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell.* 9;84(3):443-50.

Ernst, M., Najdovska, M., Grail, D., Lundgren-May, T., Buchert, M., Tye, H., Matthews, V.B., Armes, J., Bhathal, P.S., Hughes, N.R., et al. (2008). STAT3 and STAT1 mediate IL-11-dependent and inflammation-associated gastric tumorigenesis in gp130 receptor mutant mice. *J Clin Invest.* May;118(5):1727-38.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L., Postlethwait, J. (1999). Preservation of duplicate genes by complementary, degenerative mutations. *Genetics.* Apr;151(4):1531-45.

Forrai, A., Robb, L. (2003). The hemangioblast--between blood and vessels. *Cell Cycle.* Mar-Apr;2(2):86-90.

Fujiwara, Y., Browne, C.P., Cunniff, K., Goff, S.C., Orkin, S.H. (1996). Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA1. *Proc Natl Acad Sci USA;*93:12355–8.

Galloway, J.L., Wingert, R.A., Thisse, C., Thisse, B., Zon, L.I. (2005). Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Dev Cell* 8(1):109-16.

Georgopoulos, K., Winandy, S., Avitahl, N. (1997). The role of the Ikaros gene in lymphocyte development and homeostasis. *Annu Rev Immunol.*;15:155-76.

Guindon, S. and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology.* 52(5):696-704.

Hampton, M.B., Kettle, A.J., Winterbourn, C.C. (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood.* Nov 1;92(9):3007-17.

Hofer, M.J., Li, W., Lim, S.L., Campbell, I.L. (2010). The type I interferon-alpha mediates a more severe neurological disease in the absence of the canonical signaling molecule interferon regulatory factor 9. *J Neurosci.* 30(3):1149-57.

Joenje, H., Patel, K.J. (2001). The emerging genetic and molecular basis of Fanconi anaemia. *Nat Rev Genet.* Jun; 2(6): 446-457.

Jovelin, R., He, X., Amores, A., Yan, Y.L., Shi, R., Qin, B., Roe, B., Cresko, W.A., Postlethwait, J.H. (2007) Duplication and divergence of *fgf8* functions in teleost development and evolution. *J Exp Zool B Mol Dev Evol.* Dec 15;308(6):730-43.

Kalliolias, G.D., Ivashkiv, L.B. (2008). IL-27 activates human monocytes via STAT1 and suppresses IL-10 production but the inflammatory functions of IL-27 are abrogated by TLRs and p38. *J Immunol.* May 1;180(9):6325-33.

Kastner, P., Chan, S. (2008). PU.1: a crucial and versatile player in hematopoiesis and leukemia. *Int J Biochem Cell Biol.*;40(1):22-7.

Klebanoff, S.J. (2005). Myeloperoxidase: friend and foe. *J Leukoc Biol.* May;77(5):598-625.

Legendre, F., Dudhia, J., Pujol, J.P., Bogdanowicz, P. (2003). JAK/STAT but not ERK1/ERK2 pathway mediates interleukin (IL)-6/soluble IL-6R down-regulation of Type II collagen, aggrecan core, and link protein transcription in articular chondrocytes. Association with a down-regulation of SOX9 expression. *J Biol Chem.* Jan 31;278(5):2903-12.

Lejeune, D., Dumoutier, L., Constantinescu, S., Kruijer, W., Schuringa, J.J., Renauld, J.C. (2002). Interleukin-22 (IL-22) activates the JAK/STAT, ERK, JNK, and p38 MAP kinase pathways in a rat hepatoma cell line. Pathways that are shared with and distinct from IL-10. *J Biol Chem.* Sep 13;277(37):33676-82.

Long, Q., Meng, A., Wang, H., Jessen, J.R., Farrell, M.J., Lin, S. (1997). GATA1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. *Development.* Oct; 124(20): 4105-4111.

Mahboubi, K., Biedermann, B.C., Carroll, J.M., Pober, J.S. (2000). IL-11 activates human endothelial cells to resist immune-mediated injury. *J Immunol.* Apr 1;164(7):3837-46.

Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Greenlund, A.C., Campbell, D., et al. (1996). Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell.* 84(3):431-42.

Najjar, I., Deglesne, P.A., Schischmanoff, P.O., Fabre, E.E., Boisson-Dupuis, S., Nimmerjahn, F., Bornkamm, G.W., Dusanter-Fourt, I., Fagard, R. (2010). STAT1-dependent IgG cell-surface expression in a human B cell line derived from a STAT1-deficient patient. *J Leukoc Biol.* [Epub ahead of print]

Najjar, I. and Fagard, R. (2010). STAT1 and pathogens, not a friendly relationship. *Biochimie*. [Epub ahead of print]

Oates, A.C., Wollberg, P., Pratt, S.J., Paw, B.H., Johnson, S.L., Ho, R.K., Postlethwait, J.H., Zon, L.I., Wilks, A.F. (1999). Zebrafish stat3 is expressed in restricted tissues during embryogenesis and stat1 rescues cytokine signaling in a STAT1-deficient human cell line. *Dev Dyn.* 215(4):352-370.

Oikawa, T., Yamada, T., Kihara-Negishi, F., Yamamoto, H., Kondoh, N., Hitomi, Y., Hashimoto, Y. (1999). The role of Ets family transcription factor PU.1 in hematopoietic cell differentiation, proliferation and apoptosis. *Cell Death Differ.* Jul;6(7):599-608.

Ortiz-Muñoz, G., Lopez-Parra, V., Lopez-Franco, O., Fernandez-Vizarra, P., Mallavia, B., Flores, C., Sanz, A., Blanco, J., Mezzano, S., Ortiz, A., et al. (2010). Suppressors of Cytokine Signaling Abrogate Diabetic Nephropathy. *J Am Soc Nephrol.* [Epub ahead of print]

Ouaked, N., Mantel, P.Y., Bassin, C., Burgler, S., Siegmund, K., Akdis, C.A., Schmidt-Weber, C.B. (2009). Regulation of the foxp3 gene by the Th1 cytokines: the role of IL-27-induced STAT1. *J Immunol.* Jan 15;182(2):1041-9.

Pang, Q., Fagerlie, S., Christianson, T.A., Keeble, W., Faulkner, G., Diaz, J., Rathbun, R.K., Bagby, G.C. (2000). The Fanconi anemia protein FANCC binds to and facilitates the activation of STAT1 by gamma interferon and hematopoietic growth factors. *Mol Cell Biol.* Jul;20(13):4724-35.

Pang, Q., Christianson, T.A., Keeble, W., Diaz, J., Faulkner, G.R., Reifsteck, C., Olson, S., Bagby, G.C. (2001). The Fanconi anemia complementation group C gene product: structural evidence of multifunctionality. *Blood.* Sep 1;98(5):1392-401.

Patterson, L.J., Gering, M., Patient, R. (2005). Scl is required for dorsal aorta as well as blood formation in zebrafish embryos. *Blood.* May 1; 105(9): 3502-3511.

Postlethwait, J.H., Woods, I.G., Ngo-Hazelett, P., Yan, Y.L., Kelly, P.D., Chu, F., Huang, H., Hill-Force, A., Talbot WS. (2000) Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res.* Dec;10(12):1890-902.

Postlethwait, J., Amores, A., Cresko, W., Singer, A., Yan, Y.L. (2004). Subfunction partitioning, the teleost radiation and the annotation of the human genome. *Trends Genet.* Oct;20(10):481-90.

Postlethwait, J.H. (2007) The zebrafish genome in context: ohnologs gone missing. *J Exp Zool B Mol Dev Evol.* Sep 15;308(5):563-77.

Raefsky EL, Platanias LC, Zoumbos NC, Young NS. Studies of interferon as a regulator of hematopoietic cell proliferation. J Immunol. 1985 Oct;135(4):2507-12.

Rahimi, A.A., Gee, K., Mishra, S., Lim, W., Kumar, A. (2005). STAT-1 mediates the stimulatory effect of IL-10 on CD14 expression in human monocytic cells. *J Immunol.* Jun 15;174(12):7823-32.

Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T.X., Look, A.T., Kanki, J.P. (2005). Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev Cell.* 8(1):97-108.

Rose, T., Pillet, A.H., Lavergne, V., Tamarit, B., Lenormand, P., Rousselle, J.C., Namane, A., Theze, J. (2010). Interleukin-7 compartmentalizes its receptor signaling complex to initiate CD4 T lymphocyte response. J Biol Chem. [Epub ahead of print]

Schindler, C., Shuai, K., Prezioso, V.R., Darnell, J.E. Jr. (1992). Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science*. 257(5071):809-13.

Schmitt, C., Tonnelle, C., Dalloul, A., Chabannon, C., Debré, P., Rebollo, A. (2002). Aiolos and Ikaros: regulators of lymphocyte development, homeostasis and lymphoproliferation. *Apoptosis*. Jun;7(3):277-84.

Schultz, J., Koczan, D., Schmitz, U., Ibrahim, S.M., Pilch, D., Landsberg, J., Kunz, M. (2010). Tumor-promoting role of signal transducer and activator of transcription (Stat)1 in late-stage melanoma growth. *Clin Exp Metastasis*. [Epub ahead of print]

Shimozato, O., Ortaldo, J.R., Komschlies, K.L., Young, H.A. (2002) Impaired NK cell development in an IFN-gamma transgenic mouse: aberrantly expressed IFN-gamma enhances hematopoietic stem cell apoptosis and affects NK cell differentiation. *J Immunol.* Feb 15;168(4):1746-52.

Shivdasani, R.A., Mayer, E.L., Orkin, S.H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature.* Feb 2;373(6513):432-4.

Singh, K., Sinha, S., Malonia, S.K., Chattopadhyay, S. (2010). Tumor Necrosis Factor alpha (TNFalpha) regulates CD40 expression through SMAR1 phosphorylation. *Biochem Biophys Res Commun.* 391(2):1255-61.

Tajima, K., Takaishi, H., Takito, J., Tohmonda, T., Yoda, M., Ota, N., Kosaki, N., Matsumoto, M., Ikegami, H., Nakamura, T., et al. (2010). Inhibition of STAT1 accelerates bone fracture healing. *J Orthop Res.* [Epub ahead of print]

Traver, D. (2004). Cellular dissection of zebrafish hematopoiesis. *Methods Cell Biol.*; 76: 127-149

Vignais, M.L., Sadowski, H.B., Watling, D., Rogers, N.C., Gilman, M. (1996). Platelet-derived growth factor induces phosphorylation of multiple JAK family kinases and STAT proteins. *Mol Cell Biol.* Apr;16(4):1759-69.

Wang, W. (2007). Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. *Nat Rev Genet.* Oct;8(10):735-48.

Xiong, J.W. (2008). Molecular and developmental biology of the hemangioblast. *Dev Dyn.* May;237(5):1218-31.

Yan, Y.L., Miller, C.T., Nissen, R.M., Singer, A., Liu, D., Kirn, A., Draper, B., Willoughby, J., Morcos, P.A., Amsterdam, A., et al. (2002). A zebrafish sox9 gene required for cartilage morphogenesis. *Development.* 129(21):5065-79.

Young, H.A., Klinman, D.M., Reynolds, D.A., Grzegorzewski, K.J., Nii, A., Ward, J.M., Winkler-Pickett, R.T., Ortaldo, J.R., Kenny, J.J., Komschlies, K.L. (1997) Bone marrow and thymus expression of interferon-gamma results in severe B-cell lineage reduction, T-cell lineage alterations, and hematopoietic progenitor deficiencies. *Blood.* Jan 15;89(2):583-95.

Zhao, X., Ren, G., Liang, L., Ai, P.Z., Zheng, B., Tischfield, J.A., Shi, Y., Shao, C. (2010). Brief report: interferon-gamma induces expansion of Lin(-)Sca-1(+)C-Kit(+) Cells. *Stem Cells.* 28(1):122-6.