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## Enhanced transcription of complement and coagulation genes in the absence of adaptive immunity

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### Abstract

A recessive nonsense mutation in the zebrafish *recombination activating gene 1* (*rag1*) gene results in defective V(D)J recombination; however, animals homozygous for this mutation (*rag1*<sup>-/-</sup>) are reportedly viable and fertile in standard, nonsterile aquarium conditions but display increased mortality after intraperitoneal injection with mycobacteria. Based on their survival in nonsterile environments, we hypothesized that the *rag1*<sup>-/-</sup> zebrafish may possess an “enhanced” innate immune response to compensate for the lack of an adaptive immune system. To test this hypothesis, microarray analyses were used to compare the expression profiles of the intestines and hematopoietic kidneys of *rag1* deficient zebrafish to the expression profiles of control (heterozygous) siblings. The expression levels of 12 genes were significantly altered in the *rag1*<sup>-/-</sup> kidney including the up regulation of a *putative interferon stimulated gene*, and the down regulation of genes encoding *fatty acid binding protein 10*, *keratin 5* and multiple heat shock proteins. The expression levels of 87 genes were shown to be significantly altered in the *rag1*<sup>-/-</sup> intestine; the majority of these differences reflect increased expression of innate immune genes, including those of the coagulation and complement

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pathways. Subsequent analyses of orthologous coagulation and complement genes in *Rag1*<sup>-/-</sup> mice indicate increased transcription of the *complement C4* gene in the *Rag1*<sup>-/-</sup> intestine.

## Keywords

zebrafish; mouse; rag1; innate immunity; SCID

## 1. Introduction

Severe combined immunodeficiency (SCID) defines a group of genetic conditions in which individuals lack a functional adaptive immune system. In humans SCID can prove lethal when patients are exposed to a number of common pathogens as well as to a wide range of opportunistic microorganisms. SCID can be caused by mutations in multiple genes, including the *recombination activating gene 1 (RAG1)* which mediates V(D)J recombination. *RAG1*-deficient (*RAG1*<sup>-/-</sup>) humans represent ~20% of SCID patients, and present with a characteristic absence or reduction of lymphatic organs and tonsils. *RAG1*<sup>-/-</sup> SCID is associated with a loss of B and T cells but is associated with a normal level of natural killer (NK) cells resulting in a T<sup>-</sup>B<sup>-</sup>NK<sup>+</sup> phenotype. The phenotype of the *Rag1*<sup>-/-</sup> mouse model is characterized by an increased susceptibility to infection, and requires specific pathogen-free (SPF) conditions.

The zebrafish (*Danio rerio*), which has been a powerful model species for embryological studies, is emerging as a model for immunological studies (Trede et al., 2001; Yoder et al., 2002; Traver et al., 2003; Trede et al., 2004; Van Der Sar et al., 2004; Phelps and Neely, 2005; Meeker and Trede, 2008; Sullivan and Kim, 2008). Zebrafish encode genes representative of both the innate and adaptive immune response. The adaptive immune responses of fish and mammals share many similarities, and thus, zebrafish, with its unique developmental and *in vivo* imaging advantages, is becoming increasingly appreciated as a model for vertebrate immune function. Although efficient technologies are lacking for targeted disruption of genes by homologous recombination in zebrafish, target induced local lesions in genomes (TILLING) methodology is being employed routinely for generating “knock-out” zebrafish (Deiters and Yoder, 2006). A disruption of the *rag1* gene by an ENU-induced point mutation that creates a premature stop codon in the *rag1*<sup>26683</sup> allele (herein referred to the *rag1*<sup>-</sup> allele) has been identified using TILLING; this allele encodes a truncated Rag1 protein (Wienholds et al., 2002). Although zebrafish homozygous for this mutation (*rag1*<sup>-/-</sup>) are more susceptible to an injected dose of *Mycobacterium marinum* and their immunoglobulin genes fail to undergo V(D)J recombination, these immune-compromised animals appear to survive in nonsterile aquarium facilities, albeit with reduced fertility (Wienholds et al., 2002; Swaim et al., 2006). Based on these observations, we hypothesized that the *rag1*<sup>-/-</sup> zebrafish may possess an “enhanced” innate immune response to compensate for the lack of an adaptive immune system. This hypothesis is supported by earlier observations that *Rag1*<sup>-/-</sup> and other immune compromised mice can possess elevated complement and NK cell activity (Shultz et al., 1995; Shultz et al., 2000). In order to better understand the impact of this *rag1* mutation on the transcriptome of zebrafish, we compared the transcriptional state of the kidneys and intestines from these animals to normal (heterozygous) siblings and extended studies to the *Rag1*<sup>-/-</sup> mouse model. Our results establish that the expression of 12 genes are altered in the *rag1*<sup>-/-</sup> zebrafish kidney and 87 genes are altered in the *rag1*<sup>-/-</sup> zebrafish intestine. Within the *rag1*<sup>-/-</sup> zebrafish intestine numerous innate immune genes, including multiple complement and coagulation factors, are expressed at elevated levels. Furthermore, transcriptional analyses of murine complement and coagulation genes reveal that the *complement C4* gene is expressed at elevated levels in the intestine of *Rag1*<sup>-/-</sup> mice.

## 2. Methods

### 2.1. Animals

Adult zebrafish were maintained at 28 °C in a recirculating aquarium facility (Aquatic Habitats, Apopka, FL) and fed twice daily. For removal of organs, zebrafish were euthanized in a buffered solution of 0.02% Tricaine methanesulfonate (Finquel MS-222; Argent Chemical Laboratories, Redmond WA). The abdominal wall was opened and the intestine was removed. After all intra-abdominal organs were removed the kidney was visualized ventral to the spinal canal and removed.

Female 7-8 month old *Rag1*<sup>-/-</sup> (RAG1-B6.12957) and congenic (C57BL/6J) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and euthanized by CO<sub>2</sub> asphyxiation. Tissues were collected and snap frozen in liquid nitrogen.

### 2.2. Zebrafish genotyping

Zebrafish homozygous for a mutant allele (*rag1*<sup>T26683</sup>) of the *rag1* gene (Wienholds et al., 2002) and heterozygous siblings were collected from matings of heterozygous animals. Genotyping of adult animals was performed on sperm for males and fertilized eggs for females to avoid tail clip-induced injury that could lead to lethal infection in *rag1* deficient zebrafish (Swaim et al., 2006). DNA was obtained using the HotSHOT method as described previously (Meeker et al., 2007) and genotyping was performed with a derived cleaved-amplified polymorphic sequence (dCAPS) methodology (Neff et al., 2002). In brief, a partial sequence of the *rag1* gene was amplified by PCR using the forward primer *Rag1-dCAPs-F-Kpn* (GAGTCAGCAGACGAACTGCGGTAC) and the reverse primer *Rag1-dCAPs-R2* (ATTCAGTCGCATTGCCAATATCACAG). Two non-complementary bases at the 3' end of the *Rag1-dCAPs-F-Kpn* primer (underlined) create a *KpnI* site in the amplicons from the wild-type allele whereas amplicons from the mutant allele lack this *KpnI* site. Amplicons were generated using Titanium™ Taq polymerase (BD Bioscience), 1 ng/μl genomic DNA and 35 PCR cycles annealing at 60 °C degrees. The resultant PCR products were digested to completion with *KpnI* and resolved on a 3% NuSieve (3:1) agarose gel. The interpretation of the genotype was straightforward for males. Females were crossed to wild-type males and eight resulting embryos were genotyped as described above on day 1. Interpretation of the maternal genotype was: 100% of embryos heterozygous (*rag1*<sup>+/-</sup>) = homozygous for the mutant allele; 100% of embryos homozygous wild-type = homozygous wild-type; >0% but <100% of embryos heterozygous = heterozygous. The rate of mistaking a heterozygous female for a homozygous mutant under these conditions is ≤0.4% using the binomial distribution (where p=0.5, k=0, n=8). In addition, genotypes of males and females were verified post mortem.

### 2.3. RNA

Tissues from genotyped adult zebrafish were collected directly into Trizol (Invitrogen, Carlsbad, CA), homogenized using a Tissuemiser Homogenizer (Fisher Scientific, Pittsburgh, PA), stored at -80 °C, thawed within 2 months and total RNA was purified as described by the manufacturer. Frozen mouse tissues were thawed, homogenized in Trizol and total RNA was purified. The quality and quantity of the resulting RNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA.)

### 2.4. Microarray Analyses

One μg of total zebrafish RNA was converted into labeled cRNA with nucleotides coupled to the fluorescent dye (Cy3 or Cy5) using the Low RNA Input Linear Amplification Kit (Agilent Technologies) following the manufacturer's protocol. The quality and quantity of the resulting

labeled cRNA was assessed using a NanoDrop ND-1000 spectrophotometer and an Agilent 2100 Bioanalyzer. Equal amounts of Cy3 and Cy5-labeled cRNA (750 ng) from two different samples were hybridized to zebrafish microarrays (Agilent Zebrafish Oligo Microarrays, G2518A option 001) for 17 hours at 60 °C. Two biological replicates were completed for the following 4 dual hybridizations: 1) Cy3-*ragI*<sup>+/-</sup> kidney cRNA and Cy5-*ragI*<sup>-/-</sup> kidney cRNA, 2) Cy5-*ragI*<sup>+/-</sup> kidney cRNA and Cy3-*ragI*<sup>-/-</sup> kidney cRNA, 3) Cy3-*ragI*<sup>+/-</sup> intestine cRNA and Cy5-*ragI*<sup>-/-</sup> intestine cRNA, and 4) Cy5-*ragI*<sup>+/-</sup> intestine cRNA and Cy3-*ragI*<sup>-/-</sup> intestine cRNA. The hybridized arrays were then washed and scanned using an Agilent G2565BA scanner.

Data were extracted from the scanned image using Feature Extraction version 8.5 (Agilent Technologies) and imported to GeneSpring GX software version 7.3.1 (Silicon Genetics, Redwood City, CA) using enhanced Agilent FE plug-in for further analysis. The default normalization in GeneSpring was changed to the FE Normalization scenario in order to normalize imported data. To account for dye-swap-specific variation between arrays, a data transformation was executed and the signal channel and control channel measurements for dye-swapped replicates were reversed. An additional data transformation was applied to reset the row expression intensity values which were less than 0.01 to 0.01. Per Spot and Per Chip Intensity-dependent normalization was applied to rescale all genes to the same normalized value range. Finally, per gene normalization to median was completed by dividing the signal of each gene by the median of its measurements in all samples. Cross gene-error model was active based on the replicate. In order to differentiate groups of replicates, parameters were defined (kidney = K and intestine = I) and the same replicate value (K<sup>-/-</sup> vs. K<sup>+/-</sup> and I<sup>-/-</sup> vs. I<sup>+/-</sup>) was assigned to replicate samples in the same parameter. Comparisons of gene expression data were made between K<sup>-/-</sup> and K<sup>+/-</sup> as well as I<sup>-/-</sup> and I<sup>+/-</sup> separately.

“Filter on flag” was used to create quality control (QC) gene lists for each category based on flag value (present or marginal) assigned by feature extraction (FE) software during data import in at least 2 out of 4 samples using 21549 all gene list. In addition, “filter on expression” was used to create lists of genes, which display a  $\geq 1.5$ -fold difference in the *ragI*<sup>-/-</sup> samples as compared to the *ragI*<sup>+/-</sup> samples. For identification of differentially expressed genes, filter on confidence was used with *t*-test *P* value cutoff of 0.05 and fold change for the gene list was generated using filter on Volcano plot.

## 2.5. Gene List Annotation and Gene Ontology analyses

The nucleotide sequence of the Target Identity (ID) for each Agilent probe (GenBank, TIGR or ENSEMBL ID) was used to assign a transcript/gene Sequence Description and Gene Symbol for each probe. When certain TIGR or ENSEMBL IDs failed to identify a specific transcript/gene nucleotide sequence conclusively, the nucleotide sequence of the Agilent probe was used in a BLASTn search to define the Sequence Description and Gene Symbol. Probes failing to identify any transcript/gene sequence via BLAST analyses were removed from the Gene List.

DAVID software (Huang et al., 2007) was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. Zebrafish Information Network (ZFIN) IDs for each zebrafish sequence were uploaded to DAVID, converted to DAVID IDs and analyzed. As a comparative approach, the ZFIN IDs were converted to human Ensemble Gene IDs using BIOMART software (Durinck et al., 2005) and uploaded to DAVID for analyses.

## 2.6. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA (2  $\mu$ g) was annealed to oligo dT primers, reverse transcribed with Superscript III (Invitrogen), diluted 5-fold with dH<sub>2</sub>O and utilized for quantitative PCR. Primers for qRT-

PCR of zebrafish transcripts which were designed to span 70 to 100 bp and at least one intron are listed in Table 1. Optimal annealing temperatures were determined by assessing the quantity and purity of zebrafish amplicons when annealed at 55, 60, 65 and 70 °C and visualized by agarose gel electrophoresis. Primers for qRT-PCR of mouse transcripts were selected from the Primer Bank database (<http://pga.mgh.harvard.edu/primerbank>; Wang and Seed, 2003) and are listed in Table 1. All PCRs were performed using 1 µl of diluted cDNA, in a 25 µl reaction volume (iQ SYBR Green Supermix, BIO-RAD). All reactions were performed and monitored with a BioRad iCycler and iCycler iQ Optical System Software, version 3.0a. Thermal cycling parameters included an initial denaturing at 94 °C for 2 minutes followed by 45 cycles of: denaturing at 94 °C for 30 seconds, annealing at 60 °C (zebrafish primers) or 56 °C (mouse primers) for 30 seconds and extension at 72 °C for 30 seconds. Reactions from individual biological replicates were completed in triplicate and the average relative levels of expression were calculated by normalizing to levels of  $\beta$  *actin* as described (Livak and Schmittgen, 2001). When multiple biological replicates were analyzed, the average relative level of expression from each replicate was considered as a single data point and the mean (and standard error) calculated. A negative control (no cDNA) for each primer set was utilized to exclude sample contamination.

## 2.7. Histopathology

Selected *rag1*<sup>+/-</sup> and *rag1*<sup>-/-</sup> zebrafish were euthanized with Tricaine methanesulfonate (see above) and a small incision was made on the ventral body wall from the anus to the anterior extent of the main body cavity. Fish were fixed in 10% neutral buffered formalin and processed routinely. Specimens were embedded in paraffin in left lateral recumbency, and multiple step sections were cut at 5 microns. Sections were stained with hematoxylin and eosin (H&E), and analyzed via light microscopy by a single pathologist.

## 3. Results

### 3.1. Microarray analyses of *rag1*<sup>-/-</sup> zebrafish

In order to assess the impact of the *rag1* mutation on gene expression, we performed transcriptional profiling in the hematopoietic kidney (functional equivalent to bone marrow) and the intestine (major site for microbial challenge) of *rag1*<sup>-/-</sup> zebrafish and control heterozygous siblings. Microarray analyses identified 87 genes exhibiting altered transcription at 95% confidence in the *rag1*<sup>-/-</sup> intestine; twelve genes with altered transcription (95% confidence) were noted in the *rag1*<sup>-/-</sup> kidney (Table 2). Each of the genes was assigned a unique Sequence ID (“Seq. ID” in Table 2), which includes a letter designating source (e.g., i for intestine; k for kidney). Sequence IDs are indicated by brackets throughout the text (e.g. [i1]). The observed differences in transcription levels range between a ~4.2-fold increase in the intestine and a ~3.3-fold decrease in the kidney. Increased expression was confirmed by qRT-PCR for 7 of 8 selected transcripts (Figure 1).

### 3.2. Transcriptional differences between kidneys of *rag1*<sup>-/-</sup> and *rag1*<sup>+/-</sup> zebrafish

In the *rag1*<sup>-/-</sup> kidney, increased transcription was detected for 4 genes [k88-k91], only one of which, (*putative interferon stimulated gene 12*; [k90]) has a predictable function. In contrast, decreased levels of transcription are noted for 8 genes [k92-k99] including *keratin 5* (*krt5*), *fatty acid binding protein 10* (*fabp10*), *insulin-like growth factor binding protein 1* (*igfbp1*), *heat shock protein 90a2* (*hsp90a2*), *hsp70* and *hsp90a* and a gene similar to *complement C4-2*.



### 3.3. Transcriptional differences between intestines of *rag1<sup>-/-</sup>* and *rag1<sup>+/-</sup>* zebrafish

In the *rag1<sup>-/-</sup>* intestine, decreased transcription was detected for 3 genes [i85-i87], including *solute carrier family 44, member 4 (slc44a4)* and a gene similar to *core 1 synthase, glycoprotein-N-acetylgalactosamine 3- $\beta$ -galactosyltransferase, 1 (C1GALT1)*. Of the 84 genes, which are up regulated in *rag1<sup>-/-</sup>* zebrafish intestines [i1-i84], many are associated with an immune response including multiple complement factors (discussed below), antimicrobial peptides [i2], apolipoproteins [i16, i52, i66] and chitinases which are likely targeted against chitin from parasites ([i5] and [i36] are similar to chitinases from other species: Elias et al., 2005).

### 3.4. Six genes are transcriptionally altered in both *rag1<sup>-/-</sup>* kidney and intestine

Interestingly, 6 of the 8 genes that are down regulated in the *rag1<sup>-/-</sup>* zebrafish kidney were found to be up regulated in the *rag1<sup>-/-</sup>* zebrafish intestine (Figure 2). These genes are *krt5*, *fabp10*, *hsp90a2*, *hsp70*, a gene similar to *complement C4-2* and *igfbp1*. None of the genes that are up regulated in the *rag1<sup>-/-</sup>* zebrafish kidney were found to be transcriptionally altered in the *rag1<sup>-/-</sup>* zebrafish intestine. The significance of the opposing transcriptional response for these 6 genes in the kidney and intestine is unknown.

### 3.5. Gene ontology analyses

Gene ontology analyses was performed using the ZFIN ID assigned to each gene in Table 2 and DAVID software (Huang et al., 2007). Of the genes with ZFIN IDs, 77 had been assigned GO Terms and were identified by DAVID software. By restricting the results to GO Terms with six or more associated genes (from Table 2) and for which  $P < 0.01$ , 11 different GO Terms were identified (6 Biological Processes and 5 Molecular Functions), including *Response to Wounding*, *Response to External Stimulus*, and *Protease Inhibitor Activity* (Table 3).

### 3.6. Enhanced transcription of mediators of the complement and coagulation pathways in *rag1<sup>-/-</sup>* zebrafish intestine

In order to further evaluate the functional relationships of the genes with altered expression in *rag1<sup>-/-</sup>* zebrafish, their ZFIN IDs were converted to Ensembl IDs for the corresponding human gene and uploaded into DAVID software. This conversion resulted in DAVID recognizing 50 human orthologs of the 93 different zebrafish sequences shown in Table 2. Using these human sequences, DAVID software identified a single KEGG pathway ( $P < 0.01$ ) which identified 16% of the genes to be involved in the complement and coagulation pathway ( $P = 2.2E-8$ ). These genes are *carboxypeptidase b2*, *sepinc1*, *fibrinogen  $\alpha$* ,  $\beta$  and  $\gamma$  as well as complement components *C3*, *C6*, *C9*. The significance of this association is strengthened by two additional observations: (1) four additional zebrafish genes from our gene list (Table 2) are associated with this pathway (*f10*, *serpina1*, *masp2*, and *c8g*) and (2) six additional genes on our gene list are similar to genes involved in this pathway and likely play a role in the complement pathway in zebrafish (Figure 3: [i6] and [i15] are similar to complement factor B; [i24] is similar to complement factor I; [i19] is similar to C4; and [i58] and [i83] are similar to C1Q). It is noted that although [i58] and [i83] are similar to C1Q, it is possible that they may not function in immunity as seen for other C1Q-like genes (Mei et al., 2008b). In summary, 18 of the 84 (~20%) genes with increased expression in the *rag1<sup>-/-</sup>* zebrafish intestine are involved or likely involved in the coagulation and complement pathways.

### 3.7. Histopathology: Tissues from *rag1<sup>-/-</sup>* zebrafish had no remarkable abnormalities

Standard histopathology was utilized to compare the morphology and organization of the internal organs of homozygous *rag1<sup>-/-</sup>* (Wienholds et al., 2002) and heterozygous *rag1<sup>+/-</sup>* mutants. Microscopic examination of the kidneys from *rag1<sup>-/-</sup>* fish revealed normal tubules and hematopoietic cellular components but otherwise was unremarkable. Intestines in both

*rag1<sup>-/-</sup>* and *rag1<sup>+/-</sup>* animals also had no remarkable lesions, with a normal population of lymphocytes within the lamina propria. Livers in animals from both groups had normal to moderately vacuolated hepatocytes; the biliary tree was considered normal (Figure 4).

### 3.8. Complement C4 is elevated in *Rag1<sup>-/-</sup>* mouse intestine

In order to determine if the elevated levels of transcription of complement and coagulation pathway genes observed in the zebrafish extend to other vertebrate species, qRT-PCR for the corresponding genes was carried out with *Rag1<sup>-/-</sup>* and wild type (congenic) mice. No significant differences in transcription of these genes were observed in liver, the primary site of complement production in mammals (Figure 5a); however, a significant increase in transcription of *complement C4* was observed in the intestine of *Rag1<sup>-/-</sup>* mice (Figure 5b).

## 4. Discussion

Severe combined immunodeficiency (SCID) defines a group of genetic conditions, including mutations in purine metabolism, cytokine receptor subunits, T cell receptor associated protein kinases, CD45 and RAG proteins (reviewed by Buckley, 2004). *RAG1* and *RAG2* mediate V (D)J recombination (Schwarz et al., 1991; Buckley, 2004) and *RAG1*-deficient (*RAG1<sup>-/-</sup>*) humans and mice present with a loss of B and T lymphocytes, yet maintain the presence of natural killer (NK) cells resulting in a T<sup>-</sup>B<sup>+</sup>NK<sup>+</sup> phenotype (Mombaerts et al., 1992; Grundy and Sentman, 2006; Sobacchi et al., 2006). In humans SCID is lethal when patients are exposed to standard environmental pathogens. Isolation in a sterile environment represents one means to manage such individuals. Maternal bone marrow transplants, when feasible, have become a standard curative approach for SCID patients (Buckley, 2004). Similarly, the *Rag1<sup>-/-</sup>* mouse model has an increased susceptibility to infections and these animals are housed in SPF facilities. Although *rag1<sup>-/-</sup>* zebrafish originally were reported to be viable in standard, nonsterile aquaria (Wienholds et al., 2002), it has been shown that they are more susceptible to mycobacterial infection when infected by intraperitoneal injection (Swaim et al., 2006). It has not been reported if *rag1<sup>-/-</sup>* zebrafish are more susceptible to infection by immersion (rather than by injection), leaving open the possibility that lymphocyte-independent zebrafish possess a formidable barrier to environmental-based infection.

Based on observations that *Rag1<sup>-/-</sup>* mice can have elevated levels of NK cell activity depending on the background strain (Shultz et al., 2000), we hypothesized that *rag1<sup>-/-</sup>* zebrafish may possess an “enhanced” innate immune response to compensate for the lack of an adaptive immune system. This hypothesis was also supported by functional assays using the “*scid*” mouse which harbors a genetic disruption of *Prkdc*, a second murine model for SCID (Bosma et al., 1983; Shultz et al., 2000). Sera from *scid* mice can possess elevated levels of complement activity depending on the background strain (Shultz et al., 1995).

The microarray data presented here demonstrates that the transcriptional status of multiple innate immune genes is elevated in the intestine, but not the kidney of the *rag1<sup>-/-</sup>* zebrafish and includes multiple genes involved in the complement and coagulation pathway. Genes representative of both the lectin and alternative pathways of the complement cascade were shown to have elevated transcription in the *rag1<sup>-/-</sup>* intestine. For example, *masp2* and *c3*, which are transcriptionally elevated in the *rag1<sup>-/-</sup>* intestine, are essential for activating the lectin and alternative pathways, respectively. Transcriptional changes of the classical pathway in the *rag1<sup>-/-</sup>* intestine are not as compelling as bony fish encode up to 52 genes with C1q domains (Mei and Gui, 2008) and the zebrafish C1q-like genes with elevated transcription in the *rag1<sup>-/-</sup>* intestine may not be directly involved in the complement cascade (Mei et al., 2008a). Nevertheless, the increased expression of *c4* within *rag1<sup>-/-</sup>* intestine (of both zebrafish and mice) may be informative as C4 plays an early role in both the classical and lectin pathways. In addition, not all zebrafish genes involved in these pathways were represented on the Agilent

arrays leaving the possibility that additional complement and coagulation genes may be up regulated in the *rag1*<sup>-/-</sup> intestine. For example, there are 4 zebrafish genes, *hbl1*, *hbl2*, *hbl3* and *hbl4*, that are closely related to the gene encoding the mammalian mannose binding lectin (MBL in Figure 3; Jackson et al., 2007). In mammals, MBL binds carbohydrates on the surface of a wide range of pathogens and complexes with MASPs which activate the lectin pathway of the complement cascade. Although *hbl3*, which is the most similar to human MBL, was represented on the Agilent arrays and showed no significant transcriptional alteration in the *rag1*<sup>-/-</sup> intestine, it is possible that the expression of other *hbl* genes may be elevated in these animals.

The presented qRT-PCR data demonstrate an increased transcription of *complement C4* in the intestine, but not in the liver of *Rag1*<sup>-/-</sup> mice. It is interesting to note that the background strain for our murine *Rag1*<sup>-/-</sup> studies was C57BL/6J, which also was the background strain for the *scid* mice exhibiting a heightened complement activity (Shultz et al., 1995) and the background strain for gnotobiotic *Rag1*<sup>-/-</sup> studies demonstrating an increased transcription of multiple innate immune response genes (including complement genes) within the intestine *after* the introduction of bacterial species into germ free mice (Peterson et al., 2007). As the background strain of mice can influence the innate immune response in SCID models, it is possible that crossing the *rag1*<sup>-</sup> allele onto different zebrafish lines may produce animals with varying levels of innate immune function.

Multiple segments of both zebrafish T cell receptor (TCR) and immunoglobulin (Ig) genes were incorporated in the Agilent gene chips used in these studies, but no significant differences in transcriptional levels were observed between *rag1*<sup>-/-</sup> and *rag1*<sup>+/-</sup> tissues. It is possible that although V(D)J recombination is deficient in the *rag1*<sup>-/-</sup> zebrafish, transcription of TCR and Ig genes occurs normally, which could result in a preponderance of sterile transcripts. Alternatively, it may be that expression levels of these genes within the tissues studied are too low to be effectively monitored by microarray analyses. In our experiments, the majority of ~50 TCR and Ig gene sequences on these arrays were flagged as “absent” and the probes consistently producing a signal showed no significant difference between *rag1*<sup>-/-</sup> and *rag1*<sup>+/-</sup> tissues.

Three general models can be envisioned to explain the increased transcription of innate immune genes in *rag1* deficient animals and why differences are observed between zebrafish and mice. The first model invokes the genetic differences between zebrafish and mice. The second and third models rely on the microbiota of the intestine to shape the genetic response of the host.

The first model is based on whole genome sequencing efforts which indicate that after the divergence of bony fish and mammals, there was a fish-specific whole genome duplication (Hufton et al., 2008). This hypothesis is supported by the presence of 17 Toll-like receptors in zebrafish (Meijer et al., 2004). In addition, there may have been selective pressure to maintain a robust innate immune response since in certain fish, (demonstrable) antibody production can take at least 4-6 weeks, which is much longer than the time leading to host mortality for many pathogens (at LD<sub>50</sub>) and places an increased burden on the innate immune response (Ellis, 2001). It follows that fish potentially possess a more complex, and perhaps, more effective innate immune system than mammals. In this model, the robust zebrafish innate immune system is able to detect the absence of adaptive immunity via unknown mechanisms, and respond by amplifying distinct components of the innate response. The observed complexity of the intestinal versus the kidney response might be due to the substantial microbial burden within the intestinal lumen. In contrast, the less robust innate response in mice is only slightly altered in the absence of Rag1 function.



The second model is founded on the observation that the intestines of zebrafish and mouse are exposed to very different populations of commensal microorganisms (Rawls et al., 2006): the zebrafish intestinal microbiota is numerically dominated by members of the Proteobacteria and Fusobacteria phyla, while the mouse intestinal microbiota is dominated by the Firmicutes and Bacteroidetes phyla. In this model it is proposed that in the absence of Rag1 function, the zebrafish intestinal microbial community is inherently more immunogenic than the mouse intestinal microbial community, thus inducing the transcriptional response observed in the *rag1*<sup>-/-</sup> zebrafish intestine.

In the third model, it is proposed that the absence of Rag1 function results in the establishment of an intestinal microbial community with altered composition and/or activity compared to controls. This altered population of commensal microorganisms could then elicit a different set of innate immune responses than is typically evoked by the wild-type microbiota. This is supported by the observation that the expression of multiple innate immune response genes, including at least one complement gene, is altered in the intestines of gnotobiotic zebrafish when raised in the presence of different bacterial species (Rawls et al., 2004). Moreover, loss of the transcription factor T-bet in mice results in population shifts in the intestinal microbial community which are associated with spontaneous and communicable colitis (Garrett et al., 2007), suggesting that host genotype can have profound influence on the composition and activity of the intestinal microbial community. With this model, the different transcriptional responses between the intestines of *rag1*<sup>-/-</sup> zebrafish and *Rag1*<sup>-/-</sup> mice are explained by relatively larger changes in microbial community composition as a function of *rag1* genotype in fish as compared to mammals – therefore fish display a larger transcriptional response in proportion to changes in the intestinal microbiota.

It is possible that all three models may contribute to the observed transcriptional differences between the intestines of *rag1*<sup>-/-</sup> zebrafish and *Rag1*<sup>-/-</sup> mice. It will be of interest to evaluate the expression of the complement and coagulation genes in *rag1*<sup>-/-</sup> zebrafish after exposure to antibiotics or in gnotobiotic *rag1*<sup>-/-</sup> animals after controlled exposures to select microorganisms.

The observation that animal models of SCID can possess an “enhanced” innate immune response has now been documented in both mouse and zebrafish; however, the cellular and molecular mechanisms which lead to an alteration in innate immunity are unresolved. Along these lines, it is not known why the kidney of *rag1*<sup>-/-</sup> zebrafish, which is the functional counterpart to bone marrow, shows no detectable (by microscopy) lack of lymphocytes when *Rag1*<sup>-/-</sup> mice present with a severe reduction in bone marrow lymphocytes (Mombaerts et al., 1992). Although B and T cell function and the process of V(D)J recombination are strikingly similar between fish and mammals, major differences in lymphoid compartmentalization and likely specialization are noted throughout the vertebrate radiations. Furthermore, the lack of reagents with specificity for defined lymphoid markers within zebrafish confounds efforts to draw analogies between the distribution and developmental staging of lymphocytes in zebrafish versus mouse. These deficiencies preclude a more thorough assessment of the *rag1*<sup>-/-</sup> lymphocyte phenotype in zebrafish at this time.

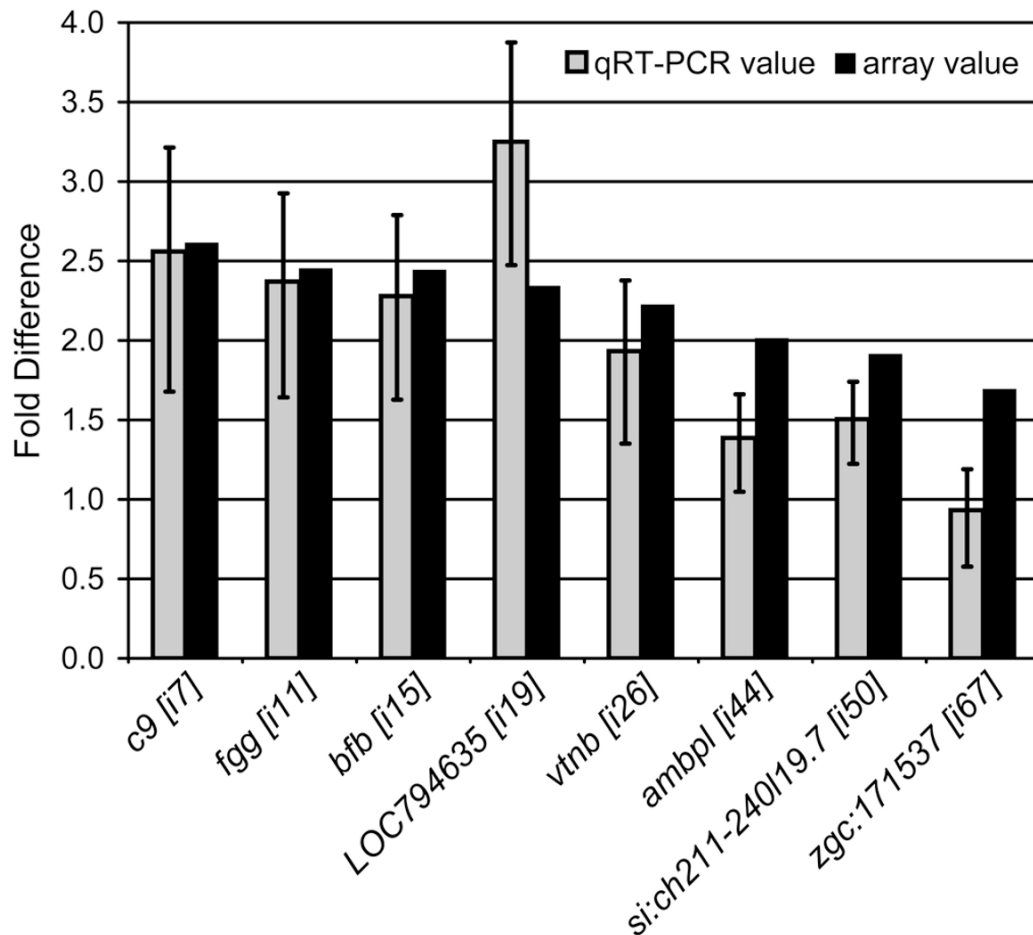
## Acknowledgments

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## Reference List

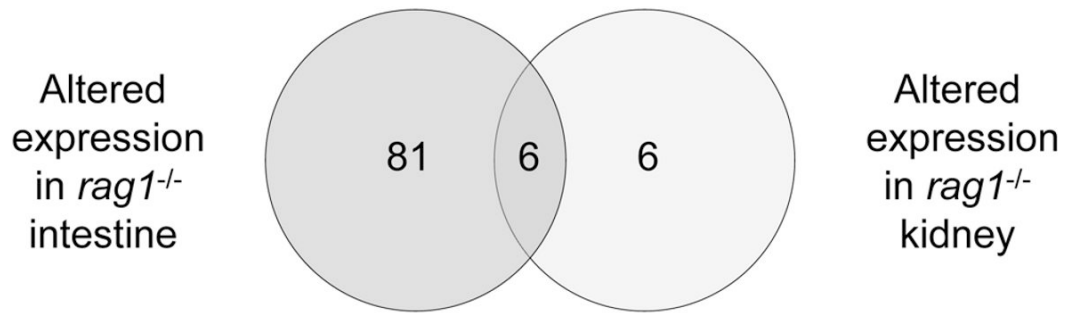
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**Figure 1. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) of select zebrafish genes**

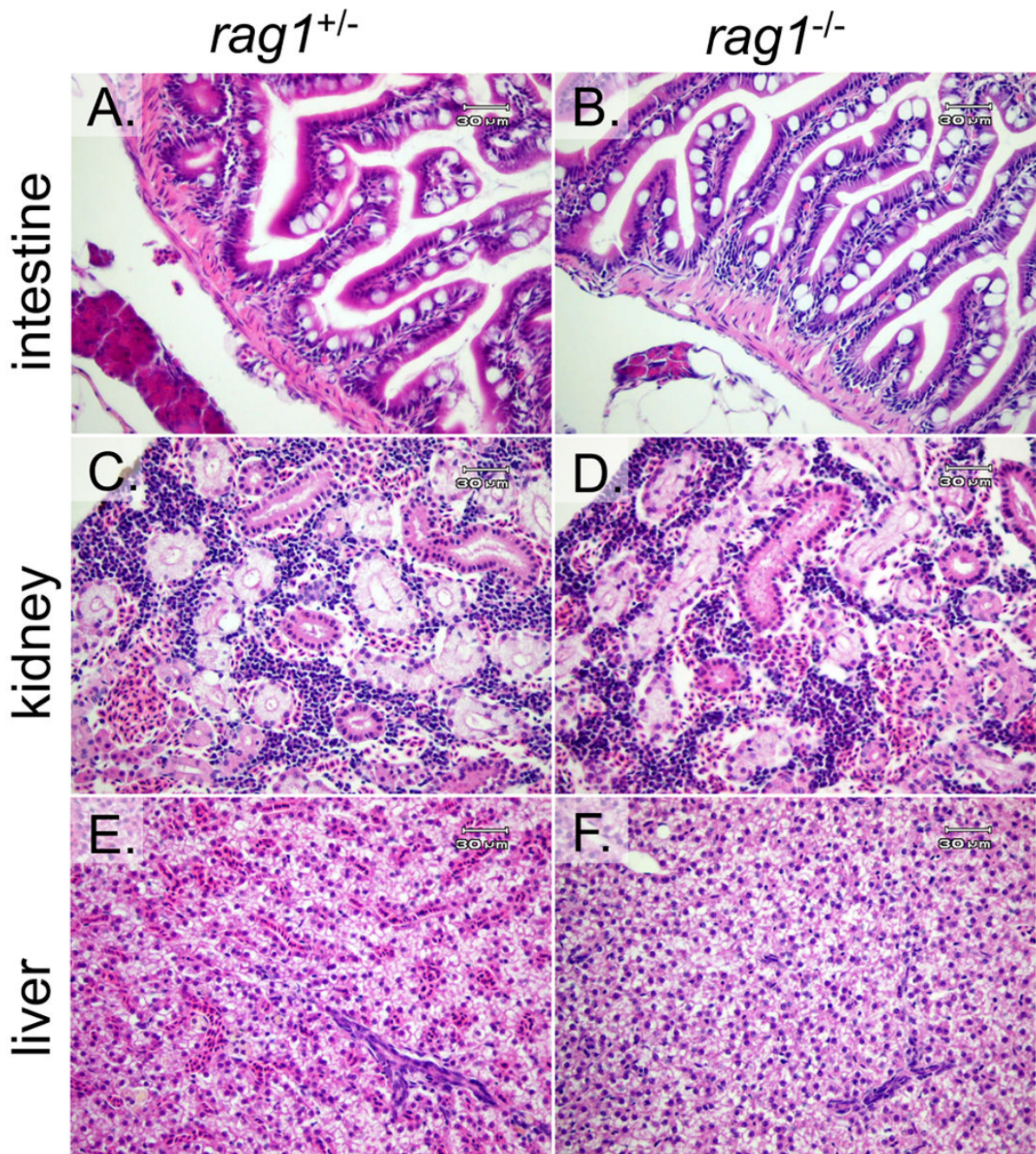
Eight genes identified as having an increased expression in the *rag1*<sup>-/-</sup> zebrafish intestine (X axis) were analyzed by qRT-PCR. C<sub>T</sub> values were normalized to  $\beta$  *actin2* and the relative fold-difference in expression was calculated ( $2^{-\Delta\Delta CT}$ ) as described (Livak et al., 2001). Values (gray bars) represent normalized expression levels in *rag1*<sup>-/-</sup> zebrafish relative to expression in *rag1*<sup>+/+</sup> zebrafish; error bars indicate standard error of the mean. Fold difference values from microarray analyses are shown (black bars) for comparison (numbers in brackets cross-reference to Table 2).



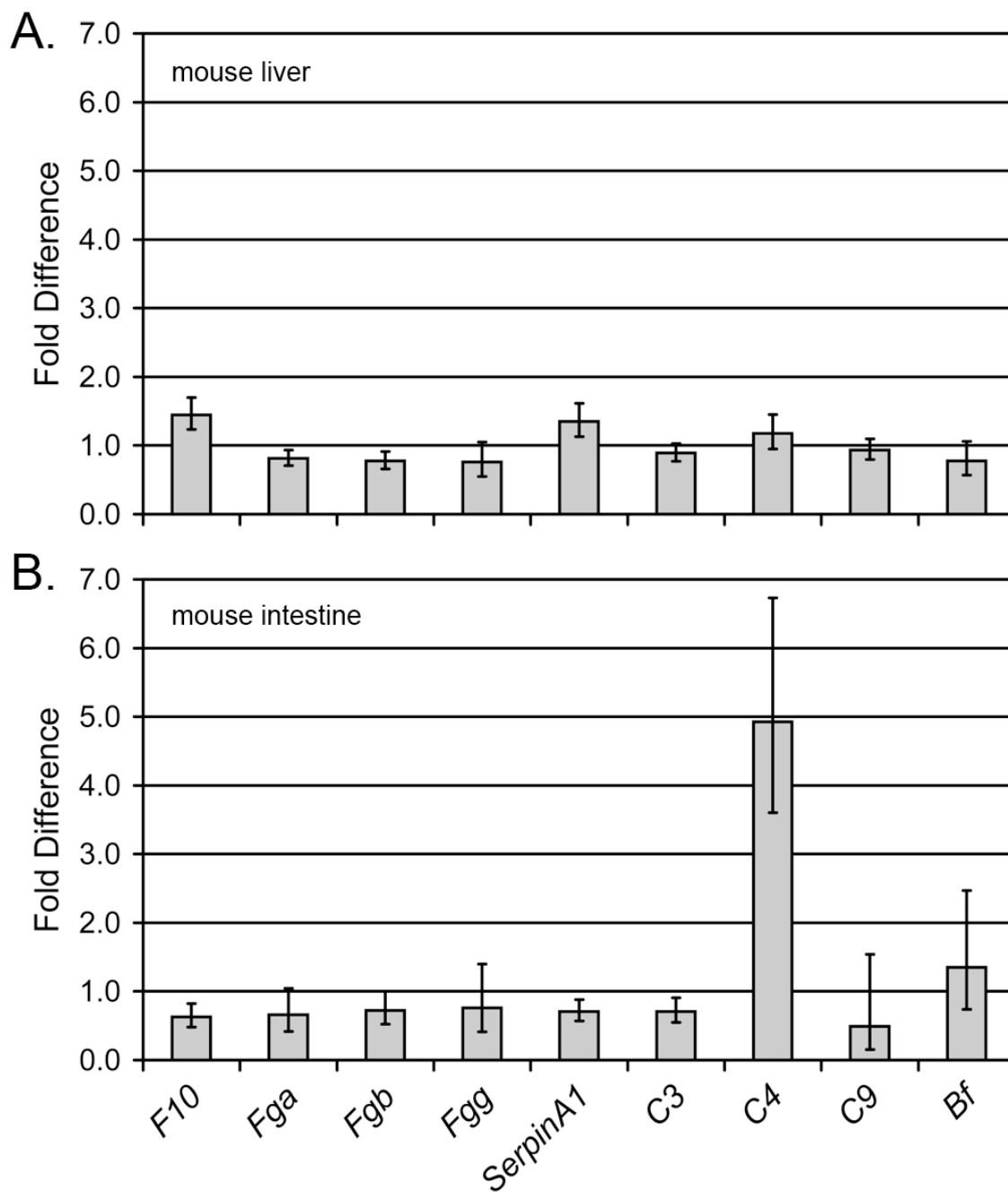
**Figure 2. Comparison of altered gene expression in intestine and kidney of *rag1*<sup>-/-</sup> zebrafish**  
Venn diagram showing the number of genes altered in *rag1*<sup>-/-</sup> zebrafish intestine and *rag1*<sup>-/-</sup> zebrafish kidney (as compared to tissues from *rag1*<sup>+/-</sup> zebrafish.) Overlapping genes demonstrated altered expression in both tissues: note that all 6 of these genes demonstrated an increased expression in *rag1*<sup>-/-</sup> zebrafish intestine and decreased expression in *rag1*<sup>-/-</sup> zebrafish kidney.







**Figure 4. Comparative histopathology of *rag1*<sup>+/-</sup> and *rag1*<sup>-/-</sup> zebrafish tissues**  
 Hematoxylin and eosin (H&E) stained tissue sections of *rag1*<sup>+/-</sup> (A, C and E) and *rag1*<sup>-/-</sup> animals (B, D and F). Tissues include intestine (A and B), kidney (C and D) and liver (E and F). No remarkable microscopic abnormalities were found in either the heterozygous or homozygous mutant zebrafish.



**Figure 5. The transcription of complement C4 is elevated in the *Rag1*<sup>-/-</sup> mouse intestine**

Nine complement and coagulation genes (X axis) were analyzed by quantitative RT-PCR from (a) liver and (b) intestine of 3 *Rag1*<sup>-/-</sup> and 3 congenic mice. Values were normalized to  $\beta$  actin and the relative fold-difference in expression was calculated ( $2^{-\Delta\Delta CT}$ ) as described (Livak et al., 2001). Values (Y axis) represent normalized expression levels in *rag1*<sup>-/-</sup> mice relative to expression in congenic *rag1*<sup>+/-</sup> mice.

Table 1

## Oligonucleotide Primer Sequences

Species	Sequence Description	ZFIN/GenBank Identifier <sup>a</sup>	Forward Primer	Reverse Primer
zebrafish	<i>complement component 9 (c9)</i>	ZDB-GENE-050522-442	CCGAGATCCTAATTCAATGG	TGTCGATGAGGCTCCCTAG
zebrafish	<i>fibrinogen gamma (fgg)</i>	ZDB-GENE-040426-1998	TGGACGTGGATGGACTGTAC	GTCATCGGGTGAGAGGTAAC
zebrafish	<i>complement component bfb</i>	ZDB-GENE-990415-34	ATGGGAAAGTGGAAATGGAAGC	GGTACGTGACTTCATCATCA
zebrafish	<i>LOC79635 similar to complement C4-2</i>	<b>XM 001334604</b>	TGGTTTGATGCTCGCAGCAG	CCATTCCAGAGAGCTTCCTC
zebrafish	<i>vitronectin b (vmb)</i>	ZDB-GENE-041116-1	CTTCACACGATACACAGATC	TGATGCCAAATCCAAGTCTTTG
zebrafish	<i>alpha-1-microglobulin/bikunin precursor, like (ambpl)</i>	ZDB-GENE-040426-1608	GTGACGACATGCCTATGTTTC	ACATCTGACAGCTCATGATG
zebrafish	<i>si:ch211-240119.7</i>	ZDB-GENE-041210-329	CGGAACCCGGAACAACCTGAG	ACAGCACAAACAGCGGGCTTTG
zebrafish	<i>zgc:171537</i>	ZDB-GENE-i080204-13	TCATCAACATTGGTAAACGCC	GTTCATGAGAAAGTCGGAACA
zebrafish	<i>β-actin2</i>	ZDB-GENE-000329-3	AGAGCTACGAGCTGCCCTGAC	TCTCGTGGATAACCCGCAAGAC
mouse	<i>Coagulation factor X (F10)</i>	<b>NM 007972</b>	GAGGGACACCTACGACTATGAT	GCCCACTTTTCTGAGGCA
mouse	<i>Fibrinogen alpha (Fga)</i>	<b>NM 010196</b>	GACCTGAGGGCCAGAAATGA	CTCCAAGGCGTTTCATGTCTAT
mouse	<i>Fibrinogen beta (Fgb)</i>	<b>NM 181849</b>	AGTGTGTGTCTACGGGATG	CTGAGGAGGTATCGGAAACAGA
mouse	<i>Fibrinogen gamma (Fgg)</i>	<b>NM 133862</b>	ACCAGAGATAACTGTTGCATCCT	CCACGTCGGTTTGGTAAAGAAG
mouse	<i>Serpinal</i>	<b>BC012874</b>	GAGCAITGGCACAGCGTTTG	AAGCGATGGTTGGATGTCAGC
mouse	<i>Complement C3</i>	<b>NM 009778</b>	CCAGTCCCCCAITTAGCTCTG	GCACCTTGCCTCTTTAGGAAAGTC
mouse	<i>complement C4</i>	<b>NM 009780</b>	TGCCAATGAAGACTACGAAGA	TGCCATTTCCGCCAGATACACA
mouse	<i>Complement C9</i>	<b>NM 013485</b>	TGTGGAAATGACTTTCAGTGTGA	GCTCTGATTCCTCCGCTACTC
mouse	<i>Complement factor b (Bf)</i>	<b>NM 008198</b>	GGAGTACCTATGTCCTCTGG	CAATCTTTTGGTCTCGGGTCTG
mouse	<i>β-actin</i>	<b>NM 007393</b>	TGTTTGAGACCTTCAACACC	TAGGAGCCAGAGCAAGTAATC

<sup>a</sup> GenBank accession numbers are listed for sequences not annotated by ZFIN.



Table 2

Zebrafish genes with altered expression in *rag1*<sup>-/-</sup> intestine and kidney

Seq. ID	Tissue	Agilent Probe	Sequence Description	Gene Symbol	ZFIN Identifier	GenBank Identifier	Fold Change
i1	Intestine	A_15_P115601	LOC555289 similar to microfibrillar-associated protein 4	<i>LOC555289</i>	n.d.	<a href="#">XM 677760</a>	4.20
i2	Intestine	A_15_P105559; A_15_P108046; A_15_P104191	hemopexin	<i>hpx</i>	ZDB-GENE-030131-5773	<a href="#">NM 001111147</a>	3.36
i3	Intestine	A_15_P110618; A_15_P103668; A_15_P105258	heat shock cognate 70-kd protein	<i>hsp70</i>	ZDB-GENE-990415-91	<a href="#">NM 131397</a>	3.35
i4	Intestine	A_15_P112162	zgc:63663	<i>zgc:63663</i>	ZDB-GENE-040426-1221	<a href="#">NM 200614</a>	3.05
i5	Intestine	A_15_P101682	zgc:55406	<i>zgc:55406</i>	ZDB-GENE-040426-2891	<a href="#">NM 213213</a>	2.85
i6	Intestine	A_15_P107518	complement factor B	<i>cfb</i>	ZDB-GENE-980526-487	<a href="#">NM 131338</a>	2.66
i7	Intestine	A_15_P112489; A_15_P110271; A_15_P103802	complement component C9	<i>c9</i>	ZDB-GENE-050522-442	<a href="#">NM 001024435</a>	2.62
i8	Intestine	A_15_P113576; A_15_P104301	zgc:92882	<i>zgc:92882</i>	ZDB-GENE-040718-176	<a href="#">NM 001002474</a>	2.56
i9	Intestine	A_15_P106533	transferrin-a	<i>tfa</i>	ZDB-GENE-980526-35	<a href="#">NM 001015057</a>	2.49
i10	Intestine	A_15_P105661; A_15_P115510; A_15_P109239	keratin 5	<i>krt5</i>	ZDB-GENE-991110-23	<a href="#">NM 131156</a>	2.48
i11	Intestine	A_15_P121055	fibrinogen, gamma polypeptide	<i>fgg</i>	ZDB-GENE-040426-1998	<a href="#">NM 213054</a>	2.46
i12	Intestine	A_15_P102047	LOC55534 similar to intelectin	<i>LOC55534</i>	n.d.	<a href="#">XM 677825</a>	2.44
i13	Intestine	A_15_P115458	inter-alpha (globulin) inhibitor H2	<i>ih2</i>	ZDB-GENE-040426-1942	<a href="#">NM 213007</a>	2.44
i14	Intestine	A_15_P103556; A_15_P112538	fibrinogen, B beta polypeptide	<i>fgb</i>	ZDB-GENE-030131-9261	<a href="#">NM 212774</a>	2.44
i15	Intestine	A_15_P106657	complement component bfb	<i>bfb</i>	ZDB-GENE-990415-34	<a href="#">NM 131241</a>	2.43
i16	Intestine	A_15_P117565	apolipoprotein A-IV	<i>apoa4</i>	ZDB-GENE-030131-1263	<a href="#">NM 001079861</a>	2.42
i17	Intestine	A_15_P111514	angiotensinogen	<i>agt</i>	ZDB-GENE-030131-1205	<a href="#">NM 198063</a>	2.40
i18	Intestine	A_15_P101542	serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1	<i>serpin1</i>	ZDB-GENE-030131-264	<a href="#">NM 182863</a>	2.38
i19	Intestine	A_15_P109004	LOC794635 similar to complement C4-2	<i>LOC794635</i>	n.d.	<a href="#">XM 001334604</a>	2.34
i20	Intestine	A_15_P110547; A_15_P105980; A_15_P119726	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antitrypsin, antitrypsin), member 1	<i>serpin1</i>	ZDB-GENE-030131-1421	<a href="#">NM 001077758</a>	2.33
i21	Intestine	A_15_P109234	complement component c3c	<i>c3c</i>	ZDB-GENE-990415-37	<a href="#">NM 001037236</a>	2.32
i22	Intestine	A_15_P120995; A_15_P111775	fibronection 1b	<i>fn1b</i>	ZDB-GENE-030131-6545	<a href="#">NM 001013261</a>	2.30
i23	Intestine	A_15_P116122	si:ch211-21910.1	<i>si:ch211-21910.1</i>	ZDB-GENE-030131-9732	<a href="#">NM 001030062</a>	2.29
i24	Intestine	A_15_P100047	LOC557557 similar to complement control protein factor I-B	<i>LOC557557</i>	n.d.	<a href="#">BCI29471</a>	2.27
i25	Intestine	A_15_P105778	insulin-like growth factor binding protein 1	<i>igfbp1</i>	ZDB-GENE-021231-1	<a href="#">NM 173283</a>	2.25
i26	Intestine	A_15_P119259	vitronectin b	<i>vmb</i>	ZDB-GENE-041116-1	<a href="#">BC055570</a>	2.22
i27	Intestine	A_15_P101717	alpha-2-HS-glycoprotein	<i>ahsg</i>	ZDB-GENE-041114-50	<a href="#">NM 212622</a>	2.22
i28	Intestine	A_15_P116686	serpin peptidase inhibitor, clade A (alpha-1 antitrypsin, antitrypsin), member 7	<i>serpin7</i>	ZDB-GENE-041010-47	<a href="#">NM 001005972</a>	2.21



Seq. ID	Tissue	Agilent Probe	Sequence Description	Gene Symbol	ZFIN Identifier	GenBank Identifier	Fold Change
i29	Intestine	A_15_P120815	carboxyl ester lipase, tandem duplicate 2	<i>cel2</i>	ZDB-GENE-061110-10	<a href="#">BC076049</a>	2.19
i30	Intestine	A_15_P107918	glucose-6-phosphatase, catalytic	<i>g6pc</i>	ZDB-GENE-031001-4	<a href="#">NM_001003512</a>	2.17
i31	Intestine	A_15_P104121	fibrinogen alpha chain	<i>fga</i>	ZDB-GENE-031010-21	<a href="#">NM_001002039</a>	2.16
i32	Intestine	A_15_P118096	zgc:92137	<i>zgc:92137</i>	ZDB-GENE-040801-179	<a href="#">NM_001003729</a>	2.15
i33	Intestine	A_15_P108361	SHC SH2-domain binding protein 1	<i>shcbl1</i>	ZDB-GENE-030131-5865	<a href="#">NM_199843</a>	2.15
i34	Intestine	A_15_P113038	si:ch73-252g14.4	<i>si:ch73-252g14.4</i>	ZDB-GENE-030131-1133	<a href="#">NM_001100029</a>	2.14
i35	Intestine	A_15_P113080	urate oxidase	<i>uox</i>	ZDB-GENE-030826-24	<a href="#">NM_001002332</a>	2.12
i36	Intestine	A_15_P102721	zgc:55941	<i>zgc:55941</i>	ZDB-GENE-040426-2014	<a href="#">NM_213249</a>	2.12
i37	Intestine	A_15_P109892; A_15_P117633	zgc:77778	<i>zgc:77778</i>	ZDB-GENE-040426-2281	<a href="#">NM_213203</a>	2.12
i38	Intestine	A_15_P105655	si:dkey-38112.3	<i>si:dkey-38112.3</i>	ZDB-GENE-030131-9563	<a href="#">BC091470</a>	2.09
i39	Intestine	A_15_P120671; A_15_P100607	LOC563048 similar to hyaluronic acid binding protein 2	<i>LOC563048</i>	n.d.	<a href="#">BC122335</a>	2.08
i40	Intestine	A_15_P113509	LOC100006895 similar to LOC567732 protein	<i>LOC100006895</i>	n.d.	<a href="#">XM_001923641</a>	2.07
i41	Intestine	A_15_P111167	zgc:158628	<i>zgc:158628</i>	ZDB-GENE-070112-2012	<a href="#">NM_001080698</a>	2.06
i42	Intestine	A_15_P117436	yippe-like 3	<i>ypl3</i>	ZDB-GENE-030516-4	<a href="#">NM_212790</a>	2.03
i43	Intestine	A_15_P117580	catechol-O-methyltransferase domain containing 1	<i>comtd1</i>	ZDB-GENE-030131-1072	<a href="#">AI477552</a>	2.01
i44	Intestine	A_15_P106260	alpha-1-microglobulin/bikunin precursor, like	<i>ambpl</i>	ZDB-GENE-040426-1608	<a href="#">NM_201118</a>	2.01
i45	Intestine	A_15_P109389	wu:fb20e08	<i>wu:fb20e08</i>	ZDB-GENE-030131-300	<a href="#">AI522512</a>	1.99
i46	Intestine	A_15_P111115; A_15_P107308	centuloplasmin	<i>cp</i>	ZDB-GENE-010522-1	<a href="#">NM_131802</a>	1.97
i47	Intestine	A_15_P100784	zgc:77825	<i>zgc:77825</i>	ZDB-GENE-040426-1887	<a href="#">NM_205643</a>	1.93
i48	Intestine	A_15_P102952	retinol binding protein 2b, cellular	<i>rpb2b</i>	ZDB-GENE-040715-7	<a href="#">NM_001002307</a>	1.93
i49	Intestine	A_15_P110951	C100002040 similar to myosin heavy chain fast skeletal type 2	<i>LOC100002040</i>	n.d.	<a href="#">XM_001339170</a>	1.91
i50	Intestine	A_15_P101461	si:ch211-240119.7	<i>si:ch211-240119.7</i>	ZDB-GENE-041210-329	<a href="#">NM_001082929</a>	1.91
i51	Intestine	A_15_P118717	mannan-binding lectin serine peptidase 2	<i>masp2</i>	ZDB-GENE-060130-154	<a href="#">BC046065</a>	1.90
i52	Intestine	A_15_P101796	apolipoprotein A-I	<i>apoal</i>	ZDB-GENE-990415-14	<a href="#">NM_131128</a>	1.88
i53	Intestine	A_15_P100991	zgc:55398	<i>zgc:55398</i>	ZDB-GENE-040426-705	<a href="#">NM_201048</a>	1.84
i54	Intestine	A_15_P109600	complement component 6	<i>c6</i>	ZDB-GENE-040426-1358	<a href="#">NM_200638</a>	1.81
i55	Intestine	A_15_P110687	solute carrier family 38, member 4	<i>slc38a4</i>	ZDB-GENE-041010-14	<a href="#">NM_001005944</a>	1.80
i56	Intestine	A_15_P101041	zgc:112493	<i>zgc:112493</i>	ZDB-GENE-050522-259	<a href="#">NM_001020703</a>	1.78
i57	Intestine	A_15_P107335	carboxypeptidase B1 (tissue)	<i>cpb1</i>	ZDB-GENE-030131-1132	<a href="#">NM_001110021</a>	1.77
i58	Intestine	A_15_P108307	LOC100150154 similar to complement component 1, q subcomponent-like 4	<i>LOC100150154</i>	n.d.	<a href="#">XM_001921998</a>	1.76

Seq. ID	Tissue	Agilent Probe	Sequence Description	Gene Symbol	ZFIN Identifier	GenBank Identifier	Fold Change
i59	Intestine	A_15_P109564	<i>zgc:152809</i>	<i>zgc:152809</i>	ZDB-GENE-060901-6	NM_001045860	1.75
i60	Intestine	A_15_P112264	<i>zgc:92753</i>	<i>zgc:92753</i>	ZDB-GENE-040718-307	NM_001002568	1.74
i61	Intestine	A_15_P107235	complement component 8, gamma polypeptide	<i>c8g</i>	ZDB-GENE-040426-1898	NM_200863	1.73
i62	Intestine	A_15_P118422	heat shock protein 90-alpha 2	<i>hsp90a.2</i>	ZDB-GENE-031001-3	NM_001045073	1.72
i63	Intestine	A_15_P111237	fatty acid desaturase 2	<i>fads2</i>	ZDB-GENE-011212-1	NM_131645	1.72
i64	Intestine	A_15_P103131	phospholipase A2, group XIIB	<i>pla2g12b</i>	ZDB-GENE-040426-2771	NM_213430	1.70
i65	Intestine	A_15_P107233; A_15_P116880	<i>zgc:77439</i>	<i>zgc:77439</i>	ZDB-GENE-031010-24	NM_212801	1.70
i66	Intestine	A_15_P112820	apolipoprotein C-II	<i>apoc2</i>	ZDB-GENE-030131-2168	AI_916586	1.70
i67	Intestine	A_15_P113156	<i>zgc:171537</i>	<i>zgc:171537</i>	ZDB-GENE-1080204-13	NM_001110109	1.69
i68	Intestine	A_15_P107222	lipase, hepatic	<i>lipc</i>	ZDB-GENE-040426-1361	NM_201022	1.68
i69	Intestine	A_15_P104732	<i>si:ch211-93f2.1</i>	<i>si:ch211-93f2.1</i>	ZDB-GENE-041014-96	BCI55642	1.68
i70	Intestine	A_15_P104772	3-oxoacid CoA transferase 1b	<i>oxct1b</i>	ZDB-GENE-060929-212	NM_001077150	1.68
i71	Intestine	A_15_P114341	<i>zgc:56326</i>	<i>zgc:56326</i>	ZDB-GENE-040426-986	NM_201167	1.67
i72	Intestine	A_15_P107695	fatty acid binding protein 10, liver basic	<i>fabp10</i>	ZDB-GENE-020318-1	NM_152960	1.66
i73	Intestine	A_15_P103003	<i>zgc:56053</i>	<i>zgc:56053</i>	ZDB-GENE-040426-1994	NM_213050	1.64
i74	Intestine	A_15_P109129	Chp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 3	<i>cited3</i>	ZDB-GENE-030425-5	NM_200078	1.61
i75	Intestine	A_15_P102267; A_15_P119634	<i>zgc:92630</i>	<i>zgc:92630</i>	ZDB-GENE-040718-449	NM_001002696	1.61
i76	Intestine	A_15_P114906	secreted immunoglobulin domain 4	<i>sid4</i>	ZDB-GENE-050726-3	NM_001034182	1.61
i77	Intestine	A_15_P106615	coagulation factor X	<i>f10</i>	ZDB-GENE-021206-9	NM_201462	1.60
i78	Intestine	A_15_P111997	<i>zgc:73355</i>	<i>zgc:73355</i>	ZDB-GENE-040426-1775	NM_200803	1.60
i79	Intestine	A_15_P118819	<i>zgc:92061</i>	<i>zgc:92061</i>	ZDB-GENE-040718-78	NM_001002383	1.59
i80	Intestine	A_15_P107520	<i>zgc:92406</i>	<i>zgc:92406</i>	ZDB-GENE-040808-53	NM_001003736	1.55
i81	Intestine	A_15_P107195	apolipoprotein Eb	<i>apoeb</i>	ZDB-GENE-980526-368	NM_131098	1.55
i82	Intestine	A_15_P114005	<i>cyp2k19</i> cytochrome P450 monooxygenase	<i>cyp2k19</i>	n.d.	BCI34885	1.55
i83	Intestine	A_15_P112103	<i>si:rp71-1g18.11 (C1ql4)</i>	<i>si:rp71-1g18.11</i>	ZDB-GENE-040724-34	NM_001030139	1.55
i84	Intestine	A_15_P116013	chymotrypsinogen B1	<i>ctrb1</i>	ZDB-GENE-030131-1171	NM_212618	1.52
i85	Intestine	A_15_P109273	<i>zgc:73262</i>	<i>zgc:73262</i>	ZDB-GENE-040426-1735	NM_200765	-1.53
i86	Intestine	A_15_P109192	solute carrier family 44, member 4	<i>slc44a4</i>	ZDB-GENE-040426-1371	NM_200413	-1.58
i87	Intestine	A_15_P103533	DKEY-202E17.1 novel protein similar to vertebrate core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1(C1GALT1)	<i>LOC555344</i>	n.d.	NM_001128331	-1.63
i88	Kidney	A_15_P113023	<i>zgc:77651</i>	<i>zgc:77651</i>	ZDB-GENE-040426-2381	NM_213247	1.89

Seq. ID	Tissue	Agilent Probe	Sequence Description	Gene Symbol	ZFIN Identifier	GenBank Identifier	Fold Change
k89	Kidney	A_15_P107584	chromosome 14 open reading frame 159	<i>c14orf159</i>	ZDB-GENE-070725-6	<u>BC155801</u>	1.89
k90	Kidney	A_15_P105717	putative interferon stimulated gene 12 (ISG12-2)	<i>zgc:152791</i>	ZDB-GENE-060901-4	<u>NM_001007133</u>	1.53
k91	Kidney	A_15_P107483	<i>zgc:63563</i>	<i>zgc:63563</i>	ZDB-GENE-030131-8928	<u>NM_200044</u>	1.52
k92	Kidney	A_15_P105778	insulin-like growth factor binding protein 1	<i>igfbp1</i>	ZDB-GENE-021231-1	<u>NM_173283</u>	-1.55
k93	Kidney	A_15_P109004	LOC794635 similar to complement C4-2	<i>LOC794635</i>	n.d.	<u>XM_001334604</u>	-1.64
k94	Kidney	A_15_P106235	<i>zgc:92533</i>	<i>zgc:92533</i>	ZDB-GENE-040801-181	<u>NM_001003445</u>	-1.92
k95	Kidney	A_15_P112150	heat shock protein 90-alpha 1	<i>hsp90a.1</i>	ZDB-GENE-990415-94	<u>NM_131328</u>	-2.47
k96	Kidney	A_15_P103668; A_15_P110618	heat shock cognate 70-kd protein	<i>hsp70</i>	ZDB-GENE-990415-91	<u>NM_131397</u>	-2.58
k97	Kidney	A_15_P118357; A_15_P118422	heat shock protein 90-alpha 2	<i>hsp90a.2</i>	ZDB-GENE-031001-3	<u>NM_001045073</u>	-2.69
k98	Kidney	A_15_P107695	fatty acid binding protein 10, liver basic	<i>fabp10</i>	ZDB-GENE-020318-1	<u>NM_152960</u>	-3.28
k99	Kidney	A_15_P105661	keratin 5	<i>ker15</i>	ZDB-GENE-991110-23	<u>NM_131156</u>	-3.34

**Table 3**  
**Genes with altered expression in *rag1*<sup>-/-</sup> zebrafish that are associated with Gene Ontology (GO) terms<sup>a</sup>**

GO Term (P value) <sup>b</sup>	Ontology <sup>c</sup>	DAVID ID <sup>d</sup>	Gene Name (DAVID)	ZFIN ID	Seq ID <sup>e</sup>
Response to Wounding (1.3E-5); Response to External Stimulus (2.1E-4)	BP	2636106	fibrinogen, b beta polypeptide	ZDB-GENE-030131-9261	i14
		2610274	fibrinogen, gamma polypeptide	ZDB-GENE-040426-1998	i11
		2611966	complement component c3c	ZDB-GENE-990415-37	i21
		2609369	coagulation factor x	ZDB-GENE-021206-9	i77
		2614980	complement factor b	ZDB-GENE-980526-487	i6
		2610608	fibrinogen alpha chain	ZDB-GENE-031010-21	i31
		2636106	fibrinogen, b beta polypeptide	ZDB-GENE-030131-9261	i14
		2610274	fibrinogen, gamma polypeptide	ZDB-GENE-040426-1998	i11
		2609392	insulin-like growth factor binding protein 1	ZDB-GENE-021231-1	k92
		2611966	complement component c3c	ZDB-GENE-990415-37	i21
Response to Stress (4.4E-5)	BP	2609369	coagulation factor x	ZDB-GENE-021206-9	i77
		2614980	complement factor b	ZDB-GENE-980526-487	i6
		2610608	fibrinogen alpha chain	ZDB-GENE-031010-21	i31
		2609941	heat shock cognate 70-kd protein	ZDB-GENE-990415-91	i3, k96
		2637387	heat shock protein 90-alpha	ZDB-GENE-990415-94	k95
		2636106	fibrinogen, b beta polypeptide	ZDB-GENE-030131-9261	i14
		2610274	fibrinogen, gamma polypeptide	ZDB-GENE-040426-1998	i11
		2613279	zgc:92406	ZDB-GENE-040808-53	i80
		2610892	transferrin-a	ZDB-GENE-980526-35	i9
		2609392	insulin-like growth factor binding protein 1	ZDB-GENE-021231-1	k92
Regulation of Biological Quality (8.0E-5)	BP	2610130	zgc:55398	ZDB-GENE-040426-705	i53
		2637785	zgc:56326	ZDB-GENE-040426-986	i71
		2609369	coagulation factor x	ZDB-GENE-021206-9	i77
		2610625	transferrin	ZDB-GENE-980526-35	i9
		2610608	fibrinogen alpha chain	ZDB-GENE-031010-21	i31
		2613213	zgc:92137	ZDB-GENE-040801-179	i32
		2637785	zgc:56326	ZDB-GENE-040426-986	i71
		2609504	inter-alpha (globulin) inhibitor h2	ZDB-GENE-040426-1942	i13
		2613123	zgc:55941	ZDB-GENE-040426-2014	i36
		Carbohydrate Metabolic Process (2.5E-3)	BP		

GO Term (P value) <sup>b</sup>	Ontology <sup>c</sup>	DAVID ID <sup>d</sup>	Gene Name (DAVID)	ZFIN ID	Seq ID <sup>e</sup>
Nitrogen Compound Metabolic Process (5.0E-3)	BP	2606698	zgc:92882	ZDB-GENE-040718-176	i8
		2610186	zgc:56053	ZDB-GENE-040426-1994	i73
		2638086	zgc:77912	ZDB-GENE-040426-2891	i5
		2637785	zgc:56326	ZDB-GENE-040426-986	i71
		2609504	inter-alpha (globulin) inhibitor h2	ZDB-GENE-040426-1942	i13
		2613123	zgc:55941	ZDB-GENE-040426-2014	i36
		2610186	zgc:56053	ZDB-GENE-040426-1994	i73
		2638086	zgc:77912	ZDB-GENE-040426-2891	i5
		2637387	heat shock protein 90-alpha	ZDB-GENE-990415-94	k95
		2642993	serine (or cysteine) proteinase inhibitor, clade c (antithrombin), member 1	ZDB-GENE-030131-264	i18
Serine-type Endopeptidase Inhibitor Activity (7.8E-6)	MF	2608876	angiotensinogen	ZDB-GENE-030131-1205	i17
		2612650	serpin peptidase inhibitor, clade a (alpha-1 antiproteinase, antitrypsin), member 7	ZDB-GENE-041010-47	i28
		2609504	inter-alpha (globulin) inhibitor h2	ZDB-GENE-040426-1942	i13
		2611808	zgc:66321	ZDB-GENE-040426-1608	i44
		2620612	serine (or cysteine) proteinase inhibitor, clade a (alpha-1 antiproteinase, antitrypsin), member 1	ZDB-GENE-030131-1421	i20
		2642993	serine (or cysteine) proteinase inhibitor, clade c (antithrombin), member 1	ZDB-GENE-030131-264	i18
		2608876	angiotensinogen	ZDB-GENE-030131-1205	i17
		2612650	serpin peptidase inhibitor, clade a (alpha-1 antiproteinase, antitrypsin), member 7	ZDB-GENE-041010-47	i28
		2609504	inter-alpha (globulin) inhibitor h2	ZDB-GENE-040426-1942	i13
		2611966	complement component c3c	ZDB-GENE-990415-37	i21
Endopeptidase Inhibitor Activity (2.6E-5); Protease Inhibitor Activity (3.4E-5); Enzyme Inhibitor Activity (6.4E-5)	MF	2609369	coagulation factor x	ZDB-GENE-021206-9	i77
		2612404	chymotrypsinogen b1	ZDB-GENE-030131-1171	i84
		2607012	alpha-2-hs-glycoprotein	ZDB-GENE-041114-50	i27
		2611808	zgc:66321	ZDB-GENE-040426-1608	i44
		2620612	serine (or cysteine) proteinase inhibitor, clade a (alpha-1 antiproteinase, antitrypsin), member 1	ZDB-GENE-030131-1421	i20
		2642993	serine (or cysteine) proteinase inhibitor, clade c (antithrombin), member 1	ZDB-GENE-030131-264	i18
		2608876	angiotensinogen	ZDB-GENE-030131-1205	i17
		2612650	serpin peptidase inhibitor, clade a (alpha-1 antiproteinase, antitrypsin), member 7	ZDB-GENE-041010-47	i28
		2609504	inter-alpha (globulin) inhibitor h2	ZDB-GENE-040426-1942	i13
		2611966	complement component c3c	ZDB-GENE-990415-37	i21
Enzyme Regulator Activity (2.5E-4)	MF	2609369	coagulation factor x	ZDB-GENE-021206-9	i77
		2612404	chymotrypsinogen b1	ZDB-GENE-030131-1171	i84
		2607012	alpha-2-hs-glycoprotein	ZDB-GENE-041114-50	i27
2611808	zgc:66321	ZDB-GENE-040426-1608	i44		
2620612	serine (or cysteine) proteinase inhibitor, clade a (alpha-1 antiproteinase, antitrypsin), member 1	ZDB-GENE-030131-1421	i20		
2642993	serine (or cysteine) proteinase inhibitor, clade c (antithrombin), member 1	ZDB-GENE-030131-264	i18		
2608876	angiotensinogen	ZDB-GENE-030131-1205	i17		
2612650	serpin peptidase inhibitor, clade a (alpha-1 antiproteinase, antitrypsin), member 7	ZDB-GENE-041010-47	i28		



GO Term (P value) <sup>b</sup>	Ontology <sup>c</sup>	DAVID ID <sup>d</sup>	Gene Name (DAVID)	ZFIN ID	Seq ID <sup>e</sup>
		2609504	inter-alpha (globulin) inhibitor h2	ZDB-GENE-040426-1942	i13
		2611966	complement component c3c	ZDB-GENE-990415-37	i21
		2609369	coagulation factor x	ZDB-GENE-021206-9	i77
		2612404	chymotrypsinogen b1	ZDB-GENE-030131-1171	i84
		2607012	alpha-2-hs-glycoprotein	ZDB-GENE-041114-50	i27
		2611808	zgc:66321	ZDB-GENE-040426-1608	i44
		2620612	serine (or cysteine) proteinase inhibitor, clade a (alpha-1 antiproteinase, antitrypsin), member 1	ZDB-GENE-030131-1421	i20
		2637387	heat shock protein 90-alpha	ZDB-GENE-990415-94	k95

<sup>a</sup>GO Terms and genes were defined by DAVID software using “Functional Annotation Chart” and selecting “Gene Ontology”. In order for a GO term to be listed in this table, at least 6 genes from Table 2 must be associated with the term with a P value # 0.01.

<sup>b</sup>GO Identity refers to Gene Ontology <http://www.geneontology.org/>.

<sup>c</sup>Ontologies were restricted to Biological Processes (BP) and Molecular Function (MF).

<sup>d</sup>DAVID identity refers to DAVID software, <http://david.abcc.ncifcrf.gov/home.jsp>.

<sup>e</sup>Zebrafish Sequence ID refers to Table 2.