Innate immune signalling of the zebrafish embryo
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Transcriptome analysis of Traf6 function in the innate immune response of zebrafish embryos

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Chapter 4

Abstract

TRAF6 is a key player at the cross-roads of development and immunity. The analysis of its *in vivo* molecular function is a great challenge since severe developmental defects and early lethality caused by *Traf6* deficiency in knock-out mice interfere with analyses of the immune response. In this study we have used a new strategy to analyse the function of Traf6 in a zebrafish-Salmonella infectious disease model. In our approach the effect of a Traf6 translation-blocking morpholino was titrated down to avoid developmental defects and the response to infection under these partial knock-down conditions was studied using the combination of microarray and next generation sequencing technology. Transcriptome profiling of the traf6 knock-down allowed the identification of a gene set whose responsiveness during infection is highly dependent on Traf6. Expression trend analysis based on the resulting data-sets identified nine clusters of genes with characteristic transcription response profiles, demonstrating Traf6 has a dynamic role as a positive and negative regulator. Among the Traf6-dependent genes was a large set of well known anti-microbial and inflammatory genes. Additionally, we identified several genes of which a role in the immune system was not previously known to be Traf6-dependent, such as the fertility hormone gene *gnrh2* and the DNA-damage regulated autophagy modulator 1 gene *dram1*. With the use of the zebrafish embryo model we have now dissected the *in vivo* function of Traf6 in the innate immune response without interference of adaptive immunity.

Introduction

Microbial infections usually elicit a rapid and strong response of the host innate immune system. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), enable the host to recognize pathogens by detecting conserved molecular patterns such as lipopolysaccharide (LPS), flagellin or peptidoglycan (1). Activation of these receptors will initiate the induction of pro-inflammatory cytokines and as a result a complex network of underlying signalling pathways is activated leading to a tailored inflammatory response with the ultimate goal of eradicating the pathogen. An essential protein transducing the signals emanating from various PRRs and cytokine receptors, including the TNF superfamily, TGFβ, IL-1/Toll-like and NOD-like receptors, is the TNF receptor-associated factor 6 (TRAF6) (2-5).

Initial studies demonstrated the ability of TRAF6 to bind to CD40, RANK and IRAK-1 and showed that NF-κB signalling via TLR4 was abolished by a dominant-negative form of TRAF6 (6-12). Analysis of TRAF6 deficient mice revealed a critical role of TRAF6 in osteoclast development and function. Furthermore, these studies indicated an essential role of TRAF6 in IL-1 signalling, as the activation of NF-
κB and JNK in response to IL-1 were absent in embryonic fibroblasts derived from TRAF6-deficient mice. Moreover, bone marrow-derived macrophages from these mutants displayed a diminished response to LPS, and dendritic cell development and function was impaired (13-15).

The molecular mechanism underlying signal transduction by TRAF6 upon infection is that TRAF6 exerts its function as a K63-specific RING finger E3 ligase. Upon activation of the TLR or the IL-1 receptor pathway, the association of MyD88 with the cytosolic part of the receptor results in the phosphorylation of IRAK-1 by IRAK-4. Subsequently, activated IRAK1 will bind to TRAF6 that will form a complex with the ubiquitin-conjugating enzymes Ubc13 and Uev1a resulting in the attachment of non-degradative K63-linked ubiquitin chains to TRAF6 itself and to NEMO, the regulatory component of the IKK complex upstream of NF-κB. Ubiquitination of TRAF6 will recruit the TAB2/3-TAB1-TAK1 complex resulting in the activation of TAK1. Subsequent activation of the IKK complex and MAP kinase cascades by TAK1 lead to the induction of pro-inflammatory cytokines by the NF-κB and AP-1 transcription factor complexes, respectively (16-19). In addition to the role of TRAF6 in innate immunity, TRAF6 function was also placed in the context of adaptive immunity. Mice containing a T-cell specific deletion of TRAF6 showed the inability to maintain CD8 memory T-cells due to defective AMP-activated kinase activation and mitochondrial fatty acid oxidation after growth factor depletion (20).

_Traf6_ deficiency in mice causes severe developmental defects and early death at 17–19 days postnatal, making _in vivo_ infection studies challenging. Therefore, we have used a zebrafish embryo model to perform _in vivo_ infection experiments. In recent years the zebrafish (_Danio rerio_) embryo system has emerged as a model to study vertebrate innate immunity, offering several advantages that complement mammalian model systems. External development and the transparent character of the zebrafish embryo, in combination with fluorescently labeled immune cells and bacteria, allows for study of host microbe interaction and inflammation processes in the living organism (21-28). Analysis of the immune system of the zebrafish revealed a fully developed innate and adaptive immune system showing significant similarities to the human equivalent (29-33). An active innate immune system is detectable already at day one of zebrafish embryogenesis (21, 34, 35). By contrast, a functionally mature adaptive immune system is not active during the first three weeks of zebrafish development establishing a clear temporal separation of the innate and adaptive immune system in the zebrafish embryo. (36-38). Therefore, the zebrafish model provides a convenient system for the _in vivo_ study of the vertebrate innate immune response to infection independently from the adaptive immune response. Furthermore, morpholino based knock-down experiments facilitate the functional analysis of genes in the zebrafish embryo that otherwise lead to lethal defects in gene knock-out studies in mice. Moreover, many infection systems for zebrafish have been developed lately, allowing the analysis of gene functions under infection conditions (32, 39, 40).
Here we report on the transcriptional analysis of the innate immune response in Traf6 knock-down and control embryos upon a bacterial infection using a previously described Salmonella enterica serovar Typhimurium (hereafter referred to as S. typhimurium) infection model (22, 33). By titrating down the concentration of a translation blocking morpholino we could avoid effects of Traf6 knock-down on embryo development and study the response to infection under these partial knock-down conditions. Multifactorial analysis of microarray data and confirmation by RNA deep sequencing allowed the identification of a gene set whose responsiveness to S. typhimurium infection is highly dependent on Traf6 function. Therefore, while our study indicates a role of Traf6 in developmental processes, it clearly illustrates its importance in the innate immune defence in the zebrafish embryo system.

Results

System for analysis of innate immune functions of Traf6

To accomplish traf6 knock-down zebrafish embryos were injected at the one cell stage with an ATG morpholino to prevent traf6 mRNA translation. Initial titration experiments of the traf6 morpholino elicited a concentration-dependent effect on embryo development showing phenotypical defects, such as body axis truncation and brain malformation, when the administered morpholino concentration exceeded 1mM. To avoid the strong interference of developmental defects, all infection assays were performed using a concentration of 1mM traf6 morpholino considered as an incomplete knock-down of traf6. To be able to discriminate between the specific effect of the traf6 knock-down and possible aspecific morpholino effects in our assay a control group was treated with a 5bp mismatch traf6 morpholino. At 27hpf both groups were either immune challenged by injection of 250 cfu of a S. typhimurium wild type strain or mock injected with PBS. The transcriptional response was subsequently analysed at 8 hours post infection (hpi) (Fig. 1A). Initial analysis of the datasets demonstrated a robust response to the infection in the control as well as in the traf6 knock-down group. At the UniGene cluster level a total of 3720 genes (p<0.05 and a fold change < -1.2 and > 1.2) were regulated upon bacterial challenge in the control group. In contrast, a reduced response showing a total of 2840 differentially regulated genes (p<0.05 and a fold change < -1.2 and > 1.2) was noticeable in the traf6 knock-down group (Fig. 1B, supplemental Table I). The S. typhimurium-induced expression signatures of both the control and the traf6 knock-down groups were consistent with the published results of Stockhammer et al. and include all genes previously validated by Q-PCR in that study (33). As shown by projection of the microarray data on a GenMapp of the TLR-signalling pathway, the S. typhimu-rium-induced gene sets of both groups included several TLR pathway components and downstream targets (Fig. 2, supplemental Table II). Furthermore, GO-analysis on the zebrafish gene identifiers by master-target testing on the level of Biological
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Process revealed among others the GO-terms immune system process and response to stimulus as significantly (p<0.05) enriched in both groups (supplemental Table III). Interestingly, genes that were clustered under the GO-term reproduction were also significantly enriched in the up-regulated fraction. In contrast, only minor differences were provoked by traf6 knock-down itself, indicating that our titration of the morpholino to avoid the developmental effect has been remarkably successful.

In total 20 genes were up- and 35 genes were down-regulated by traf6 knock-down in the absence of infection (p<0.05, fold change ≤ -1.2 and ≥ 1.2) (Fig. 1B, supplemental Table IV). Among the group of up-regulated genes we identified genes such as stc1 (stanniocalcin 1, fc=2.62), a gene involved in Ca2+ uptake in zebrafish, as well

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**FIGURE 1.** Schematic overview of the experimental setup (A) and scatter plot illustration of the transcriptional response of the various treatment groups (B). (A) Zebrafish embryos were either injected with a traf6 ATG-morpholino or a 5bp mismatch (mm) morpholino at the first cell stage. At 27 hpf both groups were immune challenged by injection of 250 cfu of a S. typhimurium strain or mock injected with PBS. The transcriptional response was subsequently analysed at 8 hours post infection (hpi) using a common reference approach. The experiment was carried out in triplicate. (B) The scatter plots on the left side and in the middle show the transcriptional response upon S. typhimurium infection in the control (mm-morpholino) and traf6 knock-down (k.d.) group respectively. The scatter plot on the right shows the response provoked by traf6 knock-down independently of the infection.
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as zgc:77734 (fc=2.57), showing similarities to the human DBI (diazepam binding inhibitor) gene. Examples of genes down-regulated by traf6 knock-down in the absence of infection are or111-3 (fc=-3.8), a member of the fish odorant receptor family, and he1a (hatching enzyme 1a, fc=-2.68) as well as rcv1 (recoverin, fc=-2.15) and anterior gradient homolog 2 (agr2, fc=-1.89) (supplemental Table IV).

**Statistical analysis of the effect of traf6 knock-down on the infection response**

In order to find those genes that were specifically differentially regulated in the traf6 knock-down group in comparison to the control group upon infection, the interaction term was analysed. Interaction is defined as the dependence of the effect of one factor (here gene knock-down by morpholino treatment) on the level of another factor (here immune challenge by infection). In terms of the analysis of variance (ANOVA) model, the interaction term measures the deviation from an expected value based on the additive combinations of the morpholino and infection means. A large positive deviation of this sort is called synergism, in which case the simultaneous morpholino and infection treatment gives rise to an expression level that deviates from the additive combination of the morpholino and infection treatment alone. A negative deviation, i.e. when the combined infection and morpholino application gives rise to a smaller effect than one could expect from the additive combination of the two effects separately, can be called interference. Synergy can be in the direction of overexpression or underexpression. In the former, a gene has a higher expression in the combined treatment than one could expect on the basis of the additive combination of the separate treatments. In the latter, a gene has an even lower expression in the combined treatment than one could expect on the basis of the additive combination of the separate treatments.

To specifically identify genes that were most highly dependent on Traf6 for their

**FIGURE 2.** GenMapp analysis of the immune response to S. typhimurium infection in the TLR pathway in control and traf6 knock-down embryos. Expression profiles of the control and traf6 knock-down groups at the 8hpi time point (infected versus non infected, FDR corrected p-value <0.05 and fold changes ≥1.2 and ≤-1.2) were simultaneously mapped on the TLR pathway. Gene boxes are colour coded with the control morpholino treatment (Control) on the left and traf6 knock-down (Traf6 k.d.) on the right side. Up-regulation is indicated in yellow, down-regulation in blue. The position of traf6 in the pathway is highlighted by a red border of the gene box. Genes that failed the fold-change cut off are depicted in gray and genes that were not significantly regulated are represented in white. Highlighting of gene boxes by red shading indicates that the S. typhimurium-induced gene expression level was lower in traf6 knock-down embryos than in control embryos, based on microarray expression trend analysis as well as RNAseq analysis. The pathway is based on knowledge of TLR signalling in mammalian species and it should be noted that most interactions remain to be experimentally confirmed in zebrafish.
transcriptional response to a bacterial infection we examined all genes that were significant in the interaction term analysis with a stringent FDR-corrected p value smaller than 0.15 (Table I). The expression profiles of 28 identified genes corresponding to this criterion are shown in a heatmap (Fig.3). For the majority of these genes (20 out of 28) the S. typhimurium-induced gene expression levels were much lower in the traf6 knock-down group than in the control group (Fig.3), indicating that the infection-mediated induction of these genes is dependent on traf6. Among these were several with a well established immune function like hamp2, mmp9, mmp13, tnfβ, il1β, ncf1, crfb4 and zgc:103580, the zebrafish ortholog of the human acute phase response gene serum Amyloid protein A. In addition to two members of the matrix metalloproteinase family (mmp9 and mmp13), the group of 20 genes with a reduced infection response in traf6 knock-down embryos also included the metalloproteinase inhibitor gene timp2b (tissue inhibitor of metalloproteinase 2b). This group also included zgc:112143, a gene homologous to the human STEAP4 gene (also known as TNFAIP9, tumor necrosis factor alpha-induced protein 9), that we previously found to be induced with alternative splice forms during Mycobacterium marinum infection in adult zebrafish (41). On the other hand, the gene group showing a reduced infection response in traf6 knock-down embryos also included genes that were pre-
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Previously not linked to immune function or TRAF6 signalling like plekhi1 (pleckstrin homology domain containing, family F), clic2 (chloride intracellular channel 2), pfsb3 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3), gnrh2 (gonadotropin-releasing hormone 2), and dram1 (DNA-damage regulated autophagy modulator 1).

In addition to the 20 genes showing a reduced infection-mediated induction in traf6 knock-down embryos, the statistical analysis also identified 4 genes (cxcl12b, atp1a1a.3, zgc:55418 and zgc:85900) that appeared to be dependent on Traf6 for their negative regulation during infection. These genes were down-regulated by infection in control embryos but not or to a lower extent in traf6 knock-down embryos (Fig.3, Table I). Four other genes showed a more complex dependency on Traf6, with opposite regulation in knock-down embryos and controls (sc5d) or with expression levels affected both in the absence and presence of infection (zgc:56292, stc1, npsn).

In conclusion, based on the interaction term analysis we identified genes that are dependent on Traf6 activity for their positive or negative regulation during S. typhimurium infection of zebrafish embryos.

Confirmation of Traf6-dependent genes by RNA deep sequencing
In order to confirm the microarray data we subjected the pooled RNA samples of

- Listed genes were identified by interaction term analysis. Significance cut off values were set to p<0.05 (FDR). All genes indicated as + were confirmed by RNAseq analysis, whereas genes indicated as – were not. Four genes indicated as not applicable (n.a.) lacked an ENSDART identifier and could therefore not be verified by RNAseq analysis. For one gene indicated as not detectable (n.d.) not enough RNA sequence reads were obtained (< 1 mapped reads per million total reads). K-means cluster identifiers refer to Figure 5.

Table I. Genes dependent on Traf6 during Salmonella infection*

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>P-value</th>
<th>UniGene ID</th>
<th>Ensembl ID</th>
<th>K-means cluster</th>
<th>RNAseq</th>
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<td>gnrh2</td>
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<td>ENSDART00000028034</td>
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<td>ENSDART00000010593</td>
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<td>+</td>
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<td>ENSDART00000013261</td>
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<td>+</td>
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<td>dros1</td>
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<td>zgc:117243</td>
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<td>chloride intracellular channel 2</td>
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<td>wu:932069</td>
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<td>DT98466</td>
<td>ENSDART00000078492</td>
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<td>zgc:103580</td>
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<td>otx2b</td>
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<td>DT98295</td>
<td>ENSDART00000053599</td>
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<td>ift8</td>
<td>intraflagellar transport 1, beta</td>
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<td>ENSDART00000054419</td>
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<td>4</td>
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<td>+</td>
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* Listed genes were identified by interaction term analysis. Significance cut off values were set to p<0.05 (FDR). All genes indicated as + were confirmed by RNAseq analysis, whereas genes indicated as – were not. Four genes indicated as not applicable (n.a.) lacked an ENSDART identifier and could therefore not be verified by RNAseq analysis. For one gene indicated as not detectable (n.d.) not enough RNA sequence reads were obtained (< 1 mapped reads per million total reads). K-means cluster identifiers refer to Figure 5.
the three biological replicates of each treatment group to Illumina RNA sequencing (RNAseq). Approximately 15 million reads were obtained for each of the four RNAseq libraries (control, control infected, traf6 knock-down, infected traf6 knock-down) and approximately 10 million reads per library could be mapped to the Ensembl transcript database based on the Zv8 genome sequence. Next we compared the sequence read counts (mapped reads per million total reads) between the treatment groups (supplemental Table VI). For 21 of the 28 genes that were significant in the interaction term analysis, we found that the RNAseq data confirmed the microarray results. This included 16 of the 20 genes positively dependent on Traf6 during infection and all 4 of the genes negatively dependent on Traf6 during infection. Representative examples of these positively (il1b, mmp9, mmp13, timp2b) and negatively (atp1a1a.3, cxcl12b) regulated genes are shown in Fig. 4. In five cases the micro-

**FIGURE 4.** RNAseq validation of microarray results. RNAseq data are shown for representative examples of genes for which the microarray expression trend was confirmed by RNAseq read counts of the four treatment groups (non-infected control, infected control, non-infected traf6 knock-down, infected traf6 knock-down). Bars indicate the relative expression of the different treatment groups based on the number of mapped sequence reads per million of total reads. For every gene the value of the uninfected control group is set to 1 and the expression level of the gene in the treatment group is calculated relative to the control value. Two different scales are used for genes in different ranges of induction level upon *S. typhimurium* infection. Cluster numbers refer to the K-means clustering in Fig.5. The complete overview of genes for which the microarray expression trend was confirmed by RNAseq is given in supplemental Table VI.
array data could not be validated by RNAseq because transcripts for these genes were not present in the Ensembl database or not enough RNAseq reads were obtained (< 1 mapped reads per million total reads). In only two cases, the RNAseq data did not confirm the microarray data. Both these cases (sc5d, npsn) were genes showing a complex dependency on Traf6 as described above. In conclusion, RNAseq analysis validated the interaction term analysis for most of the genes whose induction or repression during S. typhimurium infection was found to be dependent on Traf6.

Expression trend analysis
Since the knock-down conditions of Traf6 can be considered to be incomplete, we also wanted a broader overview of the effects of trafi6 knock-down in an expression

**FIGURE 5.** Trend analysis of the interaction term by K-means clustering. For a broader analysis of the effects of trafi6 knock-down all retrieved probes with an FDR-corrected p-value lower than 0.4 were clustered using the K-means cluster function in SPOTFIRE. All probes lacking a valid annotation were excluded from the analysis resulting in a final set of 376 probes. The identified trend in the control group is illustrated on the left side of each cluster expressed by the z-score of each probe between the noninfected (nInf con) and the infected (Inf con) control morpholino treated group. On the right side the corresponding trend of the noninfected (nInf k.d.) versus the infected (Inf k.d.) group under the trafi6 knock-down condition is illustrated. All probes that contribute to the distinct clusters are listed in supplemental table VI.
trend analysis of the microarray data using a less stringent criterion: a FDR-corrected p-value of smaller than 0.4 retrieved from the interaction term. First, we analysed these probes by enrichment analysis based on Gene Ontology (GO) annotation. A significant enrichment was demonstrated for genes clustering under the biological process GO-terms immune system process (p<0.05), response to stimulus (p<0.05), and multi-organism process (p<0.05), demonstrating a clear effect of traf6 knock-down on the immune response to S. typhimurium (supplemental Table V).

Following up the GO-term analysis, we subjected the selected probes to K-means cluster analysis, allowing for the visual discrimination between synergism and interference of the interaction term. Probes missing a valid annotation were excluded from the analysis. The remaining 376 probes, representing 233 genes, were categorized into 9 clusters (Fig. 5, supplemental table V). The microarray expression trend of all genes in the scheme was in agreement with RNAseq data.

Genes indicated by clusters 1 and 2 were respectively up- or down-regulated upon infection under the traf6 knock-down condition, whereas only minor and in the ma-
jority not significant changes were observed in the control group. These two clusters contain such genes as *zgc:1154057* (*transcriptional adaptor 2-like*) in cluster 1, and *cpox* (*coproporphyrinogen oxidase*) and *stc1* (*stanniocalcin 1*) in cluster 2 (supplemental Table VI, Fig. 4). Clusters 3 and 4 show an opposing trend in gene regulation upon infection between the control and *traf6* knock-down groups. Cluster 3 was not considered further, since regulation of genes in this cluster was generally not confirmed by the RNAseq data. Genes in cluster 4 were consistently up-regulated in the *traf6* knock-down group upon infection, while down-regulated or non-responsive in the control group, with examples such as *zgc:92061* (similar to keratin 17) and *tm7sf2* (transmembrane 7 superfamily member 2) (supplemental Table VI, Fig. 4).

A common trend in gene regulation is observed in clusters 5 to 8, where infection leads to a consistent up-regulation in the control groups and weaker up-regulation (clusters 7 and 8) or unchanged expression (clusters 5 and 6) in the *traf6* knock-down groups. Finally, cluster 9 consists of those genes that were strongly down-regulated by infection in the controls, while showing a weaker or no down-regulation in *traf6* knock-down embryos.

For further analysis we concentrated on the genes that were dependent on Traf6 for their induction (clusters 5-8) or repression (cluster 9) during *S. typhimurium* infection. From the total of 124 genes with Traf6-dependent induction and 32 genes with Traf6-dependent repression, the microarray expression trend of 105 and 22 genes, respectively, could be confirmed by the RNAseq data (supplemental table VI, with representative examples in Fig.4). We categorized these Traf6-dependent genes into functional groups based on gene ontology terms and using references to gene function of their mammalian homologs in the NCBI Entrez Gene database (Fig.6). A notable fraction of the Traf6-dependent infection-induced genes play a well established role in the immune response, for example as cytokines or interferons (e.g. *il1b, tnf, CCL-C5a, ifnphi1*), in complement activation or the acute phase response (e.g. *cfb, zgc:103580*), in prostaglandin biosynthesis (*pgts2a*), or in microbial killing (e.g. *ncf1, hamp2*). Many of the Traf6-dependent infection-induced genes are involved in signal transduction and transcriptional activation or repression. This includes the *tlr5b* gene, important for the response to flagellin, negative regulators of TLR-signaling (*irak3, socs3a*) as well as transcription factors (*atf3, jun, nfkb2*) activated by the TLR pathway (Fig.2, Fig.6). Other examples of Traf6-dependent signal transduction genes include *fas* (*TNF receptor superfamily, member 6*), *tgfb1* (*transforming growth factor, beta 1*), *ctnnb2* (beta-catenin2), *wnt10a*, and small gtpase genes (*rhogb, rhoab*). In addition to the above-mentioned Traf6-dependent members of the matrix metalloproteinase family (*mmp9* and *mmp13*) and metalloproteinase inhibitor gene (*timp2b*), the induction of several other genes involved in proteolysis was Traf6-dependent (e.g. *adam8a, agt, psme1/2, serpine1*). Finally, Traf6-dependent infection-induced gene groups were linked to apoptosis (e.g. *bcl2l, dram1*), cell adhesion (e.g. *cldn5*), transporter activity (e.g. *clic2, scl13a2*), or encoded enzymes involved in metabolic processes (e.g. *acsl4l, pfkfb3, dgat1b*) (Fig. 6).
Not only the infection-induced gene groups, but also the gene groups that were dependent on Traf6 for their repression during infection included genes for cytokines (cxcl12b, cxcl14), transporters (atp1a1a.3, slc56a11), enzymes (ca4a, sulf2) and signal transduction proteins (e.g. efnb3, nbl1). In addition, several other genes, for example vcanb, encoding a member of the hyaluronan (HA)-binding proteoglycans, were repressed by infection in a Traf6-dependent manner (Fig.6).

Taken together, based on the expression trend analysis of the microarray data and validation by RNAseq, we conclude that Traf6 has a dynamic role as a positive and negative regulator of genes responsive to S. typhimurium infection in zebrafish embryos, including a large set of well known anti-microbial and inflammatory genes as well as genes not previously linked to the immune response or to Traf6 function.

Discussion

The fact that TRAF6 is a key player at the cross-roads of development and immunity makes the analysis of its in vivo molecular function a great challenge (13, 14, 47). Severe developmental defects and early lethality caused by Traf6 deficiency in knock-out mice interfere with analyses of the immune response. In this study we have developed a new approach to analyse the function of Traf6 in a zebrafish acute infectious disease model. In this approach the effect of a Traf6 translation-blocking morpholino was titrated in such a way that developmental defects were brought back to an identifiable non-dominant factor in the transcriptome analyses. The results show that, even under partial knock-down conditions, it was possible to identify a gene set (Table I) whose responsiveness during S. typhimurium infection is highly dependent on Traf6. In addition, expression trend analysis identified nine clusters of genes with characteristic transcription response profiles, demonstrating that Traf6 has a dynamic role as a positive and negative regulator. We have confirmed the data from microarray experiments with whole transcriptome shotgun sequencing (RNA-seq). This is one of the first times that this novel deep sequencing approach has been used for quantitative transcriptome profiling (48, 49). The results show that this complementary technique gives good support for the identified Traf6-dependent infection-responsive gene set, confirming Traf6-dependent induction of 105 genes and Traf6-dependent repression of 22 genes during S. typhimurium infection. In addition, especially since this is the first time that RNA-seq technology is used for infectious disease studies, these data represent a great wealth of disease-induced transcript information that will be of great value for future studies.

Among the genes that are highly dependent on Traf6 for their induction in response to S. typhimurium infection a subset of well known immune system-associated genes such as il1b, mmp9, mmp13, hamp2, and tnfβ was found, demonstrating a specific in vivo effect of Traf6 on the innate immune response. Previously we could show that il1b and mmp9 are downstream targets of the zebrafish TLR-pathway. The
dependency of the expression of these genes on Traf6 is consistent with this study and supports the specificity of the morpholino knock-down (33). It was shown in mouse that MMP-9 can also be activated upon RANKL stimulation via TRAF6, p38 and ERK1/2 (50). RANKL is a member of the tumor necrosis factor (ligand) superfamily and is an important activator of osteoclasts, cells involved in bone resorption. Although osteoclasts only develop in zebrafish larvae after several weeks it is not unlikely that these pathways are conserved during embryonic development (51). Several of the other Traf6-dependent genes that are linked to the TLR/IL1R-pathway are also corroborated in other model systems. It was shown that the regulation of hepcidin (HAMP1) is facilitated via the TLR-pathway in mice-derived macrophages upon bacterial infection (52). Another example shown in mouse is that activation of MMP-13 through the IL1-signalling pathway was strongly impaired after traf6 knock-down (53). Interestingly, also the induction of a tissue inhibitor of metalloproteinase function, Timp2b was dependent of Traf6 during zebrafish embryo infection. The expression of timp2b was also found to be induced during mycobacterium infection in zebrafish embryos, which was suggested to function as a compensatory response to increased mmp9 activity (54). Furthermore, human TIMP2 has been linked to cancer progression and has been found to be a marker for dendritic cell response to HIV infection (55). The known immune response genes that we showed to be induced in a Traf6-dependent manner also included components of the TLR-signalling pathway, such as tlr5b and irak3. It remains a question whether the regulation of the above mentioned genes is mediated via the TLR-pathway itself or via another Traf6-directed pathway. For instance Salmonella infection also regulated tgfb1 in the TGF-beta pathway in a Traf6-dependent manner. Therefore, the poorly understood interrelatedness of the TGF-beta and TLR signaling pathways remains an important subject for future investigations.

Other Traf6-dependent infection induced genes, such as dram1 (DNA-damage regulated autophagy modulator 1) and gnrh2 (gonadotropin releasing hormone 2), have until now not been directly associated with TRAF6 function. Human homologs of dram1 are activated by p53 as a requirement to induce autophagy and damage-induced programmed cell death (56, 57). As recently highlighted in several studies, the autophagy pathway is also important for the control of intracellular pathogens and therefore the link with Traf6 function is relevant for Salmonella infection (58, 59). In the zebrafish embryo gnrh2 has been linked to central nervous system development (60). However, next to the well established function of GnRH in mammalian reproduction, an immune regulatory function was suggested as well (61, 62). In fact, Tanriverdi et al. have discussed that immune and reproductive function are intrinsically linked in a so-called hypothalamic-pituitary-gonadal axis (60). Recently it was shown that GnRH treatment of mice macrophages in vitro leads to elevated Ca2+ uptake and an impaired generation of NO and suppression of iNOS after LPS/INF-γ treatment (63). A function of Traf6 in Ca2+ homeostasis is suggested by the fact that there is an interference effect of traf6 knock-down and S. typhimurium in-
fection on *stanniocalcin* (*stc1*) regulation. The mammalian homolog of *stanniocalcin* is involved in inhibition of transendothelial migration of human macrophages and T-lymphocytes (64). In addition *stanniocalcin* was also shown to stimulate osteoblast differentiation in rat calvaria cells (65). In a broader sense, Traf6 is probably involved in other ion transport processes. For instance the induction of *chloride intracellular channel 2* (*clc2*) by Salmonella infection is highly dependent of Traf6 function. Furthermore, the negative regulation of *ATPase, Na+/K+ transporting, alpha 1a.3 polypeptide* (*atp1a1a.3*) during *S. typhimurium* infection is blocked by *traf6* knockdown.

In addition to the annotated genes we also have identified Traf6 targets of which no annotation could be derived either for zebrafish, mouse or human orthologs (Supplemental Tab. VI, Fig. 6). Even domain searches could not identify a possible function. Since the expression levels of some of these genes are both strongly affected by Salmonella induction at early time points (33) and are strongly dependent on Traf6 function, the further study of the function of these genes in the vertebrate immune system is of great interest.

Interestingly, we find the induction of several metabolic genes to be dependent on Traf6 function during Salmonella infection in one-day old embryos, suggesting a possible role in the immune response. For example, expression of *6-phosphofructo-2-kinase* (*pfkfb3*), *hexokinase 2* (*hk2*), and *diacylglycerol O-acyltransferase homolog 1b* (*dgat1b*) were induced during infection in a Traf6-dependent manner. Furthermore, the induction of an ortholog of STAP4 (*zgc:112143*), which has been shown to play a role in integration of inflammatory and metabolic responses, is also dependent on Traf6 (66). We previously found this gene also to be induced by mycobacterium infection in zebrafish (41). The expression trend analysis shows that *pfkb3*, *hk2* and *zgc:111243* cluster together with several of the above mentioned inflammatory genes such as *mmp9*, *mmp13*, *il1b* and *tnfb* (Fig. 5). Other metabolic functions might also play a role during infection since our analyses only show the minimal contribution of Traf6 to immunity. This is because functions that play an equally important role in immunity and development cannot confidently be analyzed in our method since we have titrated down the effect of the morpholino treatment to have a low effect on development.

It does not come as a surprise that after knock-down of Traf6 there is still a strong immune response to Salmonella infection, not only because the knock-down was incomplete but also because it can be expected that there are innate immune responses to Salmonella infection that are independent of Traf6, for example the chemotactic response to the bacterial infection site via G-protein coupled receptors. Furthermore, also within the TLR-dependent pathway there are possible signaling routes that might not be dependent on Traf6. For instance, the pathway of TLR4 signaling can lead to TRAF3 activation and subsequent stimulation of the interferon pathway via the IRF3 protein. It would therefore also be interesting to use our method to analyze other key factors such as TRAF3 and partners immediately downstream of the TRAF
family proteins such as TBK1 and TAB1/2/3. Such studies could show whether any of these factors might be partially redundant during the innate immune response.

**Materials and Methods**

**Bacterial strains and growth conditions**

*Salmonella typhimurium* wild type (wt) strain SL1027, containing the DsRed expression vector pGMDs3, was used for the infection of zebrafish embryos (22). Bacteria were freshly grown overnight on LB agar plates supplemented with 100μg/ml carbenicillin and resuspended in phosphate-buffered saline (PBS) prior to injection.

**Zebrafish husbandry**

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (http://ZFIN.org). Embryos were grown at 28.5 -30 °C in egg water (60μg/ml Instant Ocean sea salts). For the duration of bacterial injections embryos were kept under anaesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (tricaine, Sigma).

**Morpholino knock-down experiments**

For morpholino knockdown experiments, morpholino oligonucleotides (Gene Tools) were diluted to desired concentrations in 1x Danieu’s buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0 mM HEPES; pH 7.6] containing 1% Phenol red (Sigma). To block translation of *traf6* mRNA we injected 1 nl (1 mM) per embryo of a morpholino specifically targeting the 5’ UTR region including the start codon of *traf6* (5’ GCCATATTGGCTCGGTACGGCCTC). To control for aspecific morpholino effects we used a 5 bp mismatch morpholino (1mM, 5’ GCaATATTcGCTaGGTACaGCgTC).

**Experimental design of the infection study**

All infection experiments were performed using mixed egg clutches of ABxTL strain zebrafish. Embryos injected with the *traf6* morpholino and the 5bp mismatch morpholino were staged at 27 hours post fertilization (hpf) by morphological criteria and approximately 250 cfu of DsRed expressing *S. typhimurium* wild type bacteria were injected into the caudal vein close to the urogenital opening as described in Stockhammer et al. (33). As a control an equal volume of PBS was likewise injected. Pools of 20-40 infected and control embryos were collected 8 hours post infection (hpi). For the microarray analysis, the whole procedure was performed in triplicate on separate days.

**RNA extraction**

Embryos for RNA isolation were snap frozen in liquid nitrogen and subsequently
stored at -80°C. Total RNA from each sample was extracted using TRIZOL followed by a cleanup procedure with Rneasy Mini kit (Qiagen, Valencia, CA, USA), and a DNase treatment with RNase-Free DNase Set (Qiagen Valencia, CA, USA). The RNA concentration was measured on a nanodrop ND-100 (NanoDrop Technologies Inc., Wilmington, DE, USA) and RNA quality was checked on an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). Total RNA samples with an RNA integrity number (RIN) > 7 were used for further analysis. These assays were performed according to the manufacturer’s protocols.

**Illumina RNA sequencing**

The total RNA of the three biological samples of each treatment group, previously used for the microarray analysis, was pooled using equal amounts of RNA. To perform transcriptome sequencing, RNAseq libraries were made from 4 μg of each sample, using the Illumina mRNA-Seq Sample Preparation Kit according to the manufacturer’s instructions (Illumina, Inc. San Diego). An amount of 4 pmol of each library was sequenced in one lane with a read length of 51 nt on an Illumina GAIi instrument (Illumina, Inc. San Diego). The raw data were deposited in the GEO database under submission number GSE21024. Sequence reads were mapped to Ensembl transcripts (Zv8. 56) using the CLCbio Genomics Workbench version 3.6.5 (www.clcbio.com).

**Microarray design and hybridization**

A custom zebrafish genome 4 x 44 K microarray (Agilent) containing slight modifications in regard to a previous described design was used (accession no. GPL10042 in the GEO database) (33). In short, a total of 600 new features based on deep sequencing results were added to the existing chip design resulting in 45219 features, including 43801 well-characterized genes and 1418 controls (41). The probes of the custom manufactured Agilent array have been reannotated by mapping all probes to the Unigene 114 (unique) sequences and the Ensembl 50 and Vega 32 transcripts using the BLAST algorithm. Technical handling of the microarrays was performed at the MicroArray Department (MAD) of the University of Amsterdam (Amsterdam, The Netherlands). In short, cyanine 3 and cyanine 5 labeled cRNA samples were prepared as described in the Amino allyl message AMP II manual (Ambion) using 0.5 ug purified total RNA as template for the reaction. Test samples were labelled with Cy3 and the common reference was labeled with Cy5. The common reference was composed by combining 1 ug of cRNA from each sample and chemical coupling of this pool with Cy5. Hybridization of 825 ng of Cy3 labeled test sample and 825 ng of Cy5 labeled common reference was performed overnight according to Agilent protocols at 65° C. Images of the arrays were acquired using an Agilent DNA MicroArray Scanner (Agilent Technologies, Palo Alto, CA, USA).
**Data extraction and statistical analysis**

Spot intensities were quantified with Feature Extraction 9.5.1 (Agilent) as the foreground median signal intensity. Further processing of the data was performed using R (version 2.5.0), the Bioconductor MAANOVA package (version 1.6.0) (42) and Spotfire (version 7.3).

All slides were subjected to a set of quality control checks, i.e. visual inspection of the scans, examining the consistency among the replicated samples by principal components analysis, testing against criteria for signal to noise ratios, testing for consistent performance of the labeling dyes, and visual inspection of pre- and post-normalized data with box plots and RI plots.

The data set concerned a two-factorial Latin square design, with the factors ‘Morpholino treatment’ (2 levels: treated and not treated) and ‘Infection’ (2 levels: treated and not treated). The design was completely balanced with 3 replicates each, so the experiment involved 12 observations per gene.

After log2 transformation the data was normalized by a global LOWESS smoothing procedure. The data was analyzed using a two-stage mixed analysis of variance (ANOVA) model (43). First, array, dye, and array-by-dye effects were modeled globally. Next, the residuals from this first model were fed into a gene-by-gene model in which we took ‘Group’, ‘Array’, and ‘Dye’ as factors of which ‘Array’ was modeled as random factor. ‘Group’ is defined by each unique Morpholino and Infection treatment combination. These residuals can be considered normalized expression values and used in the graphs to depict gene expression profiles. All changes were calculated from the model coefficients. For hypothesis testing a permutation based F test, which allows relaxation of the assumption that the data are normally distributed, was used (2,000 permutations). The significance of the differences between factor level means was tested using contrasts. To account for multiple testing, all P values were adjusted to represent a false discovery rate using the method of Benjamini and Hochberg (44). The raw data were submitted to the GEO database under accession number GSE20310.

**Gene Ontology, pathway and cluster analysis**

K-means clustering was performed using Spotfire (version 7.3) Cluster initialization was set to data centroid based search and similarity measure was set to Euclidian distance. Analysis was performed on the probes retrieved by interaction term analysis with a p-value lower than or equal to 0.4. All identifiers lacking a valid annotation were excluded from the analysis leading to a dataset of 376 probes.

Gene ontology (GO) analysis was performed using the GeneTools eGOn v2.0 web-based gene ontology analysis software (www.genetools.microarray.ntnu.no) (45). Master-target analysis was performed at the level of Unigene clusters (UniGene build #105). To test for enrichment or under representation at the level of GO criteria for Biological Process (BP) we compared the UniGene identifiers retrieved from
our analysis (targets) to all identifiers present on the chip (master). Identifiers tested are listed in supplemental Table I and IV for those genes that were regulated after infection in the control and \textit{traf6} knock-down group as well as due to \textit{traf6} knock-down alone. All identifiers that were retrieved from the interaction term analysis are listed in supplemental Table VI.

Pathway analysis was performed using the GenMapp software package (www.genmap.org) (46). Analysis was done at the level of UniGene clusters (\textit{D.rerio} UniGene build #114). Significance cut-off was set at 1.2 fold change at P <0.05. Zebrafish homologs of the genes contributing to the TLR pathway were identified by either searching the ZFIN (http://zfin.org) database or the Gene and HomoloGene database of NCBI (http://www.ncbi.nlm.nih.gov) (supplemental Table II).

The following link has been created to allow review of record GSE21024:
The following link has been created to allow review of record GSE20310:

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\textbf{Supplementary Data}

Supplementary tables can be found online at: http://www.mediafire.com/?sharekey=686efof919604e919bf8d6369220dcab43aeefabc95fc71da7b01fe6e4055ae3
Traf6 function in zebrafish embryonic innate immune response

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Chapter 4

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