

5 | Transcriptome analysis of Traf6 function in early zebrafish embryogenesis

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Abstract

TRAF6 is an essential signal transduction factor that not only mediates signals emanating from the TNF-receptor family but also from the TLR/IL-1 receptors and the TGF-beta receptors. In order to analyse the function of Traf6 during early zebrafish embryogenesis, transcriptome analysis was performed at 30% epiboly using a morpholino based knock-down approach. This approach turned out to be very challenging since non-specific effects of control morpholinos also appeared to trigger many responses at the transcriptome level. However, using different morpholinos directed against *traf6* mRNA and by comparisons to two control morpholinos we were able to identify a large gene set that is specifically controlled by Traf6 during embryogenesis. Using GO and pathway analyses we were able to give new insights into the diverse functions of Traf6. We compared the gene set that we found to be dependent on Traf6 during early embryogenesis with a previously identified set of genes dependent on Traf6 in the context of infection of one-day-old embryos and found that only a set of 14 genes was overlapping. This limited overlap shows that the function of Traf6 is modulated from a control factor of developmental genes to a control factor of a largely different set of genes involved in functions in infectious disease. In addition, we can conclude that there is a general effect of all morpholino injections on *tlr3* expression that is not dependent on Traf6. In contrast, there is a response of *tlr4a*, *tlr4b* and *tlr9* to morpholino injections in general that appears to be dependent on Traf6. These results prompt further investigations into the function of Traf6 in mediating responses to immunogenic stimuli at very early stages of embryogenesis.

Introduction

The members of the tumor necrosis factor receptor-associated factor (TRAF) family are essential signal transduction proteins that play a role in a wide range of physiological processes ranging from cell growth to immune responses and to programmed cell death. They were originally discovered as adapter proteins that mediate signals emanating from the TNF-receptor family. To date, seven members, named TRAF1 to TRAF7, have been identified in mammals (1, 2). All TRAF proteins are characterised by a TRAF domain at the carboxyl-terminus of the protein that can be further subdivided into a highly conserved TRAF-C domain and a less conserved coiled-coil domain referred to as TRAF-N domain. In addition all TRAF proteins, with the exception of TRAF1, contain a RING finger domain and several zinc finger motifs at the N-terminus. Self-association and interaction with TNF-receptors and other adaptor proteins is mediated by the TRAF-domain whereas downstream signalling is relying on the conserved domains of the N-terminus (2).

Among all members of the TRAF-family, TRAF6 holds a unique position, as TRAF6 is not only mediating signals emanating from the TNF-receptor family but

also from the TLR/IL-1 receptors and the TGF-beta receptors, eventually leading to the activation of the AP-1 and NF- κ B transcription factor complexes (3, 4). Analysis of TRAF6 function by gene knock-down studies in mice revealed a critical role of TRAF6 in lymph node and exocrine gland organogenesis as well as in apoptosis and osteoclast development and function (5-8). TRAF6 was shown to bind to the receptor activator of NF- κ B (RANK) and upon stimulation of RANK by the associated ligand RANKL, TRAF6 forms a complex with TAB1, TAB2 and TAK1, eventually leading to the activation of NF- κ B, JNK and p38, thereby promoting osteoclast differentiation, function and survival (9-11). Similarly, following TLR receptor activation, TRAF6 also activates NF- κ B through TAB1-TAB2-TAK1 complex formation. Furthermore, it was demonstrated that JNK and p38 activation is also mediated by TRAF6 upon activation of the TGF-beta receptor as well as the TLR-pathways (3, 12, 13). To activate JNK and p38 after TLR stimulation TRAF6 forms a complex with the protein ECSIT (12). In mouse embryonal carcinoma (EC) cells ECSIT was shown to be involved in BMP signalling by forming complexes with nuclear Smads on the *Tlx2* enhancer (13). TGF-beta signalling via TRAF6 was shown to be Smad-independent. However, whether TRAF6 is also involved in Smad-dependent BMP signalling events via ECSIT is still unknown. Recently it was demonstrated that TRAF6 also functions as a transcriptional cofactor in osteoclasts promoting FHL2 gene expression, further demonstrating the diverse functions of TRAF6 (14).

The zebrafish embryo is an extensively used model organism to study the molecular processes underlying vertebrate developmental, organogenesis and immune function. We could previously demonstrate the role of *Traf6* in the immune response towards a bacterial infection of zebrafish embryos (Chapter 4). Initial experiments of morpholino mediated *traf6* knock-down revealed developmental defects during zebrafish embryogenesis. To investigate the role of *Traf6* in early zebrafish development and to elucidate the implication of *traf6* knock-down on the transcription profile we performed a microarray based transcriptome analysis during early zebrafish embryogenesis. By using several control morpholino experiments we could show that *Traf6* directs the transcription of a large set of genes during early embryogenesis. These sets were further characterized using ontology and pathway analyses, showing that *Traf6* has a role in many fundamental biological processes. We have also compared our results with the infection data obtained in chapter 4.

Results

Phenotypic alterations after *traf6* knock-down

For functional analysis of *traf6* in zebrafish embryogenesis we designed two non-overlapping translation-blocking morpholinos targeted against the 5' UTR of the *traf6* transcript and tested these in at doses of 0.5 mM, 1 mM and 2 mM. Phenotypic effects of *traf6* knock-down at 30% epiboly were only observed after *traf6*-mor-

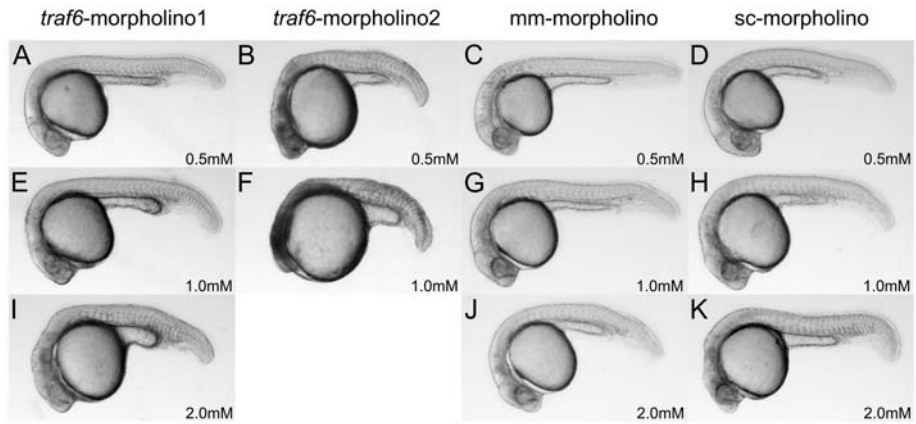


FIGURE 1. Overview of representative phenotypes upon morpholino treatment. Zebrafish embryos were injected with two translation-blocking morpholinos specifically targeting *traf6* (*traf6*-morpholino1 and 2) and two control morpholinos; 5bp mismatch (*mm*) and standard control-morpholino (*sc*) (A-K). (A, E, I) Morphology of zebrafish embryos injected with 0.5 mM, 1 mM and 2 mM of *traf6*-morpholino-1 and (B, F) *traf6*-morpholino-2 at 24hpf. Injection of 2 mM *traf6*-morpholino-2 resulted in 100% lethality at 24hpf. (C, G, J) Morphology of *mm*-morpholino injected embryos and (D, H, K) *sc*-morpholino injected embryos at 24 hpf. Zebrafish embryos are shown in lateral view with the anterior side to the left and dorsal to the top.

pholino-2 injection at the highest concentration. At this stage we observed a delay of at least 2 hours in epiboly progression as compared to normal development. However, at 24 hpf phenotypic changes were observed with both morpholinos as shown in figure 1. The results show that injection with *traf6*-morpholino-2 results in high mortality at the 2 mM concentration and shortening of the anterior-posterior (AP) axis and strong necrosis in the developing nervous system at the lower concentrations (Fig. 1 B, F). With *traf6*-morpholino-1 we observed a shortening of the AP axis at 1 and 2 mM (Fig. 1 E, I). We have also designed a control morpholino that contains 5bp mismatches (*mm*) as compared to *traf6*-morpholino-1. We furthermore tested a standard control morpholino (*sc*) that has previously been used in other studies and which is assumed not to target zebrafish genes (20). Control experiments with these morpholinos at various concentrations did not show phenotypes at 24 hpf that were comparable to the observed effects of *traf6*-morpholinos-1 and -2 treatment at 0.5 and 1 mM concentrations (Fig. 1 C, D, G, H). However, at 2 mM concentration necrosis in the head area and malformation of the somites posterior to the yolk sac extension was observed (Fig. 1 J, K)

Transcriptome analysis of Traf6 knock-down compared to control experiments

As studies in human and mammalian models have shown TRAF6 to be involved as

Transcriptome analysis of Traf6 function in early zebrafish embryogenesis

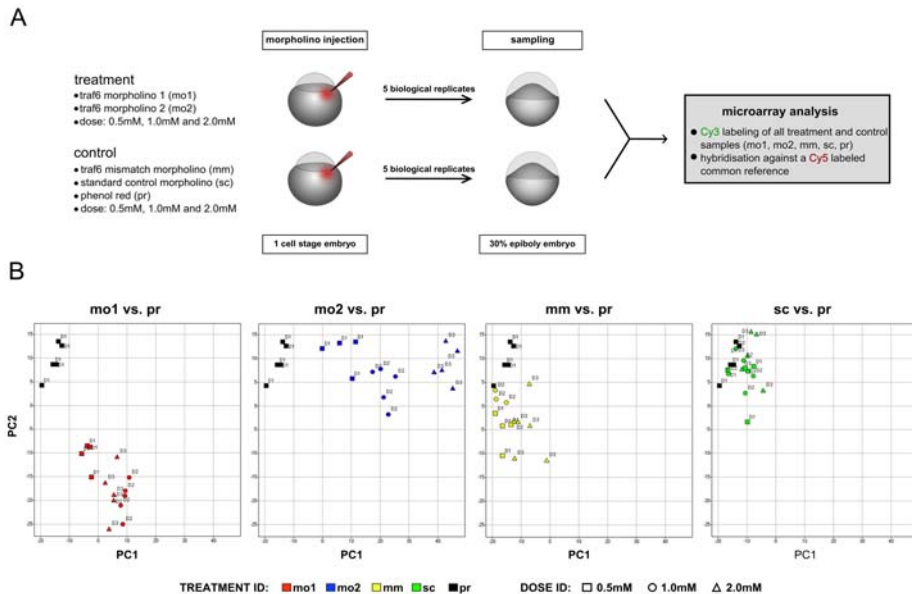


FIGURE 2. Schematic overview of the experimental setup (A) and principal component analysis (PCA) of the transcriptome responses (B). (A) Zebrafish embryos were injected at the one cell stage with two morpholinos specifically targeting *traf6* mRNA indicated as *traf6*-morpholino₁ and *traf6*-morpholino₂ respectively (treatment). In addition a 5bp mismatch morpholino (mm) and a standard control morpholino (sc) were injected to control for general morpholino effects and phenol red (pr) as a control for the injection process in general. The transcriptional response was analysed at 30% epiboly using a common reference approach. The experiment was carried out in quintuplicate. (B) Transcriptome responses after morpholino and control treatment were analysed by PCA.

a signal transducer in various pathways, the observed knock-down phenotypes are hard to investigate with *in situ* gene pattern analyses methods. In order to get an overview of the signal transduction processes that are affected by *traf6* knock-down we decided to perform transcriptome expression profiling using custom-designed Agilent micro-arrays containing 43,371 probes. As above, zebrafish embryos at the one cell stage were injected with *traf6*-morpholinos-1 and -2 and with the mismatch and standard control morpholinos at concentrations of 0.5, 1, and 2 mM. In addition we also injected Phenol red as a general control for the injection per se (Fig.2 A). The assay was performed in biological quintuplicate and analysis of the transcriptome took place at 30% epiboly to minimise the accumulation of secondary effects caused by *traf6* knock-down. Principal component analysis (PCA) demonstrated good separation of the biological replicates of the two specific *traf6*-morpholinos (*traf6*-morpholino-1, *traf6*-morpholino-2) along the concentration range (Fig.2 B). Treatments with *traf6*-morpholino-2 showed a good correlation between the strength of response and the increase of the treatment dose whereas for *traf6*-morpholino-1 we found the

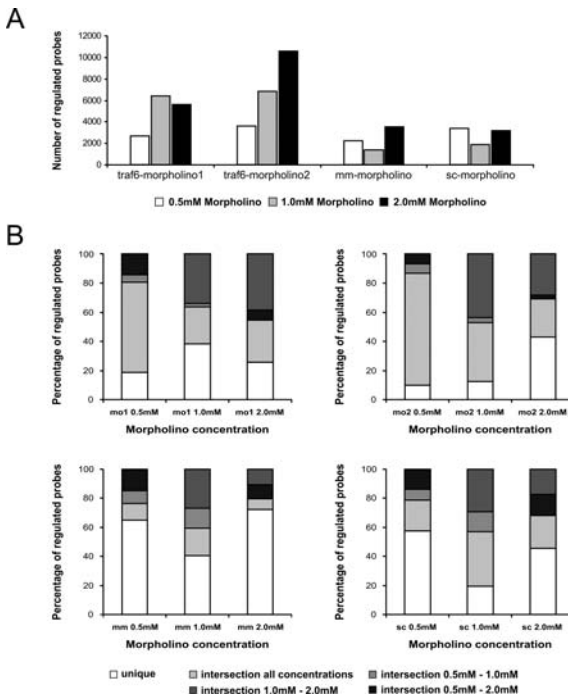


FIGURE 3. Analysis of the transcriptional response. (A) Bar graph representation of the dose-response relationship after *traf6*- and control morpholino treatment. The transcriptional response of *traf6*- and control morpholino treatment groups was analysed against the phenol red transcriptome response. Significance cut-off was set to $p < 0.05$. To account for multiple testing, all P values were FDR adjusted. (B) Illustration of relative overlap in each treatment group along the concentration range. mo1, *traf6*-morpholino1; mo2, *traf6*-morpholino2; mm, 5bp mismatch morpholino; sc, standard control morpholino treatment

strongest effect to be elicited at a concentration of 1 mM. In contrast, only a poor separation was shown for the 5bp mismatch morpholino and no separation for the standard control morpholino, indicating a concentration independent effect at that stage. For the control morpholino treatments, in particular for the sc-morpholino treated group, we observed less deviation from the phenol red treatments.

In agreement with the results of the PCA analysis we found an increasing number of probes in the signature set of *traf6*-morpholino-2 treatment, ranging from 3578 at 0.5 mM up to 10539 at 2 mM (Fig.3 A, Supplemental Tab. I). The strongest response after *traf6*-morpholino-1 treatment was observed at a dose of 1 mM with a total of 6358 probes in the signature set. At a dose of 0.5 mM the signature set contained 2624 regulated probes whereas it contained 5623 probes at a dose of 2 mM. The two control groups were very similar to each other regarding the number of regulated genes and the distribution over the concentration range. In both cases we observed a decrease in the number of regulated genes at 1 mM, followed by an increase at the 2 mM dose (Fig.3 A).

In order to define a *traf6* knock-down specific transcription profile we first identified subsets of the individual treatment groups that were consistently regulated (FDR adjusted P-value < 0.05 for at least two concentrations) along the concentration range (Fig.3 B). Analysis of the *traf6*-morpholino treatment groups demonstrated that a high percentage of the probes were overlapping along the concentration range, indicating a specific effect of the morpholinos. For *traf6*-morpholino-2 treatment we

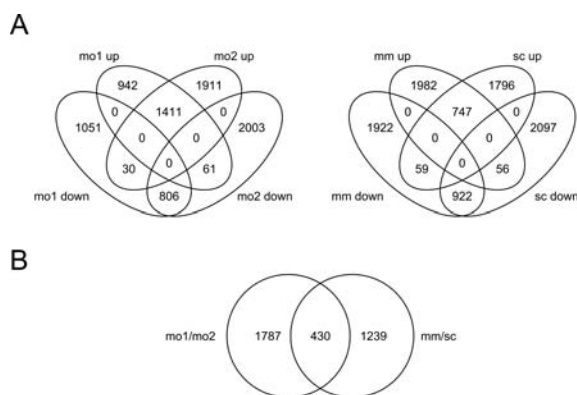


FIGURE 4. Identification of specific *traf6* knock-down and general morpholino effects. (A) Upper Venn diagram illustrates the overlap of the response signatures between *traf6* specific morpholino-1 and -2. Lower Venn diagram illustrates the overlap between the mm- and sc-morpholino response signatures. Overlap of *traf6* specific morpholino-1 and -2 response signature was defined for all

probes that were significantly regulated ($p < 0.05$) in at least 2 out of 3 concentrations. Overlap of mm- and sc-morpholino response signature was defined in a less stringent way by taking all probes that were significant in at least one concentration of each treatment. (B) Identification of *traf6* knock-down specific expression profile and general morpholino response profile. mo1, *traf6*-morpholino1; mo2, *traf6*-morpholino2; mm, 5bp mismatch morpholino; sc, standard control morpholino treatment; mo1/mo2, overlap *traf6*-morpholino1 and -2 response ; mm/sc, overlap mm- and sc-morpholino response

found an overlap ranging from a total of 90% of all significantly regulated probes at 0.5 mM over 88% at 1 mM down to 57% at 2 mM. In the case of *traf6*-morpholino-1 treatment the smallest overlap was observed at 1 mM with 62% and the maximum at 0.5 mM with 81% of all regulated probes. For both treatments we furthermore observed that the expression of large probe sets was affected by the morpholino treatment at all concentrations: 1628 probes for *traf6*-morpholino-1 and 2760 probes for *traf6*-morpholino-2 (Supplemental Tab. II and III). An overall much weaker response was observed for the control groups and there was also considerable smaller percentage of regulated probes overlapping along the concentration range. In the mm-morpholino as well as the sc-morpholino treated groups the highest overlap was present at the 1 mM dose reaching 60% after mm-morpholino treatment and 80% upon sc-morpholino treatment. However, a substantially smaller overlap was evident at 0.5 mM and 2 mM in both cases, ranging between 35 and 55%. Furthermore, the expression of only a small probe set of 260 for the mm-morpholino group and 706 for the sc-morpholino group was consistently changed after treatment with each concentration of morpholinos (Supplemental Tab. IV and V).

Definition of specific sets for *traf6* knock-down and control effects

We specified the common signature of *traf6*-morpholino1 and 2 by determining the overlap of the above described subsets, i.e. the number of probes consistently changed in the same direction for both morpholinos and at least two concentrations for each morpholino (Fig. 3 B). We found a total of 1411 probes to be commonly up-regulated

Table I. Master-target test of GO analysis for Biological Process of the overlap between mismatch and standard control morpholino*

GO-term	Name	MASTER	UP	DOWN
GO:0008150	biological_process	5265	136	158
GO:0065007	biological regulation	1009	24	32
GO:0009987	cellular process	3042	79	108
GO:0051301	cell division	42	1	4
GO:0044237	cellular metabolic process	2053	51	76
GO:0032502	developmental process	742	22	29
GO:0009790	embryonic development	217	9	12
GO:0007389	pattern specification process	133	6	10
GO:0051234	establishment of localization	574	15	13
GO:0040007	growth	53	0	2
GO:0002376	immune system process	92	2	6
GO:0006955	immune response	55	1	5
GO:0051179	localization	648	18	15
GO:0051641	cellular localization	145	8	3
GO:0008152	metabolic process	2402	60	90
GO:0009058	biosynthetic process	1041	21	42
GO:0044237	cellular metabolic process	2053	51	76
GO:0044238	primary metabolic process	2022	49	74
GO:0051704	multi-organism process	24	0	2
GO:0032501	multicellular organismal process	714	18	29
GO:0048519	negative regulation of biological process	98	5	4
GO:0043473	pigmentation	11	1	1
GO:0048518	positive regulation of biological process	89	2	1
GO:0050789	regulation of biological process	922	23	30
GO:0000003	reproduction	21	0	1
GO:0050896	response to stimulus	282	8	11
GO:0006955	immune response	55	1	5

* A master-target statistical test using eGOn software was performed with input gene lists of zebrafish UniGene identifiers. The master input lists contained all UniGene identifiers present on the microarray. The target lists contained the UniGene identifiers that were retrieved by the intersection analysis of the mismatch and standard-control morpholino response signatures with an FDR adjusted P-value lower than 0.05. The table indicates the number of genes in each list that are associated with the indicated GO-terms. Numbers highlighted in grey are significantly enriched in the target list compared to the master whereas all numbers indicated in black demonstrated a significant underrepresentation in the target list compared to the master ($p < 0.05$).

upon *traf6*-morpholino treatment and 806 to be commonly down-regulated (Fig.4 A). The response of a small group of 91 probes was anti-correlated (Supplemental Tab. VI). In addition, we were interested in a probe set that was responsive both to the mismatch and standard-control morpholinos, reasoning that this set would most likely characterize a general response to morpholino treatments. Taking into consideration the low consistency of the control groups along the concentration range, and the therefore largely dose-independent response, we decided to take a less restrictive selection criterion than for the comparison between *traf6*-morpholinos and compared all probes that were significantly (FDR adjusted P-value < 0.05) regulated by the mismatch and standard control morpholinos at minimally one of the applied doses. A total of 747 commonly up-regulated probes and 922 commonly down-regulated probes were found (Fig.4 A). In addition we observed 115 anti-correlated probes (Supplemental Tab. VII). In a next step we determined the overlap between the common *traf6* knock-down and control signature and subtracted this set, leading to a *traf6* knock-down signature of 1787 regulated probes representing 868 genes (Fig. 4B, supplemental Tab. VIII).

Table II. Master-target test of GO analysis for Biological Process for the overlap between traf6 morpholino1 and z*

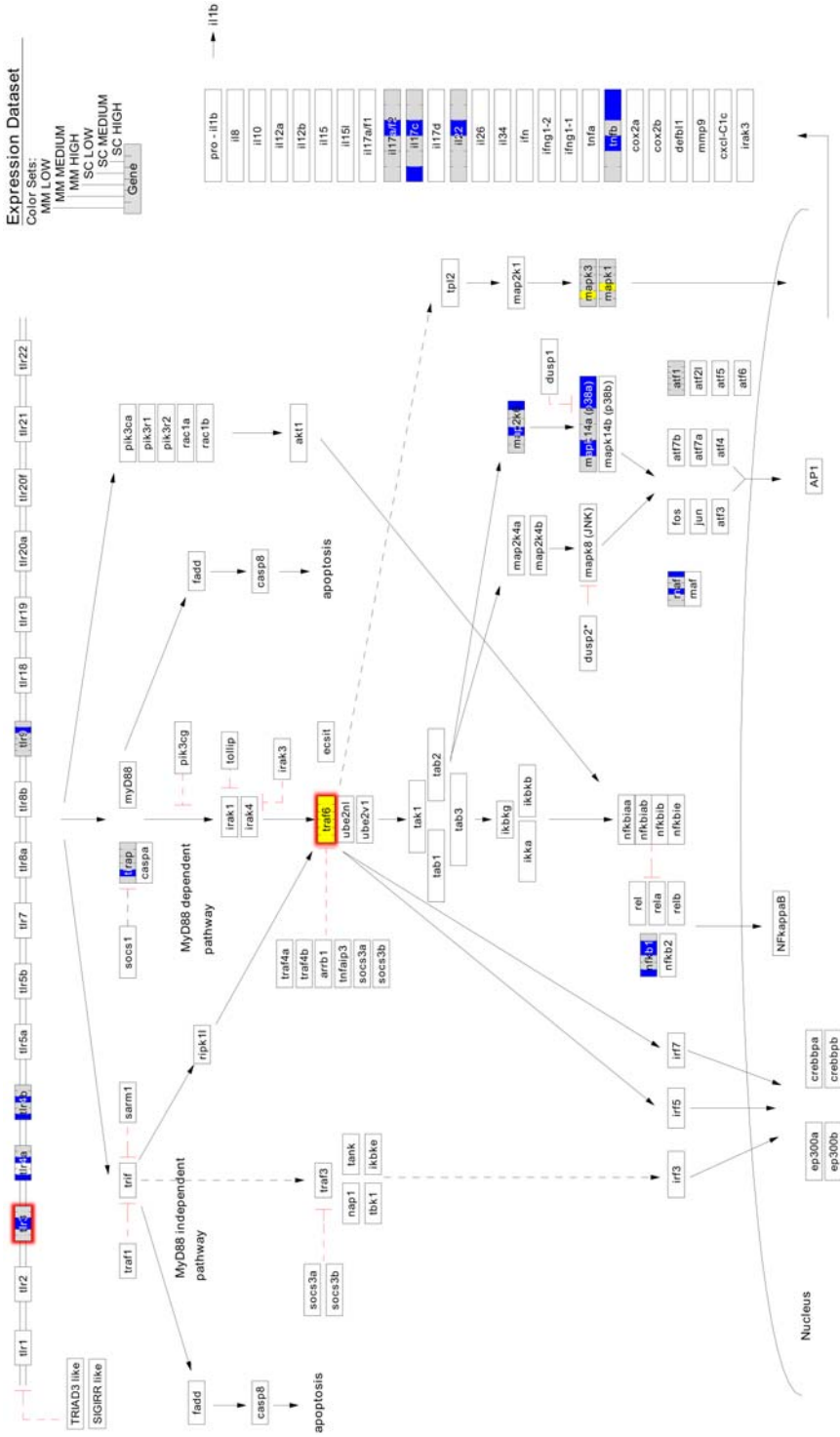
GO-term	Name	master	UP	DOWN
GO:0008150	biological_process	5265	286	116
GO:0022610	biological adhesion	83	2	1
GO:0065007	biological regulation	1009	47	19
GO:0009987	cellular process	3042	151	75
GO:0010467	gene expression	921	29	23
GO:0050794	regulation of cellular process	864	35	17
GO:0032502	developmental process	742	30	19
GO:0048856	anatomical structure development	506	16	10
GO:0016265	death	81	10	4
GO:0007275	multicellular organismal development	648	19	14
GO:0051234	establishment of localization	574	40	9
GO:0040007	growth	53	1	1
GO:0002376	immune system process	92	1	2
GO:0051179	localization	648	43	10
GO:0008152	metabolic process	2402	129	57
GO:0009056	catabolic process	139	17	5
GO:0019222	regulation of metabolic process	711	26	13
GO:0051704	multi-organism process	24	0	0
GO:0032501	multicellular organismal process	714	20	15
GO:0007275	multicellular organismal development	648	19	14
GO:0048519	negative regulation of biological process	98	2	1
GO:0043473	pigmentation	11	1	0
GO:0048518	positive regulation of biological process	89	5	3
GO:0050789	regulation of biological process	922	39	18
GO:0000003	reproduction	21	1	2
GO:0019953	sexual reproduction	14	1	2

* A master-target statistical test using eGOn software was performed with input gene lists of zebrafish UniGene identifiers. The master input lists contained all UniGene identifiers present on the microarray. The target lists contained the UniGene identifiers that were retrieved by the intersection analysis of the traf6-morpholino1 and 2 response signatures with an FDR adjusted P-value lower than 0.05. The table indicates the number of genes in each list that are associated with the indicated GO-terms. Numbers highlighted in grey are significantly enriched in the target list compared to the master whereas all numbers indicated in black demonstrated a significant underrepresentation in the target list compared to the master ($p < 0.05$).

Ontology and pathway analysis of general morpholino effects

We initially tested the control signature for enrichment of gene ontology groups using eGOn, a web-based data mining tool for transcriptome data (www.genetools.micrarray.ntnu.no) (18). The analysis revealed several enriched GO terms in the down-regulated fraction, for instance “embryonic development”, “pattern specification process” and “metabolic process” (Tab. I). Interestingly, we also found the GO term “immune response” to be significantly enriched in the control group, comprising genes of various TLRs such as *tlr3*, *tlr4a*, *tlr4b* and *tlr9*.

To analyse the general morpholino effect on the TLR-pathway in more detail we used the GenMapp software package (genmapp.org) (19). Analysis of the TLR-pathway revealed several more members to be regulated (Fig.5). Beside the Toll-like receptors we also found *toll-interleukin 1 receptor (TIR) domain containing adaptor protein (tirap)*, one of the TLR adaptor proteins, as well as *nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (nfkb1)* and several mitogen activated protein kinases (MAPK). Interestingly *traf6* was up-regulated in the control and *traf6*-morpholino treatments suggesting that *traf6* is induced by morpholino treatments



in general.

Ontology and pathway analysis of *traf6* knock-down signatures

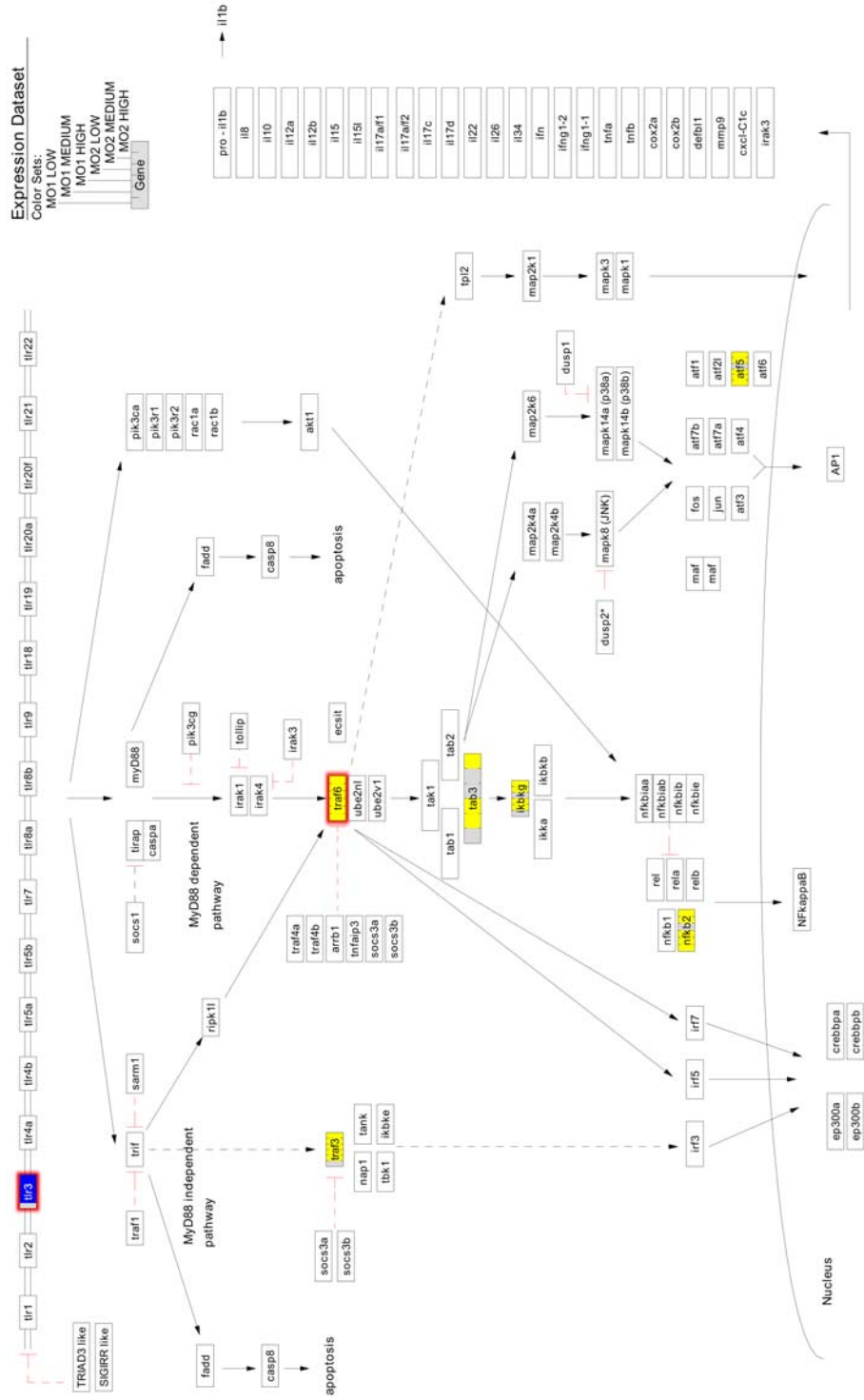
Analysis of the *traf6* knock-down signature with eGOn revealed the GO terms “death” and “catabolic process” to be significantly enriched in the up-regulated fraction (Tab. II). Under the “death” category we found such genes as the serine/threonine kinase *ripk2* and members of the BCL-2 protein family (*bad*, *bokb* and *bax*), all promoting programmed cell death. In agreement with this notion we also found genes that are counteracting programmed cell death down-regulated. For instance *baculoviral IAP repeat-containing 2 (birc2)*. Additionally we found GO-terms such as “gene expression” and “multicellular organismal development” showing significant underrepresentation in the up-regulated fraction.

Traf6 plays a pivotal role in the signal transduction of the TLR-pathway. We were therefore interested in the effect of *traf6* knock-down on members of this pathway. Projection of the *traf6* knock-down signature demonstrated an induction of *traf3* upon *traf6* knock-down (Fig. 6). Furthermore, the zebrafish homolog of TAB3, an interacting partner of TRAF6, was up-regulated. Likewise the regulatory subunit of the IKK-complex, *nemo (ikbkg)*, was induced after *traf6* knock-down. NEMO was shown to be an ubiquitination target of TRAF6 in mice fibroblast cells and ubiquitination of NEMO by TRAF6 is involved in IL-1 mediated activation of NF- κ B (21). Furthermore, the transcription factors *nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (nfbk2)* and *activating transcription factor 5 (aft5)* were induced upon *traf6* knock-down. We also performed pathway analysis of the TGF beta pathway. The results (not shown) showed only one gene in this pathway, namely *transforming growth factor, beta receptor II (tgfbr2)*, to be up-regulated after *traf6* knock-down and not after control morpholino treatments.

Traf6 dependent genes in early embryogenesis and immunity

We previously indentified a set of genes that was dependent on Traf6 function in the context of a bacterial challenge (Chapter 4). To clarify whether there are genes that

FIGURE 5. Toll-like receptor (TLR) pathway analysis of the transcriptional effect that was elicited by morpholino treatment in general. Genes that were regulated due to the general morpholino effect were mapped on the TLR pathway. Gene boxes are colour coded from left to right with 0.5 mM (LOW), 1 mM (MEDIUM) and 2 mM (HIGH) expression data of the mm-morpholino (MM) and the standard control morpholino (SC). Up-regulation is indicated in yellow, down-regulation in blue. Genes that failed the fold-change cut off (>1.2 and <-1.2) were indicated in grey. White denotes genes that were not passing the significance cut-off value (FDR adjusted P-value <0.05) or were not represented on the array platform. 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.



were dependent on Traf6 function during early embryogenesis and immune defence we compared the Traf6 dependent infection signature with the *traf6* knock-down signature of this study. Furthermore, we included a previously identified embryonic infection response signature in the analysis (Chapter 4). We identified 628 common regulated probes between the early embryonic *traf6* knock-down signature and the embryonic infection signature (Fig. 7, Supplemental Tab. IX). We classified the commonly shared probes into common up- and down-regulated and anti-correlated expressed probe sets and subsequently tested for enrichment of gene ontology groups using eGOn (www.genetools.microarray.ntnu.no). We found the GO terms “death” and “regulation of localization” to be significantly enriched in the common up-regulated group (Supplemental Tab. X.). In the shared down-regulated fraction we could identify GO terms linked to metabolic processes like “macromolecule metabolic process” and “catabolic processes” as significantly enriched. Probes that were up-regulated in the early embryonic knock-down signature and down-regulated in the embryonic infection signature were enriched for the GO terms “cell cycle” and “cell division”.

In addition we found 15 probes (14 genes) to be regulated in all three signatures and 1 (*coproporphyrinogen oxidase, cpox*) to be common between the early embryonic *traf6* knock-down signature and the Traf6 dependent infection signature (Tab. III). The probes that were regulated in all three signatures in common contained genes such as *DNA-damage regulated autophagy modulator 1 (dram1)*, which was shown to be activated by p53 and promotes p53 induced autophagy as well as *rad17*, important for DNA-damage-induced cell cycle G2 arrest (22). Another gene of this group was *stx11a*, a homolog of the human *SYNTAXIN11* gene. SYNTAXIN11 was demonstrated to play a role in phagocytosis in human macrophages (23).

Discussion

As shown in chapter 4 the Traf6 protein is an important signal transduction protein in the innate immune system of vertebrates. In addition TRAF6 plays a role in various cellular developmental processes in mice (6, 7, 14). In this chapter it is shown for the first time that Traf6 is also involved in the regulation of a large gene set during early embryogenesis. In order to analyse the function of Traf6, transcriptome

FIGURE 6. Toll-like receptor (TLR) pathway analysis of the specific *traf6* knock-down effect. Traf6 knock-down specific expression profiles were mapped on the TLR pathway. Gene boxes are colour coded for 0.5 mM (LOW), 1 mM (MEDIUM) and 2 mM (HIGH) of the *traf6*-morpholino1 (M1) and *traf6*-morpholino2 (M2) expression data as described in figure 5. 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.

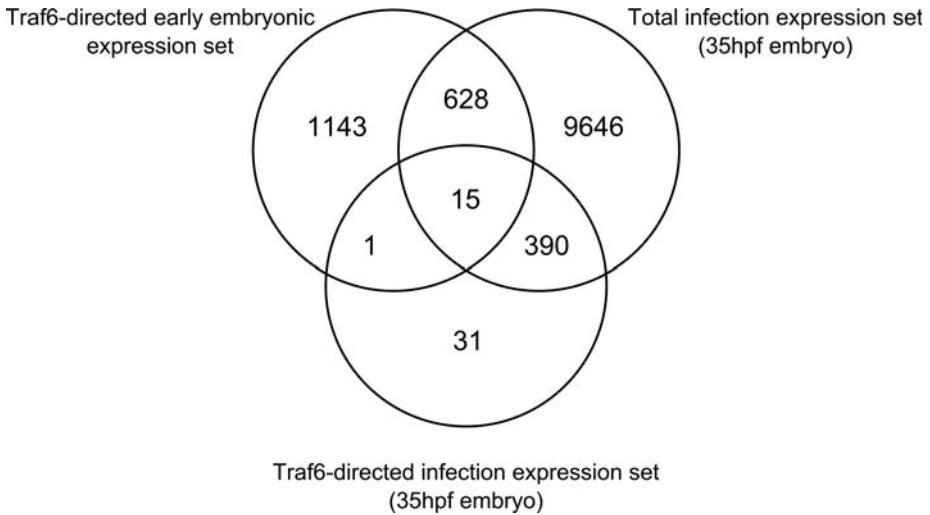


FIGURE 7. Traf6-controlled genes in early embryogenesis and embryonic immune response. Traf6-controlled probes in early embryogenesis were compared to a total infection expression set and to a Traf6-dependent infection expression set. Total and Traf6-controlled expression sets were retrieved from a previously performed Salmonella infection assay (Chapter 4). In short, *traf6* knock-down and mm-morpholino treated embryos were infected with Salmonella at 27 hpf and the infection response was analysed after an incubation time of 8 hours. The Traf6-dependent infection expression set was retrieved by interaction term analysis.

analysis at 30% epiboly was performed using a morpholino based knock-down approach. This approach turned out to be very challenging since non-specific effects of control morpholinos also appeared to trigger many responses at the transcriptome level. However, using different morpholinos directed against *traf6* mRNA and by comparisons to two control morpholinos we were able to identify a large gene set that is specifically controlled by Traf6 during embryogenesis. Using GO and pathway analyses we were able to give new insights into the diverse functions of Traf6 in transcriptional control.

Knock-down of *traf6* leads to a strongly altered transcriptome profile with each of the tested morpholinos, even at concentrations as low as 0.5 mM. Apparently the response at the transcriptome level at 30% epiboly is far stronger than the observed morphological alterations at later stages would suggest (Fig. 1). For instance, injection of *traf6*-morpholino-1 at a concentration of 0.5 mM led to no detectable morphological changes at 1 dpf, whereas a significant change of expression of 2624 probes was observed at the 30% epiboly stage. Although the two morpholinos were very different in their dose response at the morphological level at 1 dpf, they showed strong similarity in the response at the transcriptome level at 30% epiboly. We could identify a stringent overlap set of the effects of the two morpholinos of 868 genes

Table III. Traf6 controlled genes in early embryogenesis and embryonic immune response*

Probe ID	UniGene ID	Symbol	Description	traf6 k.d.		
				early embryogenesis (fc)	immune response (fc)	
LOLS08715	Dr.11530	zgc:152863	zgc:152863	1.5, 1.7, 1.8; 1.4, 2.1, 3.1	-1.4	n.s.
LOLS06846	Dr.13838	zgc:153057	zgc:153057	-1.2, -1.2, -1.2; -1.1, -1.2, -1.3	2.2	1.5
A_15_P115394	Dr.31220	rad17	RAD17 homolog (S. pombe)	1.3, 1.6, 1.6; 1.1, 1.2, 1.4	-1.4	-1.1
LOLS07060	Dr.75603	h3f3a	H3 histone, family 3A	-1.2, -1.5, -1.5; -1.5, -1.9, -3.1	-1.2	n.s.
A_15_P113277	Dr.7614	slc25a25	solute carrier family 25	1.3, 1.8, 1.6; 1.3, 1.7, 2.2	1.6	n.s.
A_15_P110827	Dr.77486	lass5	LAG1 homolog, ceramide synthase 5	1.0, 1.2, 1.3; 1.1, 1.2, 1.3	1.1	n.s.
A_15_P111539	Dr.77501	dram1	DNA-damage regulated autophagy modulator 1	-1.2, -2, -1.6; -1.4, -1.9, -2.9	2.2	n.s.
A_15_P103389	Dr.78554	cpox	coproporphyrinogen oxidase	1.2, 1.4, 1.5; 1.1, 1.3, 1.6	n.s.	-1.5
LOLS07574	Dr.79180	srpr	signal recognition particle receptor (docking protein)	-1.3, -1.3, -1.3; -1.2, -1.2, -1.1	1.5	n.s.
A_15_P114256	Dr.81309	psme1	proteasome activator subunit 1	-1.1, -1.4, -1.6; -1.2, -1.6, -2.2	5.1	2.1
LOLS00214	Dr.81309	psme1	proteasome activator subunit 1	-1.2, -1.5, -1.5; -1.2, -1.7, -2.3	3.9	1.8
LOLS01729	Dr.83170	stx11a	syntaxin 11a	1.3, 1.4, 1.3; 1.1, 1.4, 1.6	2.5	1.6
A_15_P101112	Dr.90156	f3b	coagulation factor IIIb	1.4, 1.6, 1.4; 1.2, 1.5, 2.0	2.8	1.7
A_15_P113317	n.a.	n.a.	n.a.	-1.2, -1.2, -1.3; -1.2, -1.2, -1.1	1.5	n.s.
LOLS09680	n.a.	n.a.	n.a.	-1.1, -1.3, -1.2; -1.2, -1.2, -1.3	1.4	n.s.
A_15_P113108	n.a.	n.a.	n.a.	1.2, 1.2, 1.2; 1.0, 1.2, 1.3	3.9	2.4

* For *traf6* knock-down during early embryogenesis the fold-changes (fc) are indicated for mo1 and mo2 with increasing concentrations (0.5mM - 2mM); probes that were not significantly changed are indicated as n.s. (p-value > 0.05)

after correction for general morpholino effects (Fig. 4). The ontology analyses of these genes, as shown in the results (Tab. I), indicate diverse functions ranging from catabolic processes (e.g. glycolysis) to gene regulation. Since the function of Traf6 in early embryogenesis in vertebrates has not yet been analysed, it is difficult to identify specific ontology classes that can be linked to functions of Traf6 previously described in the literature except for functions in apoptosis. Traf6 knock-down led to the induction of several genes involved in the regulation of programmed cell death. Genes such as *BCL2-antagonist of cell death (bad)* and *bcl2-associated X protein (bax)*, known to promote apoptosis, were induced whereas genes promoting cell survival such as *birc2* showed decreased expression levels (24-26). Our results are in line with a recent publication of Yoon et al. where it was demonstrated that TRAF6 has an anti-apoptotic effect in mice cell lines (27). In these cell lines TNF stimulation caused sustained JNK activation through the accumulation of reactive oxygen species (ROS) eventually leading to cell death. It was shown by Yoon et al. that TRAF6 deficiency leads to the inactivation of GSK3 β and in turn to impaired NF- κ B dependent transcription.

The TLR signalling pathway has been shown to be crucial for embryogenesis in insects and nematodes and it might therefore be expected that Traf6 plays a role in early development of vertebrates as well. Considering the early stage of embryogenesis that we have studied we can expect that many of the genes affected by *traf6* knock-down are likely to play a role in development. We identified a large overlap in transcriptional gene response between the early embryonic *traf6* knock-down effect and embryonic infection responses, indicating that early development and infectious disease processes have many molecular mechanisms in common. However, it is notable that only a set of 14 annotated genes showed a Traf6 controlled response in early embryogenesis and in the context of an embryonic infection response at 35 hpf.

These genes include *DNA-damage regulated autophagy modulator1 (dram1)*, a target gene of p53 (28). This limited overlap shows that the functions of Traf6 in early embryogenesis differ from its functions in innate immunity at later stages of embryogenesis. This is remarkable considering the fact that many key partners of Traf6 play a role in both development and infectious disease. Further studies at a biochemical level might show how Traf6 function is modulated from a control factor of developmental genes to a control factor of genes involved in functions in infectious disease.

In addition to the analysis of the function of Traf6 we could identify a gene set whose transcription is controlled by morpholino injections in general. We could show that several genes previously linked to development or metabolism, are also triggered by morpholinos in general. By identifying these general effects of morpholinos at the transcriptome level we provide a reference set for morpholino off-target effects. Considering the general use of morpholinos in the studies of zebrafish development, immunity and metabolism this will be an important resource for the scientific community. Interestingly several of these genes are linked to the immune system. We could show that a group of four Toll-like receptors, namely *tlr3*, *tlr4a*, *tlr4b* and *tlr9* were down-regulated in response to morpholino treatments. In mammals, activation of the innate immune response by nucleic acids is known to be mediated via TLR3, TLR7 and TLR9 (29). However, it has not been yet reported that morpholinos can also activate these pathways. Furthermore, TLR4 is thus far not known as a receptor for DNA or RNA. From the *traf6* knock-down results we can conclude that the general response of *tlr3* to morpholino injections is not dependent on Traf6. In contrast the response of *tlr4a*, *tlr4b* and *tlr9* appears to be dependent on Traf6. It is very interesting that these pathways are activated in the early stages of embryogenesis and therefore we would like to further study the signal transduction pathways involved in the sensing of morpholinos. For instance it would be of interest to find out why these receptors were down-regulated. An explanation for the negative regulation of these receptors might be a negative feedback loop since the injection of morpholinos was approximately 5 hours prior to the analyses of the transcriptome response. The regulation of these Toll-like receptors in early embryogenesis indicates that also other players of this pathway are functionally present in the early embryo. This is consistent with previous results demonstrating that several TLR receptors and downstream signal transduction components are expressed at very early stages of embryogenesis (30). This includes *tlr3*, *tlr4a* and *tlr4b* as well as the adaptor proteins *tirap* and *trif*. However, there is nothing known on functional aspects of the innate immune system at the very early stages of embryogenesis and based on our results it would be of interest to test responses of early embryos to other immunogenic compounds and infectious agents at the very early stages of embryogenesis.

Materials and Methods

Zebrafish husbandry

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (<http://ZFIN.org>). All experiments were performed on mixed egg clutches from several pairs of AB strain zebrafish. Embryos were grown at 28,5 °C in egg water (60µg/ml Instant Ocean sea salts).

Morpholino knock-down experiments

For morpholino knockdown experiments, morpholino oligonucleotides (Gene Tools) were diluted to desired concentrations in 1x Danieü'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). Translation of *traf6* was blocked by two non-overlapping morpholinos specifically targeting the 5' UTR region of *traf6* (*traf6*-morpholino-1, 5'GCCATATTGGCTCGGTACGGCCTC and *traf6*-morpholino-2, 5'GCCTATACTGCTGCTTCCTGTAAAG). To control for aspecific morpholino effects, a 5 bp mismatch morpholino (5'GCaATATTcGCTaGGTACaGCgTC) of *traf6*-morpholino-1 and a standard control morpholino (www.gene-tools.com) were injected. Embryos were injected at the one cell stage with 1 nl of either one of the above mentioned morpholinos with the following concentrations: 0.5, 1 or 2mM. In addition, 1x Danieü's buffer containing only Phenol red was injected to control for the injection effect per se. All injections were done in random order and the experiment was performed in quintuplicate.

RNA extraction

Pools of 20-30 embryos of each treatment group were collected at 30% epiboly for RNA isolation. Embryos 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.

Microarray design and hybridization

A custom zebrafish 4 x 44 K microarray (Agilent) that was previously described was used (accession no. GPL7735 in the GEO database) (15). 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 labelled 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 labelled 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 labelled test sample and 825 ng of Cy5 labelled 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 procedure

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) (16).

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 labelling dyes, and visual inspection of pre- and post-normalized data with box plots and RI plots.

The data set concerned a two-factorial design, with the factors ‘Treatment’ (5 levels: ‘Phenol Red’, ‘Standard Control’, ‘Mismatch’, ‘Morpholino 1’ and ‘Morpholino 2’) and ‘Dose’ (3 levels: ‘Low’, ‘Middle’ and ‘High’), with the factor ‘Dose’ being applicable only to the morpholino injections, not to the Phenol Red control. After log₂ transformation the data was normalized by quantile normalization. The data was analyzed using a two-stage mixed analysis of variance (ANOVA) model (17). First, array, dye, and array-by-dye effects were modelled globally. Next, the residuals from this first model were fed into a gene-by-gene model in which we took ‘Group’, ‘Array’, and ‘Injection’ as factors of which ‘Array’ and ‘Injection’ were modelled as random factors. ‘Group’ is defined by each unique treatment and dose combination. For hypothesis testing an F_s test was used and 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 of 5%.

Gene Ontology, pathway and cluster analysis

Gene ontology (GO) analysis was performed using the GeneTools eGOn v2.0 web-based gene ontology analysis software (www.genetools.microarray.ntnu.no) (18). 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).

Pathway analysis was performed using the GenMapp software package (www.genmap.org) (19)116116116116. Analysis was done at the level of UniGene clusters (*D. rerio* UniGene build #114). Significance cut-off was set at >1.2 and <-1.2 fold change at an FDR adjusted P-value <0.05. Zebrafish homologs of the genes contributing to

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the TLR and TGF-beta superfamily 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>).

Supplementary Data

Supplementary tables can be found online at: <http://www.mediafire.com/?sharekey=686efof919604e919bf8d6369220dcab43aecfab95fc71d759e682a8cd2154a>

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