Mecp2 regulates \textit{tnfa} during zebrafish embryonic development and acute inflammation

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Abstract

Mutations in \textit{MECP2} cause Rett syndrome, a severe neurological disorder with autism-like features. Duplication of \textit{MECP2} also causes severe neuropathology. Both diseases display immunological abnormalities that suggest a role for MeCP2 in controlling immune and inflammatory responses. Here, we used \textit{mecp2}-null zebrafish to study the potential function of Mecp2 as an immunological regulator. Mecp2-deficiency resulted in an increase in neutrophil infiltration and upregulated expression of the pro- and anti-inflammatory cytokines Il1b and Il10 as a secondary response to disturbances in tissue homeostasis. In contrast, expression of the pro-inflammatory cytokine tumor necrosis factor alpha (Tnfa) was consistently downregulated in \textit{mecp2}-null animals during development, representing the earliest developmental phenotype described for MeCP2-deficiency to date. Expression of \textit{tnfa} was unresponsive to inflammatory stimulation, and was partially restored by re-expression of functional \textit{mecp2}. Thus, Mecp2 is required for \textit{tnfa} expression during zebrafish development and inflammation. Finally, RNA sequencing of \textit{mecp2}-null embryos revealed dysregulated processes predictive for Rett syndrome phenotypes.
Introduction

The human X-chromosomal gene methyl-CpG-binding protein 2 (MECP2) was identified as an epigenetic factor capable of binding to methylated DNA (Lewis et al., 1992). Mutations in human MECP2 lead to Rett syndrome (RTT) (Amir et al., 1999), a severe neurological disorder associated with autistic features and motor skill regression after an apparently normal early development (Lyst and Bird, 2015). RTT patients often also display growth retardation (Tarquinio et al., 2012), gastrointestinal and biliary tract disorders (Motil et al., 2012), and oxidative stress (Filosa et al., 2015). Conversely, over-expression of human MECP2 caused by duplication of its genetic locus (Xq28) results in severe mental retardation and progressive neurological symptoms (Van Esch et al., 2005). Although neurological defects are the most striking clinical presentation of RTT and MeCP2-duplication syndrome, both diseases display immunological abnormalities that point towards a role for MeCP2 in regulating immune and inflammatory responses.

Disturbances in tissue homeostasis are detected by pattern recognition receptors (PRRs), such as the family of Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Beg, 2002; Matzinger, 2002; Medzhitov and Janeway, 2000). Activation of TLRs by infection or cellular damage initiates a signaling cascade that leads to the production of pro-inflammatory cytokines and chemokines (Akira and Takeda, 2004). The primary function of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL1B), is to initiate an appropriate cellular or humoral immune response to neutralize the disturbance. Anti-inflammatory cytokines, including interleukin 10 (IL10) and transforming growth factor beta (TGFβ), balance the activity of pro-inflammatory cytokines by stimulating resolution of inflammation and tissue repair. Alterations in the balance between pro- and anti-inflammatory cytokines are potentially harmful, as prolonged inflammation can be damaging to tissues, while inadequate immune responses leave the body vulnerable to infections.

RTT patients showed a dysregulated cytokine and chemokine profile and displayed subclinical inflammation (Cortelazzo et al., 2014; Pecorelli et al., 2016). Data obtained using a mouse model of RTT demonstrated that MeCP2 regulates microglia and macrophage responsiveness to inflammatory stimulation, hypoxia, and glucocorticoids (Cronk et al., 2015). Transplantation of wild type microglia has even been suggested as a therapeutic strategy for RTT patients based on findings obtained using RTT mice (Derecki et al., 2012), but these findings have since been disputed by others in the field (Wang et al., 2015). While investigations concerning the role of the immune system in the onset of RTT are still ongoing, MeCP2 duplication syndrome is linked to immunodeficiency with increased susceptibility to infections for reasons that remain to be uncovered (Bauer et al., 2015). An emerging
theme is that MeCP2 normally regulates the immune response towards inflammatory stimuli and other stress factors.

The zebrafish was originally employed as model organism to study vertebrate embryogenesis due to its external fertilization and development, genetic tractability, and optical transparency allowing non-invasive intravital imaging (Kimmel et al., 1988). These characteristics have also helped to develop the zebrafish as a useful model for the study of vertebrate immunity (Renshaw and Trede, 2011; van der Vaart et al., 2012). A recently described mecp2-null zebrafish mutant showed altered motor behaviors (Pietri et al., 2013), and mecp2 was found to be required for normal zebrafish brain development (Gao et al., 2015). Zebrafish mecp2 was broadly expressed early in embryonic development, after which it became enriched in the brains of zebrafish larvae (Gao et al., 2015). This is similar to the distribution of Mecp2 in mice, where it is highly expressed in neurons, but also ubiquitously found at lower levels in other cell types (Song et al., 2014).

Here, we studied the potential function of zebrafish Mecp2 as an immunological regulator during development and inflammation. We found that mecp2-null zebrafish display several previously unappreciated phenotypes also present in RTT patients, including growth retardation, gastrointestinal tract phenotypes and dysregulated expression of cytokines. The gene expression levels of the pro- and anti-inflammatory cytokines il1b and il10 showed a peak during development, but were not hyper-responsive to inflammatory stimulation in mecp2-null larva. We therefore suggest that the increased expression levels of these inflammatory cytokines during development were a response to a disruption of tissue homeostasis in the absence of Mecp2. Remarkably, we found that gene expression levels of zebrafish tnfa were profoundly downregulated during the first hours of embryonic development in mecp2-null, preceding the first noticeable disease phenotypes. To the best of our knowledge, this finding represents the earliest developmental phenotype associated with MeCP2-deficiency. The lower tnfa expression levels persisted throughout larval development, and tnfa was unresponsive to inflammatory stimulation in mecp2-null larvae. Finally, the expression of tnfa in mecp2-null embryos could be partially restored by enforced expression of wild type mecp2. However, re-expression of tnfa in mecp2-null embryos was not sufficient to rescue the observed RTT phenotypes. Based on these findings, we conclude that zebrafish Mecp2 is required for tnfa expression during development and inflammation. To assess the earliest changes attributable to loss of Mecp2 function, we utilized RNA sequencing to analyze the transcriptome of mecp2-null embryos shortly after initiation of embryonic transcription (Kane and Kimmel, 1993). Strikingly, this revealed disrupted biological processes that are highly predictive of RTT phenotypes that develop much later in human patients. Further exploration of this transcriptome data and its changes over time may generate novel insights into additional developmental functions of MeCP2.
Results

*mecp2*-null zebrafish displayed growth retardation, gastrointestinal tract phenotypes and systemic inflammation

To study the function of Mecp2 during zebrafish development, we used a mutant line containing a premature stop codon in the mecp2 gene (*mecp2*Q63*') that truncates the protein before the methyl binding domain (MBD) and transcriptional repression domain (TRD), both vital to its function (Lyst and Bird, 2015; Pietri et al., 2013). Although adult *mecp2*-null zebrafish are viable and fertile with no overt phenotypes, these animals display behavioral alterations during their larval development (Pietri et al., 2013). Upon further characterization, we found that developing *mecp2*-null embryos displayed growth retardation at 2 days post fertilization (dpf) (Figure 1A and B). However, no significant difference in total body length was discernible between *mecp2*-null and wild types at 7 dpf (Figure 1C and D). Around 4 dpf, green/yellow discoloration was observed in the gastrointestinal (GI) tracts of *mecp2*-null larvae (Figure 1E), indicative of an accumulation of or disruption in flow of bile (Delous et al., 2012). At 7 dpf, dark yellow droplets were regularly observed in the GI tracts of *mecp2*-null larvae (Figure 1F), consistent with bile overproduction. To investigate whether these phenotypes are preceded or accompanied by systemic inflammation, we analyzed gene expression of the inflammation marker C reactive protein (*crp*) by quantitative real-time PCR (qPCR) (Okamura et al., 1990). In the first 3 days of zebrafish development, we found no difference in *crp* expression between wild type and *mecp2*-null larvae, but *crp* levels were significantly elevated in *mecp2*-null larvae by 4 and 5 dpf (Figure 1G). This demonstrates that *mecp2*-null larvae mount an inflammatory response at 4 and 5 dpf that is detectable at a whole-organism level, after an early developmental period with no overt signs of systemic inflammation. Together, these results show that *mecp2*-null zebrafish display several RTT features during their development, including growth retardation, GI tract phenotypes, and systemic inflammation.

Neutrophil numbers and mobilization confirm the presence of inflammation in *mecp2*-null larvae

To further investigate and characterize the possible inflammatory response in *mecp2*-null larvae suggested by increased *crp* levels, we first analyzed neutrophil numbers. Neutrophils are among the first innate immune cells that respond to disturbances in tissue homeostasis; increased tissue infiltration has previously been used to mark inflammation in zebrafish models of wounding, infection, and inflammatory bowel disease (Brudal et al., 2014; Brugman et al., 2009; Oehlers et al., 2011; Renshaw et al., 2006). We used Tg(*mpx:eGFP*) animals (Renshaw et al., 2006), in which neutrophils are fluorescently labeled, to assess the number and distribution of neutrophils in the *mecp2*-null background over several developmental time points. Correlating with our *crp* results, we
did not find any difference in neutrophil number between wild type and mecp2-null larvae at 3 dpf, but total neutrophil numbers were significantly increased in mecp2-null larvae at 4 and 5 dpf (Figure 2A). These findings reproduce the neutrophilia observed in MeCP2-null mice displaying RTT phenotypes, and underscore the conserved function of MeCP2 in lower vertebrates (Cronk et al., 2015).

Neutrophilic granulocytes begin to accumulate in the caudal hematopoietic tissue (CHT) of developing zebrafish embryos following initiation of circulation at 26hpf (Bertrand et al., 2007; Le Guyader et al., 2008; Stachura and Traver, 2011). A large number of neutrophils continue to reside in the CHT in uninflamed larvae, from which they can be mobilized to migrate towards inflamed tissues when needed (Yoo and Huttenlocher, 2011). We therefore aimed to approximate the source of inflammation in mecp2-null larvae by determining which tissues displayed increased neutrophil infiltration. Although the head region of mecp2-null larvae contained a slightly increased number of neutrophils at 5 dpf, we did not observe any significant infiltration of neutrophils into the brains of mecp2-null animals (Supplementary figure 1A, B, and C). Starting at 2 dpf, we observed increases in neutrophil numbers associated with the GI tract of mecp2-null larvae (Figure 2C and D), indicating this tissue as a potential source of inflammation. We reproduced this observation by using a previously characterized anti-sense morpholino oligonucleotide approach designed to block initiation of zebrafish Mecp2 protein translation (Supplementary figure 1D) (Gao et al., 2015). These findings are in agreement with our previous observation of GI tract phenotypes during mecp2-null larval development.

Since microglia and macrophages have previously been implicated in RTT-like etiology in mice and became depleted with disease progression (Cronk et al., 2015), we also assessed their number and localization by using Tg(mpeg1:eGFP) animals with fluorescently labeled microglia and macrophages (Ellett et al., 2011). At 3 dpf, mpeg1-expressing microglia have colonized the brain and are capable of mounting a functional immune response (Herbomel et al., 2001; Svahn et al., 2013). However, we found no distinguishable difference in microglia or macrophage numbers or localization between mecp2-null and wild type larvae from 3 to 5 dpf (Figure 2E, F, and G). In summary, our results indicate that disrupting MeCP2 function during zebrafish development leads to a systemic immune response that appears to originate from the GI tract, based on observed GI tract phenotypes and neutrophil influx into this tissue.

**Expression of central pro- and anti-inflammatory cytokines is dysregulated in mecp2-null larvae**

Because inflammation is mainly controlled by the expression and activity of pro- and anti-inflammatory cytokines and chemokines, we queried whether the expression of these regulatory
molecules is affected by Mecp2-deficiency. We used qPCR to analyze the gene expression levels of a panel of zebrafish inflammatory cytokines and chemokines in mecp2-null and wild type larvae during the first 7 days of development. The panel consisted of the pro-inflammatory cytokines il1b, interleukin 6 (il6), and tnfa; the pro-inflammatory chemokine interleukin 8 (cxcl8a); and the anti-inflammatory cytokines il10 and tgtb1. At each time point analyzed over the 7-day time course, tnfa was expressed at dramatically lower levels in mecp2-null compared to wild type embryos of the same age (Figure 3A). Even at 6 hpf, the earliest time point with clearly detectable tnfa expression in wild type embryos, its expression was significantly reduced in mecp2-null animals (Figure 3A). In contrast, we found no significant difference in expression levels of il6, cxcl8a, and tgtb1 between mecp2-null and wild type over the developmental time course of 7 days (Supplementary Figure 2A, B, and C). We did detect a significant increase in whole-organism il1b and il10 expression in mecp2-null at 5 dpf, after being expressed at wild type levels for the first 4 days of development (Figure 3B and C). While il1b reverted back to wild type levels over the next two days (Figure 3B), the significantly increased expression of il10 peaked at 6 dpf, after which it also trended downwards (Figure 3C). The expression levels of il1b and il10 indicate a temporal increase in inflammatory signaling, followed by resolution of inflammation.

We sought to confirm the specific downregulation of tnfa by confocal microscopy imaging of wild type and mecp2-null larvae carrying a Tg(tnfa:eGFP) reporter that expresses eGFP under control of tnfa regulatory sequences (Supplementary figure 3) (Marjoram et al., 2015). Wild type larvae of 3 dpf expressed eGFP in brain regions (Figure 3D and E), posterior gut epithelium (Figure 3F), and dorsal root ganglion neurons (Figure 3G). In contrast, 3 dpf mecp2-null larvae had no detectable expression of GFP in any of these tissues (Figure 3H, I, J, and K). Since the Tg(tnfa:eGFP) reporter-construct introduced an additional tnfa promoter region at a random location in the zebrafish genome (Marjoram et al., 2015), the lack of eGFP expression caused by Mecp2-deficiency appears linked to the DNA sequence of the tnfa promoter, rather than its chromosomal location. The decreased expression of tnfa precedes any observable phenotype, suggesting that this is not part of a secondary inflammatory response, but rather due to genetic dysregulation.

mecp2-null larvae are unable to activate tnfa expression during an acute inflammatory response

Our finding that tnfa is downregulated in mecp2-null as early as 6hpf is highly suggestive for a direct effect of Mecp2 on tnfa expression. To test whether the tnfa gene has lost its responsiveness to inflammatory stress signals, we designed an acute inflammation assay by injecting the yeast cell wall particle zymosan, a TLR2-ligand (Underhill et al., 1999), into the brains of 3 dpf zebrafish larvae (Figure 4A). Fluorescently labeled zymosan injected into the brains of wild type larvae was rapidly
phagocytosed by Tg(mpeg1:eGFP)-positive microglia (Figure 4B), and all zymosan particles were cleared from the brain tissue at 4 hours post injection (hpi) (Figure 4C). The clearance of zymosan is accompanied by an acute inflammatory response characterized by an initial upregulation of the pro-inflammatory cytokine genes *il1b* and *tnfa*, followed by an upregulation of the anti-inflammatory cytokine genes *il10* and *tgfb1* (Figure 4D). The gene expression levels of these inflammatory cytokines returned to baseline levels at 4 hours post injection of zymosan (Figure 4D). The inflammatory response to zymosan injected into the brain is strongest in dissected heads of wild type larvae, but its effects on *il1b* gene expression can also be detected in whole animal preparations of injected zebrafish larvae (Supplementary figure 4A).

We confirmed that Tg(mpeg1:eGFP)-positive cells present in the brain of wild type and mecp2-null larvae phagocytosed zymosan at comparable rates (Supplementary figure 4B and C). We then used qPCR to compare the gene expression levels of *il1b*, *il10*, and *tnfa* between mecp2-null and wild type larvae over a 4-hour time course after injection of zymosan into the brain. Strikingly, mecp2-null larvae were unable to increase the gene expression level of *tnfa* in response to zymosan injection, unlike the wild type control group (Figure 4E). In comparison, we found no difference in gene expression levels of *il1b* and *il10* between mecp2-null and wild types during this inflammatory response (Figure 4E). This demonstrates that the genetic regulation of *il1b* and *il10* in response to a danger signal is not disturbed by Mecp2-deficiency, and suggests that their upregulation during mecp2-null larval development is part of an inflammatory response to disturbances in tissue homeostasis. In contrast, we conclude that zebrafish *tnfa* was unresponsive to inflammatory stimulation in the absence of functional Mecp2. These results show that even during an acute stress event, mecp2-null larvae cannot activate *tnfa* expression, and further suggests that Mecp2 is required for proper expression of *tnfa*.

**Re-expression of mecp2 in mecp2-null zebrafish embryos partially rescues tnfa gene expression**

Since mecp2-null larvae were unable to express *tnfa* at wild type levels during development or during an acute inflammatory response, we asked whether re-expression of wild type mecp2 was sufficient to restore *tnfa* gene expression levels in mecp2 mutants. For this purpose, we injected full-length mecp2 mRNA into mecp2-null or wild type zygotes. Injection of mecp2 mRNA resulted in a 15 to 20-fold overexpression of mecp2 at 1 dpf (Figure 5A), or approximately 50-fold overexpression at twice the dose (Supplementary figure 5). Even at the highest dose tested, we were unable to detect increased mecp2 expression levels at 3 dpf (Supplementary figure 5), suggesting a rapid decay of the injected mecp2 mRNA. We analyzed the effect of mecp2 overexpression on *tnfa* and *il1b* gene expression levels by qPCR. We found that overexpression of wild type mecp2 in mecp2-null could
partially rescue \textit{tnfa} gene expression levels at 1 dpf, while it did not affect \textit{tnfa} gene expression in wild type embryos (Figure 5B). Overexpression of wild type \textit{mecp2} mRNA had no noticeable effect on gene expression levels of \textit{il1b} in either \textit{mecp2}-null or wild type embryos (Figure 5C). The lower \textit{tnfa} expression throughout embryonic and larval development, combined with the unresponsiveness of \textit{tnfa} to inflammatory stimulation in \textit{mecp2}-null suggested a direct effect of Mecp2-deficiency on \textit{tnfa} gene expression. Based on the \textit{mecp2} mRNA re-expression experiments, we conclude that Mecp2 is required to allow normal expression of \textit{tnfa} in zebrafish embryos and larvae. However, overexpression of \textit{mecp2} mRNA in wild type embryos did not alter \textit{tnfa} expression, indicating that Mecp2 alone is not sufficient to induce \textit{tnfa} expression. This suggests a mechanism where Mecp2 allows additional transcriptional regulators to be recruited to modulate \textit{tnfa} gene expression.

**Re-expression of \textit{tnfa} in \textit{mecp2}-null zebrafish embryos does not rescue RTT phenotypes**

We observed that \textit{tnfa} expression is significantly reduced in \textit{mecp2}-null zebrafish during embryonic and larval development. The posterior gut epithelium is a prominent source of \textit{tnfa} expression in wild type larvae (Figure 3F), whereas \textit{mecp2}-null animals had no detectable \textit{tnfa} expression in this tissue (Figure 3J). Since dysregulated \textit{tnfa} expression has previously been implicated in the onset of inflammatory bowel disease in zebrafish larvae (Marjoram et al., 2015), we hypothesized that the lack of \textit{tnfa} expression might contribute to the development of inflammatory phenotypes in the GI tract of \textit{mecp2}-null zebrafish. To test for the potential involvement of reduced \textit{tnfa} expression in the development of RTT phenotypes, we injected a previously described morpholino oligonucleotide targeting \textit{tnfa} expression into wild type zygotes (López-Muñoz et al., 2011). We found that knockdown of \textit{tnfa} resulted in a significant increase in the number of GI tract associated neutrophils compared to control injected individuals (Figure 5D), as well as a significant decrease in total body length (Figure 5E). Both these phenotypes are also observed in \textit{mecp2}-null larvae. Next, we attempted to rescue the GI tract neutrophil infiltration and growth reduction observed in \textit{mecp2}-null larvae by re-expressing \textit{tnfa}. For this purpose, we injected plasmid encoding full-length \textit{tnfa} mRNA into \textit{mecp2}-null or wild type zygotes (Supplementary figure 6). Enforced expression of \textit{tnfa} did not reduce the number of neutrophils associated with the GI tract in \textit{mecp2}-null larvae (Figure 5F), nor did it restore the reduced body length of \textit{mecp2}-null larvae (Figure 5G). In summary, while knockdown of \textit{tnfa} mimicked the phenotypes observed in \textit{mecp2}-null larvae, restoring \textit{tnfa} expression was not sufficient to rescue the growth retardation and GI tract inflammatory phenotypes observed in \textit{mecp2}-null animals.
RNA sequencing reveals early developmental effects of mecp2-deficiency and predicts RTT phenotypes

In this study, we have demonstrated that Mecp2 regulates tnfa gene expression levels during early zebrafish embryonic development. Even at 6 hpf, when low levels of tnfa expression can first be detected in wild type embryos, mecp2-null embryos express significantly reduced levels of tnfa. At this time point of development, zebrafish embryos are undergoing epiboly and gastrulation, which initiate formation of the basic body plan. While we were able to detect the effect of mecp2-deficiency at this early developmental stage in zebrafish embryos, RTT patients do not display phenotypes until at least 6 months after birth. Therefore, we reasoned that the zebrafish embryo could be highly informative regarding the earliest effects of disrupted MeCP2-function that ultimately result in disease phenotypes. To assess the genes that are disrupted during early development of mecp2-null, we performed RNA sequencing to reveal whole-transcriptome differences between 6 hpf mecp2-null and wild type embryos. For the mecp2-null group, embryos were derived from homozygous mecp2-null parents to avoid the confounding influence of maternally deposited wild type mecp2 RNA. The three biological replicates of each condition clustered closely together after DESeq2 analysis (Figure 6A). At 6 hpf, 3837 transcripts are significantly upregulated in mecp2-null versus wild type embryos, while 4217 transcripts are significantly downregulated (Figure 6B). Although the raw counts for tnfa were lower in mecp2-null compared to wild type embryos, the average number of raw counts for tnfa in wild type embryos was too low to demonstrate significance (data not shown).

For an unbiased assessment of potentially disrupted biological processes in mecp2-null embryos at 6 hpf, we submitted the subsets of differentially up- or down-regulated genes to gene ontology (GO) analysis. GO analysis revealed that genes associated to a large range of biological processes were significantly enriched in the differentially expressed subsets, illustrating that mecp2-deficiency has a broad effect on transcription. We limited our further analysis to GO terms with at least 2-fold enrichment, and only included the most specific GO term for groups of hierarchically clustered terms (Figure 6C and D). This strict GO term analysis revealed significantly enriched biological processes that are linked to known MeCP2-functions, like epigenetic regulation of transcription and mRNA splicing (Lyst and Bird, 2015). Importantly, GO analysis also identified enriched processes at this early developmental stage, which become relevant to RTT phenotypes at later stages, including neurological development; craniofacial development; vascular dysfunction; redox homeostasis; developmental growth; myeloid cell differentiation; and hepatobiliary system development. Finally, GO analysis identified enriched biological processes that, to the best of our knowledge, have not been previously linked to MeCP2-function or RTT, including dorsal/ventral pattern formation;
protein folding; and intracellular protein targeting. The analysis of the RNA sequencing data underscores the relevance of the zebrafish model for the study of MeCP2-function and RTT, while potentially identifying new biological processes of interest.

At the same time, the unbiased analysis of enriched GO terms appears predictive for the growth retardation, myeloid cell number disruption (neutrophilia), and hepatobiliary dysfunction that occurs later during development. For the GO term ‘developmental growth’, 44 genes out of a total of 187 linked to this biological process were significantly downregulated in 6 hpf mecp2-null versus wild type embryos. For the GO terms ‘myeloid cell differentiation’ and ‘hepaticobiliary system development’, 32 out of a total of 133 or 111 genes linked to this process (respectively) were significantly upregulated in mecp2-null embryos. To investigate the extent of individual gene dysregulation in the absence of Mecp2, we plotted the normalized fold-change of all differentially expressed genes linked to these three GO terms in a heatmap (Figure 7A). While Mecp2-deficiency affected the gene expression level of a large number of genes related to these biological processes, approximately 75% of these genes were up- or downregulated at 2-fold change or less (Figure 7B). The same observation was made at a genome wide scale for all significantly upregulated genes in mecp2-null, or to a lesser degree for all significantly downregulated genes (Figure 7C). Notable exceptions to this general tendency for small differences in gene expression levels are bbs4 and nos1 (GO term ‘developmental growth’); sptb, smad9, and casp3b (GO term ‘myeloid cell differentiation’); and sfrp5 and a2ml (GO term ‘hepaticobiliary system development’). While the neuronal expressed Nos1 (Nitric oxide synthase 1) protein is well-known for its role in neurotransmission, mutations in genes from the Bardet-Biedl syndrome (BBS) family, such as bbs4, result in an autosomal recessive disorder characterized by mental retardation and other severe symptoms. The anti-inflammatory adipocytokine Sfrp5 modulates metabolic dysfunction during obesity in mice (Ouchi et al., 2010), and A2ml (Alpha2 macroglobulin-like) was shown to be essential for liver development in zebrafish (Hong and Dawid, 2008). A potential role for Sptb, Smad9 and Casp3b in neutrophilia is not directly clear. For all genes with relatively high differential expression between mecp2-null and wild type embryos at 6 hpf, it will be interesting to investigate whether their dysregulation extends into developmental phases when RTT phenotypes first arise.
Discussion

The large body of literature on MeCP2 and RTT contains evidence that mutations in MeCP2, as well as its over-expression caused by duplication of its genetic locus, result in abnormal functioning of the immune system (Bauer et al., 2015; Cortelazzo et al., 2014; Cronk et al., 2015; Derecki et al., 2012; Leoncini et al., 2015; Pecorelli et al., 2016). Furthermore, since RTT patients acquire disease symptoms after an apparently normal early development, we hypothesized that misregulated responses to external or internal inflammatory stimuli encountered during development may play a key role in the onset of RTT. We therefore set out to test the potential function of zebrafish Mecp2 as an epigenetic regulator of immune and inflammatory responses during development.

Indeed, gene expression levels of the inflammation marker *crp* and mobilization of neutrophils provided evidence for the presence of inflammation in *mecp2*-null larvae after an inflammation-free early development. Increased gene expression levels of *il1b* and *il10* measured at a whole organism level were found to be involved in this inflammatory response. We hypothesized that lacking the epigenetic regulator Mecp2, the zebrafish genes encoding Il1b and Il10 were hyper responsive to inflammatory stimulation. By submitting both wild type and *mecp2*-null larvae to an acute inflammation assay, we were able to disprove this hypothesis. Gene expression levels of *il1b* and *il10* were regulated at a similar fashion in response to inflammatory stimulation in wild type and *mecp2*-null larvae. Combined with the fact that *il1b* and *il10* were expressed at wild type levels in *mecp2*-null during early development, we suggest that the peak in expression of these pro- and anti-inflammatory cytokines was a response to a disturbance in tissue homeostasis in the absence of Mecp2.

We observed an increased infiltration of neutrophils into the GI tract of *mecp2*-null, combined with GI tract phenotypes and a potential disturbance of bile production or flow. These observations are relevant, since RTT patients frequently display gastrointestinal tract phenotypes, including gastrointestinal dismotility (Baikie et al., 2014; et al., 2012). Additionally, cholesterol metabolism is altered in RTT patients (Segatto et al., 2014), and limiting cholesterol biosynthesis alleviated RTT symptoms and increased the survival of *mecp2*-null mice (Buchovecky et al., 2013). While bile acids, a major component of cholesterol, have immunomodulatory effects (Brestoff and Artis, 2013), inflammation can also suppress the expression of bile transporters and thereby reduce the flow of bile (Kosters and Karpen, 2010). With the proven contribution of zebrafish larval and embryonic models to the study of liver diseases and inflammatory bowel diseases (Goessling and Sadler, 2015; Love et al., 2007), the zebrafish *mecp2*-null mutant may be ideally suited to illuminate the role of inflammation in the gastrointestinal tract of RTT patients.
The most striking result obtained during this study was the fact that zebrafish *tnfa* was not expressed at normal levels in the absence of functional Mecp2 during embryonic and larval development, or during an acute inflammatory response. Combined with our finding that re-expression of wild type Mecp2 can partially rescue *tnfa* expression in *mecp2*-null embryos, we conclude that zebrafish Mecp2 influences the transcriptional potential of *tnfa*. Importantly, the dysregulated expression levels of *tnfa* at 6 hpf precede any of the developmental phenotypes observed in the absence of functional Mecp2, and could potentially be a causative factor for RTT features displayed later during development. Indeed, knockdown of *tnfa* gene expression induced neutrophilic infiltration into the GI tract of zebrafish larvae, a phenotype resembling that observed in *mecp2*-null individuals. In this light, it’s interesting to note that genetic inhibition of Tnfa and Tnfr2 in zebrafish previously resulted in the mobilization of neutrophils to the skin, revealing a crucial role for the TNFα/TNFR2 axis in the protection against Duox1-mediated oxidative stress (Candel et al., 2014). RTT patients often display oxidative stress and we identified the GO term ‘redox homeostasis’ as one of the biological pathways altered in *mecp2*-null embryos. The potential link between reduced *tnfa* expression in the GI tract and inflammation caused by increased oxidative stress is therefore an interesting topic for further study in *mecp2*-null zebrafish embryos and larvae.

However, we found that re-expression of *tnfa* did not alleviate the phenotypes observed in *mecp2*-null zebrafish. Transcriptome analysis revealed that a total of 8,054 genes are differentially expressed between *mecp2*-null embryos and wild types at 6 hpf. Even if the enforced expression of *tnfa* could be titrated to match wild type endogenous levels – which differ per tissue and circumstance –, it indeed seems unlikely that re-expression of only one dysregulated gene would be sufficient to alleviate the observed RTT features.

The observation that overexpression of *mecp2* in wild type embryos did not raise *tnfa* gene expression levels indicates that the presence of Mecp2 alone is not sufficient to increase transcription of *tnfa*. The experiments performed in this study also provide clues into which aspect of the diverse Mecp2 functions might be involved in the regulation of *tnfa* (Lyst and Bird, 2015). The Tg(*tnfa*:eGFP) construct (Marjoram et al., 2015), introducing an additional copy of the *tnfa* promoter in the genome, did not drive expression of eGFP in the absence of Mecp2, indicating that the regulatory sequences of the *tnfa* transgene are critically important for its regulation by Mecp2. It is possible that sequence-specific DNA-binding of Mecp2 results in chromatin remodeling that increases the transcriptional potential of the zebrafish *tnfa* gene (Ballestar et al., 2000; Baubec et al., 2013; Yusufzai and Wolffe, 2000). Another plausible explanation is that Mecp2 is involved in the transcriptional activation of *tnfa* by recruiting the co-activator CREB1, since the CREB-binding protein
(CBP)/p300 was shown to play a stimulus-dependent role in T cell receptor-activated TNFα gene expression (Falvo et al., 2000).

Several in vivo and in vitro models exist for the study of RTT and MeCP2 function, including Mecp2-null mutant mice (Chen et al., 2001; Guy et al., 2001); Xenopus laevis with truncated MeCP2 (Stancheva et al., 2003); induced pluripotent stem cells (iPSCs) from RTT patients’ fibroblasts (Marchetto et al., 2010); mecp2-null mutant zebrafish (Pietri et al., 2013); and most recently transgenic monkeys overexpressing MeCP2 (Liu et al., 2016). The results obtained using these different models are sometimes conflicting and MeCP2 function varies between different tissues or cells of the same organism. For instance, the NFκB-pathway component Irak1 was specifically upregulated in cortical callosal projection neurons in Mecp2-null mice, but not in distinct organs like the lungs, heart, spleen, or kidney (Kishi et al., 2016). Even when the same model organism and experimental conditions are used, results can still differ fundamentally (Derecki et al., 2012; Wang et al., 2015). In this regard, while we consistently found zebrafish tnfa to be downregulated in mecp2-null animals, Cronk et al. found an increase in Tnfa-induced transcriptional signature genes specifically in isolated Mecp2-null microglia (Cronk et al., 2015). The different cell source utilized in these experiments might explain the conflicting results, making it worthwhile to analyze tnfa transcript levels in isolated zebrafish mecp2-null microglia and other immune cells.

With the sometimes-conflicting findings on the effect of MeCP2-deficiency under differing conditions and from various model systems, it is challenging to reach a unified and evolutionary conserved conclusion on MeCP2 function. Nonetheless, we believe that contributions from each individual model system will ultimately help to understand the function of MeCP2 in health and disease. We have used the zebrafish embryonic and larval system to demonstrate that Mecp2 is required for tnfa expression during zebrafish development and inflammation. Besides this, our RNA sequencing results provide insights into the earliest genetic alterations that occur in the absence of MeCP2 function, which ultimately could result in RTT phenotypes. Furthermore, zebrafish embryos are amenable to high-throughput screening for drugs with the potential to remedy these phenotypes (Tan and Zon, 2011). We believe that these findings have the potential to instruct future studies in zebrafish and other model systems to increase our understanding of MeCP2-function and its role in RTT pathogenesis.
Materials and methods

Zebrafish husbandry and maintenance

Zebrafish (*Danio rerio*) were maintained according to the guidelines of the UCSD institutional Animal Care and Use Committee. The following zebrafish lines have been used during this study: AB (wild type strain); *mecp2*<sup>Q63*</sup> mutants (Pietri et al., 2013); Tg(*mpx*:eGFP)<sup>i114</sup> (Renshaw et al., 2006); Tg(*mpeg1*:eGFP)<sup>gl22</sup> (Ellett et al., 2011); Tg(*tnfa*:eGFP) (Marjoram et al., 2015). Genotyping of *mecp2*<sup>Q63*</sup> mutants occurred as previously described (Pietri et al., 2013) When needed for experimental purposes, zebrafish were anesthetized using Tricaine (200 µg/ml).

Microscopy

For stereomicroscopy, embryos and larvae were mounted in E3 medium containing 3% methyl cellulose (Sigma-Aldrich). Bright field images were acquired using a Leica MZ16 stereomicroscope with Leica DFC295 camera (Leica Microsystems). Epifluorescence images were acquired using a AxioZoom.V16 stereomicroscope (Zeiss). For confocal microscopy, larvae were mounted in E3 medium containing 0.5% low melting point agarose (Sigma-Aldrich). Confocal micrographs were acquired using a Leica SP5 confocal system (Leica Microsystems). Images were created using Imaris (Bitplane) and ImageJ software.

Quantitative real-time PCR

mRNA was isolated using the RNeasy mini kit according to the manufacturer’s instructions (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit according to the manufacturer’s instructions (BioRad). Quantitative real-time PCR (qPCR) was performed using iQ SYBR Green Supermix (BioRad) and the BioRad CFX96 real-time system according to the manufacturer’s instructions. Gene expression levels were calculated relative to the expression of the housekeeping gene *tata-binding protein* according to the 2<sup>ΔΔCt</sup> method. Primers used for qPCR analysis of gene expression are listed in Supplementary table 3.

Microinjection of zymosan particles

Zebrafish larvae of 3 dpf were positioned with the dorsal side up to allow injection of 1 nl PBS containing 100 – 150 Alexa594-labeled Zymosan A (*S. cerevisiae*) BioParticles (Molecular Probes) into the brain. As a control for a potential wounding effect, 1 nl of sterile PBS was injected in a similar way. The percentage of zymosan particles phagocytosed by Tg(*mpeg1*:eGFP)-positive cells was determined based on confocal micrographs of the brain.
Microinjection of mRNA, plasmids, and antisense oligonucleotide morpholinos

A gBlock (Integrated DNA Technology) containing full-length zebrafish mecp2 cDNA (ENSDART00000040672) was cloned into a Zero Blunt TOPO PCR vector according to the manufacturer’s instructions (Life Sciences). Zebrafish mecp2 mRNA was synthesized using the mMessage mMachine SP6 Transcription Kit according to the manufacturer’s instructions (Invitrogen). 50 or 100 pg of mecp2 mRNA was injected into the yolk of 1-cell stage zebrafish embryos. The antisense oligonucleotide morpholino targeting mecp2 expression was injected as described by Gao et al. (2015), while the antisense oligonucleotide morpholino targeting tnfa expression was injected as described by Lopez-Munoz et al (2011). Control plasmid (pCS2+) and Tnfa plasmid (Roca et al., 2008) (20 pg) were injected into the yolk sac of 1-cell stage embryos.

RNA sequencing

mRNA was isolated using the RNeasy mini kit according to the manufacturer’s instructions (Qiagen). Library preparation and sequencing was performed by the Institute for Genomic Medicine Center at the University of California, San Diego. RNA sequencing was performed on an Illumina HiSeq4000 platform using single reads of 50 bases in length. RNA sequencing data was mapped to the zebrafish genome (version Zv9) using TopHat 2.1.1 (https://ccb.jhu.edu/software/tophat/index.shtml). Raw counts were submitted to DESeq2 analysis using the Galaxy website (https://usegalaxy.org/). Gene ontology analysis was performed using the Gene Ontology website (http://geneontology.org/). The heatmap displaying differential gene expression was created using Gene-E software (Broad Institute). RNA sequencing data is accessible under Gene Expression Omnibus accession number GSE80348.

NB: GEO private reviewer link:


Statistical analysis

Except for the RNA sequencing, all data (mean ± SEM) were analysed (Prism 5.0, GraphPad Software) using unpaired, two-tailed t-tests for comparisons between two groups and one-way ANOVA with Tukey’s Multiple Comparison method as a post-hoc test for other data (***: p<0.001; **: p<0.01; *: p<0.05; ns: not significant).
Acknowledgements

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Competing Interests

The authors declare that no competing interests exist.
References


Figure 1: mecp2-deficient zebrafish display inflammation during larval development

(A and C) Representative stereo microscopy images of 3 and 7 dpf wild type and mecp2-null zebrafish larvae and (B and D) their total body length as measured in millimeters (mm) (n = 15 per condition; Student T-test; ***: p<0.001; data representative of three individual experiments). (E and F) Stereo microscopy images of 4 and 7 dpf wild type and mecp2-null zebrafish illustrating the gastrointestinal (GI) tract phenotypes regularly observed (indicated by arrowheads). The frequency...
of these phenotypes is shown in relation to the total number of examined animals (19 out of 48 mecp2-null animals at 3dpf; 22 out of 46 mecp2-null animals at 7 dpf). (G) Quantitative real-time PCR was performed to determine the whole-organism gene expression level of the inflammation marker crp relative to the expression of the housekeeping gene tbp. Wild type and mecp2-null samples (n=3 with 20 embryos or larvae pooled per sample) were taken every day for the first 5 days of development. The relative fold change versus gene expression in 1 dpf wild type is shown. (One-way ANOVA with Tukey’s post hoc test; **: p<0.01; ns: not significant; data representative of two individual experiments).
Figure 2: Neutrophil number and distribution confirm the presence of inflammation in mecp2-null larvae

(A) Total numbers of Tg(mpox:eGFP)-positive neutrophils were counted in 3, 4, and 5 dpf wild type and mecp2-null larvae using stereo fluorescent microscopy (n=12 larvae per condition pooled from two individual experiments; larvae were scored for three consecutive days). (B) Representative stereo microscopy images of 4 dpf Tg(mpox:eGFP) wild type and mecp2-null larvae. (C) Numbers of Tg(mpox:eGFP)-positive neutrophils associated with the gastrointestinal tract of 2 to 5 dpf wild type and mecp2-null larvae were counted (n≥12 embryos per condition; data representative of three individual experiments). (D) Representative confocal micrographs (maximum projection) of the gastrointestinal tracts of 5 dpf Tg(mpox:eGFP) wild type and mecp2-null larvae in which the gastrointestinal tract has been delineated with a white dashed line based on the transmitted light images. (E) Representative confocal micrographs (maximum projection) of the brain region of 3 dpf Tg(mpox:eGFP) wild type and mecp2-null larvae. (F) Brain-associated Tg(mpeg1:eGFP)-positive cells were counted for 3 dpf wild type, heterozygous, and mecp2-null larvae (n=7, n=11, n=6, respectively; One-way ANOVA with Tukey’s post hoc test; ns: not significant; data representative of two individual experiments). (G) Total numbers of Tg(mpeg1:eGFP)-positive cells were counted in 3, 4, and 5 dpf wild type and mecp2-null larvae using stereo fluorescent microscopy (n=11 and 12 embryos per condition, respectively; data representative of two individual experiments). A Student T-test was used for all statistical analyses, except for the data analyzed in (F), by comparing wild type and mecp2-null numbers per day (***: p<0.001; **: p<0.01; *: p<0.05; ns: not significant).
Figure 3: Expression of central inflammatory cytokines is dysregulated in mecp2-null larvae

Quantitative real-time PCR was performed to determine the whole-organism gene expression level in wild type or mecp2-null animals of (A) tnfα from 4 hpf to 7 dpf, or (B and C) of il1b and il10 from 1 to 7 dpf. Gene expression is related to the expression of the housekeeping gene tbp, where the fold change relative to gene expression in 1 dpf wild type embryos is shown (n=3 with 20 embryos or larvae pooled per sample for 1 – 7 dpf; 30 embryos were pooled per sample for the 4 – 12 hpf time points; data representative of two individual experiments). A One-way ANOVA with Tukey’s post hoc test was used for all statistical analyses (**: p<0.01; *: p<0.05; ns: not significant). (D – K) Representative confocal micrographs of 3 dpf Tg(tnfa:eGFP) wild type and mecp2-null larvae showing the eGFP expression pattern in (D and H) brain regions in a lateral view, (E and I) brain regions in a dorsal view, (F and J) posterior gut epithelium in a lateral view, and (G and K) dorsal root ganglion neurons in a lateral view.
Figure 4: mecp2-null larvae are unable to increase tnfa expression during an acute inflammatory response

(A) Schematic of the injection of Alexa-594 labeled zymosan into the brains of 3 dpf zebrafish larvae. (B) The percentage of zymosan particles phagocytosed by Tg(mpeg1:eGFP)-positive cells in wild type larvae was determined using confocal microscopy of samples fixed every 5 minutes after injection (n=5 larvae per time point). (C) Representative confocal micrograph of a wild type Tg(mpeg1:eGFP) larvae at 4 hours post injection. An asterix (*) indicates a zymosan particle phagocytosed by Tg(mpeg1:eGFP)-positive cells. (D) Quantitative real-time PCR was performed to determine the whole-organism gene expression level of il1b, tnfa, il10, and tgfβ1 relative to the expression of the
housekeeping gene \( tbp \). Samples (n=3 with 10 embryos per sample) were taken at 1, 2, 3, and 4 hours post injection of zymosan or PBS as a control. The relative fold change of zymosan versus PBS injected samples is shown for each time point to account for a possible wounding effect by the injection itself. (E) Quantitative real-time PCR was performed to determine the whole-organism gene expression level of \( il1b, tnfa, il10 \), and \( tgfb1 \) relative to the expression of the housekeeping gene \( tbp \). Wild type or \( mec2p \)-null samples (n=3 with 10 embryos per sample) were taken at 1, 2, 3, and 4 hours post injection of zymosan. The relative fold change of zymosan injected versus uninjected wild type controls is shown for each time point to not exclude a potential different response in \( mec2p \)-null towards the wound caused by the injection. A One-way ANOVA with Tukey’s post hoc test was used for all statistical analyses (**\( p < 0.001 \); **\( p < 0.01 \); *\( p < 0.05 \); ns: not significant; data representative of at least two individual experiments).
Figure 5: Re-expression of mecp2 in mecp2-null zebrafish embryos partially rescues tnfa gene expression, while enforced expression of tnfa does not alleviate phenotypes caused by Mecp2-deficiency
Wild type and mecp2-null 1 cell stage embryos were injected with 50 pg of full length mecp2 mRNA. Quantitative real-time PCR was performed to determine the whole-organism gene expression level of (A) mecp2, (B) tnfa, and (C) il1b relative to the expression of the housekeeping gene tbp. Wild type and mecp2-null samples (n=3 with 30 embryos pooled per sample) were taken at 24 hours post fertilization. The relative fold change of each condition versus uninjected wild type controls is shown. (D and E) Oligonucleotide morpholino targeting tnfa expression was injected as previously described by Candel et al. (2014). (D) Numbers of Tg(mpox:eGFP)-positive neutrophils associated with the gastrointestinal tract of 2 dpf control and tnfa morpholino injected larvae were counted (n≥9 embryos per condition). (E) Total body length as measured in millimeters (mm) of 2 dpf control and tnfa morpholino injected larvae (n≥25 per condition). (F and G) Wild type and mecp2-null 1 cell stage embryos were injected with tnfa cDNA containing plasmids as previously described by Lopez-Munoz et al (2011). (F) Numbers of Tg(mpox:eGFP)-positive neutrophils associated with the gastrointestinal tract of 3 dpf wild type or mecp2-null larvae (injected with control or tnfa mRNA containing plasmids) were counted (n≥12 embryos per condition). (G) and their total body length (mm) was measured (n≥26 embryos per condition). A One-way ANOVA with Tukey’s post hoc test was used for all statistical analyses involving more than two groups. Student T-test was used for all statistical analyses comparing two groups (***: p<0.001; **: p<0.01; *: p<0.05; ns: not significant; data representative of at least two individual experiments).
Figure 6: RNA sequencing reveals early developmental effects of mecp2-deficiency relevant to RTT

RNA sequencing was performed on RNA isolated from groups of 6 hpf wild type and mecp2-null embryos (n=3 biological replicates per condition with 30 embryos pooled per replicate). DESeq2 analysis was performed using https://usegalaxy.org/. (A) A sample-to-sample distances plot for the three biological replicates per condition was used to detect potential outliers. (B) An MA-plot of differential expression caused by Mecp2-deficiency is shown. The log2 fold change is plotted on the y-axis and the average of the counts normalized by size factor is shown on the x-axis. Each gene is represented with a dot. Genes with an adjusted p value below 0.05 are shown in red. (C and D) Enriched gene ontology (GO) processes for significantly up- or down-regulated genes in mecp2-null
versus wild type embryos are listed in the tables. Only GO terms with at least 2-fold enrichment are shown. For hierarchically clustered GO terms, only the most specific term is included in the list.
Figure 7: Heatmap of differentially expressed genes in mecp2-null embryos

(A) A heatmap displays the extent of differential gene expression between 6 hpf mecp2-null versus wild type embryos. The genes incorporated in the heatmap represent all differentially expressed...
genes that belong to the gene ontology terms ‘developmental growth’ (downregulated genes), ‘myeloid cell differentiation’ (upregulated genes), and ‘hepaticobiliary system development’ (upregulated genes). For all genes, the positive or negative normalized fold change (nFC) for mecp2-null embryos versus wild type embryos is shown. (B) The graph displays the percentage of significantly differentially expressed genes in mecp2-null versus wild type with a fold change equal to or less than 2 (≤ 2 fold change) or higher than 2 (> 2 fold change). The following groups are shown: significantly differentially expressed genes belonging to the gene ontology terms ‘developmental growth’, ‘myeloid cell differentiation’, and ‘hepaticobiliary system development’; genome wide significantly upregulated genes; genome wide significantly downregulated genes; and all significantly differentially expressed genes.
Supplementary figure 1: Neutrophil numbers in the head and brain region of wild type and mecp2-null larvae

(A) Tg(mpox:eGFP)-positive neutrophils were enumerated in the head region of 5 dpf wild type and mecp2-null larvae using stereo fluorescent microscopy (n=12 embryos per condition). (B) Numbers of Tg(mpox:eGFP)-positive neutrophils present in the brain were counted for wild type and mecp2-null larvae at 5 dpf (n=12 embryos per condition). (C) Representative stereo microscopy images of the head region of 5 dpf Tg(mpox:eGFP) wild type and mecp2-null larvae in which the brain tissue has been indicated with a white dotted line. A Mann–Whitney U test was used for statistical analysis (*: p<0.05; ns: not significant). (D) Oligonucleotide morpholino targeting mecp2 expression was injected as previously described by Gao et al. (2015). Numbers of Tg(mpox:eGFP)-positive neutrophils associated with the gastrointestinal tract of 2 dpf control and mecp2 morpholino injected larvae were counted (n≥30 embryos per condition). Student T-test was used for the statistical analyses (***: p<0.001).
Supplementary figure 2: Expression levels of *il6*, *cxcl8a*, *tgfb1*

Quantitative real-time PCR was performed to determine the whole-organism gene expression level of (A) *il6*, (B) *cxcl8a*, and (C) *tgfb1* in wild type or *mecp2*-null animals. Gene expression is related to the expression of the housekeeping gene *tbp*, while the fold change relative to gene expression in 1 dpf wild type embryos is shown (n=3 with 20 embryos or larvae pooled per sample). A One-way ANOVA with Tukey’s post hoc test was used for all statistical analyses (ns: not significant).
Supplementary figure 3: Confirming the presence of Tg(tnfa:eGFP) in mecp2-null larvae

Since mecp2-null larvae display reduced tnfa gene expression levels and reduced Tg(tnfa:eGFP) encoded GFP expression, the presence of the Tg(tnfa:eGFP) construct in mecp2-null larvae was confirmed by genotyping with the following primers: ACGACGGCAACTACAAGACC (forward) and GTCCTCCTTAGTCAAGGCTG (reverse). The resulting PCR product was detected using gel electrophoresis. The mecp2-null larvae were positive for the Tg(tnfa:eGFP) transgene, which corroborates the residual GFP expression observed in mecp2-null larvae with confocal microscopy (Figure 3).
Supplementary figure 4: Microinjection of zymosan into the brain of zebrafish larvae

(A) Zymosan was injected into the brain of 3 dpf wild type larvae. Quantitative real-time PCR was performed to determine gene expression level of il1b relative to the expression of the housekeeping gene tbp in the dissected heads and bodies of injected larvae. Samples (n=3 with 10 heads or bodies per sample) were taken at 1 hour post injection of zymosan or PBS as a control. The relative fold change of zymosan versus PBS injected samples is shown to account for a possible wounding effect by the injection itself. A One-way ANOVA with Tukey’s post hoc test was used for statistical analysis (*: p<0.05; ns: not significant).

(B) The percentage of zymosan particles phagocytes by Tg(mpeg1:eGFP)-positive cells for wild type and mec2-null larvae using confocal microscopy of samples fixed 30 minutes after injection (n=5 larvae per condition). A Student T-test was used for statistical analysis (ns: not significant).

(C) Representative confocal micrographs of a wild type and mec2-null Tg(mpeg1:eGFP) larvae at 30 minutes post injection. An asterix (*) indicates zymosan phagocytosed by Tg(mpeg1:eGFP)-positive cells.
Supplementary figure 5: over-expression of *mecp2* in wild type zebrafish embryos

Wild type 1 cell stage embryos were injected with 100 pg of full length *mecp2* mRNA. Quantitative real-time PCR was performed to determine the whole-organism gene expression level of (A) *mecp2*, (B) *tnfa*, and (C) *il1b* relative to the expression of the housekeeping gene *tbp*. Wild type and RNA-injected samples (n=3 with 30 embryos pooled per sample) were taken at 1 and 3 days post fertilization. The relative fold change of each condition versus uninjected wild type controls is shown. A One-way ANOVA with Tukey’s post hoc test was used for all statistical analyses (***: p<0.001; ns: not significant).
Supplementary table 1: Primers used for quantitative real-time PCR

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Supplementary figure 6: over-expression of tnf in mec2p-null zebrafish embryos

Wild type (wt) and mec2p-null (mec2p-/-) 1-cell stage embryos were injected with 20 pg of plasmid containing full length mec2p cDNA (+pCS2+_Tnfa), or an empty plasmid as control (+pCS2+). Plasmid-injected samples (n=10 embryos pooled per sample) were taken at 2 days post fertilization. RNA was isolated from these samples and DNaseI treatment was performed to prevent carryover of plasmid DNA (+ DNaseI). Quantitative real-time PCR was performed to determine the whole-organism gene expression level of tnf relative to the expression of the housekeeping gene ef1a. Non-reverse transcriptase treated samples (- RT) were taken along as a control to exclude plasmid DNA contamination.