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Authors
Paraiso, Kitt D
Blitz, Ira L
Zhou, Jeff J
et al.

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Morpholinos do not elicit an innate immune response during early *Xenopus* embryogenesis

Kitt D. Paraiso[1,2], **Ira L. Blitz**[1], **Jeff J. Zhou**[1], and **Ken W.Y. Cho**[1,2,3,*]

[1]Developmental and Cell Biology, University of California, Irvine

[2]Center for Complex Biological Systems, University of California, Irvine

[3]Lead Contact

SUMMARY

Gentsch et al. (2018) recently reported that a common side effect of translation-blocking morpholino antisense oligonucleotides is the induction of a set of innate immune response genes in *Xenopus* embryos, and that splicing-blocking morpholinos lead to unexpected off-target mis-splicing events. Here we present an analysis of all publicly available *Xenopus* RNA-seq data in a reexamination of effects of translation-blocking morpholinos on the innate immune response. Our analysis does not support the authors' general conclusion, which was based on a limited number of RNA-seq datasets. Moreover, the strong induction of an immune response appears to be specific to the *tbxt/tbxt2* morpholinos. The more comprehensive study presented here indicates that using morpholinos for targeted gene knockdowns remains of considerable value for the rapid identification of gene function.

eTOC BLURB

Gene expression interference by morpholinos has been questioned due to unwanted side-effects, including immune response induction by a limited set of morpholinos. By performing a metaanalysis of available transcriptomic datasets, Paraiso et al. show that induction of an immune response is not a general side-effect of morpholinos during early embryogenesis.

*Correspondence: kwcho@uci.edu.

AUTHOR CONTRIBUTIONS

K.D.P., K.W.Y.C., and I.L.B. wrote the manuscript. K.D.P. and J.J.Z. performed bioinformatics analyses. All authors participated in wet bench experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Table S3 *(Related to Figure 3,4) List of innate immune response genes identified by gene ontology or through literature by the work of Robert and Ohta.*

Keywords

translational blocking morpholino; innate immune response; *tbxt*; *brachyury*; loss of function; reverse genetics; gene knockdown; *Xenopus*; zebrafish

INTRODUCTION

Morpholino antisense oligonucleotides (MO) have been used widely for nearly two decades in both the *Xenopus* and zebrafish research communities to transiently knockdown the function of targeted genes (Heasman et al., 2000; Nasevicius et al., 2000). The method is relatively inexpensive and quite rapid, as the analysis of morphants can be directly performed in injected F0 embryos. However, use of MOs in zebrafish was suggested to induce unwanted side effects including the induction of cell death in the nervous system and expression of *tp53* transcripts derived from an alternative promoter (Robu et al., 2007), whereas no evidence of such issues has been reported in *Xenopus* or other species. In addition, the appropriateness of MOs as a loss-of-function (LOF) tool has been questioned, because the majority of phenotypes resulting from a subset of MO knockdown experiments in zebrafish were not seen in corresponding genetic LOF mutants (Kok et al., 2015). Others have suggested that these differences could be explained by genetic compensation in LOF mutants (Rossi et al., 2015). The utility of MOs as a genetic tool has been met by opposing views in both *Xenopus* and zebrafish (Blum et al., 2015; Stainier et al., 2015).

In the January 2018 issue of *Developmental Cell*, a report using RNA-seq analysis suggested that a side effect of the use of translation-blocking MOs targeting *tbxt/brachyury* paralogs in *Xenopus tropicalis* embryos caused induction of a significant number of genes involved in the innate immune response, and that injection of splice-blocking MOs led to off-target splicing defects (Gentsch et al., 2018). This study examined a limited set of published RNA-seq datasets from MO-mediated LOF and concluded that the induction of an innate immune response by translation-blocking MOs is a *common* side effect. The earliest time point whereby embryonic cells can induce an innate immune response is unclear. Induction of innate immune response related genes *tp53, tp53inp1* and *c3ar1* by MOs in RNA-seq datasets generated as early neurula stage/stage 14 suggest that relevant immune cells might not be required as migrating myeloid progenitor and hemangioblast progenitor cells only appear at stage 14 and stage 18, respectively (Briggs et al., 2018). This study suggested that this immune response is cell intrinsic and can be activated in all embryonic cells; and that this initiates at least as early as neurula stage. We are interested in the function of maternal effect gene products in early stage embryos and these proteins are synthesized from spliced mRNAs deposited in the egg during oogenesis. Therefore we re-examined whether translation blocking MOs cause induction of innate immune genes, and not whether spliceblocking MOs result in off-target mis-splicing. Because the published genome-wide analysis was based only on a limited number of MO knockdown experiments, we wished to address whether the effects of MOs on an innate immunity response are indeed a common occurrence. Since the previous analysis was restricted to embryos of mid-neurula and later stages, we also wanted to determine whether induction of an innate immune response occurs in the period between the onset of zygotic transcription and neurula stages. The question is

fundamentally important, as both the *Xenopus* and zebrafish research communities have used MOs to uncover the function of many genes. Contrary to Gentsch et al., our analysis of 54 publicly available *Xenopus* MO knockdown datasets with their corresponding control datasets demonstrates that cohorts of *Xenopus* innate immune response genes are not commonly activated by translation-blocking MOs, but we did find infrequent activation of a few genes reported. Based on currently available transcriptomic datasets, we suggest that the strong effects observed by Gentsch et al. are confined to the use of *tbxt/tbxt2* (formerly known as *t/brachyury* and *t2/brachyury2*), and that the use of translation-blocking MOs remains a useful approach to uncovering the biological function of genes during early *Xenopus* embryogenesis.

RESULTS

Strong induction of *tp53, tp53inp1*, and *c3ar1* genes is confined to the injection of *tbxt/tbxt2* MO oligonucleotides

To validate the results by Gentsch et al. (2018) we first searched for all current publicly available *X. tropicalis* and *X. laevis* RNA-seq datasets in the NCBI Sequence Read Archive (SRA), the European Nucleotide Archive (ENA), and the DNA Databank of Japan Sequence Read Archive (DRA) for data involving MO knockdown experiments. We found 16 projects comprised of 48 *X. laevis* and 91 *X. tropicalis* RNA-seq datasets (Table S1). All 16 projects used MOs except for the Gazdag et al. (2016) datasets, which used alternative stabilized antisense oligonucleotides. Among the 16, 10 projects (Kwon et al., 2014; Chiu et al., 2014; Yasuoka et al., 2014; Marlétaz et al., 2015; Dichmann et al., 2015; Nakamura et al., 2016; Campbell et al., 2016; Noiret et al., 2016; Ding et al., 2017; Gentsch et al., 2018) contained experiments where the GeneTools standard control or experimental MO injected sample could be compared with a non-MO injected control (i.e., uninjected or water injected). We used only the datasets that had these controls for our analysis. The extent of our analysis includes morphant sequencing datasets ranging from stage 9 through stage 36, encompassing those experiments analyzed by Gentsch et al. from stage 14 through stage 36. A majority of the sequencing datasets we analyzed overlapped with stages analyzed by Gentsch et al. during neurula (N = 18), early tailbud (N = 14) and late tailbud (N = 6). We extended the analysis to early embryogenesis by including datasets from blastula (N = 2) and gastrula (N = 14) stages.

Among the innate immune response genes induced by MOs, the expression of *tp53inp1*, *tp53*, and *c3ar1* were those most extensively studied in Gentsch et al., and therefore we sought to reproduce their results in our initial analyses by examining the expression of each of these genes in the newly collected MO knockdown data (Figure S1). Gentsch et al. reported that these three genes were induced not only following an injection of a *tbxt/tbxt2* quadruple MO cocktail (Figure 1A), but also after control MO injection, although the inductions were weaker than in the *tbxt/tbxt2* MO injections. To further validate these results, we performed our own microinjections of the standard control MO into *X. tropicalis* embryos in biological replicates. Contrary to their findings, RT-qPCR shows that *tp53* and *tp53inp1* are generally not induced across all biological replicates, regardless of

developmental stage (Figure 1B). *c3ar1* induction, on the other hand appears consistent with the published findings.

We then examined the expression of these genes among all other available *X. tropicalis* and *X. laevis* datasets and found that the inductions of *tp53inp1* and *tp53* were clearly weaker than in the expression data reported by Gentsch et al., i.e., mean inductions < 1.5-fold. For *c3ar1*, the mean induction was < 2-fold (Figure 1C, D). Because we aligned the reads to the version 9 *X. tropicalis* genome assembly using Bowtie2 and RSEM while the published study aligned to the version 7 assembly using STAR, we also examined the possibility that discrepancies between conclusions might have arisen based on the use of different bioinformatics analysis protocols. The fold changes reported in Gentsch et al. were comparable to those in our experiments, with the exception of *X. laevis c3ar1.L* (the *c3ar1* homeologous gene copy found on the long chromosome subset of the allotetraploid *X. laevis* genome). This difference with *c3ar1.L* was likely due to its low expression levels in the *exosc9* MO experiment, resulting in high variance in fold change quantitation (Table S2). Overall, our experiments and meta-analysis of public RNA-seq datasets suggest that there is no induction of *tp53* and *tp53inp1*, while the induction of *c3ar1* is variable.

A cohort of innate immune response genes are not commonly activated by morpholino oligonucleotides

As we find little evidence of an innate immune response by assaying for the expression of *tp53inp1*, *tp53*, and *c3ar1*, we wondered whether we could detect this biological process from the transcriptomic datasets by looking at a larger cohort of genes. Because a list of the innate immune response genes in *Xenopus* was not available in the Gentsch et al. paper, we performed differential expression analysis using the same software and parameters as used in their study. We compared available RNA-seq datasets from *tbxt/tbxt2* morphants and *tbxt*$^{-/-}$;*tbxt2*$^{-/-}$ mutant embryos and identified 1,154 genes that were specifically activated in the morphants compared to their respective controls. Among these genes, Gene Ontology (GO) analysis identified three innate immune response-related terms: ‘innate immune response’ (GO:0045087), ‘regulation of innate immune response’ (GO:0045088), and ‘positive regulation of innate immune response’ (GO:0045089). We combined the genes corresponding to these three GO terms and generated two gene lists (one for *X. tropicalis* and the other for *X. laevis*, Table S3), and used these lists for subsequent analyses. The 77 *X. tropicalis* and 120 *X. laevis* lists are comprised of genes involved in various subsystems of the innate immune response including complement system genes such as *c1r, c1s, c3, c4a*, and *c9*; the signaling molecule *nfkb1* (which regulates cytokine production); and interferon regulatory transcription factor genes such as *irf1*, *irf7*, and *irf9*.

We then determined whether any of the other publicly available RNA-seq datasets involving MO experiments showed activation of the genes from our combined list for *Xenopus* innate immune response discussed above. These innate immunity genes are generally not activated in either the *X. tropicalis* or *X. laevis* datasets when a 1.5-fold expression level difference is used as a cutoff value (Figure 2A,B). While a few datasets showed statistically significant activation, that was not a consistent occurrence among biological replicates. On the contrary, and as expected, the majority of the *tbxt/tbxt2* datasets did show an up-regulation of the

cohort of innate immunity genes in stage 26 and stage 34 embryos (Figure 2C). The standard control MO injection at stage 34 displayed a weak up-regulation that, while the median was < 1.5 fold, was nevertheless statistically significant (Figure 2C). We conclude that gene cohort analysis using the GO-identified genes does not detect statistically significant induction of innate immune response genes resulting from the injection of MOs.

The analysis we performed thus far might not provide a complete view of the induction of innate immune response genes. Large cohort analysis can carry a risk of minimizing the contributions of specific genes in the analysis pipeline. Additionally, the innate immune genes induced in *tbxt/tbxt2* morphants might be inductions specific to this MO cocktail, but might not reveal a set of innate immune genes that are induced by other MOs. Therefore, we employed two additional analyses. First, because Robert and Ohta (2009) had provided an annotated list of innate immune response genes conserved between mammals and *Xenopus*, we worked from that list to identify corresponding gene models in the *X. tropicalis* v9.0 and *X. laevis* v9.2 genome assemblies by means of both gene name matching and BLAST alignments. That analysis identified a set of 53 *X. tropicalis* gene models and 81 *X. laevis* gene models. The lists included categories such as leukocyte receptors, signaling molecules, cytokines, cytotoxic killing genes, antibacterial peptides, and the complement system (Table S3). When these lists were compared with the set of genes from the previous GO-identified cohort, only 13/53 of *X. tropicalis* and 15/81 *X. laevis* gene models overlapped. Therefore, using the Robert and Ohta gene collection expands our analysis beyond the list derived from GO annotations. We then determined whether any of the innate immunity genes from the Robert and Ohta were induced in the available MO-injected datasets. We did not detect significant activation (p-value of < 0.01) of innate immunity genes with the exception of four samples (Figure 3A,B). The literature-identified cohort of innate immune response genes was again seen to be most activated by the *tbxt/tbxt2* MO cocktail at stage 34, and less strongly at stage 26 (Figure 3C).

In a second analysis, we examined the list of differentially expressed genes from each of the available datasets to determine whether different subsets of innate immunity genes were significantly induced by different MOs. Analysis was performed on all datasets containing at least two replicates to obtain lists of genes that are differentially expressed in individual MO-injected samples relative to uninjected (or water injected) sibling embryos. We applied the same cutoff criteria described by Gentsch et al. (2018) of 1.5-fold change with an adjusted p-value of < 0.1, to create these gene lists. GO enrichment analysis was then performed on each gene list. GO terms related to innate immune response are significantly enriched in the *tbxt/tbxt2* MO dataset at stage 34, but less significantly at stage 26 (Figure S2). The control MO from Gentsch et al. showed some enrichment of GO terms related to innate immune response at stage 34, but not at stage 26. When we performed similar differential expression analyses with all the other available datasets we were unable to detect any enrichment of GO terms related to innate immune response (Figures S3, S4). Taking these observations together with other analyses, we conclude that innate immune response induction is not a common feature of a MO-injected transcriptome. The robust induction of the innate immune seems to be specific to the *tbxt/tbxt2* MO. We did not observe the excessive induction of an immune response by control MO prior to stage 34 (Figure 2A, 2C, 3A, 3C).

tbxt/tbxt2 MOs are unusual in inducing a subset of innate immune response genes

GO term enrichment analysis did not reveal innate immune induction, with the exception of the *tbxt/tbxt2* MOs and control MOs (but only at stage 34). Therefore, we next examined whether individual genes other than *tp53inp1*, *tp53*, and *c3ar1* were consistently activated by MO injection. If MO injections generally induce innate immune responses, then a key set of innate immunity genes should be up-regulated across embryos injected with different MOs. We combined the GO-identified innate immune genes with those identified by Robert and Ohta (Table S3) and searched for genes meeting the following two criteria: a t-test p-value < 0.01, and a fold change up-regulation > 1.5. Among all the *X. tropicalis* datasets, five genes *(rab7b, riok3, irg1*, *ripk2*, and *c1s*) in the GO-identified cohort and four genes *(il1*, *c1q, c1s*, and *c5)* in the Robert and Ohta cohort were up-regulated (Figure 4A,B). However, if the *tbxt/tbxt2* MO datasets are removed from the analysis, none of these genes are upregulated in a statistically significant manner. These results indicate a strong contribution from the *tbxt/tbxt2* datasets to the outcome. As this effect is not seen in other MO injection experiments, we suggest that the strong upregulation of select innate immune response genes is not a general phenomenon related to MO injection, but rather is a peculiarity associated with *tbxt/tbxt2* datasets (Figure 1).

A similar analysis was performed for the five *X. laevis* MO datasets using the combined gene lists from the GO-identified genes and those identified by Robert and Ohta (Tables S3). Among these, activation of only two genes, *ptafr.L* (platelet activating factor receptor), *socs3.L* and *socs3.S* (suppressor of cytokine signaling 3), were statistically significant (Figure 4C,D). Neither of these were found in the analysis of the *X. tropicalis* datasets above. At present, the role of *Xenopus ptafr.L* in innate immunity is not well understood. Much of what is known about the *socs3* gene concerns its role during regeneration following wounding wherein *socs3* is induced after epithelial (Kuliyev et al., 2005) and retinal ganglion optic nerve (Whitworth et al., 2017) wounding, as well as in spinal cord (Lee-Liu et al., 2014) and limb (Grow et al., 2006) regeneration models. Thus, a common set of genes does not appear to be upregulated between *X. tropicalis* and X. *laevis* as part of an immune response.

DISCUSSION

The combined use of MOs and RNA-seq has become a powerful tool in assaying genome-wide functions of developmental genes. Particularly, as we are interested in establishing gene regulatory networks in the early embryo using these technologies, the findings by Gentsch et al. (2018) whereby MOs can induce innate immune response from early embryos, has been a cause of concern in the analysis of transcriptomic datasets. However, when we examined publically available RNA-seq datasets generated from multiple labs including our own, we find no compelling evidence that MOs cause an innate immune response prior to late tailbud stages/stage 34. The only strong effects we identified appear to be particular to the *tbxt/tbxt2* quadruple MO experiments. Interestingly, Gentsch et al. have suggested that the induction of an immune response could be dependent on the GC content of MOs as stronger induction of innate immune response genes was detected with MOs having higher GC content. However, we note that our analysis using *foxh1* and *gsc* MOs

having relatively high GC content, 60% and 56%, respectively, did not induce immune response genes during gastrula stages (compared to the standard control MO GC content of 32%). Therefore, we believe that MOs still remain a powerful knockdown tool with proper controls in assaying for gene function, especially combined with the use of RNA sequencing methods.

Stage dependence of an immune response

Because later stage samples from the standard control MO and *tbxt/tbxt2* MOs by Gentsch et al. showed induction (Figure 2A,C), this finding suggests that there is stage dependence in eliciting an immune response. Consistent with this finding, the standard control MO experiments by Marlétaz et al. (2015), Nakamura et al. (2016), and Yasuoka et al. (2015) which were performed at stage 14 or earlier, did not show a strong induction of innate immune response genes. Most of the available *Xenopus* datasets we analyzed had been generated on or prior to stage 14 except for three from *X. laevis*. But when we analyzed these three later-stage *X. laevis* datasets, one from stage 20 *(rfx2* MO) and two from stage 26 *(ptbp1* and *exosc9* MOs), none of the three showed any statistically significant induction of innate immune response genes (Figure 2C).

The lack of innate immune response in early embryonic stages is consistent with the biology of the early immune system in *Xenopus* embryos. Functional primitive myeloid cells are reported to be first detected during early tailbud stages (stage 26) (Costa et al., 2008). In addition, recent single cell RNA-seq datasets (Briggs et al., 2018) have shown that the initial appearance of migrating myeloid progenitor and hemangioblast progenitor cells occurs during neurulation, at stages 14 and 18, respectively. At present, it is unclear as to whether these progenitor cells are competent to perform immune-related functions during these stages. We note that our analysis largely considers the effects of MOs on embryos at neurula or earlier stages due to the scarcity of transcriptomic datasets in later stages. Therefore, the induction of an innate immune response and the mechanism thereof during these later stages is still unknown.

Explaining the discrepancy

How then can we explain the discrepancy between our conclusions and that of Gentsch et al.? As shown here, the *tbxt/tbxt2* MO cocktail's effects are an outlier when compared to other MOs. While some MOs can up-regulate a small number of genes related to an innate immune response, a larger scale genomewide effect seen with the *tbxt/tbxt2* MOs is not seen in these other experiments.

Why are *c3ar1, socs3* and *ptafr*, genes associated with innate immune responses, up-regulated in some MO experiments? Of these three genes, when examining *X. tropicalis* RNA-seq datasets, only *c3ar1* is found to be up-regulated (after *tbxt/tbxt2* datasets are excluded from analysis), albeit in inconsistent manner. Interestingly, *c3ar1* expression is upregulated by MOs targeting mesodermally-active transcription factors during gastrula stages (e.g., Cdx1, Cdx2, Cdx4, Gsc), but not regulators of epidermal development during tailbud stages (e.g., Ptbp1, Rfx2, or Exosc9). Perhaps perturbation of TFs in the mesoderm leads to up-regulation of *c3ar1*, which is broadly expressed in this tissue during gastrula

stages (McLin et al., 2008). C3ar1 is a chemotactic receptor that, along with its ligand, C3, plays a role in numerous developmental events where morphogenetic movements require chemotaxis. *c3ar1.L* is required for radial intercalation during epiboly and cohesive migration of neural crest cells (Carmona- Fontaine et al., 2011; Szabo et al., 2016). *c3ar1* (and *c3)* is also expressed in the developing eye, otic placodes, and in the presumptive liver of the tailbud embryo (McLin et al., 2008)., Thus, disruption of various processes during early development might result in induction of *c3ar1*, independent of this gene's role in innate immunity.

When analyzing *X. laevis* (Figure 4C-E), but not *X. tropicalis*, datasets, injection of *ptbp1, rfx2*, or *exosc9* MOs leads to up-regulation of both homeologs of *socs3*, and *ptafr.L*, but again only inconsistently. During normal development, *socs3* is expressed at tailbud stages in neural tube, neural crest cells, the dorsal epidermis, and somites, suggesting a developmental role for this factor in these ecto- and mesodermal derivatives (Yan et al., 2015). This finding is interesting in that *ptbpl*, *rfx2*, and *exosc9* are all involved in normal epidermal development. Phenotypically, *ptbp1* and *exosc9* morphants exhibit blister formation underneath the dorsal fin of tailbud embryos and display disruptions of epidermal layer formation (Noiret et al., 2016). The *rfx2* gene encodes a critical transcription factor involved in regulation of ciliogenesis in the epidermis (Chung et. al, 2014; Kwon et al., 2014). Thus, induction of *socs3.L* after *ptbpl*, *rfx2*, or *exosc9* MO injections is likely the result of perturbations to normal epidermal development. A role for *ptafr* in early *Xenopus* development has not been reported. Thus, while we see infrequent activation of a handful of genes by MO injection, these could be due to developmental regulation, rather than an immune response.

How can the differences in expression of numerous genes (including *c3ar1*)between *tbxt/tbxt2* MO knockdowns and mutants in the Gentsch et al. study be explained? Currently, it is difficult to answer this question decisively, however there are a number of possible explanations. First, it is tempting to speculate that a compensation mechanism, as has been proposed in zebrafish to explain reported discrepancies between some morphants and their mutant counterparts (Rossi et al., 2015), might be operational here. Other alternatives might include the efficacy of the *tbxt/tbxt2* MOs by the tailbud stages where RNA-seq was performed, or how different genetic backgrounds of the mutant and morphant embryos contributes to the observations reported.

Like Gentsch et al., a recent MO experiment in zebrafish (doi: https://doi.org/10.1101/479188) has noted increased expression of a selected group of interferon-stimulated genes, particularly during segmentation stage (equivalent to *Xenopus* tailbud stage). Hence, it remains possible that MOs may induce an immune response during later development, and should be used with proper controls. In addition, as available transcriptomic datasets are largely generated for early embryonic development, neither ours nor Gentsch et al.'s findings are conclusive to determine whether an immune response is induced by MOs specifically during later embryonic development. Based on our extensive analysis, we conclude that MOs do not elicit an innate immune response during early *Xenopus* embryogenesis

STAR METHODS

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ken W.Y. Cho (kwcho@uci.edu).

Experimental model and subject details

Xenopus tropicalis adults were obtained either from NASCO (University of Virginia stock) or raised in the laboratory; and were maintained in agreement with the University of California, Irvine Institutional Animal Care Use Committee (IACUC). *X. tropicalis* females were injected with 10 units of Chorulon HCG (Merck and Co.) 1-3 nights prior to embryo collection, and 100 units of HCG on the day of embryo collection. Eggs were collected in dishes coated with 0.1% BSA in 1/9× MMR. Sperm suspension in 0.1% BSA in 1/9× MMR was obtained from sacrificed adult *X. tropicalis* males and the eggs were *in vitro* fertilized with sperm suspension (Ogino et al., 2006). The embryos were dejellied with 3% cysteine in 1/9x MMR pH 7.8 for 10 minutes after fertilization, and were then ready for manipulation. Embryos were staged using the Nieukwoop-Faber developmental table (Nieuwkoop and Faber, 1958; Khokha et al., 2002).

Method Details

Standard control MO microinjection—The standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') was obtained from GeneTools, LLC. *X. tropicalis* embryos were injected with 20 ng of the standard control MO at 1-2 cells stage. RNA is harvested from whole embryos at either stage 10 or stage 36 based on the NF developmental table using previously described methods (Chomczynski et al., 1987). RNA samples were reverse transcribed, and gene expression was assayed with qPCR using the Roche Lightcycler 480 II and the Roche SYBR green I master with the default SYBR green protocol. Fold change in gene expression between uninjected and control MO injected was calculated using the ΔΔCp approach.

Identification of cohorts of innate immune response genes using Gene Ontology—The RNA-seq datasets from Gentsch et al. (2018) were obtained from NCBI GEO using the accession number GSE96655. The reads were aligned to the *X. tropicalis* genome v9.0 (Hellsten et al., 2010; Karimi et al., 2018) using Bowtie2 v2.2.7 (Langmead and Salzberg, 2012) and RSEM v1.2.12 (Li and Dewey, 2011). Differential expression was performed using DEseq2 (Love et al., 2014) using the cutoffs of > 1.5 fold change and < 10% FDR. The control MO and tbxt/tbxt2 MO RNA-seq experiments were compared to their respective sibling uninjected controls; while the $tbxt^{-/-};tbxt2^{-/-}$ mutant RNA-seq experiments were compared to their respective wild type controls. From this analysis, we identified the list of genes that are upregulated in the control MO or the tbxt/tbxt2 MOs, that are not upregulated in the $tbxt^{-/-}tbxt2^{-/-}$ mutants. Gene Ontology analysis was performed using the Gene Ontology Consortium online tool (Ashburner et al., 2000; The Gene Ontology Consortium, 2017) and obtained three GO terms related to innate immune response. From these three terms, we obtained a list of genes in our differential expression analysis that are associated either one of the three GO terms.

Identification of cohorts of innate immune response genes from Robert and Ohta—*Xenopus* genes that are associated with innate immunity were identified from Robert and Ohta (2009). We then searched for their corresponding gene models in the *X.tropicalis* genome v9.0 (Hellsten et al., 2010; Karimi et al., 2018) and the X. *laevis* genome v9.2 (Session et al., 2016; Karimi et al., 2018).

Meta-analysis of published RNA-seq datasets using MOs—We searched for RNA-seq datasets that involved the use of knockdown technologies in *X. tropicalis* and *X. laevis* in the NCBI Sequence Read Archive (SRA), the European Nucleotide Archive (ENA), and the DNA Databank of Japan Sequence Read Archive (DRA). We obtained datasets from 16 projects (Table S1) (Tandon et al., 2013; Gentsch et al., 2013; Kwon et al., 2014; Chiu et al., 2014; Yasuoka et al., 2014; Marlétaz et al., 2015; Dichmann et al., 2015; Wills et al., 2015; Nakamura et al., 2016; Campbell et al., 2016; Gazdag et al., 2016; Gao et al., 2016; Noiret et al., 2016; Ding et al., 2017; Gentsch et al., 2018; Skariah et al., 2018). We aligned the reads to the appropriate the *X. tropicalis* genome v9.0 (Hellsten et al., 2010; Karimi et al., 2018) or the *X. laevis* genome v9.2 (Session et al., 2016; Karimi et al., 2018) using Bowtie2 v2.2.7 (Langmead and Salzberg, 2012) and RSEM v1.2.12 (Li and Dewey, 2011) to obtain the expression pattern in transcripts per million (TPM) or normalized read counts. Data figures were generated using the functions *boxplot, plot* and *barplot*; and statistical significance of fold changes was tested using the function *t.test* in R v3.1.0, all using the expression in TPM (R Core Team, 2014). For Gene Ontology analysis, we first performed differential expression using DEseq2 (Love et al., 2014) using the cutoffs of > 1.5 fold change and < 10% FDR. Metascape (Tripathi et al, 2015) was used to perform Gene Ontology analysis and visualize enrichment results, with default parameters whereby significant GO terms were identified with a minimum overlap of 3, p-value > 0.01, and a minimum enrichment of 1.5. Datasets that did not yield any GO terms due to low number of differentially expressed genes were not reported.

Quantification and statistical analysis

Data quantification and statistical analysis are described in the method details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Analyzed publicly available *Xenopus* morphant RNA-seq datasets
- Innate immune response gene induction is not a general effect related to morpholinos
- Strong induction of an immune response is specific to the *tbxt/tbxt2* morpholinos

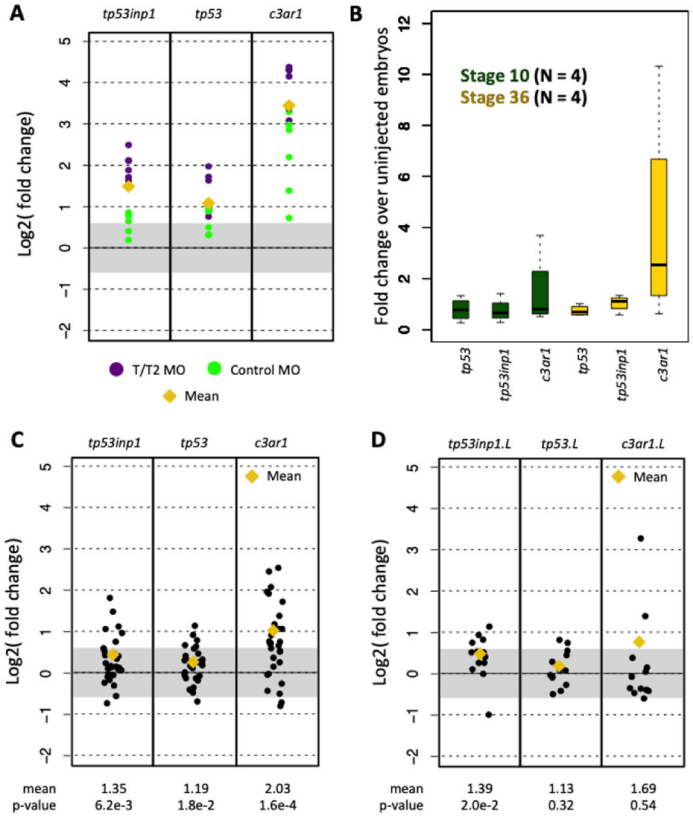

Figure 1. Expression of innate immune response genes in X. tropicalis and X. laevis RNA-seq datasets.

(A) Fold change in induction caused by the tbxt/tbxt2 MOs and the control MOs. (B) Fold change caused by control MO in biological replicates at stages 10 and 36 using RT-qPCR. (C,D) Fold change induction of innate immune response genes in *X. tropicalis* (C) and *X. laevis* (D) datasets.

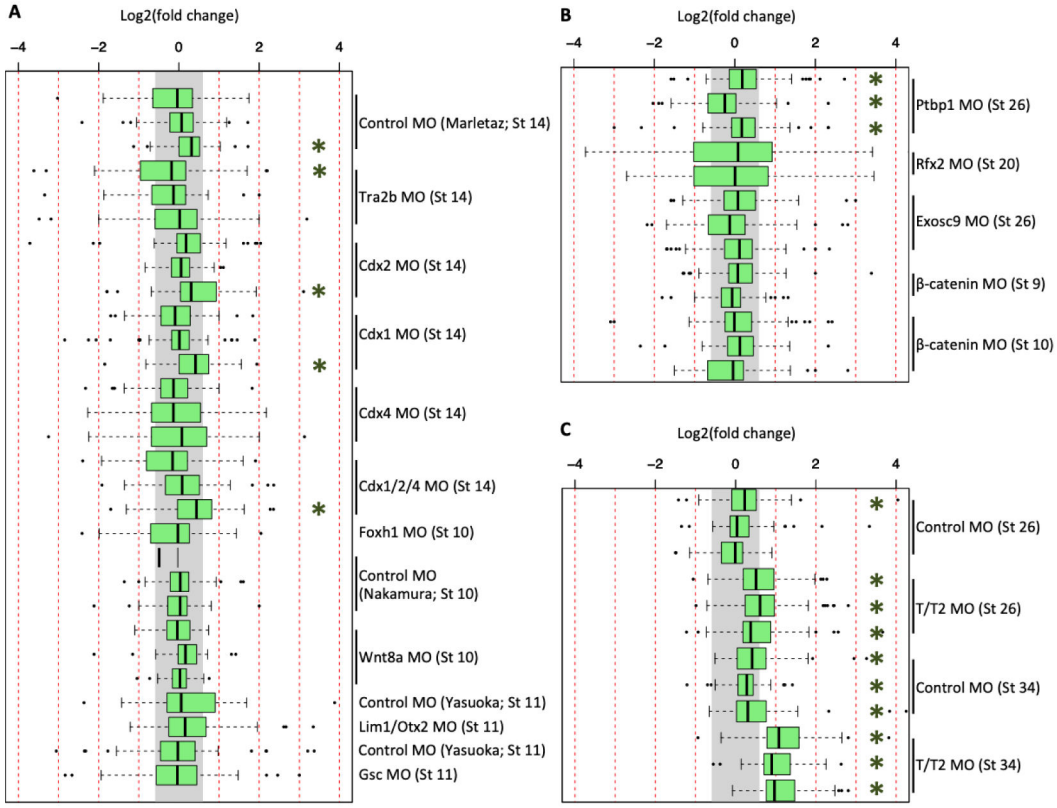

Figure 2. Expression of GO-identified innate immune response genes in X. tropicalis and X. laevis RNA-seq datasets.

Fold change expression of innate immune response genes across 29 datasets in *X. tropicalis* (A), 13 datasets in *X. laevis* (B), and in 12 the tbxt/t2 MO datasets (C). Gray region indicates fold change of < 1.5x. Green asterisk (*) indicates a T-test p-value of < 0.01.

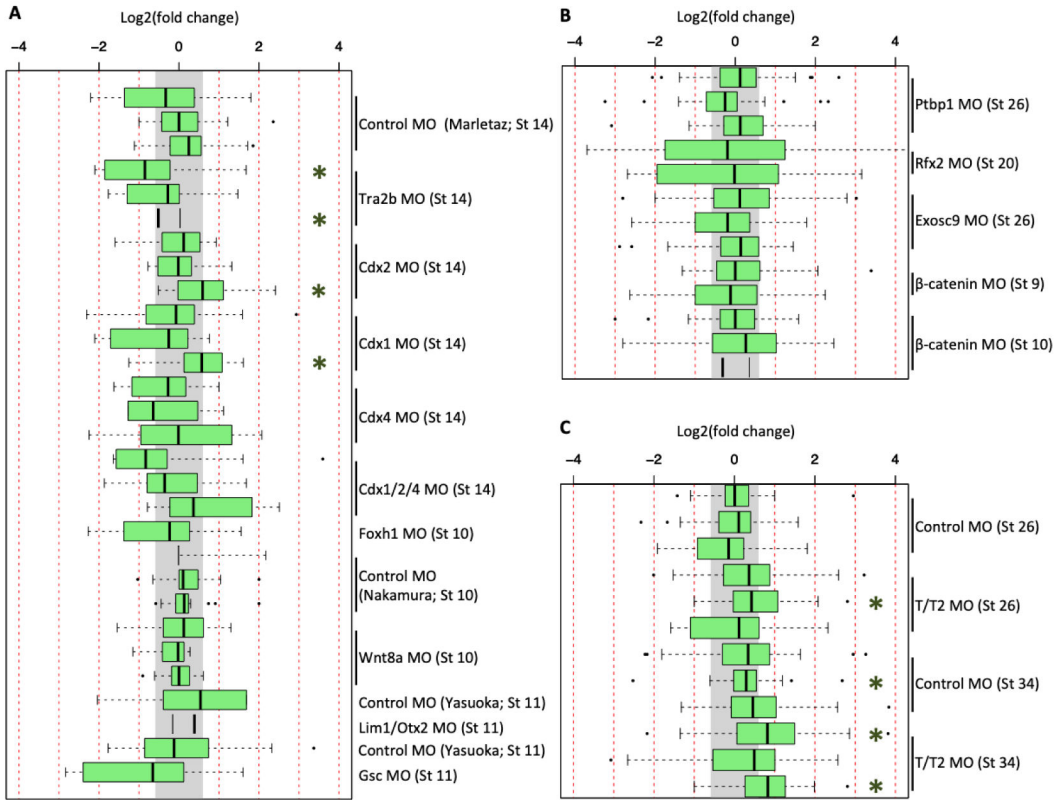

Figure 3. Expression of literature-identified innate immune response genes in X. tropicalis and X. laevis RNA-seq datasets.

Fold change expression of innate immune response genes across 29 datasets in *X. tropicalis* (A), 13 datasets in *X. laevis* (B), and in 12 the tbxt/t2 MO datasets (C). Gray region indicates fold change of < 1.5x. Green asterisk (*) indicates a T-test p-value of < 0.01.

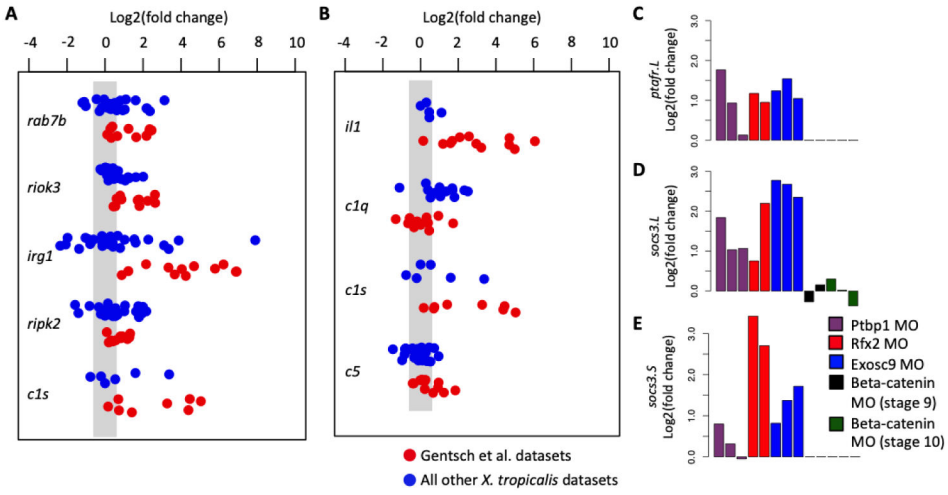

Figure 4. Specific induction of innate immune response genes.

Fold change expression of genes which were identified to be significantly activated in the *X. tropicalis* datasets in both the GO-identified (A) and the literature-identified (B) cohort of genes. Fold change expression of *X. laevis* genes *ptafr.L*/gene13059 (C), *socs3.L*/gene3766 (D) and *socs3.S*/gene50103, which were identified to be significantly activated. We used the criteria p-value < 0.01 and fold change > 1.5 to define significant.

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
mMessage mMachine Sp6 Transcription Kit	Thermo Fisher Scientific	Cat#AM1340
Deposited Data		
Wnt8a morpholino and control RNA-seq	Nakamura et al., 2016	GEO: GSE72657
Foxh1 morpholino and control RNA-seq	Chiu et al., 2014	GEO: GSE53654
E2a morpholino and control RNA-seq	Wills et al., 2015	GEO: GSE56169
Lim/Otx2 morpholino, Gsc morpholinos and control RNA-seq	Yasuoka et al., 2014	DRA: DRA000516, DRA000517, DRA000518, DRA001093, DRA001094, DRA001095
Mov10 morpholino and control RNA-seq	Skariah et al., 2018	GEO: GSE86382
Beta-catenin morpholino and control RNA-seq	Ding et al., 2017	GEO: GSE93195
Tbp/Tlf/Tbp2 morpholino, Gcn5 antisense DNA and control RNA-seq	Gazdag et al., 2015	GEO: GSE76995
Ascl1 morpholino and control RNA-seq	Gao et al., 2016	GEO: GSE76915
Rfx2 morpholino and control RNA-seq	Kwon et al., 2014	GEO: GSE50593
Tcf21 morpholino and control RNA-seq	Tandon et al., 2013	GEO: GSE45786
Cdx1, Cdx2, Cdx4 and Cdx1/2/4 morpholinos, and control RNA-seq	Marlétaz et al., 2015	GEO: GSE71006
Tbxt/Tbxt2 morpholino and control RNA-seq	Gentsch et al., 2013	GEO: GSE48663
Foxn4 morpholino and control RNA-seq	Campbell et al., 2016	GEO: GSE89271
Ptbp1 morpholino, Exosc9 morpholino and control RNA-seq	Noiret et al., 2016	GEO: PRJEB8711
Tra2b morpholino and control RNA-seq	Dichmann et al., 2015	GEO: PRJNA266550
Tbxt/Tbxt2 morpholino and control RNA-seq	Gentsch et al., 2018	GEO: GSE96655
X. tropicalis genome version 9.0	Hellsten et al., 2010; Karimi et al., 2018	RRID:SCR_003280; URL:http://www.xenbase.org/
X. laevis genome v9.2	Session et al., 2016; Karimi et al., 2018	RRID:SCR_003280; URL:http://www.xenbase.org/
Experimental Models: Organisms/Strains		
X. tropicalis, out-bred Nigerian	University of Virginia, NASCO	URL:https://www.enasco.com/
Oligonucleotides		
X. tropicalis smn2 RT primer forward: AAATTCCCAGGACCAAAAGG	Integrated DNA Technologies	N/A
X. tropicalis smn2 RT primer reverse: ACACGTGTCGCCTACTCTCC	Integrated DNA Technologies	N/A
X. tropicalis tp53 RT primer forward: CCCTCAACTGAGGATTACGC	Integrated DNA Technologies	N/A
X. tropicalis tp53 RT primer reverse: CTTGTTGAGGTCGGTGGAGT	Integrated DNA Technologies	N/A
X. tropicalis tp53inp1 RT primer forward: CCCAGCCCTGATAGAACAGA	Integrated DNA Technologies	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
X. tropicalis tp53inp1 RT primer reverse: TTTCATTCGAGCAGCAAGAG	Integrated DNA Technologies	N/A
X. tropicalis c3ar1 RT primer forward: CAATATCAGGAATGGGACGAA	Integrated DNA Technologies	N/A
X. tropicalis c3ar1 RT primer reverse: TTCACTTCCGGTAACGTGCT	Integrated DNA Technologies	N/A
Standard control morpholino: 5'-CCTCTTACCTCAGTTACAATTTATA-3'	GeneTools	N/A
Software and Algorithms		
RSEM v.1.2.12	Li and Dewey, 2011	RRID:SCR_013027; URL:http://deweylab.biostat.wisc.edu/rsem/
Bowtie 2 v2.2.7	Langmead and Salzberg, 2012	RRID:SCR_016368; URL:http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
DEseq2	Love et al., 2014	RRID:SCR_016533;URL:https://github.com/PF2-pasteur-fr/SARTools
R v3.1.0	R Core Team, 2014	RRID:SCR_001905; URL:http://www.r-project.org/
Metascape	Tripathi et al., 2015	RRID:SCR_016620;URL:http://metascape.org/gp/index.html#/main/step1
Gene Ontology	Ashburner et al., 2000; The Gene Ontology Consortium, 2017	RRID:SCR_002143;URL:http://www.geneontology.org/