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Dual oxidase in the intestinal epithelium of zebrafish larvae has anti-bacterial properties ☆

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ABSTRACT

Reactive oxygen species (ROS) function in a range of physiological processes such as growth, metabolism and signaling, and also have a pathological role. Recent research highlighted the requirement for ROS generated by dual oxidase (DUOX) in host-defence responses in innate immunity and inflammatory disorders such as inflammatory bowel disease (IBD), but *in vivo* evidence to support this has, to date, been lacking. In order to investigate the involvement of Duox in gut immunity, we characterized the zebrafish ortholog of the human *DUOX* genes. Zebrafish *duox* is highly expressed in intestinal epithelial cells. Knockdown of Duox impaired larval capacity to control enteric *Salmonella* infection.

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1. Introduction

The epithelial lining of metazoans, including the skin and all mucosal surfaces, serves as a primary barrier against pathogens. Maintenance of this epithelial integrity, especially in the very concentrated microbial milieu of the intestine of vertebrates, requires coordinated intervention by the intestinal immune system. Intestinal epithelial cells (IECs) recognize bacterial components through Toll-like receptors (TLRs) and the nucleotide oligomerization domain (Nod)-like receptors, Nod1 and Nod2 [1]. Upon sensing of pathogens, IECs evoke an innate inflammatory response, primarily by cytokine and chemokine signaling. A breach of this intestinal host–microbe homeostasis contributes to the pathogenesis of inflammatory bowel disease (IBD), commonly manifested as Crohn's disease or ulcerative colitis [2]. Mutations in the human *NOD2* gene have the highest disease-specific risk association for IBD [3].

Another potential bactericidal mechanism of the gut epithelial barrier is the generation of reactive oxygen species (ROS) and nitric oxide [4]. Overproduction of ROS is thought to exacerbate tissue damage in IBD [5]. Dual oxidase (DUOX), a member of the NADPH-oxidase family, catalyzes ROS production. First cloned from human thyroid tissue, the *DUOX* genes *DUOX1* and *DUOX2* are also highly expressed in mucosal surfaces of the lungs and intestine, respectively [6–8]. Whereas genetic and molecular evidence *in vivo* demonstrate roles for the TLR and Nod pathways in mammalian intestinal immunity, this data is still lacking in the case of ROS mediation. Evidence that Duox-generated ROS functions in intestinal microbial control has come from studies in *Drosophila* [9–11]. Recently, zebrafish Duox in epidermal cells has been reported to be involved in paracrine signaling to resolve wound inflammation [12]. This provided the first *in vivo* evidence that Duox-derived hydrogen peroxide signals to leukocytes in tissues in a vertebrate. In the present study, we begin to investigate if Duox has an antimicrobial function in the zebrafish intestinal epithelium. A molecular evolutionary study has identified only a single zebrafish *duox* gene, in contrast to two loci, *Duox1* and *Duox2*, in mammals [13]. We isolated and characterized the zebrafish ortholog of the human DUOX genes. We show that zebrafish *duox* displays a conserved tissue expression pattern when compared with mammalian *Duox*, with robust expression in the

Abbreviations: ROS, reactive oxygen species; Duox, dual oxidase; IBD, inflammatory bowel disease; IECs, intestinal epithelial cells; TLRs, Toll-like receptors; NOD, nucleotide oligomerization domain; hpf, hours post-fertilization; dpf, days post-fertilization; hpi, hours post-infection.

☆ Accession numbers used in text: zebrafish *duox:* Q08JS2_DANRE (ENSDART00000090727) http://www.ensembl.org/Danio_rerio/Transcript/Summary?db=core;g=ENSDARG00000062632;h=BLAST_NEW:BLA_1YEqVecHB!!20100726;r=25:3412 9147-34133351;t=ENSDART00000090727. Human *DUOX1*: NM_017434 (RefSeq).

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thyroid and mucosal surfaces including the intestinal epithelia. Infection experiments with the enterobacterium *Salmonella* in zebrafish larvae demonstrate a role for Duox in gut immunity.

2. Materials and methods

2.1. Zebrafish stocks and embryo collection

Wild-type zebrafish (*Danio rerio*) embryos were obtained from natural spawnings, raised in embryo medium (E3) at 28.5 °C [14], and developmentally staged as described [15]. Research was conducted with approval from the University of Auckland Animal Ethics Committee.

2.2. Cloning and characterization of the zebrafish duox gene

A candidate zebrafish *duox* gene was identified by TBLASTN analysis using the human DUOX2 amino acid sequence. A transcript fragment of this gene has been predicted on zebrafish chromosome 25 by Ensembl v8 being Q08JS2_DANRE (ENSDART00000090727). Based on the 4799 coding region human sequence for *DUOX1* (NM_017434), we cloned a 4584 bp putative zebrafish *duox* gene. This full coding sequence was cloned by RT-PCR of three component fragments (primer sequences available upon request), and overlapping sequences were ligated together and cloned into The SstI and EcoRI sites of pBlueScript KS+ vector to reconstruct the full-length zebrafish *duox* coding sequence. Phylogenetic analysis was performed using MacVector 9 software. Zebrafish amino acid sequences were compared with orthologs and other related proteins from mammalian, lower vertebrate and invertebrate phyla. Where available, proteins from other teleost fish species were included to account for fish-specific sequence variation. Under default setting, sequences were aligned using the ClustalW method, from which best trees reconstructed using the Neighbor-joining method and arbitrarily out-grouped to a related, but distinct protein. Genes were located *in silico* within the zebrafish genome by searching the zebrafish Ensembl database with derived peptide sequences using the TBLASTX program.

2.3. Whole-mount in situ hybridization and histology

RNA probes were synthesized using a DIG RNA labeling kit (Roche) from the RNA polymerase binding sites in the pBlueScript KS+ vector. Whole-mount *in situ* hybridizations were performed essentially as described [16] and imaged under a Leica MZ16FA fluorescence stereomicroscope with a DC490 camera. Histological sections were performed as described [17] and slides were imaged with a Leica DMR compound microscope and a DC420 camera.

2.4. Duox loss-of-function assay

Functional zebrafish Duox was transiently depleted from larvae by microinjection of antisense morpholino oligonucleotides (MO) into 1–4 cell stage embryos as previously described [18]. A MO targeting the first exon–intron boundary (Duox MO 5′-TAGATTACTA CTCACCAACAGCTTA-3′) and a standard control MO (Control MO) were synthesized by GeneTools, LLC (Philomath, Oregon, USA). The effectiveness of the splice-blocking Duox MO to disrupt pre-mRNA processing was assessed by RT-PCR as described [19].

2.5. Larval infection assays

Larvae (4 dpf) injected with either standard control MO or *duox* MO were housed in groups of 24 in Petri dishes and infected by static immersion in 7–10 $\times$ 10^8 CFU/ml of GFP-labeled *Salmonella enterica* serovar Typhimurium [20]. In this sub-lethal dose, wild-type larvae survive at least for 72 h post-infection (hpi), the latest timepoint tested. Exposures were performed in triplicate with a parallel treatment groups for sampling. Microbial persistence was measured at 0.5 and 22 hpi. For determination of bacterial load, 10 embryos per treatment group were taken from the parallel sample sets and euthanized in an ice bath. Each sample was then washed three times in PBS to remove non-adherent bacteria. Larvae were then homogenized in 100 μl PBS in a fresh microfuge tube. A 50 μl aliquot of homogenate was diluted for duplicate plating onto LB agar and grown overnight at 37 °C. The intracellular bacterial load was determined by a modification of the gentamicin exclusion assay [21]. The remaining 50 μl of homogenate was incubated in 200 μg/ml gentamicin at 28 °C for 1 h, washed and diluted for duplicate plating on LB agar and overnight growth at 37 °C. GFP-expressing CFU were counted by UV illumination (LAS-3000) with a green fluorescence filter.

3. Results and discussion

3.1. Orthology of the zebrafish duox gene

The existence of a single zebrafish *duox* gene has been identified by bioinformatic and evolutionary analyses [13]. Expression of this gene in zebrafish epidermal cells where it functions in wound healing has only been analyzed by semi-quantitative RT-PCR. Here, we cloned a putative 4584 bp zebrafish *duox* gene. To confirm the identity of this gene, the derived amino acid sequence was compared against orthologs for related NADPH oxidases, NOX 1–5, lactoperoxidase and myeloperoxidase from mammalian, vertebrate, and invertebrate phyla. Consistent with previous analysis [13], the extended phylogram shows that the zebrafish *duox* groups to the dual oxidase cluster, within which it diverges on an independent branch from the invertebrate and parallel mammalian Duox1 and Duox2 clades (data not shown). Reminiscent of their dual catalytic domains, the Duox clades diverged on a separate branch from the peroxidases, and from the rest of the NADPH-oxidase family members [13]. A best tree generated by the Neighbor-joining method with the zebrafish monoamine oxidase used as an outgroup sequence is shown here for a subset of proteins with highest similarity with the putative zebrafish Duox (Fig. 1A). The phylogram demonstrates that zebrafish *duox* is an ortholog for DUOX1 and DUOX2.

To independently establish orthology between zebrafish *duox* and *DUOX1* and *DUOX2*, analysis of conserved syntenies was conducted. Zebrafish *duox* maps to chromosome 25 and was found to be syntenic with *scamp2, duoxa, ckmt1* and *foxb1.2* (Fig. 1B; Ensembl, Zv8 [22]). The orthologs for these genes; *SCAMP2, DUOXA1* and *DUOXA2*, and *FOXB1* all map with the human *DUOX1* and *DUOX2* genes to human chromosome 15. While humans have two Duox and two dual oxidase activator genes (*DUOXA1* and *DUOXA2*), zebrafish have only one of each (*duox* and *duoxa*) [13]. These activator genes fall in the immediate vicinity of the Duox orthologs in both zebrafish and humans. Taken together, these data strongly suggest that we have cloned the zebrafish ortholog of mammalian *Duox* genes.

3.2. Conservation of pattern of expression between zebrafish duox and mammalian Duox genes

Tissue-specific expression profiles have been extensively reported for Duox orthologs by RT-PCR, *in situ* hybridization, and Northern and Western analyses. The *DUOX* genes were first cloned in the human thyroid [23,24]. Orthologs have since been identified in mice, rats, pigs, fruit flies, nematode worms and sea urchins.

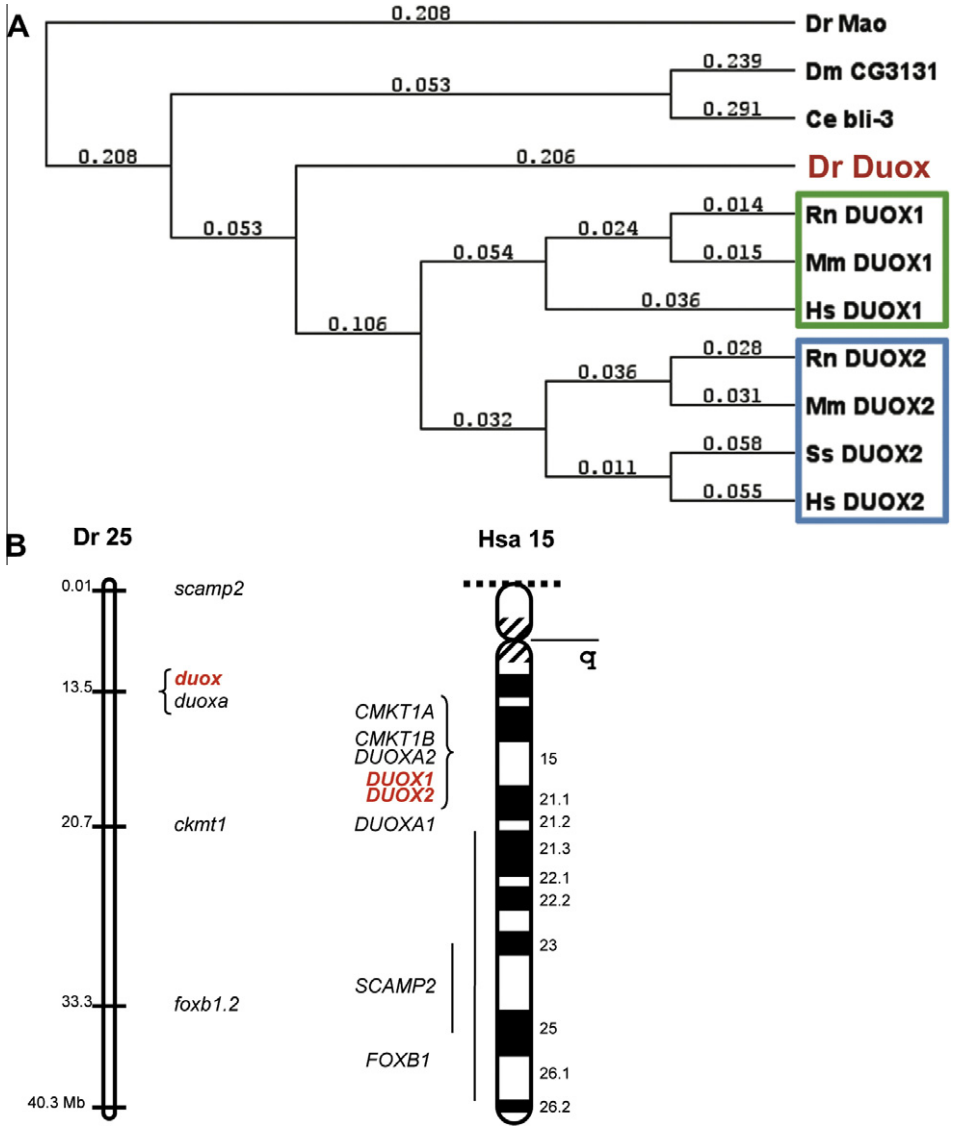

Fig. 1. Orthology of zebrafish *duox*. (A) Phylogenetic analysis of zebrafish Duox with invertebrate and mammalian Duox proteins. The Neighbor-joining method was used to produce a best tree of the zebrafish duox (red) phylogenetic relationships from an alignment with mammalian orthologs. Although zebrafish (*Danio rerio*, Dr) duox diverges on an independent branch from the clustered Duox1 (green box) and Duox2 (blue box) clades, it is more closely related to these mammalian paralogs (Hs, human; Mm, mouse; Rn, rat; Ss, pig) than to the single orthologs from invertebrates (Ce, nematode worm; Dm, fruit fly). (B) Syntenic analysis of the zebrafish and human *duox* genes. Zebrafish *duox* maps to linkage group 25, within a region that is syntenic with other zebrafish genes that exhibit conserved syntenies on human chromosome 15 where the human paralogs, *DUOX1* and *DUOX2*, are located. Only the long arm (q) of Hsa 15 is presented here. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DUOX gene expression also localize to many extra-thyroid tissues, principally those of mucosal origin such as the secretory and barrier epithelia in the respiratory, salivary and gastrointestinal organs, where exposure to exogenous microflora is greatest [7,11,25,26].

DUOX1 expression in respiratory mucosa predominates over that of *DUOX2*, and is not detected in intestinal tissues [7,26]. Conversely, *DUOX2* is strongly expressed in the IECs along the entire gastrointestinal tract [7,25]. *DUOX2* expression in the gut is polarized across the mucosa to the terminal ends of glands and intestinal folds, and the apical poles of highly differentiated cells, but is absent from intestinal crypts [25].

The expression patterns of zebrafish *duox* were examined by whole-mount *in situ* hybridisation using digoxigenin-labeled riboprobes in embryos from 24 h post-fertilization (hpf) up to 5 days post-fertilization (dpf). It appears that in zebrafish, as in mammals, Duox is present in mucosal surfaces in immediate contact with microbes. Transcripts were detected in epidermal tissue across the entire embryo trunk at 24 h, in a pattern outlining individual cells (Fig. 2A and B). This is consistent with the recently described role for epidermal zebrafish Duox in wound healing [12]. From 48 hpf, expression became prominent in the brain region (Fig. 2C), with patches of expression in epidermal tissues (Fig. 2D). As embryo development progressed, the intensity of the staining weakened in the brain while it became stronger in the swim bladder and gut (Fig. 2E–H). The swim bladder is regarded as a homologous organ to the mammalian lung, having similar developmental and molecular ontogeny [27]. Robust intestinal bulb expression at 4 dpf was similar to the expression of *cdx1b* and *ifabp* in differentiated IECs [28]. At this stage the gut is completely functional and larvae have begun autonomous feeding [29,30]. Expression within the intestinal tract was restricted to the anterior third of the intestine; transcripts were not detected in anorectal tissues (Fig. 2E).

In mammals, both *DUOX1* and *DUOX2* are expressed in the thyroid [23]. The mammalian mature thyroid gland is made up of follicular tissue producing the thyroid hormones and the

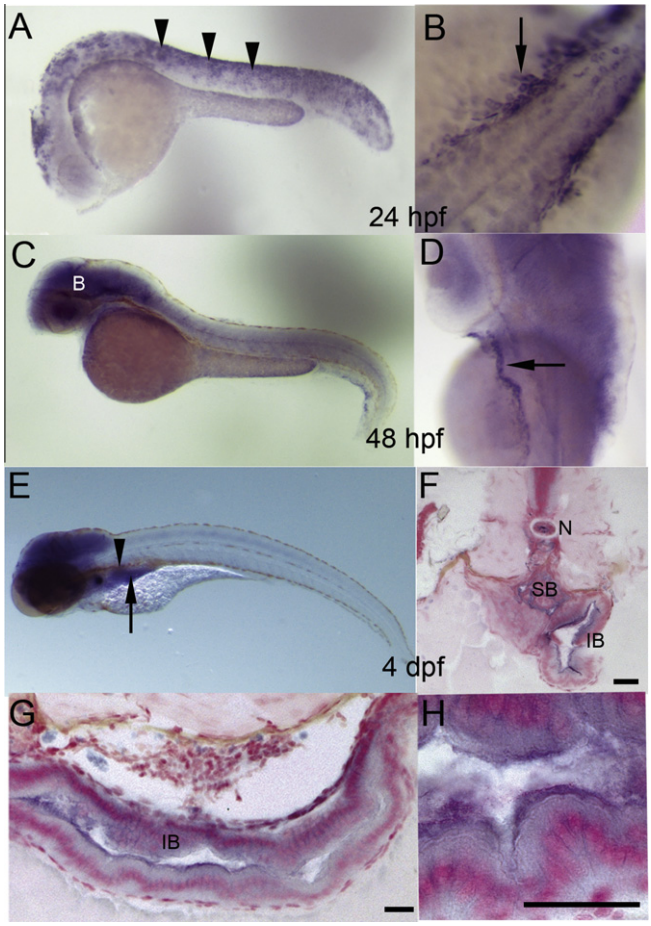

Fig. 2. Zebrafish *duox* is expressed in mucosal epithelia. (A, B) 24 hpf; epidermal expression, lateral view in A (arrowheads); higher magnification of dorsal view in (B) (arrow). (C, D) 48 hpf; expression within the brain (B), lateral view in (A); dorsolateral view in (D); Epidermal expression (arrow). (E–H) 4 dpf; lateral view in (E), transverse section in (F); sagittal section in (G) and (H); expression in swim bladder (arrowhead in (E), SB in (F)) and intestinal bulb (arrow in (E), IB in (G); higher magnification in (H)). N, notochord. Scale bars: 50 μm.

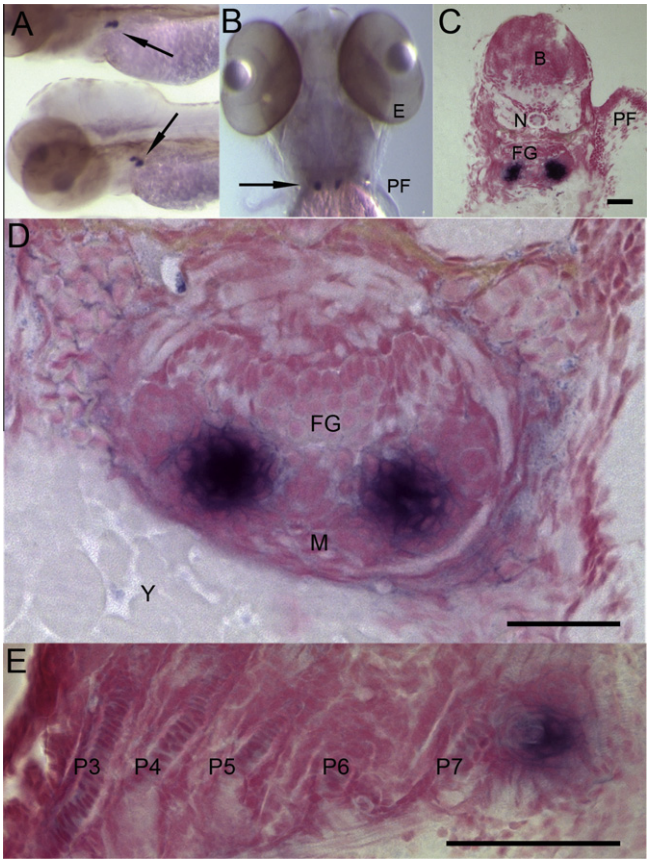

Fig. 3. Zebrafish *duox* is expressed in the thyroid-associated ultimobranchial bodies. (A–E) 4 dpf; lateral view in (A); ventral view in (B); transverse section in (C) with higher magnification in (D); lateral section in (E). Expression in paired ultimobranchial bodies (arrows in (A, B)). E, eye; PF, pectoral fin; B, brain; N, notochord; FG, foregut; M, muscle; Y, yolk; P3–P7, pharyngeal arches. Scale bars: 50 μm.

ultimobranchial bodies that produce calcitonin. However, in fish, amphibians and birds, the ultimobranchial bodies form independently of the thyroid follicles [31]. In zebrafish, these paired ultimobranchial bodies are located just posterior to the pharyngeal arches, on either side of the heart [32]. Consistent with this, we detected pronounced expression of *duox* in bilateral dots posterior to pharyngeal arch 7 in 4 dpf larvae (Fig. 3A and B). Detailed tissue sections displayed the expression of *duox* in the ultimobranchial bodies adjoining the muscles ventral to the foregut region (Fig. 3C–E).

3.3. Duox can limit microbial persistence in the zebrafish gut

To investigate whether Duox in the zebrafish intestine has an innate immune defensive role against pathogen incursion, we depleted Duox by morpholino knockdown technology. Embryos injected with Duox MO did not display gross morphological abnormalities, at least until the start of infection experiments (4 dpf). Effectiveness of splice-blocking MO was assessed by RT-PCR by standard methods (data not shown). Larvae were infected with enteric bacteria, *S. enterica* serovar Typhimurium that has been tagged with GFP. In previous studies, we have shown in immersion assays that this GFP-labeled bacterial strain colonized the larval intestine [20]. Larvae injected with Control MO had survival rates comparable with untreated siblings after 2 days of infection, whereas survival of Duox MO-injected larvae was less than 50% (data not shown). As Duox-depleted larvae survived within 24 h of infection, bacterial persistence assays were performed at 0.5 and 22 h post-infection. The colony forming units reported represent the intracellular bacterial load of triplicate sample groups. We observed that bacterial persistence was consistently more than 2-fold higher in Duox MO-injected larvae compared to the cohort injected with Control MO (Fig. 4). This inability to efficiently clear pathogenic bacteria is likely to contribute to poor survival of larvae with lengthened duration of infection. The oxidative burst resulting from Duox-generated ROS can induce microbial cell death. Recently, it has been demonstrated that this production of anti-bacterial ROS by Duox is directly controlled by Nod2 [33]. NOD2 physically interacts with DUOX2 in the plasma membrane to induce rapid generation of ROS in human intestinal cells *in vitro*. Of clinical relevance, IECs dissected from inflamed tissues of IBD patients have elevated levels of DUOX2 protein when compared with non-inflamed tissues from the same patient [33]. These data open up another level of complexity around the function of NOD2 in intestinal homeostasis, and strengthens the contribution of DUOX in IBD pathogenesis.

4. Conclusion

We isolated and characterized a gene from zebrafish chromosome 25 that encodes zebrafish Duox. By a combination of genomic, molecular and expression analyses, we confirm that this

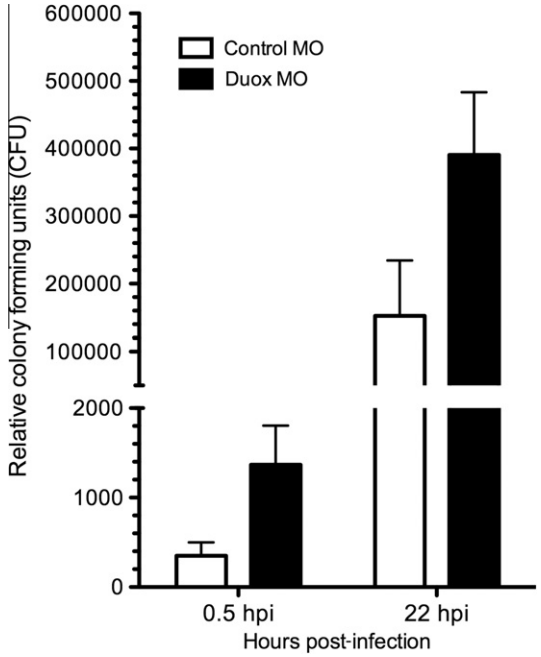

Fig. 4. Knockdown of zebrafish Duox reduces the capacity of larvae to clear *Salmonella* infection. The graph represents relative numbers of intracellular *Salmonella* colony forming units (CFU) per 4 dpf larva at 0.5 and 22 hpi. Bars represent average bacterial load from three biological replicates, with at least 10 larvae per sample group. Error bars denote SD.

gene is the single ortholog of human *DUOX1* and *DUOX2*. Zebrafish larvae depleted of Duox were less effective in controlling persistence of the enteric pathogen *Salmonella*, suggesting that Duox-derived ROS has bactericidal function in the intestine *in vivo*.

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