



## Modeling the Novel Gene in Zebrafish: EXD2 (Exonuclease 3'-5' Domain Containing 2)

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### Abstract

EXD2 (Exonuclease 3'-5' Domain Containing 2) is a known gene and reported to be an essential tool in repairing DNA double-strand breaks (DSBs) by homologous recombination through its 3'-5' exonuclease activity. At this time, there is no report indicating any relation to human disease resulting from alteration in the EXD2 gene. However, several laboratories works illustrated that EXD2 has critical roles in cell function. Zebra fish facilitated the functional study of novel genes as a model for human diseases by utilizing morpholinos as knocking down agents. Disturbing the EXD2 gene showed multiple deformities in the body, which may reflect the critical role in animal tissues and development at the early stages. The expression profile of the *exd2* differed in several organs from an adult AB-zebra fish, and also the expression of *exd2* increased with an advance in age. Disturbing *exd2* caused multiple defects and embryos dead at five-days old. Human RNA suppressed the phenotypes and helped the embryos to grow normally.

**Keywords:** EXD2; Morpholinos; Zebrafish; Expression; Eyes; Curvature body; Arrested development.

### Introduction

*Danio rerio*, generally known as zebra fish, has become a favorite model of choice in the biomedical research covering a broad array of topics ranging from developmental biology and morphogenesis to neurosciences, regeneration, and aging in the past decades. The study of human genetic variation provides opportunities to examine the structure and function of genes and their defects. The field has widely spread to include studies of genome-wide polymorphic autosomal microsatellite variation [1] and single-nucleotide variation [2]. Understanding genetic variations in the DNA by utilizing animal models such as mice and zebra fish provides fruitful information about genotype/phenotype association and biological mechanisms of disease [3,4].

There are several studies which have been performed to utilize zebra fish as a model for human diseases. Lu et al. showed the effect of *Tbx20* gene in the development and maintenance of the heart [5]. Another group demonstrated that introduction of MAPK10 mutations into ret

heterozygotes enhanced the ENS deficit, supporting MAPK10 as an HSCR susceptibility locus [6]. Also, an interesting study showed the role of miRNA-9 in Brain Development, the authors indicated that in the forebrain, miR-9 is necessary for the proper development of dorsoventral telencephalon by targeting marker genes expressed in the telencephalon. It regulates proliferation in telencephalon by regulating *Foxg1*, *Pax6*, *Gsh2*, and *Meis2* genes [7].

EXD2 (Exonuclease 3'-5' Domain Containing 2) is reported to be an essential tool in repairing of DNA double-strand breaks (DSBs) by homologous recombination through its 3' -5' exonuclease activity [8,9]. Recently, Sliva demonstrated that EXD2 is required for mitochondria integrity and efficient mitochondrial translation. Furthermore, reduction of EXD2 led to defect in the metabolic process in the cell, including decreasing respiration and increasing reactive oxygen species (ROS). Both human and Zebra fish were identified in a genome-wide. Structurally, the EXO and HNH-like domains are both crucial for EXD2 function. It was proposed that the HNH-like motif influences stable binding of EXD2 to ssRNA and the 28S subunit [10,11].

Anti-sense gene knockdown *via* Morpholino oligonucleotides has been extensively used in zebra fish because of the ease with which these reagents can be applied to the embryo, and their effectiveness. Although this procedure is not a genetic manipulation, Morpholinos have proven to be a valuable tool for assessing gene function in development, as first described in *Xenopus* [12], especially in systems where alternative reverse genetic strategies were not immediately accessible. In this report, we present new findings in knocking down the EXD2 gene by using morpholinos in the context of reverse genetic approaches. We observed new phenotypes which might relate to some of the phenotypes in human.

### Materials and Methods

#### Zebra fish

Zebra fish were raised at 28.5°C on a 14/10 light/dark cycle. Embryos obtained from natural mating were staged according to hour post-fertilization (hpf).

#### Morpholino injections

Embryos at 1-2 cell stage were pressure injected with 1 nL of the EXD2 blocking morpholinos (MO) targeted to the splice site at the boundary of exon/intron 4 and exon/intron 6, designed and synthesized by Gene Tools (Philomath, OR). The sequence for the EXD2-e4i4 was 5'-GTAAGCAGTGTGTACCTCCGTCTGA-3', and EXD2-e6i6 5'-ATCCTCCAGCTCTCATTACCTGG-3'; and for the standard control MO (CMO), the sequence was 5' - CCTCTTACCTCAGTTACAATTTATA-3'.

#### Measure of eye size

Embryos were dechorionated and anesthetized with Tracine. Eye measurements were taken from the anterior to the posterior edge. Significant differences between groups were analyzed by Student's t-test (Prism; GraphPad, San Diego, CA).

## RT-PCR

First-strand cDNA was generated using 1 µg total RNA, 200 ng random hexamers (AppliedBiosystem), 1 mL of 10 mM dNTP (NEB), 4 mL of 5x first strand buffer (Invitrogen), 2 mL of 0.1 M DTT (Invitrogen), 1 mL of RNase inhibitor (Promega), and 1 mL of Super-script III reverse transcriptase (Invitrogen) in a 20 µL reaction. PCR was performed using 2 µL of cDNA from the RT reaction, with 30 rounds of amplification with an annealing temperature of 60°C and 90°C extension time. The primer sequence used in this report is based on the ensemble ID (ENS DART00000111389.4); forward: 5' - CTCACGATATCCTGCTGCTG-3' ; reverse: 5' - CACCACCTTCAGTCCATGTG-3'.

## Quantitative RTPCR

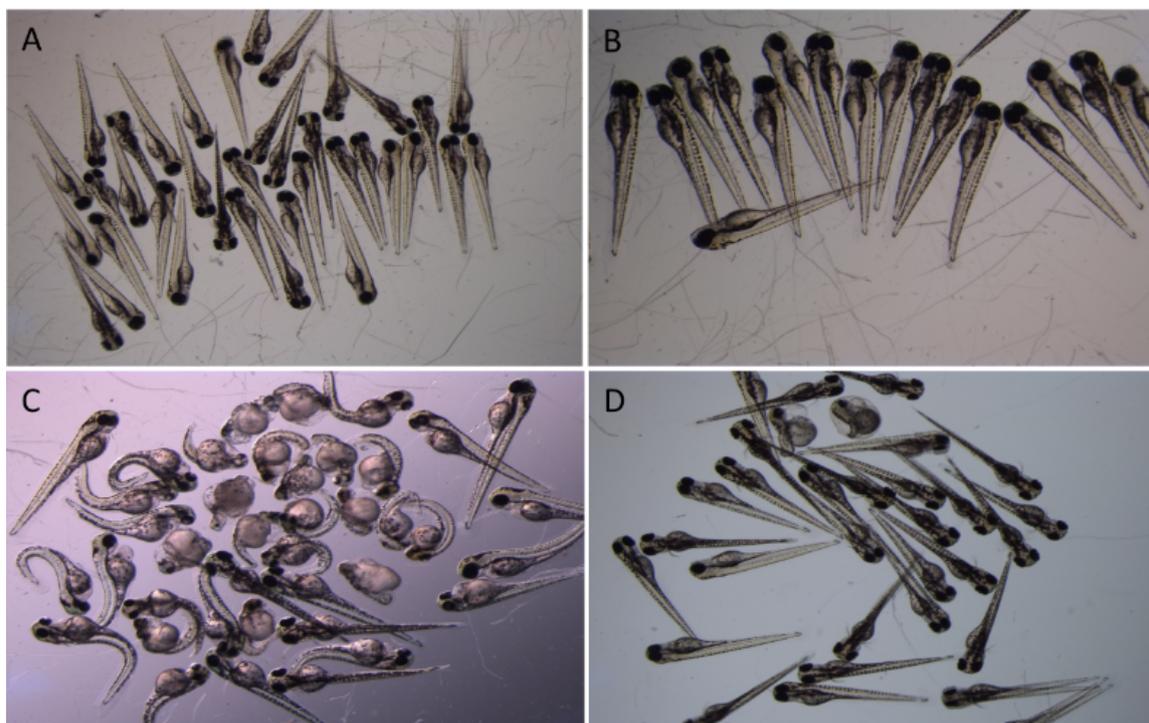
For the qRTPCR, we followed the manufacturing protocol using the following kit: RT<sup>2</sup> SYBR Green qPCR Mastermix, Qiagen (Cat No. : 330500), the thermal program is started with initial denaturation at 95°C for 15 mins, and followed with 35 cycles of denature at 95°C for 45 second, anneal at 58°C for 30 second, and extend at 60°C for 1 min/kb and finally hold at 4°C.

## RNA synthesis and rescue experiment

RNA was synthesized utilizing the mMessage mMachine transcription kit (Ambion). The rescue experiment was done in three trials (n=3, mean number embryos per group=80) after different sets of different RNA concentrations. The optimal concentration was found to be 8 µg and was injected into 1-2 cell embryos. Phenotypes were categorized as 'severe curvature body with small eyes' or 'mild', or 'normal.'

## Results

Knockdown of *exd2* using a MO that targets both splice sites (e6i6, e4i4) results in interesting phenotypes compared to control MO and un-injected embryos. The characterization of the changes in the body is summarized in Figure 1. These include slow development, heart and abdomen edema, seizure movement, coloboma, spinal curvatures, and small eyes. The most observed phenotypes are the spinal curvature, then heart and abdomen edema (Figure 1). Additionally, rescue experiments in the zebra fish have shown that human *EXD2*-RNA is sufficient to suppress the *exd2* splice site MO-induced physical structure of the larva (Figure 2).



**Figure 1:** A) Wild type AB zebrafish (72hpf), untreated B) AB-ZF (72hpf) treated with control MO C) ZF-Embryos (72hpf) injected with KD-MO targeting splice site D) Rescue exp. AB-ZF (72hpf). Wild type RNA co-injected with MO.

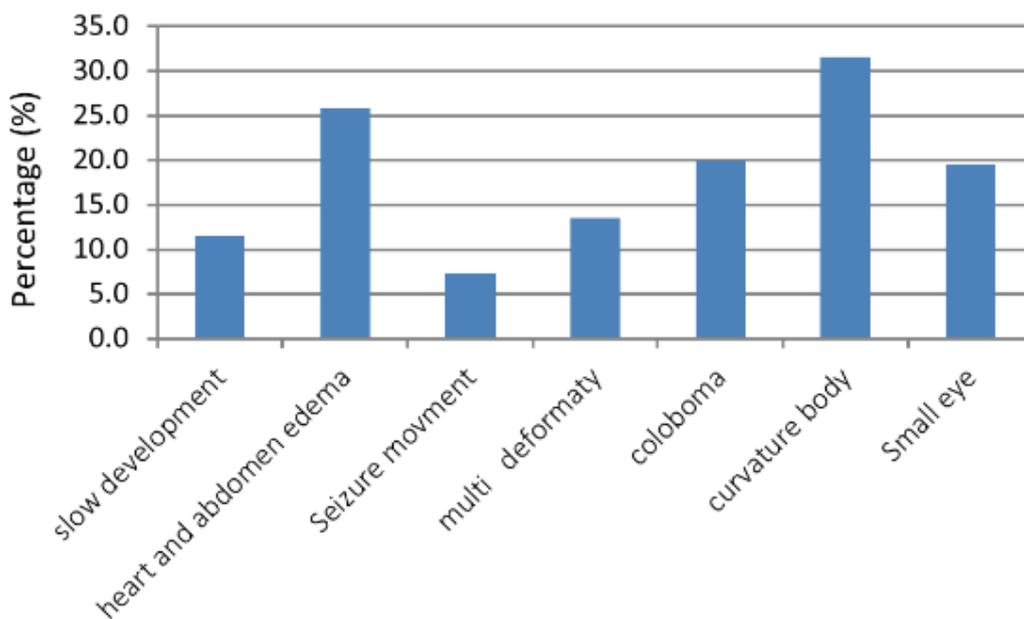


Figure 2: Distribution of the phenotypes in different tissues observed at 72 hpf.

We also observed that the size of the eye orbit is shorter in the embryos with KD exd2 comparing to wild-type and control MO. The size of the eye was noted in the larva and measured by the computer. The KD embryos presented smaller eyes compared to ctl MO and un-

injected embryos. Unfortunately, the embryos did not survive after the fourth day; therefore we were not able to assess startle vision (Figure 3).

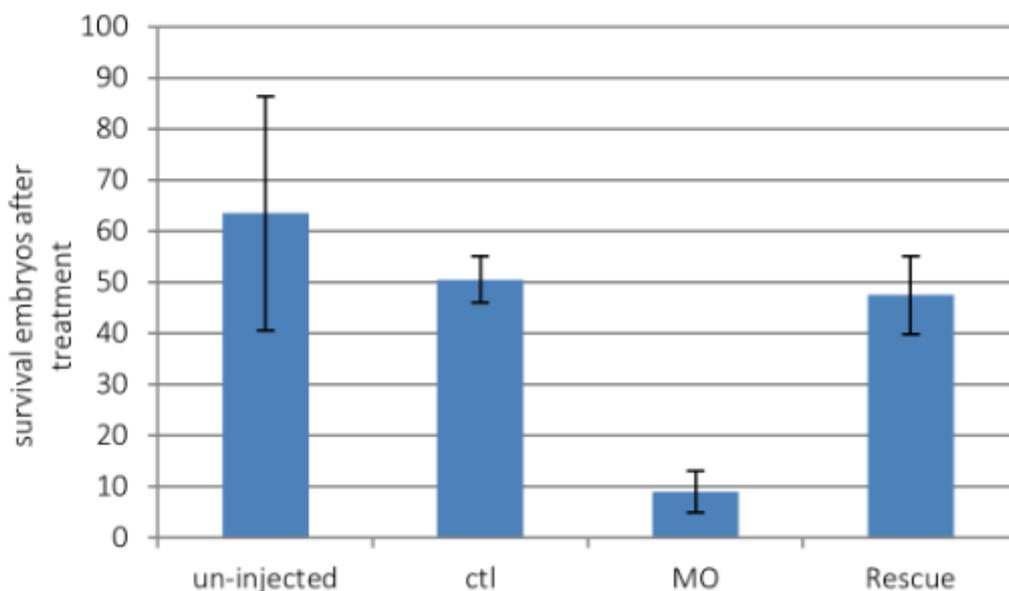
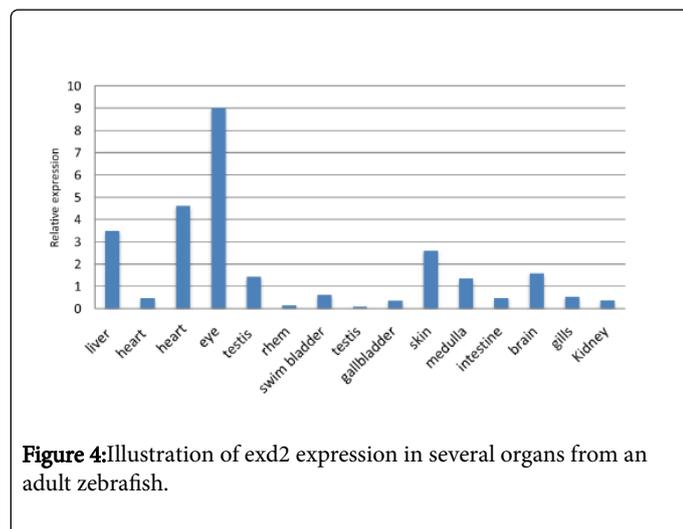


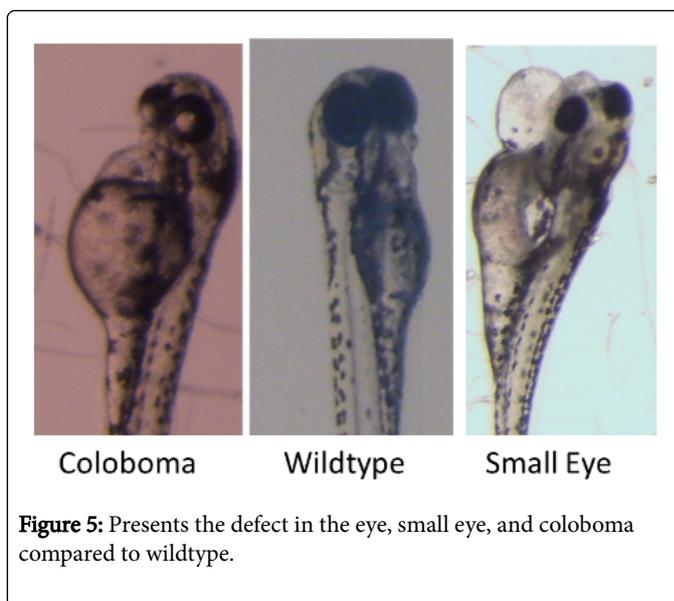
Figure 3: Graphical representation of exd2 gene knockdown. Human EXD2-RNA can rescue the healthy.

The expression profile of the *exd2* in the adult zebra fish organs showed heterogeneity across the tissues. Figure 4 presents the expression of the *exd2* in the designated tissues. We further examined the *exd2* expression at the early stages of development at 24 hpf, 36 hpf, and 72 hpf. The expression of the gene is progressing with time, and the highest expression observed at 72 hpf.



## Discussion

The present study employs zebra fish to investigate the effect of knocking down the EXD2 gene on the development of the embryos. Although the EXD2 gene has no links to human diseases, several studies showed the importance of the EXD2 protein in cellular functions [8,10,13]. Our preliminary finding is based on the morpholinos approach as the option recommended by Stainier et al. [14]. The current study showed arrested development of the embryos entirely at an early stage, on the fourth day. This is probably because of disturbing the structure of the *exd2* protein by using blocking splice sites with morpholinos. *Exd2* protein is assumed to be involved in DNA double-strand breaks repair in the presence of Mer11 and Exo1 to execute resection in G1 [9]. Also, EXD2 is one of the essential factors for the mitochondrion process. Therefore, inactivation of EXD2 in *Drosophila melanogaster* showed several dysfunctions in cellular biological functions such as a defect in mitochondrial translation, reduced ATP production, developmental delay, and impaired respiration [10]. The above data confirmed our finding when knocking down the EXD2 gene using zebra fish, which result in developmental delay and arrested embryos at 96 hpf, and in addition to the phenotypes mentioned in Figure 5 These observations developed aggressively as the expression of the *exd2* increased in tissues by time; several tissues were affected, which reflect the importance of the *exd2* in the developed cells. In this report, we presented and characterized various changes in the embryos of zebra fish resulting from utilizing morpholinos and also presented the expression of the *exd2* in several tissues and timing manner in embryos. The expression data is in agreement with the observed phenotypes, which draw attention to the importance of *exd2* in the affected tissues. In summary, this report catalogs the observations in zebra fish embryos resulting from damaging the EXD2 gene, and illustrates EXD2 expression in various tissues from an adult zebra fish and also measuring the expression in the embryos at early stages, 24, 48, and 72 hpf.



## Conclusion

Morpholinos-mediated knockdown of EXD2 in zebrafish confirms that *exd2* is one of the fundamental genetics elements for healthy development. As there is no human disease(s) yet associated with the *exd2* gene, the current approach, in combination with mRNA rescue, has empowered us to generate a model that might help to explain and divining some similar phenotype(s) observed in humans.

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