INVESTIGATING THE ROLE OF *wdr8* IN EYE AND MUSCLE DEVELOPMENT USING ZEBRAFISH AS A MODEL ORGANISM

BY

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(22MT0169)

Dissertation

SUBMITTED TO

INDIAN INSTITUTE OF TECHNOLOGY

(INDIAN SCHOOL OF MINES), DHANBAD

For the award of the degree of

MASTER OF TECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY
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ABSTRACT

The proteincoding gene *wdr8*, also known as "WD Repeat Domain 8," is essential to a many different biological functions.WD repeat protein family is known for their involvement in protein-protein interactions and the regulation of essential biological functions. *wdr8* contains *wd40* repeats, which are protein domains involved in mediating diverse cellular activities, such as signal transduction, cell cycle regulation, and chromatin assembly. *wdr8* is a centriolar protein essential for the formation of primary cilia, growth of the axonemal microtubules, and for recruiting other members for ciliogenesis during cell division. A recent study identified a novel mutation in the *wdr8* gene that results in Isolated Microspherophakia, a rare genetic eye disorder characterised by improper lens development. When *wdr8* is mutated using morpholinos, zebrafish (Danio rerio)exhibit smaller eyes and fewer cell in the lens and retina.They also exhibit cell cycle arrest, an increase in retinal cell death, and muscle distortion. Here the effect of CRISPR-Cas9 based knockout of *wdr8* on eye and muscle development, and attempt to decipher the other molecular players that may be involved in mediating the link between *wdr8* and eye development.

CHAPTER 1- INTRODUCTION

1.1WD-repeats

The term "WD-repeats" or "WD-40 repeats" refers to domains containing 40–64 amino acids that contain a conserved Tryptophan-Aspartate (WD) dipeptide at the end (C-terminus) and a conserved Glycine-Histidine (GH) dipeptide at around 11–24 amino acids from the start of the sequence (N–terminus) (Fig.1.1). This domain was first discovered in the G-protein Retinal Transducin's β-subunit, and tens of thousands of other proteins from various eukaryotes have since been found to contain it. There is extensive evidence of WD-40 repeat like domains even in prokaryotes [1], pointing towards their early evolutionary origin. Proteins with at least one WD repeat domain (which typically consisting of 6-8 WD-repeats) are referred to as WD- repeat containing proteins, or WDR proteins.

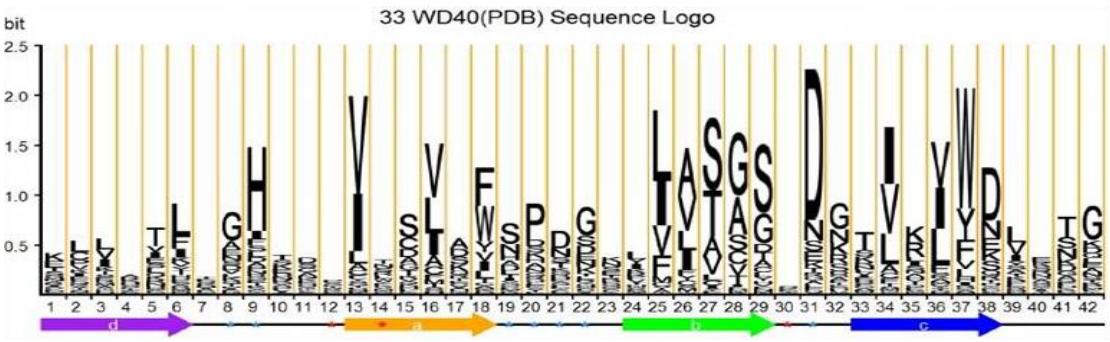


Fig 1.1—Sequence map of the WD40 repeat sequence. Size of the letters shows extent of conservation of residue at the particular position (Yang et al., 2013

1.2 The WDR protein family

WDR proteins typically consist of 4-16 WD-40 repeats, though there is evidence of proteins with as high as 35 repeats. Till date, around 6,00,000 WDR proteins from 4426 species have been recorded in the WDSPdb (the WD40-repeat protein Structures Predictor database; http://www.wdspdb.com/wdsp/). WDR proteins perform a variety of roles; they are frequently crucial parts of multiprotein complexes engaged in a broad range of signaling pathways, such as those related to immune response, cell cycle control, ubiquitin signaling and protein degradation, DNA damage detection and repair, and epigenetic regulation of gene expression and chromatin organization. [2]. This ability of WDR proteins to display such variability in function can be attributed in part to the unique structure of the WDR domains and the variable regions within the repeat sequences.

1.3 Structure of WDR domains—the β-propeller

Only a small percentage of the approximately 6,00,000 WDR proteins that have been identified to date have had their crystal structures clarified. Till date, all predictions for the tertiary structure of WDR proteins indicate that they form a propeller structure; the overall three-dimensional organization of the propeller depends on the number of WD-repeats and the length of the intervening sequences [5]. The β-subunit of Retinal Transducin was the first to be structurally characterized [3]; it consists of a single WDR domain with seven contiguous WD-repeats which are arranged into a highly symmetric seven-bladed β-propeller fold (Fig. 1.2). Each blade in this fold consists of four anti-

parallel β-strands; three of these strands are composed of residues from one WD-repeat, and the fourth strand consists of residues from the preceding WD-repeat. This sharing of one sequence between two blades is believed to stabilize the molecule [4].

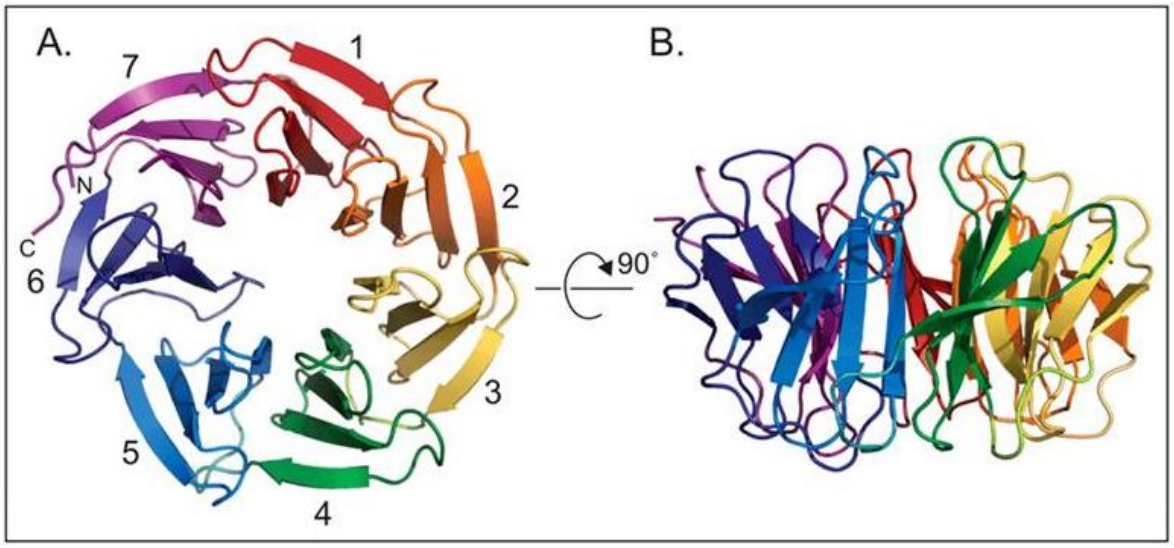


Fig 1.2— A. β-propeller fold adopted by the WDR domain B. Side view of the β-propeller fold, showing the top, bottom and circumference residues allowed for protein interaction. (Guarnaccia et.al, 2018)

The WDR domains themselves do not possess any catalytic activity; the evolutionary pressure to conserve the sequence is apparently so that the propeller structure is formed. This structure is advantageous, as it provides extensive surface area — upper, lower, and the circumference— for several simultaneous protein-protein interactions [6]. This makes WDR domains one of the most abundant classes of protein-protein interacting domains, making WDR proteins crucial members of a large number of signalling pathways.

The variety in function of WDR proteins can be attributed to the arrangement of variable versus conserved residues in the β-propeller. The conserved GH-WD cores of the repeat contribute predominantly to inner strands a-c of each blade, while the variable amino-terminal portions of each repeat form the cd loop and much of the outer d strand [3]. This distribution enables the variable residues of each WDR protein to occupy the circumference of the toroidal structure, enabling each WDR protein to interact with a different set of proteins.

1.4 WDR proteins in human diseases

Thus far, the WD-repeat containing domain has been recognized as the fourth most abundant domain in the human proteome [7]. Given that these proteins are crucial members of several pathways fundamental to the survival and development of an organism like cell cycle regulation and DNA repair, dysfunctional WDR proteins often contribute to diseases. Mutations in several genes coding for WDR proteins have been identified that are linked with diseases. A study by Kim et al. in 2020 assessed 360 WDR genes in humans, and reported that 79 of them were associated with human pathologies.

The majority of these genes (40.5%) were associated with neurological conditions such as various cognitive deficiencies, Parkinson's disease (due to the LRRK2 gene), LIS1 or PAFAH1B1-caused Lissencephaly, microcephaly (due to the WDFY3, WDR62, and NUP37 genes), and microcephaly (because to the LIS1 gene) (Fig.1.3). In addition, a sizable percentage of genes (21.5%) were connected to ciliopathies, a class of diseases characterized by abnormalities in the structure and function of both motile and non-motile cilia.A small percentage of these proteins were also involved in other disorders like skeletal disorders, eye disorders and cancers (Fig. 1.3)

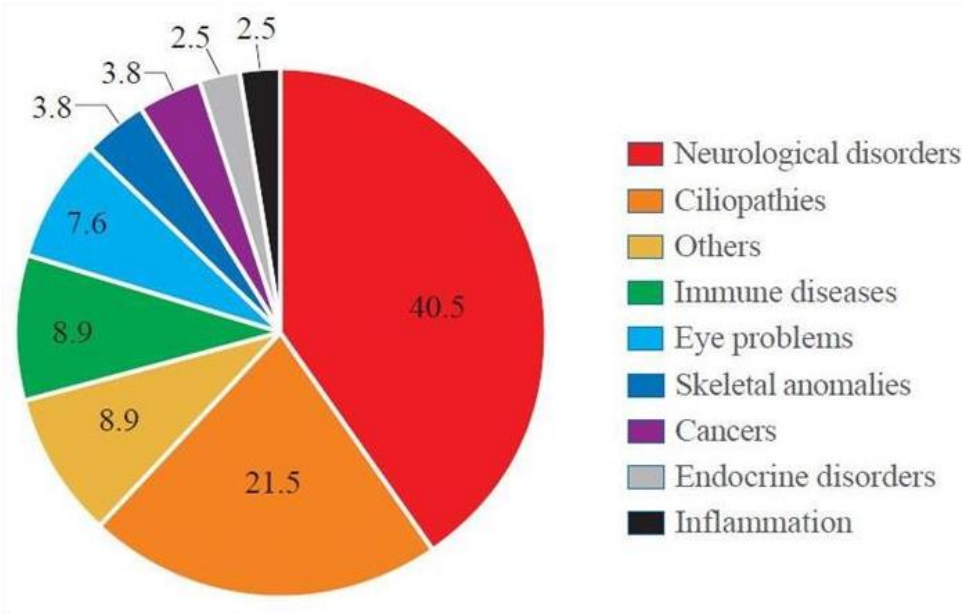


Fig 1.3— Representation of categories of disorders linked with WDR proteins in humans (Kim et.al., 2020)

However, this list is far from exhaustive; several WDR proteins in humans have not been functionally characterised. Often, how these mutations in the identified genes contribute to disease – even in case of those proteins that have been characterised – is not clear.

1.5 *wdr8* and Microspherophakia

In 2021, a novel mutation within the *wdr8* gene was identified to be linked to Isolated Microspherophakia. Microspherophakia (MSP) is a rare genetic eye disorder where the eye lens becomes smaller, thicker and more spherical [8]. It can manifest alone or in conjunction with other genetic disorders, such as autosomal dominant and recessive variants of Weill-Marchesani syndrome and Marfan syndrome. [8]. This defect is generally bilateral, and results in myopia that becomes pronounced by the age of 11 or older. Often, there can be secondary complications like glaucoma and ectopic lentis (Fig. 1.4), which is the displacement of the lens through the dilated pupil into the anterior chamber resulting in blindness [9].

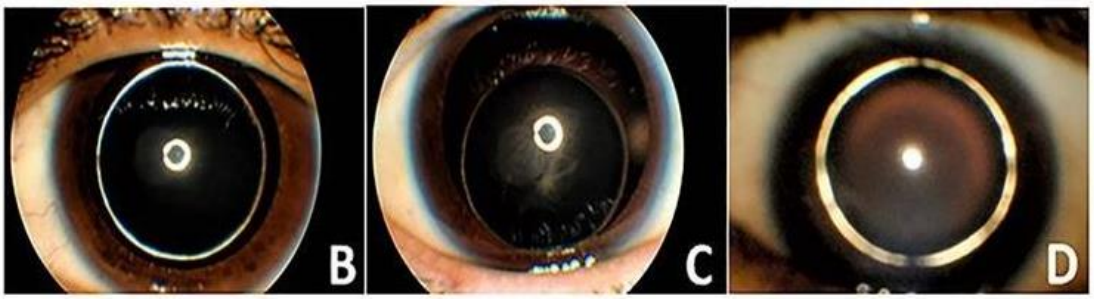


Fig 1.4— Microspherophakia as seen in three Indian MSP families – B and C show a cataractous change in centre of the lens. (Madhangi et.al., 2021)

In a consanguineous Indian family, a whole-genomic linkage scan revealed a homozygous duplication (insertion) mutation in the LTBP2 gene's last exon, which was connected to isolated microspherophakia [8]. Affected individuals from a different family under investigation, however, lacked this LTBP2 mutation. In this and a separate consanguineous MSP family, homozygosity mapping and whole exome sequencing identified the *wdr8* gene as a second causal gene for isolated microspherophakia [10]. The *wdr8* protein experiences a missense mutation as a result of this mutation, with a leucine replacing the proline at position 383. (p.P383L, Fig. 1.5).

Homo sapiens	(NP_060288.3)	QKLRLFAVLEQLSPVRAFQWDPQQPRLAIC
Pan troglodytes	(XP_001151905.1)	QKLRLFAVLEQLSPVRTFQWDPQQPRLAIC
Pongo abelii	(XP_002811628)	QKLRLFAVLEQLSPVRAFQWDPQQPRLAIC
Callitrix jacchus	(XP_002750245)	QKLRLFAVLEQLSPVRTFQWDPKQPRLAIC
Macaca mulatta	(XP_001084011)	QKLRLFAVLEQLSPVRTFQWDPQQPRLAIC
Bos taurus	(XP_002750245)	RKLRPLAVLEQLCTVRAFQWGPQQPRLAIC
Canis lupus familiaris	(XP_536722.2)	QKLKLFVVLEQLSPVRAFQWDPQQPRLAIC
Mus musculus	(NP_067474)	QKLKLFVVLEHMSPVRSFQWDPQQPRLAIC
Rattus norvegicus	(NP_001014284.1)	QKLKLFVVLEHMSPVRSFQWDPRQPRLAIC
Gallus gallus	(NP_001006299)	QKLKLFVVLEQLCAIQSFQWDPRQPRLAVC
Meleagris gallopavo	(XP_003212252.1)	QKLKLSVVLEQLCAIQSFQWDPRQPRLALC
Xenopus laevis	(NP_001089991)	QKMRLFVVLEQTSAVRAFHWDPVQPRLAIC
Danio rerio	(NP_956187)	QKFSLLAVLEQTAPVRCFVWDPHLPRLALC
Amphimedon queenslandica	(XM_003389008)	-KASLVALLIHINPIRDFQWDPIQSRLAIC
Oryza sativa	(NP_001065400)	CRLDLAAVLVQKDPIRAAAWDPNCPRLVFC
Arabidopsis thaliana	(NP_196376)	RLLEVAAILVQKEPIRAAVWDPTCTRLLLC

Fig 1.5— The Proline at the 383rd position is conserved across various species. (Image: Madhangi)

1.6 *wdr8*

wdr8 is a member of the WDR protein family, with a single predicted WDR domain with 8 WD-repeats in humans (Fig.1.6). This protein is essential for the formation of primary cilia, and for growth of the axonemal microtubules during cell division [11]. It is recruited to the centrioles in a Cep135-dependent manner, and is subsequently important for targeting of centriolar satellite proteins to the centrosomes [11]. Depletion of WDR8 was found to reduce the levels of PCM1, SSX2IP, Cep290 and Cep90 near centrioles [11]. Conversely, low levels of SSX2IP or PCM1 led to a reduction in the amount of satellite-localised WDR8, implying interdependency in satellite localisation between PCM1,

SSX2IP and WDR8 [11]. These satellite proteins including SSX2IP, PCM1 and Cep290 are important for proper assembly and function of primary cilia. The WDR8-hMsd1/SSX2IP centrosomal complex in particular is conserved across species, from budding yeast to humans. This complex has a crucial role during cell division; they are required for assembly of proper bipolar spindles, and is responsible for the maintenance of proper spindle length, orientation probably through anchoring the minus end of the mitotic spindles to the centrosome [12]. In addition to its role in ciliogenesis, *wdr8* is also reported to have a role in ossification of cartilage and osteogenesis [13].

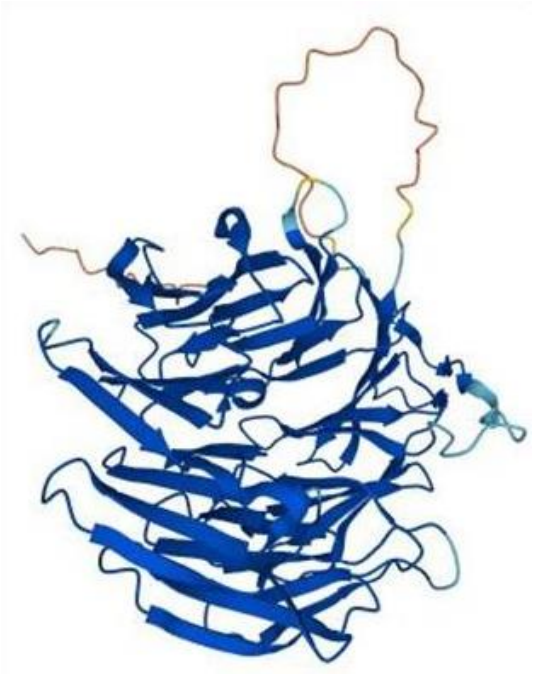


Fig 1.6— A. *wdr8* structure predicted by Alpha Fold.

A novel maternally essential protein called *wdr8* has been found to be necessary for the assembly of the centrosome during the development of the medaka (Oryzias latipes) embryo [14]. Following a CRISPR-Cas9-based knockout of the *wdr8* gene, a cross of heterozygous parents produced *wdr8*-/- children who displayed normal development and were phenotypically indistinguishable from fish of the wild type from infancy to maturity. On the other hand, crossings between wild type dads and *wdr8*-/-mothers produced significant phenotypic abnormalities. On the other hand, the development of zygotes derived from *wdr8*−/− dads was identical to that of zygotes of the wild type. This suggested that faithful cleavages in the big blastomeres of the early fish embryo depend on *wdr8* supplied by the mother [14].

1.7 Summary of findings; the MSP-*wdr8* story

The following were the major findings of a study by Madhangi et al. [10] from our lab:

• Morpholino-based knockdown of *wdr8* in zebrafish results in decreased eye size, lens size and number of cells in the lens and retina.

• This knockdown of *wdr8* caused cell cycle arrest in retinal cells, and these later undergo apoptosis.

CHAPTER 2- LITERATURE REVIEW

2.1 Zebrafish as a model system

Danio rerio or zebrafish has been a popular model organism of choice for the study of the eye and associated disorders in recent times. Listed here are some reasons for choosing to perform our studies using zebrafish:

• Zebrafish procreate widely and are simple to care for. They have a brief generation period of two to four months, during which a single mating pair can yield up to 200 fertilized eggs every week. Ex utero fertilization occurs, and the developing embryo is transparent, making it simple to see how it is developing early on [15].

• In terms of morphology, physiology, and function, the zebrafish and human eyes are extremely similar. Zebrafish have cone-rich eyes, much like human eyes, because of their diurnal lifestyle [15] (Fig.2.1). This results in color vision with a cone density that is similar to that of humans.

• The zebrafish and humans share a large amount of genetic and metabolic similarities; at least one orthologue can be found in 70% of human genes, and 84% of known human disease-causing genes have a zebrafish equivalent [16]. *wdr8*, which has a single ortholog in the zebrafish, is 89% identical to *wdr8* in humans.

• Genetic manipulations like knockouts or knockdowns are highly efficient in zebrafish due to ease of microinjection into the 1-celled embryo, unlike in case of mice.

• Phenotypic analysis and imaging post injection is easy due to the translucent nature of embryos.

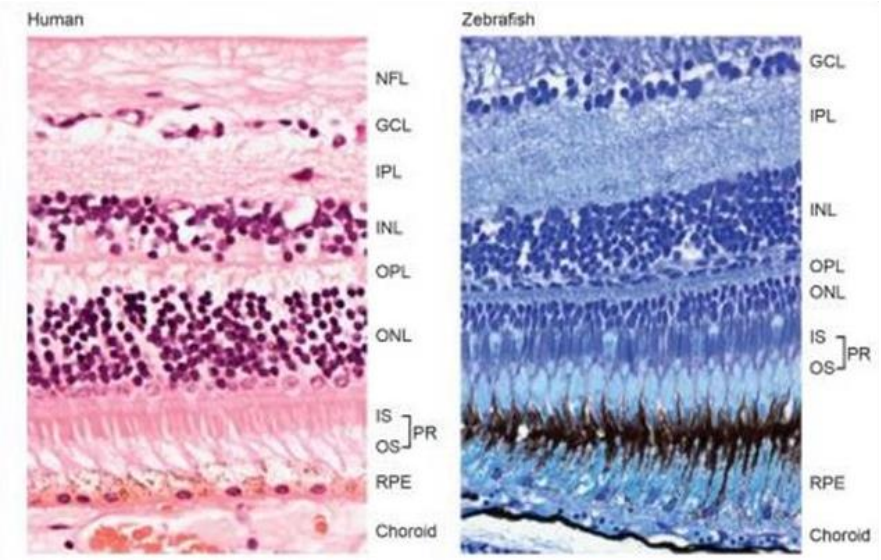


Fig 2.1— Histological cross-section of the retinas of humans and zebrafish reveals similarities in the cell organization and structural characteristics of the retinal layers. This diagram shows the following: PR stands for photoreceptor; ONL stands for outer nuclear layer; OPL for outer plexiform layer; INL for inner nuclear layer; GCL for ganglion cell layer; and NFL for nerve fiber layer (Richardson et al., 2017).

2.2 Generation and characterisation of *wdr8* knockout zebrafish

CRISPR-Cas9 as a gene editing tool

A potent tool for eukaryotic cell genome editing is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system, which was borrowed from the bacterial adaptive immune system.

The modern-day CRISPR toolkit consists of two major components — the single guide RNA or sgRNA, and the Cas9 nuclease. The sgRNA consists of a 20-base pair sequence complimentary to the region in the genome to be targeted, and a second loop region which recruits and interacts with the Cas9 nuclease at the target site. The conserved HNH and RuvC nuclease domains are then used by the Cas9 nuclease to perform strand-specific cleavage [17]. A 2-6 bp sequence called the Protospacer Adjacent Motif or PAM sequence is required for the Cas9 nuclease to cut, and is generally found 3-4 nucleotides downstream from the cut site. The target DNA of the widely used CRISPR-Cas9 system, which is derived from Streptococcus pyogenes, needs to come before a 5′-NGG PAM.

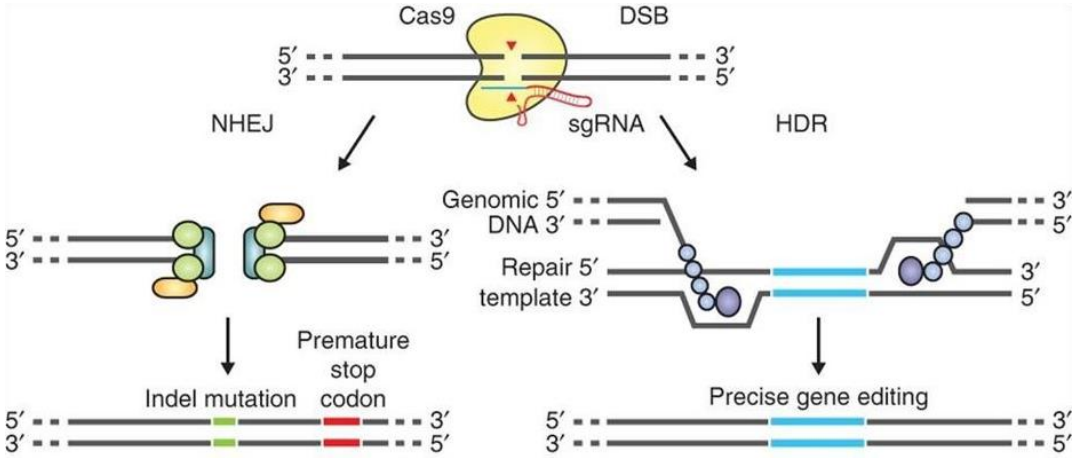


Fig 2.2— Schematic showing the essential components of the CRISPR-Cas9 editing system, and the events following a DNA double strand break (DSB). This can either be repaired using the NHEJ pathway or the HDR pathway, with different outcomes — this is leveraged for specific manipulations like generating knockouts or knock-ins (Ann Ran et al.,2013).

There are two possible repair pathways for a double strand break caused by Cas9 at the target site: the error-prone Non-Homologous End Joining (NHEJ) pathway and the Homology Directed Repair (HDR) pathway [17] (Fig 1.8). Utilizing the NHEJ process for DNA repair, gene knockouts can be produced. The broken ends of DNA are simply re-ligated through NHEJ in the absence of a repair template, leaving scars in the form of insertion/deletion (indel) mutations (Fig. 1.8). This can be used to control gene knockouts, since premature stop codons and frameshift mutations can result from indels that occur within a coding exon [18]. Larger deletions in the genome can be mediated by a number of sgRNAs and the resulting breaks in DNA.

The HDR pathway can be exploited to perform directed manipulations of the genome, like knocking- in our sequence of interest at specific locations in the genome. An exogenously provided DNA sequence flanked by sequences complimentary to the region on either side of the cut site can allow for introduction of desired DNA fragment into the genome through these flanking homologous sites (Fig.2.2).

The former strategy was employed to knockout WDR8 in zebrafish embryos to study the effects of absence of WDR8 on phenotypical and cellular events.

2.3 Structure of Zebrafish Eye

Zebrafish have large eyes compared to their overall size; by 72 hours post fertilisation (hpf), the retina of the fish is visually receptive and mimics the adult retina in terms of anatomy and function, which is similar to that of humans [15]. Like the structure of the human eye, the retina of zebrafish is made up of three layers: retinal, plexiform, and retinal. The outer nuclear layer, which is in charge of converting light into biological signals, is home to the photoreceptor cells, or rods and cones. The inner nuclear layer is occupied by Müller glial cell bodies and amacrine interneurons. The layer closest to the eye lens is called the ganglion cell layer, and it contains the ganglion cell bodies. The outside and interior synapses that connect these cell layers are made up of plexiform layers.

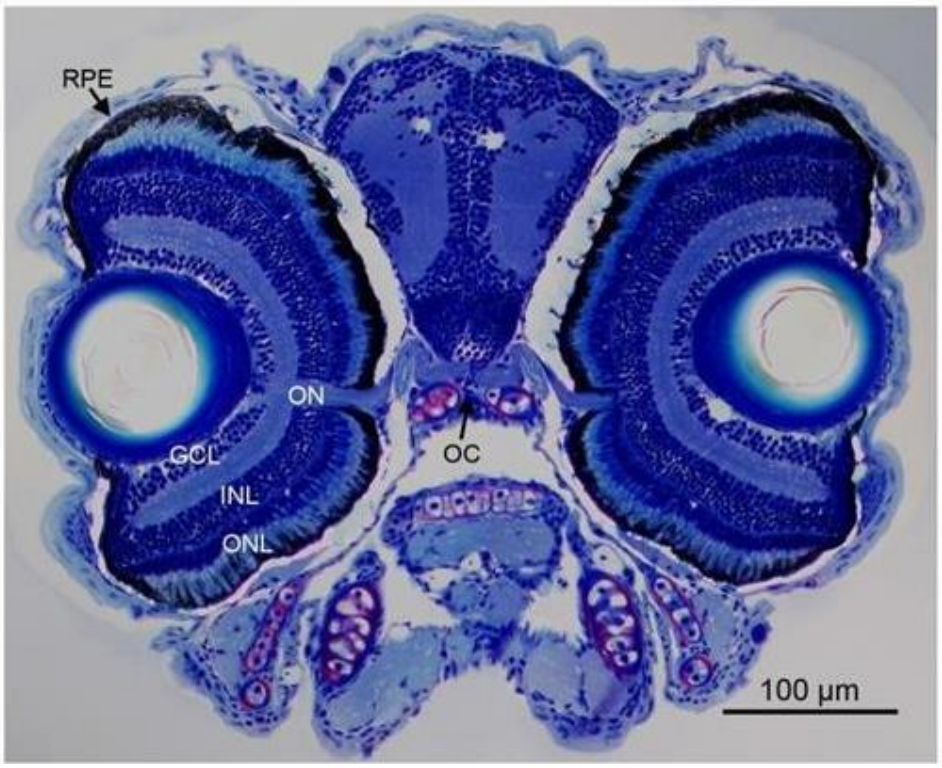


Fig 2.3— Zebrafish eye at 6dpf. Histology shows the vertebrate typical layering of the larval zebrafish retina with the optic nerve and chiasm. ONL, outer nuclear layer; INL, inner nuclear layer; GLC, ganglion cell layer; ON, optic nerve; RPE, retinal pigment epithelium; OC, optic chiasm. (Gestri et.al., 2012)

2.4 Muscular structure of zebrafish

The arrangement of zebrafish muscles follows a pattern that is characteristic of most vertebrates. Here is a simplified alignment of the structure and arrangement of zebrafish muscle:

1. Myotomes: Zebrafish muscles are organized into repeating segments called myotomes. Myotomes are blocks of muscle tissue that run along the length of the fish's body. Each myotome consists of several individual muscle fibers or myofibers.[20]

2. Epaxial and Hypaxial Muscles: Within each myotome, zebrafish have two main groups of muscles: the epaxial muscles and the hypaxial muscles.

• Epaxial Muscles: The epaxial muscles are located dorsal (toward the back) to the vertebral column. They include the muscles responsible for controlling movements of the fish's dorsal fin and maintaining posture.

•Hypaxial Muscles: The hypaxial muscles are situated ventral (toward the belly) to the vertebral column. These muscles are involved in generating bending movements and controlling the fish's lateral movements.[20]

3. Horizontal Skeletal Muscles: In addition to the myotomes, zebrafish have horizontal skeletal muscles that connect the vertebrae and provide stability and flexibility to the fish's body. These horizontal muscles run perpendicular to the myotomes and contribute to the overall locomotion and manoeuvrability of the fish.

4. Fin Muscles: Zebrafish possess various muscles associated with their fins. These muscles include the muscles that control the movement of the caudal fin (tail fin), dorsal fin, pectoral fins (located on the sides), and pelvic fins (located near the belly).

5. Heart Muscle: The heart of the zebrafish consists of cardiac muscle, which forms the muscular walls of the atrium and ventricle. The cardiac muscle contracts rhythmically to pump blood throughout the fish's circulatory system.[21][22]

6. Smooth Muscles: Smooth muscles are found in various organs and tissues of zebrafish, including the gastrointestinal tract, blood vessels, and reproductive organs. These muscles are responsible for involuntary movements and control functions like digestion and circulation.[23]

CHAPTER 3- MATERIALS AND METHODS

3.1 Zebrafish maintenance

In accordance with OCED requirements, adult fish were kept at 28 ± 2o C in a light-controlled environment with a 10-hour light and 14-hour dark cycle, with darkness from 8 PM to 10 AM. Fish were kept in RO water that had sea salt added to it (0.06 g of ocean sea salt/L of RO water). Twice a day, dry flake fish food was given to adult fish. The fish and larvae were handled with extreme caution to prevent unnecessary suffering to the animals.

3.2 Design and preparation of sgRNA

Three sgRNAs were designed against Exon 3 and Exon 4 of the *wdr8* gene. We expect that the cuts introduced would result in removal of a large chunk of protein coding sequence from the genome; hence, functional *wdr8* protein can no longer be produced (Fig 3.1).

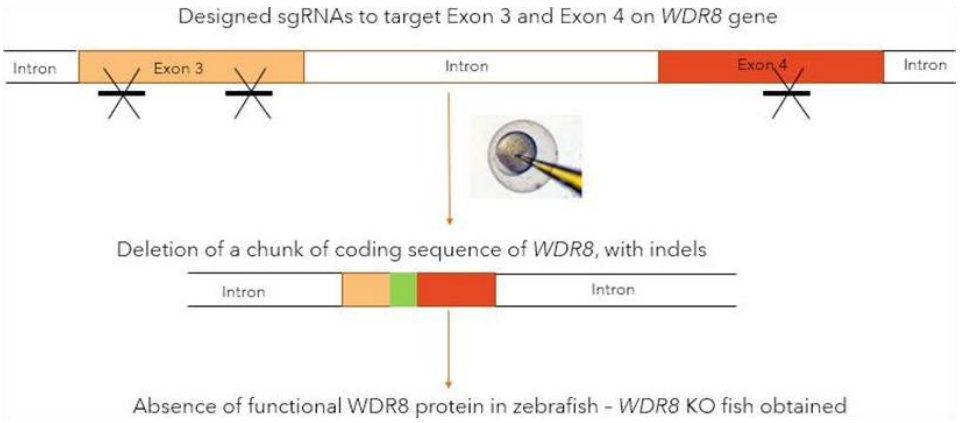


Fig 3.1— Design for CRISPR-Cas9 based knockout of *wdr8.*

Sequences of sgRNA:

sgRNA 1	TGCCGATGTCCCGAACCACC (PAM)	AGG
sgRNA 2	CGTGCACCTGTACACATGCC (PAM)	TGG
sgRNA 3	TTGGACTAGTCTCGTCACGC (PAM)	TGG

sgRNA were prepared using the abm *GeneCraft-R CRISPR sgRNA Synthesis and ScreeningKit (S. aureus)* (Fig 2.5).

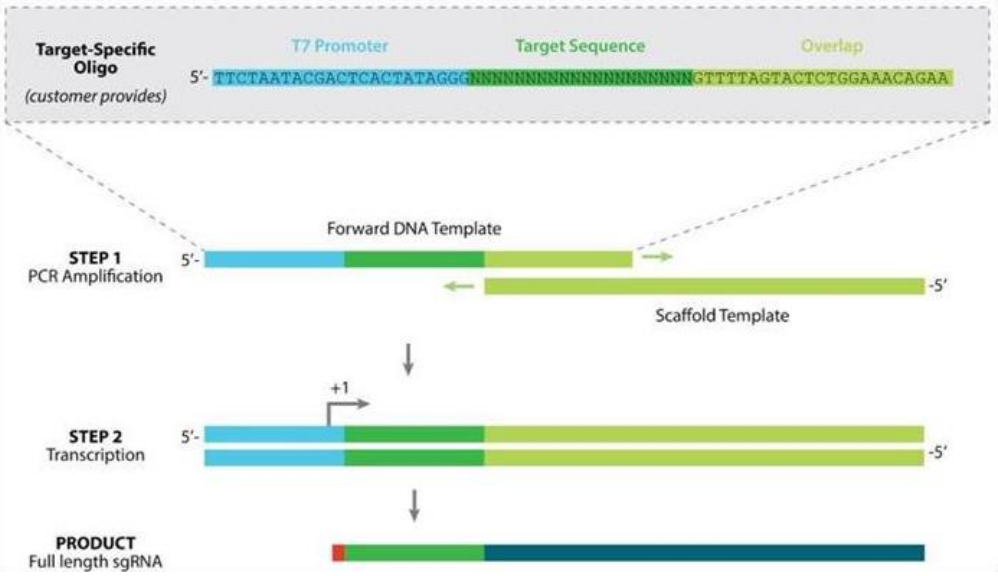


Fig 3.2— Workflow for synthesis of sgRNA

Sequences of oligos designed for sgRNA synthesis:

Oligo 1	5'- TTCTAATACGACTCACTATAGGGTGCC GATGTCCCGAACCACCGTTTTAGAGCTAGAAAT AGCAAG- 3'
Oligo 2	5'- TTCTAATACGACTCACTATAGGGCGTGCACCTGTACACATGCCGT TTTAGAGCTAGAAAT AGCAAG- 3'
Oligo 3	5'- TTCTAATACGACTCACTATAGGGTTGGACTAGTCTCGTCACGCGT TTTAGAGCTAGAAAT AGC AAG- 3'

These sgRNA oligos were then PCR amplified using the following protocol and cycling conditions:

Component	Amount
Nuclease Free Water	23 µL
GeneCraft-R Scaffold Template & Primer	1 µL
Target-Specific or sgRNA Control Oligo (10 µM)	1 µL
2X PCR Bestaq™ MasterMix	25 µL
Total	50 µL

Cycling conditions:

Step	Temperature	Time	Cycle
Initial denaturation	95°C	5 minutes	1
Denaturation	95°C	30 seconds	35 cycles
Annealing	55°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	5 minutes	1
Hold	4°C	Hold	1

Once amplified, sgRNA were synthesised using the following reaction protocol incubated at37°C overnight:

Component	Amount
2X Classic sgRNA Synthesis SuperMix	10 µL
Amplified oligo	6 µL
OneScribe Enzyme Mix	4 µL
Total	20 µL

The synthesised sgRNA was then subjected to DNAse treatment for 15 minutes at 37°C.

3.3 Microinjection into zebrafish embryo

Zebrafish embryos were injected with equal volumes of sgRNA, purified Cas9 protein,

Cas9 expressing plasmid and phenol red (to visualise the injection), and were then washed and keptin E3 medium post injection. Some un-injected eggs were similarly kept in E3 medium to serveas a control. Rescue experiments were performed in a similar manner; additionally, the p3X- FLAG-dr*wdr8* construct was injected to allow for expression of zebrafish *wdr8* in knockoutbackground.

Embryos after injection were maintained in 1X E3 medium in 8 cm petri plates.

Composition of 50X E3

Component	Amount (in g)
NaCl	34.8
KCl	1.6
CaCl2.2H2O	5.8
MgCl2.7H2O	9.78
Water	Up to 1L

3.4 DNA isolation from whole larvae

DNA isolation was done from larvae at 3-4 days post fertilisation (dpf) using the QIAGEN DNeasy Blood & Tissue Kit.

3.5 Screening and validating knockout

Validation of *wdr8* was first done using PCR to check for presence of a 4.1kb gene fragmentconsisting of the region with both Exon 3 and Exon 4 of *wdr8*—primers were designed upstream of Exon 3 and downstream of Exon 4 to verify the deletion of this chunk of DNA from the genome (Fig. 2.6). PCRs were run with Phusion DNA Polymerase (Thermo Fischer),with an annealing temperature of 66°C, with an extension time of 130s.

FORWARD PRIMER: GGGGAAAATATACAGGGGGCTAATAAGTGA

REVERSE PRIMER: CAGGGTGGACTCCTCCTACAGCCATAC

3.6 Eye measurement of Embryos

The ImageJ software was used to measure the body length for each embryo . Eye length was determined as the longest line through eye, which refers to as the outer diameter and

then the longest line through retina is called retinal diameter. The ratio of outer diameter to retinal diameter as measured and quantified.

3.7 Light microscopy of Embryos

At particular developmental periods (48 and 72 hours post fertilization), uninjected and morphant embryos were killed and preserved in 4% paraformaldehyde for the night. The embryos were photographed after fixation and cleaned with PBS (Phosphate Saline Buffer). Images of the entire embryo's lateral view were taken using an Olympus SZX12 stereo microscope equipped with a fixed magnification Leica DFC300FX camera.

3.8 Swimming Assay

A swimming assay plate was created to test zebrafish embryos' capacity to swim. Concentric circles with radii spaced 0.5 cm apart were drawn at the bottom of an 8 cm plastic petri dish that had 1X E3 medium in it. The swimming assay involved releasing 30 embryos per set of 4dpf embryos at the center circle and touching them with a single hairbrush up to three times in order to elicit an escape response. Zebrafish embryos naturally gravitate toward the periphery from the center. Using a Nikon L120 camera, all of the swimming experiments were captured for further examination. Using the graphpad prism 5.0 program, the total distance traveled by embryos and the amount of time needed to complete the task were measured and plotted.

3.9 Heartbeat Count

Heartbeat was counted in 3dpf embryos (15 embryo each set) after placing them under ZEISS SteREO Discovery.V12 microscope fitted with Axiocam ICm 1 camera. Videos were captured using ZEN system software and heartbeat as manually counted for 30second in each embryo. A blind study was setup to prevent bias in the results. Five researchers from the lab volunteered in a blinded experiment to count Zebrafish heartbeat. Heartbeat counted for 30 seconds were multiplied by to get heartbeat/min.

3.10 Annexin V-PI staining for apoptosis detection

Using the FITC Annexin V apoptosis detection kit I (BD Biosciences), the amount of apoptosis in the retinal cells was measured. To produce a single cell suspension, whole embryos at 3dpf were homogenized, treated with collagenase, and preserved in 4% paraformaldehyde. Following two PBS washes, the cells were pelleted and resuspended in 100 µL of 1X Binding Buffer. The positive control cells were those that were exposed to H2O2 for 20 minutes. Following that, these samples were incubated for 15 minutes at 25°C in the dark with 5 µL of FITC Annexin V and PI. Each tube was then filled with an additional 400 µL of 1X Binding Buffer, and the samples were examined using a BD AccuriTM C6 cytometer (BD Biosciences).

3.11 JC-1 dye staining for analysis of status of mitochondrial membrane

potential

To produce a single cell solution, whole embryos were homogenized and treated with collagenase. After two PBS washes, the cells were resuspended in 2 μM JC-1 dye and allowed to sit at room temperature for 15 to 20 minutes. As a positive control, rotenone-treated cells were also incubated in 2 μM JC-1 dye for 15–20 minutes. After being resuspended in PBS, the cells were examined with a BD AccuriTM C6 cytometer (BD Biosciences).

3.12 Statistical Analysis

All statistical tests were performed using GraphPad Prism, using a two-tailed students'.

CHAPTER 4 – RESULTS

4.1 Knockout of *wdr8* confirmed by PCR

At the end of 3dpf, most of the injected larvae showed reduced eye size as compared to control uninjected fish (Fig. 3.1 A, B). These larvae were separated out, and a few of them were randomly picked for DNA isolation to test for presence or absence of the 4.1kb gene fragment containing Exon 3 and Exon 4 using PCR. DNA from these larvae showed bands at less than 500bp, indicating that a portion of the gene corresponding to Exon 3 and Exon 4 was missing (Fig. 4.1 C, D).

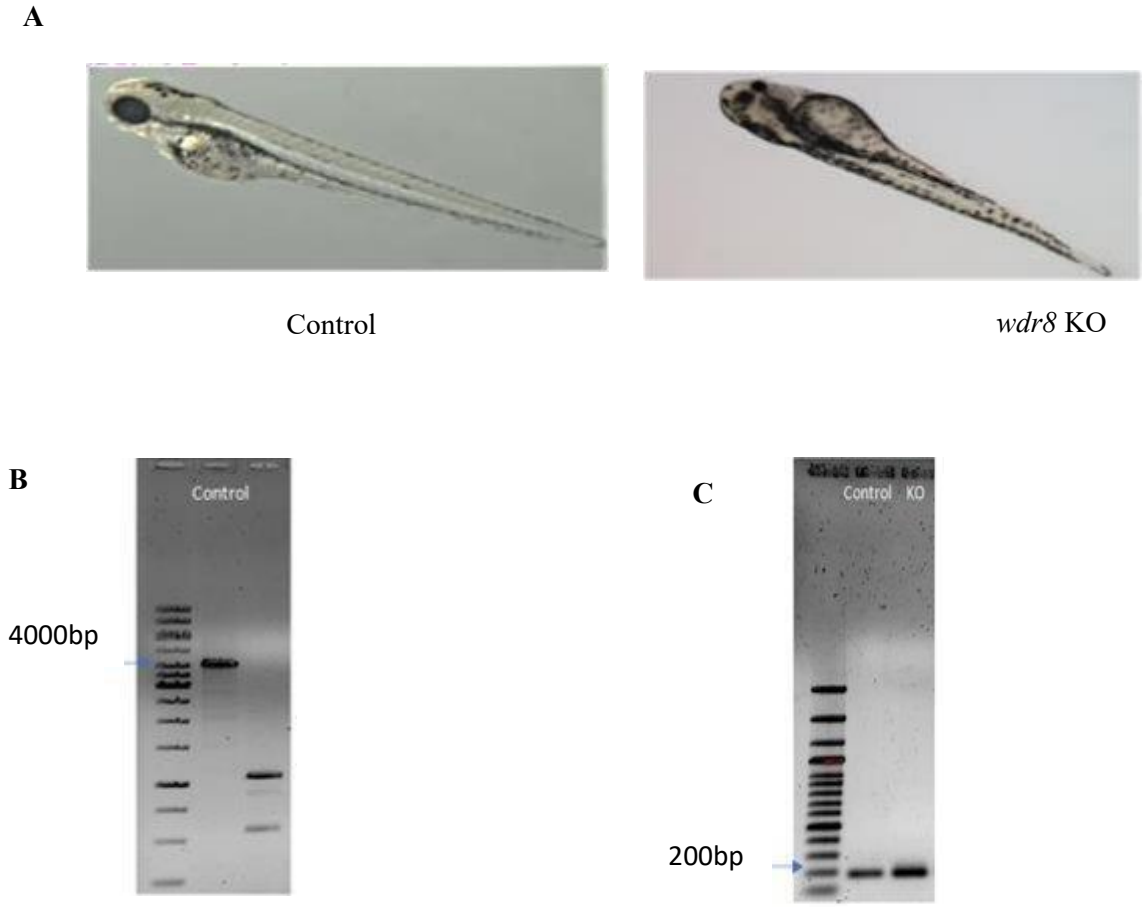


Fig 4.1— A. Control embryos, *wdr8* knockout embryos, and *wdr8* showing differences in eye size. B. PCR for 4.1kbp fragment containing Exon 3 and Exon 4 with DNA from control and *wdr8* knockout embryos. Control shows a crisp 4.1kb band, while this is absent in KO lane. C. PCR for EF1α (Elongation Factor 1 alpha, eukaryotic elongation factor) with DNA from control and *wdr8* knockout embryos. Both show a crisp band at ~200bp, indicating that DNA integrity of the KO fish is unaffected.

4.2 *wdr8* knockout fish show reduced eye and lens size

wdr8 knockout fish showed a clear reduction in eye size as compared to uninjected

rols.While the average eye size was 883.6 ± 54.7 µm for uninjected control, the mean eye size of *wdr8* knockout fish reduced to 652.7 ± 83.7 µm. A similar trend was seen in case of lens size, with WDR8 knockout fish showing significantly smaller lens (236.9 ± 31.6 µm) as compared to uninjected controls (313.8 ± 28.8 µm).

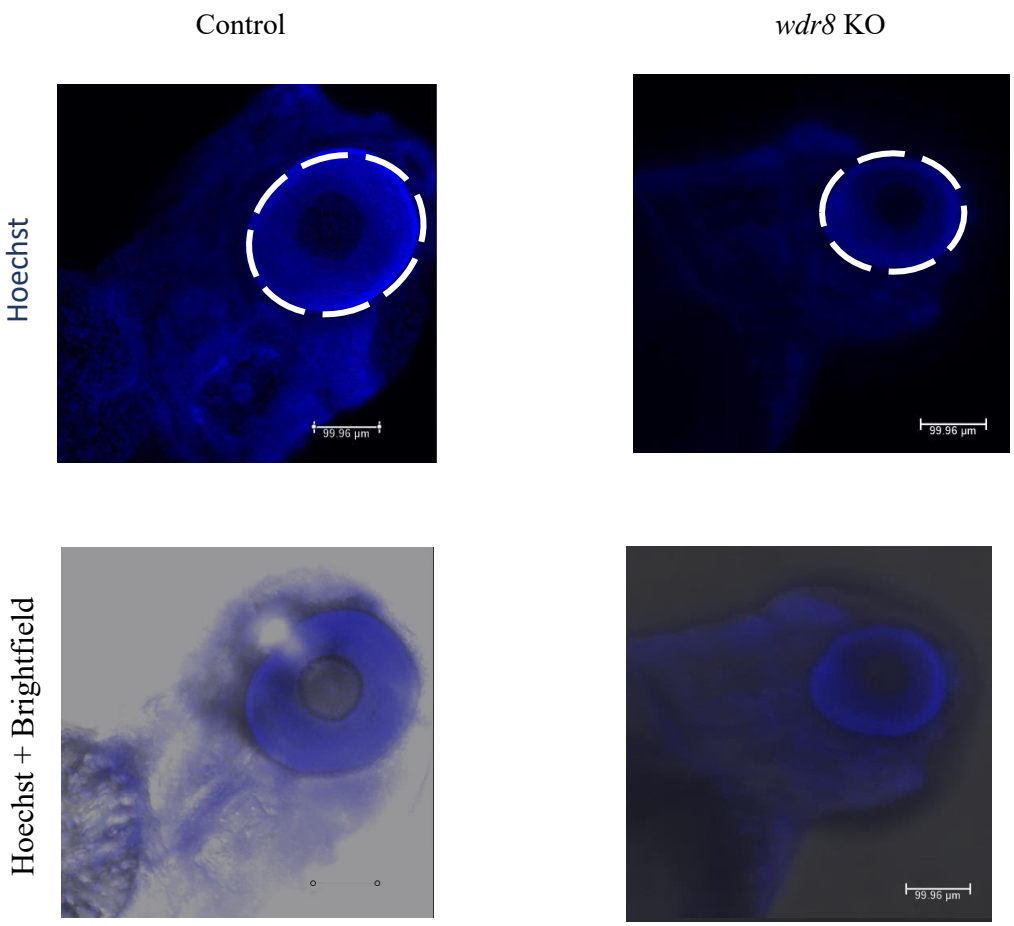


Fig. 4.2— Confocal images of control embryos, *wdr8* knockout embryos, showing differences in eye size. Blue is used to represent Hoechst staining, which is a nuclear marker.

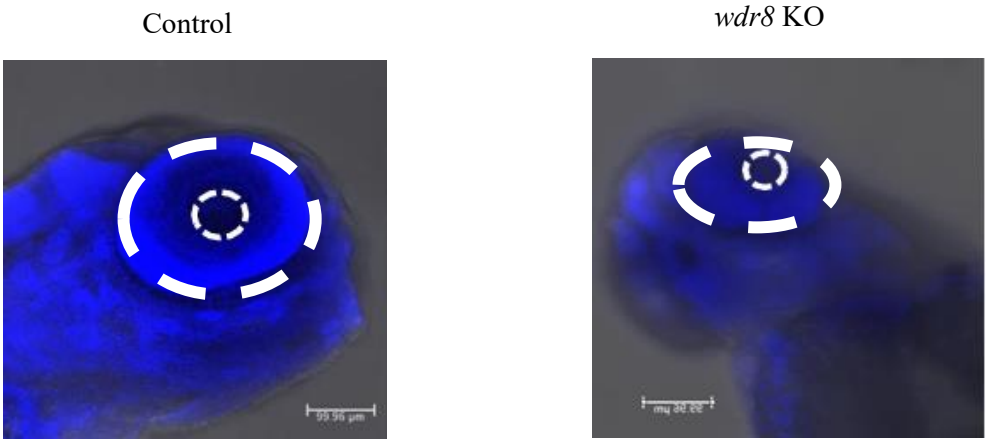
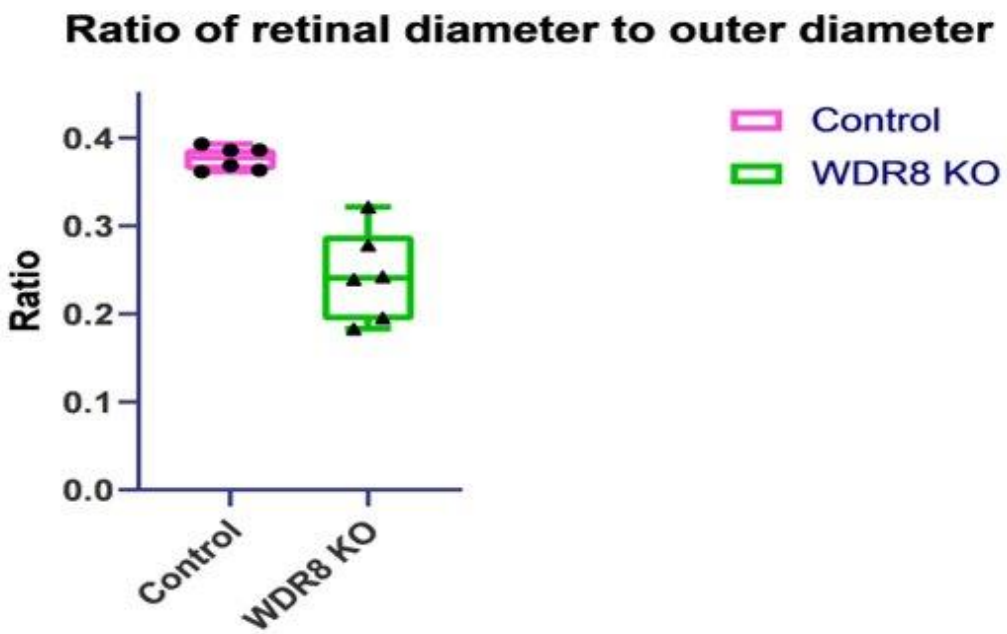


Fig 4.3 – Confocal images of control and *wdr8* KO embryos, showing differences in retinal and equatorial diameter. Blue is used to represent Hoechst staining, which is a nuclear marker.



(N =6)

Fig. 4.4 - Quantification of ratio of equatorial and retinal diameter of control and *wdr8* KO embryos.

4.3 Knockout of *wdr8* does not affect organisation of retina

Though the number of cells in the retina appears to be reduced in *wdr8* knockout larvae, the structure of the retina and all the retinal layers are unaffected (Fig. 4.5). The number

of cells in each layer and the thickness of each layer, however, appears to be reduced in case of knockout of WDR8 (Fig. 4.5).

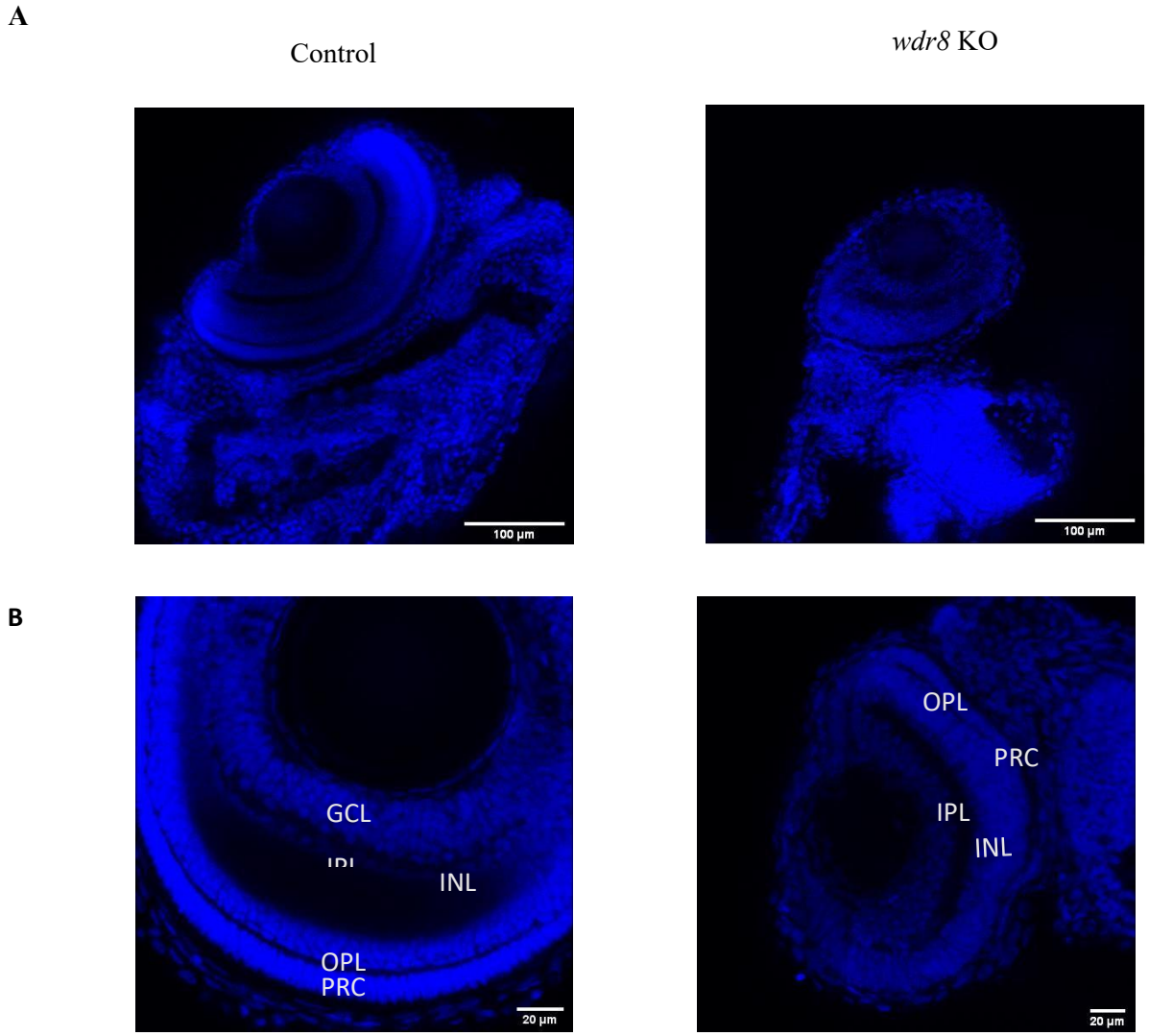


Fig 4.5— A. Confocal images of control embryos and *wdr8* knockoutbryos showing differences in number of cells and thickness of retinal layers. Blue is used to represent Hoechst staining, which is a nuclear marker. As is evident from the image, *wdr8* KO fish have very sparse distribution of cells in the retina. B. Magnified images showing retinal layers in control and *wdr8* KO embryos, with retinal layers marked. GCL-Ganglion Cell layer, IPL- Inner Plexiform Layer, INL- Inner Nuclear Layer, OPL-Outer Plexiform Layer, PRC-Photoreceptor cells. (Bar- 20 µm)

4.4 *wdr8* knockout fish show defective muscle arrangement

In addition to eye defects, *wdr8* knockout larvae show improper organisation of muscles. In particular, the vertical myosepta — structures that connect adjacent muscle bundles together and allow for motility through translation of forces through the bundles — is

very poorly developed in case of *wdr8* knockout fish (Fig. 4.7). This might also explain the reduced motility observed in case of *wdr8* knockout larvae.

A

B

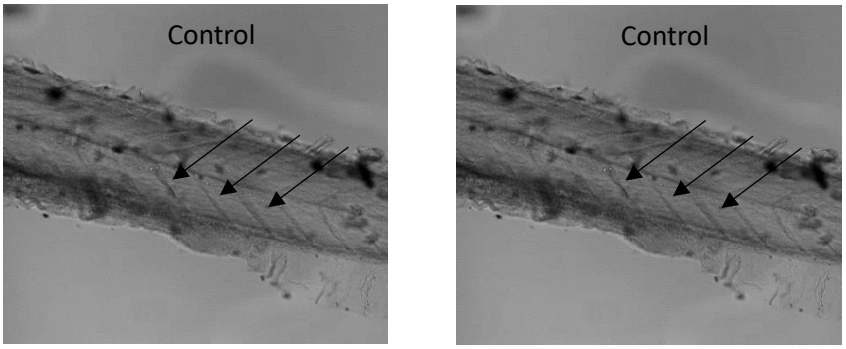


Fig 4.6— A. Control and *wdr8* knockout fish showing difference in muscle arrangement. B. Magnified images showing complete lack of development of vertical myosepta fibres in the *wdr8* knockout larvae (myosepta in control indicated with black arrows) (Bar- 100 µm).

4.5 *wdr8* KO results in slower muscular movement: Swimming Assay

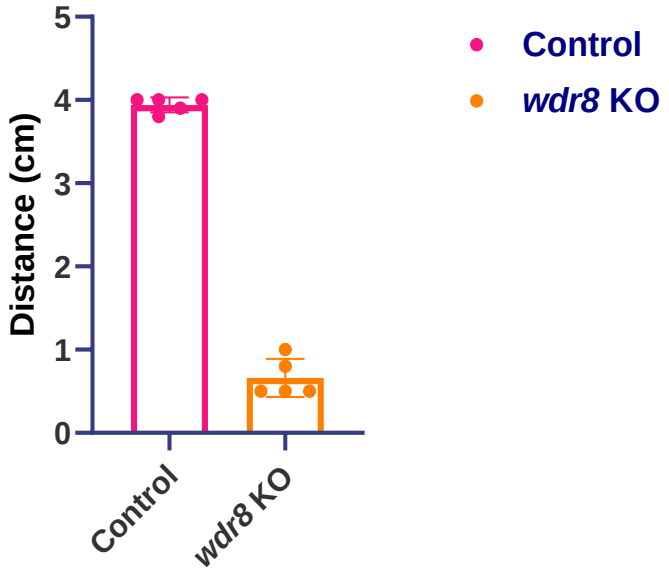


Fig 4.7- Graphical representation showing distance travelled in 2 seconds by control and *wdr8* KO larvae.

Average distance travelled by a uninjected control larvae in 2 second is **3.94cm** whereas average distance travelled by a *WDR8* KO(3dpf) larvae in 2 second is **0.67cm**. (N = 5)

4.6 *wdr8* KO results in less Heartbeat counts per minute

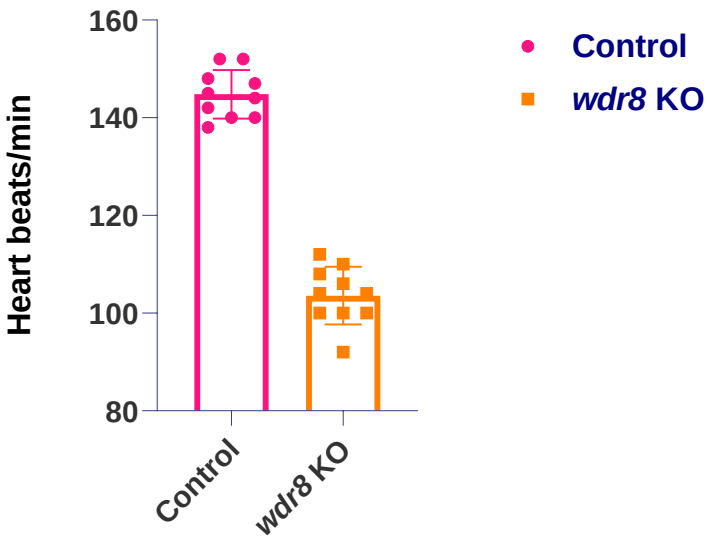


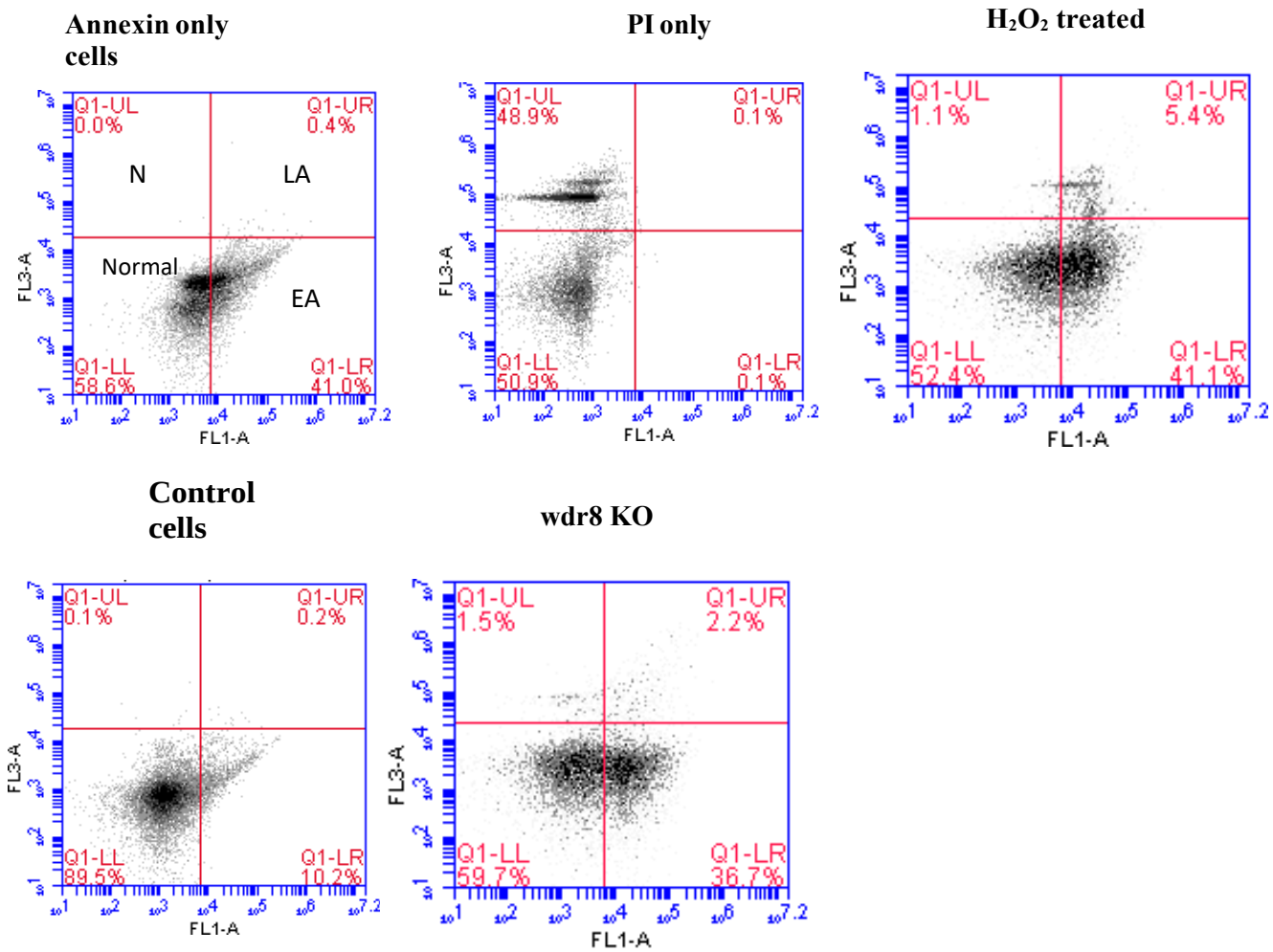
Fig 4.8- Graphical representation showing heartbeats/min of uninjected control larvae and *wdr8* KO larvae.

An average heart beats/min of **145** is observed in control fishes whereas an average heart beats/min of **104** is observed in *wdr8* KO fishes. (N = 10)

4.7 *wdr8* knockout fish show increased apoptosis

Since *wdr8* knockout larvae show reduced number of cells in the lens and retina, along with defective development of the myosepta, the levels of apoptosis in larvae at 3 dpf was checked to identify the cause of these developmental deficits using Fluorescence Activated Cell Sorting (FACS) using Annexin V-PI staining. The number of apoptotic cells in *wdr8* knockout larvae was found to be higher (38.9%) as compared to that in uninjected controls (11.2%). Exogenous expression of zebrafish *wdr8* in the knockout background restored the levels of apoptosis to that in uninjected controls (10.4%) .

A.



B.

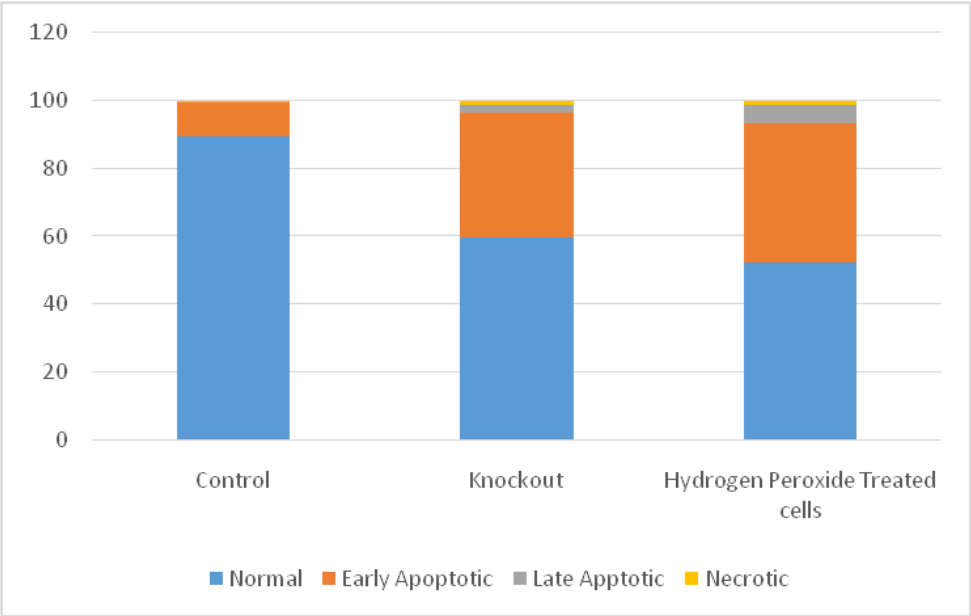
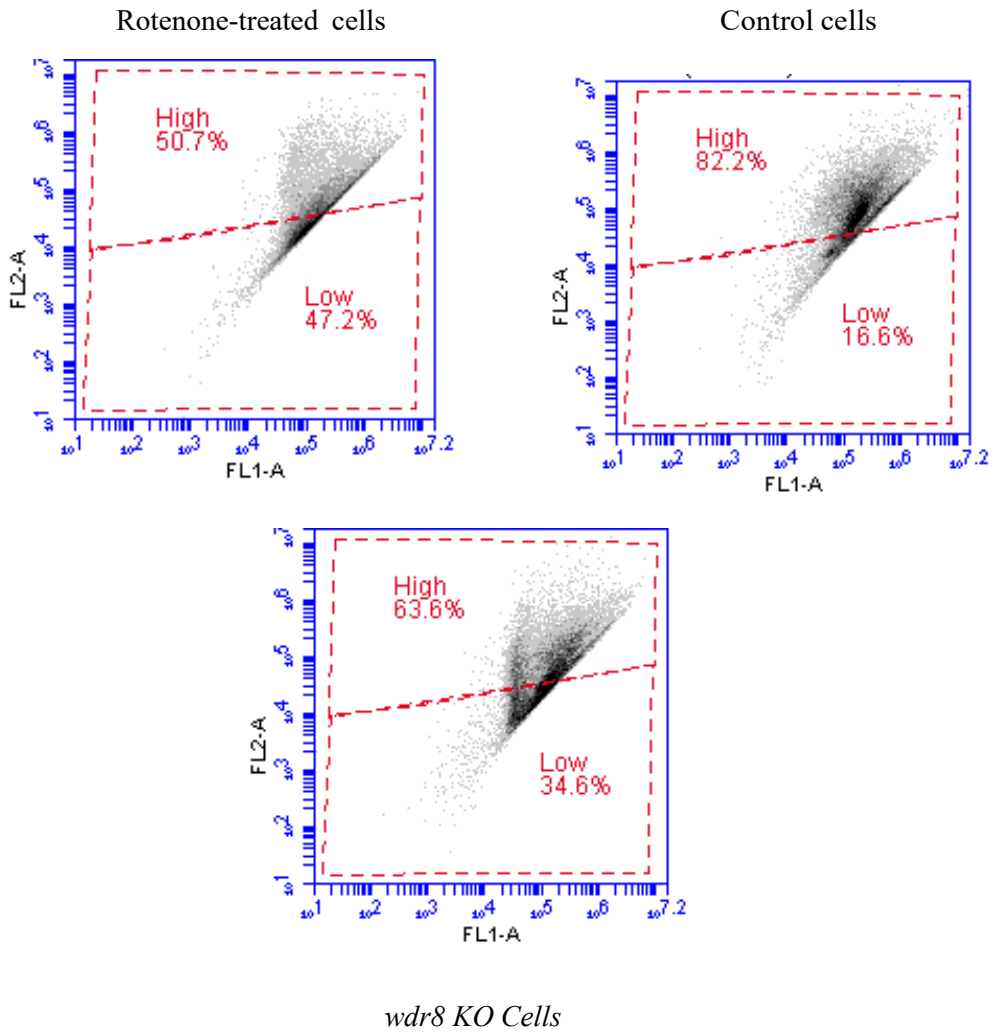


Fig 4.9- A. FACS data showing the number of cells under different stages of apoptosis. Normal refers to the normal healthy cells, EA refers to early apoptotic cells, LA refers to late apoptotic cells and N refers to necrotic cells. The number of cells in each quadrant is indicated at the corners. H_2O_2 was used a positive control, as it is known to induce apoptosis. **B**. Graphical representation of proportion of normal, apoptotic and necrotic cells in uninjected control, *wdr8* KO and *wdr8* KO.

4.8 *wdr8* knockout fish show disrupted mitochondrial membrane potential

The status of mitochondrial membrane potential in case of knockout of *wdr8* was assessed; this is often the earliest indicator of impending cell apoptosis. JC-1 was used as an indicator of mitochondrial health — this is a cationic dye which permeates and accumulates within healthy mitochondria to form aggregates which emit a red fluorescence. When membrane integrity is compromised, JC-1 no longer accumulates in sufficient concentrations in the mitochondria to form aggregates; it hence stays in its monomeric form, which emits a green fluorescence. The ratio of red versus green fluorescence can hence be taken as an indicator of mitochondrial health. The cells from 3-day old *wdr8* knockout fish showed a marked shift towards low red fluorescence (34.6% cells in the low red fraction as opposed to 16.6% of cells in uninjected control) when treated with JC-1. The number of cells with low red fluorescence fell to 27.4 % when exogenous *wdr8* was supplied in the knockout background. This indicates that knockout of *wdr8* could hamper mitochondrial membrane potential.

A.



B.

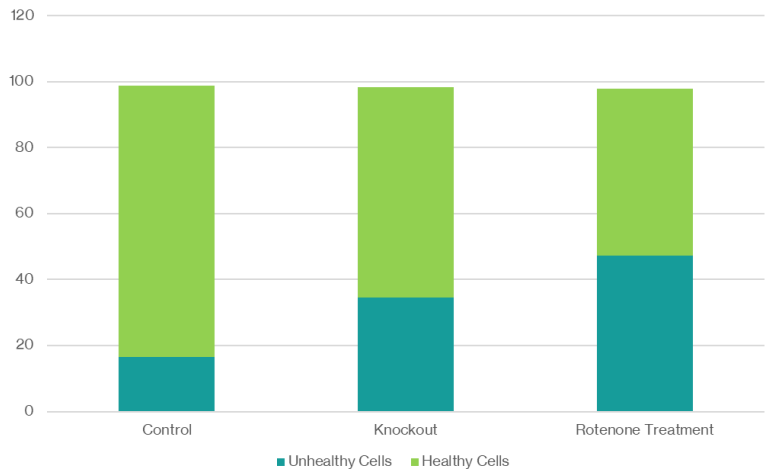


Fig 4.10- A. FACS data showing the distribution of healthy and unhealthy cells. The number of cells in each quadrant is indicated at the corners. Rotenone was used a positive control, as it is known to disrupt mitochondrial membrane potential. **B**. Graphical representation of proportion of healthy and unhealthy cells in uninjected control, *wdr8* KO and *wdr8* KO.

CHAPTER - CONCLUSION

Using zebrafish as a model, this study has attempted to test the effects of absence of functional *wdr8*on overall development, with a focus on eye development. The *wdr8* knockout fish show similar eyephenotypes and cellular signatures as *wdr8* morphants did — they show reduced eye and lens size and reduced number of cells in eyes and retinal layers. All the layers of the retina are well developed,indicating that *wdr8* does not have a role to play in delamination of retinal layers. *wdr8* knockout larvae also show additional defects in muscle organisation — they show reduced motility and poor organisation of the vertical myosepta and a weaker cardiac muscle. Preliminary results indicate that these fish also show increasedapoptosis and compromised mitochondrial membrane integrity — this could be a result of cell divisiondefects, given the role of *wdr8* in regulating ciliogenesis

CHAPTER 6- FUTURE SCOPE

Using zebrafish as a model organism for studying genetic mutations like *wdr8* knockout (KO) and its resulting muscular and eye defects holds significant promise for future research. Here are some potential future scopes:

o Elucidating Molecular Mechanisms: Zebrafish can provide insights into the molecular mechanisms underlying muscular and eye defects caused by *wdr8* knockout. Researchers can use techniques like CRISPR/Cas9 to precisely manipulate the zebrafish genome, allowing them to study how *wdr8* disruption affects cellular processes involved in muscle and eye development.

o Drug Screening: High-throughput drug screening can be performed using zebrafish models to find possible therapeutic molecules for the treatment of muscular and ocular abnormalities caused by *wdr8* knockout. This strategy can hasten the identification of novel medications and treatment targets for diseases with comparable characteristics in humans.

o Gene Therapy Development: Zebrafish models can aid in the development and optimization of gene therapy strategies for treating genetic disorders caused by *wdr8* mutations. Researchers can test the efficacy and safety of gene editing techniques such as CRISPR/Cas9-mediated gene correction or gene replacement therapies in zebrafish models before translating them to clinical applications.

o Understanding Human Diseases: Studying *wdr8* knockout in zebrafish can provide valuable insights into human diseases with similar phenotypes, such as muscular dystrophies and retinal degenerative disorders. Zebrafish share many genetic and physiological similarities with humans, making them a powerful model for understanding the pathogenesis of these conditions.

o Developmental Biology Research: Zebrafish embryos develop rapidly and are transparent, allowing researchers to observe developmental processes in real-time. Studying WDR8 knockout in zebrafish can shed light on the role of *wdr8* in embryonic development and tissue morphogenesis, contributing to our understanding of fundamental biological processes.

o Gene-Environment Interactions: Zebrafish models offer the opportunity to investigate how genetic mutations interact with environmental factors to influence disease phenotype. By manipulating environmental variables such as temperature, light exposure, or chemical exposures, researchers can explore gene-environment interactions that modulate the severity of muscular and eye defects in *wdr8* knockout zebrafish.

o Overall, the future scope of using zebrafish as a model organism for studying *wdr8* knockout and its associated muscular and eye defects is vast and holds great potential for advancing our understanding of genetic diseases and developing novel therapeutic interventions.

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ABSTRACT

The proteincoding gene WDR8, also known as "WD Repeat Domain 8," is essenti al to many different biological functions. WD repeat protein family is known for their involvement in protein-protein interactions and the regulation of essential biological functions. WDR8 contains WD40 repeats, which are protein domains involved in mediating diverse cellular activities, such as signal transduction, cell cycle regulation, and chromatin assembly. WDR8 is a centriolar protein essential for the formation of primary cilia, growth of the axonemal microtubules, and for recruiting other members for ciliogenesis during cell division. A recent study identified a novel mutation in the *wdr8* gene that results in Isolated Microspherophakia, a rare genetic eye disorder characterised by improper lens development.

When wdr8 is mutated using morpholinos, zebrafish (Danio rerio) exhibit smaller eyes and fewer cells in the lens and retina. They also exhibit cell cycle arrest, an i ncrease in retinal cell death, and muscle distortion. Here, we explore the effect of CRISPR-Cas9 based knockout of *WDR8* on eye and muscle development, and attempt to decipher the other molecular players that may be involved in mediating the link between WDR8 and eye development.

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