

# **The modelling of RECQL4-related human diseases on zebrafish**

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# Abbreviations

BER – Base Excision Repair

BLM – Bloom Syndrome Helicase

Cas9 – CRISPR associated protein 9

CRISPR – clustered regularly interspaced short palindromic repeats

crRNA – CRISPR targeting RNA

DNA – Deoxyribonucleic acid

Dpf – Days post fertilisation

DSB – Double-strand breaks

HRDC – Helicase and RNase D-like C-terminal

mtDNA – Mitochondrial DNA

NER – Nucleotide excision repair

PCR – Polymerase chain reaction

RNA – Ribonucleic acid

RNP – ribonucleoprotein

RQC – RecQ C-terminal

WRN – Werner Syndrome Helicase

## Rezümé

A munkám során a RECQL4 helikázzal foglalkoztam. Ez a fehérje részt vesz a DNS hibajavításban, rekombinációban és replikációban, és a mitokondriális genom fenntartásában is. A CRISPR/Cas9 rendszer segítségével több *recql4* mutáns allélt generáltunk zebrahalban (*Danio rerio*), és megkezdtük a feltérképezésüket. Eredményeim azt mutatják, hogy a *recql4* mutánsok egyedi morfológiai elváltozásokat mutatnak, csökkent mozgási képességeik vannak és alacsonyabb a várható élethosszuk.

## Összefoglaló

A RecQ helikázok, melyeket „a genom őrzői” -nek is neveznek, kritikus szerepet játszanak a genom szerkezetének fenntartásában és stabilizálásában. A legtöbb gerinces esetén öt darab ilyen helikázt kódoló gént találunk a genomban, ezek a *RECQL1*, a *BLM*, a *WRN*, a *RECQL4* és a *RECQL5*. Fontosságukat egyértelműen mutatja, hogy az öt gén közül háromhoz (*BLM*, *WRN* és *RECQL4*) köthető súlyos tünetekkel járó monogén recesszív emberi betegség. E szindrómák molekuláris etiológiájának megértéséhez elengedhetetlen a helikázok pontos funkcióinak feltérképezése. Diákköri kutatómunkám fő célja egy új zebrahal modell létrehozása a *RECQL4* gén mutációjával kapcsolatos betegségekre. Ennek a génnek a mutációjának legérdekesebb aspektusa, hogy három különböző szindróma köthető hozzájuk: Rothmund-Thomson szindróma, RAPADILINO szindróma és Baller-Gerold szindróma. Egyelőre nem értjük, mi okozza ezt a sokféleséget, de ennek feltárása fontos orvosi és diagnosztikai alkalmazáshoz vezethet. A *RECQL4* részt vesz a DNS-javításban, rekombinációban és replikációban, és egyedülállóan a RecQ helikázok közül mitokondriális lokalizációt is mutat, ami arra utal, hogy fontos lehet a mitokondriális genom fenntartásában is. A CRISPR/Cas9 rendszer segítségével több *recql4* mutáns allélt generáltunk zebrahalban (*Danio rerio*), és megkezdjük molekuláris és fenotípusos jellemzésüket. Eredményeim azt mutatják, hogy a *recql4* mutánsok egyedi morfológiai elváltozásokat mutatnak, amelyek a humán betegségek tüneteire hasonlítanak, mozgási képességeik csökkentek és alacsonyabb a várható az élettartam.

## Summary

RecQ helicases, also known as the “guardians of the genome”, play critical roles in genome maintenance and stability. Most vertebrate genomes, encode for five such helicases: RECQL1, BLM, WRN, RECQL4 and RECQL5. Their importance is clearly shown by the fact that mutations in three of the five genes (*BLM*, *WRN* and *RECQL4*) result in monogenic recessive diseases with severe symptoms in humans. To understand the molecular etiology of these syndromes, it is essential to map the exact functions of these helicases. The main goal of my research is to establish novel zebrafish models for the diseases that are related to the mutation of the *RECQL4* gene. The most interesting aspect of such mutations is that three different syndromes can be linked to them: Rothmund-Thomson syndrome, RAPADILINO syndrome and Baller-Gerold syndrome. We do not understand yet, what causes this diversity, but revealing it could have important medical and diagnostic implications. RECQL4 participates in DNA repair, recombination and replication and uniquely among RecQ helicases it also exhibits mitochondrial localization suggesting that it might be important in the maintenance of the mitochondrial genome. Using the CRISPR/Cas9 system we generated multiple mutant alleles of *recql4* in zebrafish (*Danio rerio*) and started their molecular and phenotypic characterization. My results show that *recql4* mutants show unique morphological disparities, reminiscent of the human patients, impaired movement and reduced lifespan.

# Introduction

DNA is the foundation that all living organisms are built on, its code dictates how they develop and function. Therefore, the integrity and maintenance of the genome is a crucial point in the life of a cell. There are several proteins or protein families that have a role in protecting the structure of the DNA. Our research group is especially interested in one of these, called RecQ helicases. These helicases have critical roles in genome maintenance and stability and thanks to this they are also often called as the “guardians of the genome”.

The RecQ helicase family consist of 5 members in humans: RECQL1, BLM, WRN, RECQL4 and RECQL5. Each of them has a unique and important role in keeping the genome safe besides their helicase activity. Their importance is clearly shown by the fact that mutations in three of the five genes that code these helicases cause monogenic recessive diseases with severe symptoms in humans (*BLM*, *WRN* and *RECQL4*). Mutations in *BLM* and *WRN* lead to Bloom syndrome and Werner syndrome, respectively. The case of *RECQL4* is more intriguing as the mutations that affect this gene can lead to three different kind of syndromes: Rothmund-Thomson syndrome, RAPADILINO syndrome and Baller-Gerold syndrome. We do not understand yet, what causes this diversity, but revealing it could have important medical and diagnostic implications (Croteau et al., 2014). During my student research project, I focused on *RECQL4*.

The structure of RecQ helicases shows a high level of conservation (Figure 1). They have three distinctive domains: the core helicase domain, the RecQ C-terminal (RQC) domain and the Helicase and RNase D-like C-terminal (HRDC) domain. While the first two domains can be found in all of the five helicases, the HRDC domain can only be found in BLM and WRN. The main role of the core helicase domain is the trademark ATP-dependent helicase activity, the unwinding of the two DNA strands. The RQC domain also participates in the helicase activity, but its specific role is to bind G4 quadruplexes and other DNA structures (Croteau et al., 2014). The putative role of the HRDC domain is to interact with other proteins to recognize DNA damages (Kitano, 2014).

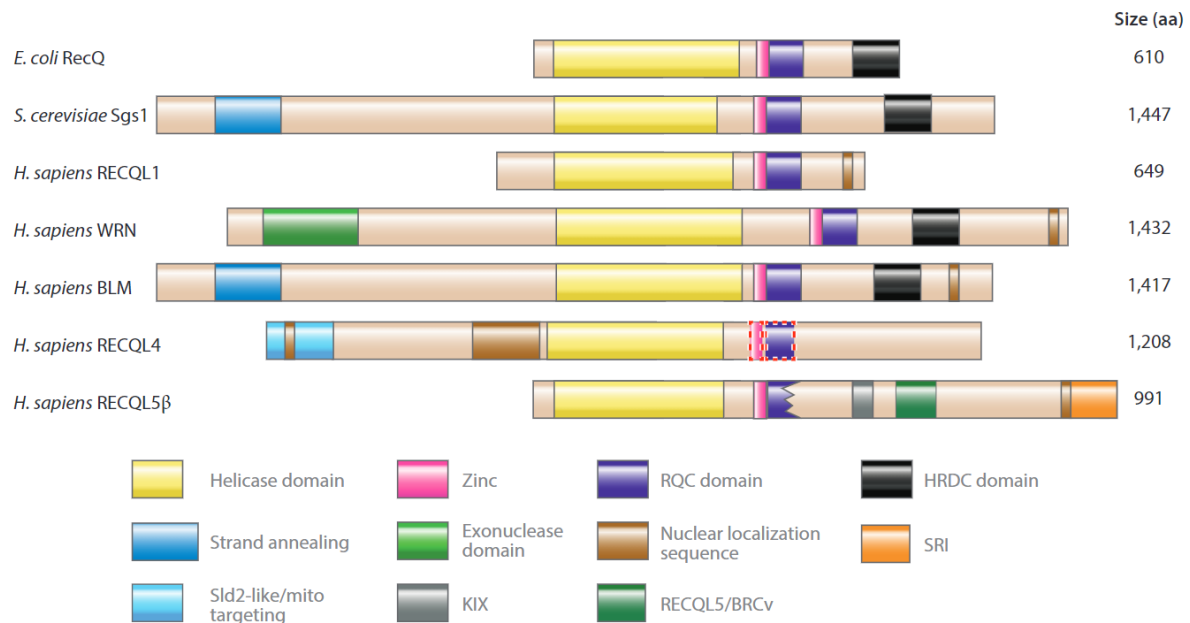


Figure 1: The domain structure and the size of the different RecQ helicases that can be found in humans, in *Escherichia coli* and in *Saccharomyces cerevisiae* (Croteau et al., 2014)

RECQL4 has multiple important roles in genomic maintenance (Figure 2). It can interact with the Shelterin complex to resolve D-loops and G4 structures, but it also has a role in replication initiation. It is involved in numerous DNA repair processes, such as the role in the double-strand break (DSB) repair, base excision repair (BER), nucleotide excision repair (NER) and it also interacts with p53 to mediate mtDNA synthesis. This latter aspect is a unique property among the RecQ helicases as the others do not contain a mitochondrial localization sequences (Luong & Bernstein, 2021).

The most important for us from these processes are the roles of RECQL4 in the replication initiation and its importance in the mitochondrial integrity.

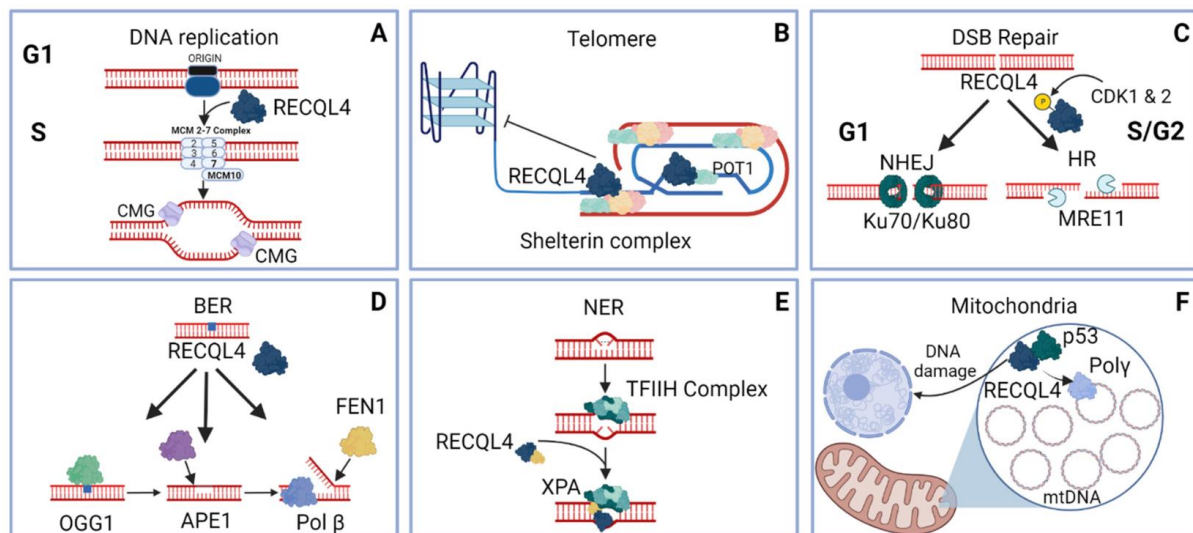


Figure 2: The genomic maintenance roles that *RECQL4* participates in. (A) Has a role in the replication initiation, (B) interacts with the Shelterin complex to protect the telomeres, (C) mediates DSB repair in two different ways, (D) stimulates several BER proteins, (E) Partakes in the Nucleotide excision repair, (F) Helps the synthesis of mtDNA with p53 (Luong & Bernstein, 2021)

A so far unmapped aspect of the *RECQL4* mutations is that three different autosomal recessive diseases can be linked to them. Several mutations can cause each of these syndromes and mutations for all three syndromes can be found along the full coding region of *RECQL4* (Figure 3).

Rothmund-Thomson syndrome is a rare disease, with only 500 reported cases so far. Its most common symptoms are cataracts, increased osteosarcoma incidence, skeletal deformities, short stature and it also has a characteristic facial rash called poikiloderma (Luong & Bernstein, 2021). They identify two types of Rothmund-Thomson syndrome, from which only the type II contains a mutation in the *RECQL4* gene. Type I is caused by the mutation of *ANACP1* (Larizza et al., 2010) (Ajeawung et al., 2019). Some results show that Rothmund-Thomson syndrome is a progeroid disease which can possibly be addressed to the malfunction of the mitochondria (Zirn et al., 2021) (Luong & Bernstein, 2021). Also, there have been experiments that showed that the depletion of *RECQL4* increases the level of mitochondrial DNA damage, and decreases the oxygen consumption rates (Croteau et al., 2012).

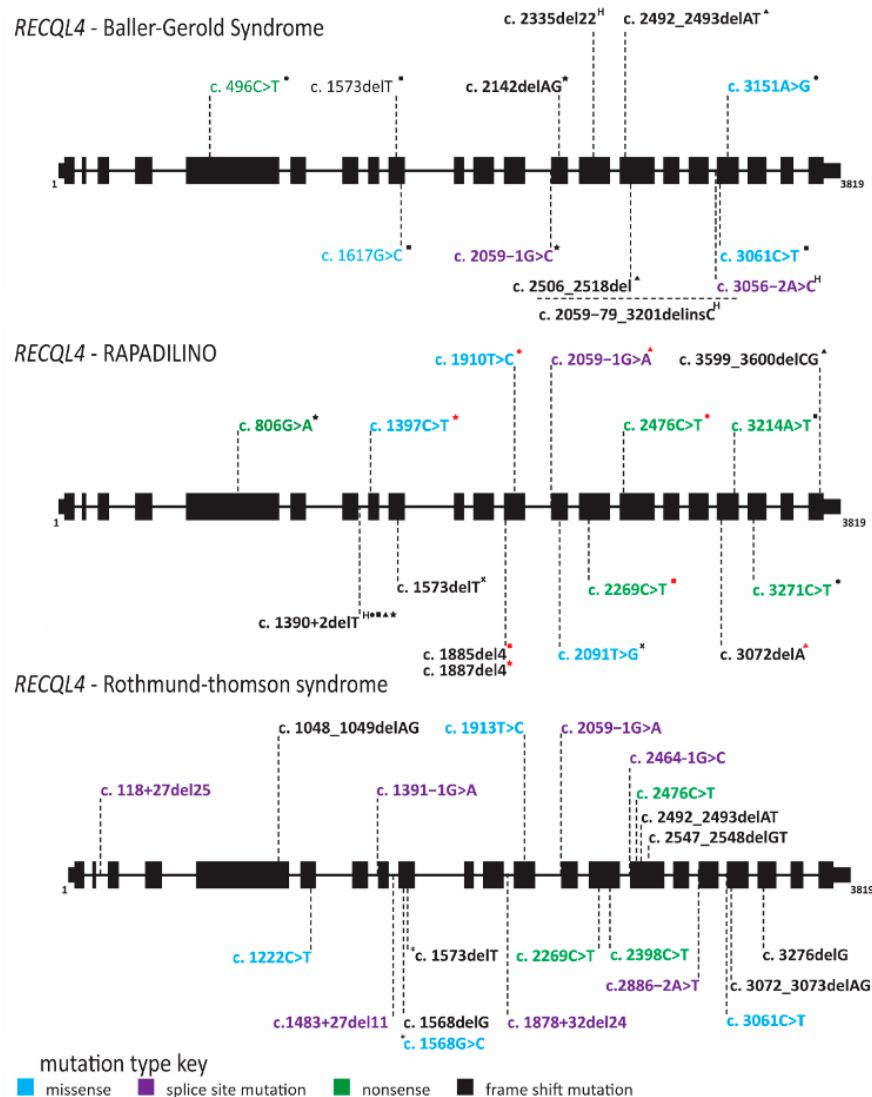


Figure 3: Mutations that cause specific *RECQL4*-related diseases (Luong & Bernstein, 2021).

Baller-Gerold syndrome is also an extremely rare disease, with fewer than forty cases have been reported so far. It has similar symptoms as Rothmund-Thomson syndrome, for example poikiloderma, and developmental abnormalities, but it has a unique feature because it is associated with coronal craniosynostosis (Luong & Bernstein, 2021).

RAPADILINO is an acronym, which shortens the related symptoms. RA for radial ray malformations, PA for patellae hypoplasia and cleft or high arched palate, DI for diarrhea and dislocated joints, LI for little size and limb abnormalities, and NO for slender nose and normal intelligence. RAPADILINO syndrome shares symptoms with Rothmund-Thomson syndrome, but this is the only one that associated with palatal abnormalities also this is the only disease that can only be caused by the mutation of *RECQL4* (Luong & Bernstein, 2021).

Numerous animal models have been established to study Recql4 function. There has been a high incidence of embryonic lethality in the case of mice (*Mus musculus*) and flies (*Drosophila melanogaster*) and has an essential role in the DNA replication in the case of frog (*Xenopus laevis*) eggs, which demonstrates how important this protein is (Hoki et al., 2003; Sangrithi et al., 2005; Wu et al., 2008). My goal was to create and characterize a novel zebrafish model as well.

## Aims

The main goal of my student research project is to establish a new zebrafish model for *RECQL4*-related diseases. Hitherto no mutant alleles for *recql4* have been described in this species (widely considered one of the top biomedical models), therefore we hope that our new mutant lines will lead to interesting novel insights into these syndromes. Previously in our lab we have successfully established a zebrafish model for Bloom syndrome (Annus et al., 2022) and I will rely on this know-how during my work.

As no detailed expression data is available for *recql4*, first I wanted to examine the expression pattern of *recql4* in embryos during development. I used an *in silico* approach to find *recql4* expression in online databases which contain single-cell RNA sequencing (scRNA-Seq) timecourse datasets (<https://zebrahub.ds.czbiohub.org>, <http://bis.zju.edu.cn/ZCL/>) (Farnsworth et al., 2020; Jiang et al., 2021). I complemented these results with whole mount *in situ* hybridisation experiments as well, using embryos from several developmental stages.

To generate mutant *recql4* alleles we used the CRISPR/Cas9-based genome editing method. We targeted different exons of the genes, so I will be able to compare different mutant alleles during my research.

One of my major tasks was to find adult fish that carry deleterious germline mutations in their *recql4* gene, and create pure lines where the effects of these alleles can be observed. Genotyping PCR protocols need to be established for each of these alleles.

After successfully establishing stable heterozygous lines, I will analyze in detail their distinctive phenotypes.

Finally, once stable lines are established and maintained, I will continuously monitor the different genotypes, to see if the animals that carry mutant alleles are prone to particular diseases (e.g. cancer), or have reduced lifespan.

# Methods and Materials

## Fish husbandry

Our fish lines are maintained at the Animal Facility of the Biology Institute at ELTE Eötvös Loránd University under standard conditions (Aleström et al., 2020; Westerfield, 2000). During our experiments we used wild type (AB) and *recql4* mutant fish. All experimental procedures were approved by the Hungarian National Food Chain Safety Office (Permit Numbers: PE/EA/290-2/2018, PE/EA/2025-7/2017).

## Collection embryos

Fish were put into classical breeding tanks and after successful mating the embryos were collected with a net and raised in plastic Petri-dishes containing E3 medium at 28.5°C (Westerfield, 2000).

## Generating new mutant lines

So far, we have generated two mutant alleles for *recql4*. The first contains an 8 bp long deletion in the 5<sup>th</sup> exon of the gene, while the second one contains a 7 bp long deletion in the 16<sup>th</sup> exon. The mutagenesis was done using the CRISPR/Cas9 methodology (Kroll et al., 2021) Targeting efficiency was assayed in the injected embryos by PCR amplifying and Sanger sequencing the targeted loci. Mosaic animals in the P0 generation that carry mutant *recql4* alleles in their germline were identified by crossing injected fish with wild type ones and genotyped their offspring (see below). The mutagenesis was carried out by my supervisor, Dr. Máté Varga.

## DNA isolation

DNA was either isolated from adult fish or from embryos (Meeker et al., 2007). In the case of adult animals, I clipped a small part of their tail fin added 100 µl 50 mM NaOH at 95°C for 15 minutes and after that I added 10 µl pH 8.0 Tris-HCl solution equivalent to 10% of the total volume. In the case of embryos and larvae, I had to use the whole animal to extract the DNA.

## Primer design

We designed primers using the help of NCBI Primer-Blast and the “Primer Wizard” function of the Benchling (<http://benchling.com/>) electronic notebook. We generated primers for genotyping PCR, sequencing PCR and for *in situ* hybridisation. Later the PCRs were optimised for the melting temperature of the primers and the length of the DNA section that we were trying to multiply.

| Primer name           | Sequence                            | Primer Type                |
|-----------------------|-------------------------------------|----------------------------|
| recql4-ex5-F          | 5' CGCCGAACACCACAAAAGCTAA<br>3'     | Forward                    |
| recql4-outer-F        | 5' CAGAAGAGCGTGGAGCGAG 3'           | Forward                    |
| recql4-ex5-F2         | 5' TCCAGTTCCTGAGGCAGTCC 3'          | Forward                    |
| recql4-WT-F           | 5' AAGCAAATCTGGCTCCCCG 3'           | Forward                    |
| recql4-8bpdel-inner-F | 5' TGTGGAAGCAAATCCGGGGA 3'          | Forward                    |
| recql4-8bpdel-F       | 5' TGGAAGCAAATCCGGGGAC 3'           | Forward                    |
| recql4-8bpdel-F2      | 5' GCAAATCCGGGGACTCATGGTG 3'        | Forward                    |
| recql4-ex5-R          | 5' ATGTTACCCACCTGACCCTGAC 3'        | Reverse                    |
| recql4-ex5-R2         | 5' GACCCTGACCGTGTTCCTTCAT 3'        | Reverse                    |
| recql4-outer-R        | 5' TGACCCTGACCGTGTTCCTCA 3'         | Reverse                    |
| recql4_ex16-F         | 5'<br>GCCAGGAAGAAGATCCGCAAGCC<br>3' | Forward                    |
| recql4_ex16-R         | 5' AGAAACAGGTGGCAGTGTGCGG<br>3'     | Reverse                    |
| recql4_insitu_F       | 5' AAGCAGCAACTCCTCGCCGAAC 3'        | ( <i>in situ</i> ) Forward |
| recql4_insitu_R       | 5' GAAGCCACCGAAACGACCCCTG<br>3'     | ( <i>in situ</i> ) Reverse |

Table 1: The list of primers that I used during the experiments. In the first column we can see their name, in the second the sequence and in the third their type

## Genotyping

Genotyping was done by Sanger sequencing of the PCR products using the Eurofins Genomics (<http://eurofinsgenomics.eu>) TubeSeq service.

## RNA isolation

To create a cDNA library, total RNA was isolated from live embryos at four different developmental stages: 4 hours post fertilisation (hpf), 6 hpf, 14-20 somite stage and 1 day post fertilisation (dpf). For the isolation we used Trizol (ThermoFisher Scientific, 15596026) according to the manufacturer's protocol.

## Anti-sense *in situ* probe synthesis

As a first step total RNA was used to create a cDNA using reverse transcriptase (ThermoFisher Scientific, 18090010), according to the manufacturer's protocol. The cDNA library was used to PCR-amplify a 1218 bp long sequence specific for exons 5-7 of *recql4*. The PCR product was ligated it into pGEM®-T Easy vector (Promega, A1360) overnight. I transformed *Escherichia*

*coli* DH5 $\alpha$  competent cells using this plasmid and grew the bacterium overnight at 37°C. I isolated the plasmids from these cells using a standard miniprep kit (Zymo Research, D4020) and tested how successful was the transformation using EcoRI digestion enzyme (ThermoFisher Scientific, ER0275). If everything was successful, I linearized the plasmid and used SP6 RNA polymerase to synthesise the anti-sense probe at 37°C in a mixture containing H<sub>2</sub>O, Digoxigenin labelled NTP (Fisher Scientific 11209256910) and the RNase inhibitor Ribolock (ThermoFisher Scientific, EO0381). As a final step I cleaned my probe using 3M sodium acetate and stored it in H<sub>2</sub>O at -20°C (Thisse & Thisse, 2008).

### ***In situ* hybridisation**

I performed the whole mount *in situ* hybridisation reactions according to the protocols of the Thisse laboratory (Thisse & Thisse, 2008). Embryos from six different developmental were collected and fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) solution and stored in 100% methanol at -20°C. Six stages were used: 50% epiboly, 16 somite stage, 1,2,3 and 5 dpf. Embryos were stored in 80% glycerol at 4°C after the hybridisation.

### **Cartilage staining**

5 dpf embryos were fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) solution and stored in 100% methanol at -20°C. Next day at the start of the staining I washed the embryos with 50 % ethanol and added 1 ml 0,02% Alcian Blue and 25  $\mu$ l 10M MgCl<sub>2</sub>. The embryos were stained overnight and on the second day I used 0,5 ml 3% H<sub>2</sub>O<sub>2</sub> and 0,5 ml 2% KOH to fade them. Embryos were stored in 80% glycerol at 4°C.

### **Statistical analysis and visualization**

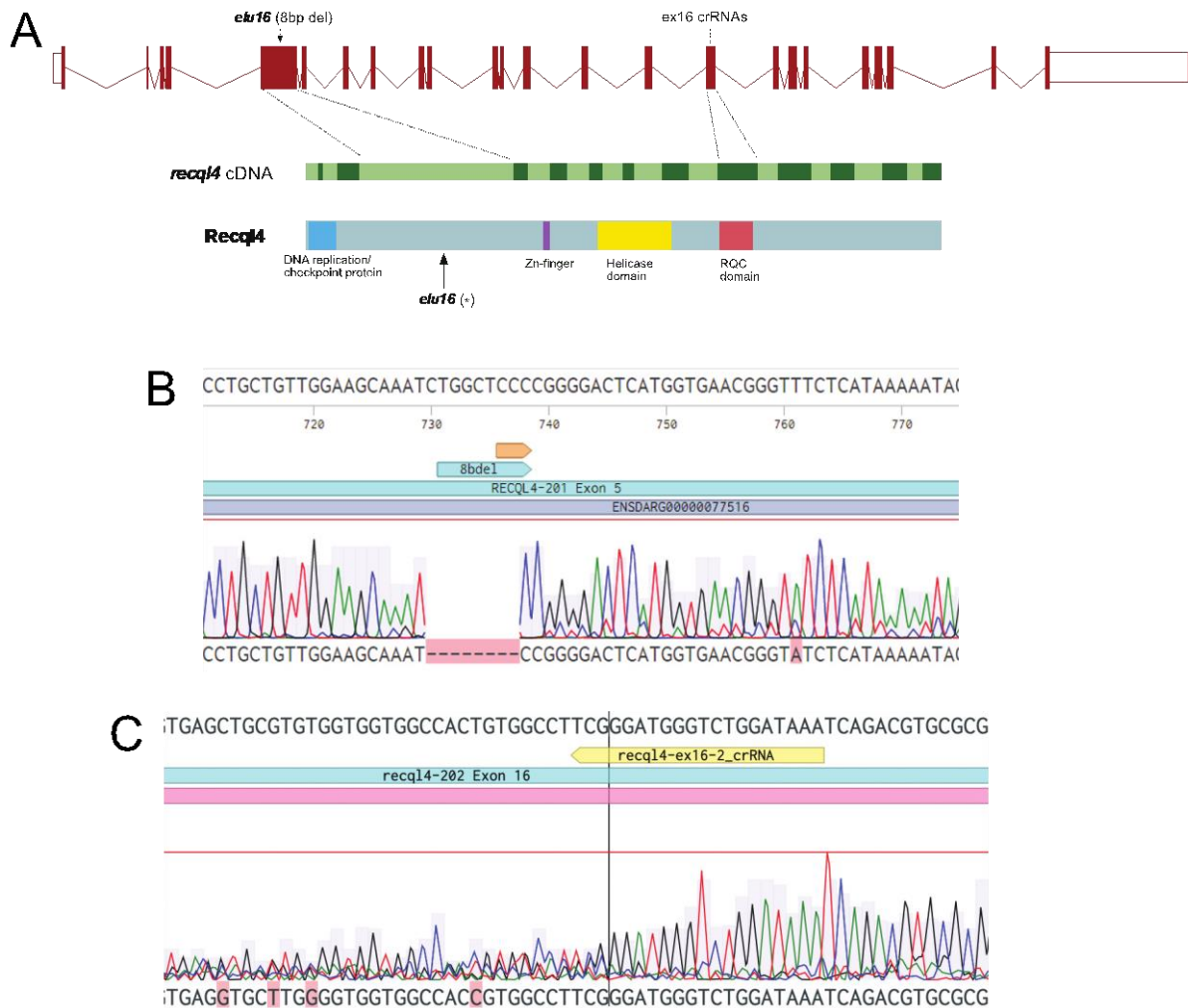
For visualisation and statistical evaluation of the data from the cartilage staining experiments and for the lifespan analysis, I used RStudio with the help of the *survival* and *survminer* libraries (Therneau, 2022) and *ggplot2* (Wickham H, 2016).

## Results and Discussion

### Creating novel *recql4* zebrafish mutant lines

To generate mutations similar to the ones that cause for example Rothmund-Thomson syndrome we applied the CRISPR/Cas9 system (Figure 4A) to generate loss of function mutants. First, we targeted exon 5 of *recql4* and in the injected fish we were able to identify some mosaic carriers of an 8 bp deletion (c.807\_814delTGGCTCCC) which affects the helicase ATP-binding domain (Figure 4A,B). We call this allele *elu16*. To create fish heterozygous for this mutation, we had crossed the mosaic carriers with wild type animals and created an F1 generation. Using the heterozygous animals from this generation I performed an incross and started an F2 generation. I observed a very prominent larval lethal phenotype in this offspring (see below in the Phenotyping section), that was present in Mendelian ratios and genotyping (also see below) confirmed that the fish showing the phenotype were indeed *elu16* homozygotes.

As known human *RECQL4* mutations are embryonic viable (and indeed in our lab's previous work, loss-of-function alleles of *blm* were also viable (Annus et al., 2022)) we were somewhat surprised by the severity of this phenotype. Therefore, we decided to also establish a second allele for the gene, targeting exon 16. A mutation here would affect the helicase C-terminal of Recql4, but other N-terminal domains would be intact (Figure 4A). Sequencing after the injection of the corresponding Cas9 targeting RNPs showed that the targeting was successful and indel mutations can be seen in the respective region (Figure 4C).



**Figure 4: Targeting the *recql4* gene to create new alleles.** (A) The schematic representation of the *recql4* gene (top) and *Recql4* protein (bottom). The position of the *elu16* mutation in exon 5 and of the targeted region in exon 16 are highlighted. (B) Sanger sequencing shows that the *elu16* allele contains an 8 bp deletion. (C) Sanger sequencing of *recql4* exon 16 in Cas9 RNP injected “cripsant” shows successful genome editing. (Note the collapse of the sequenogram in the proximity of the crRNA binding site, indicative of the presence of indel mutations.)

## Genotyping *recql4* mutant alleles

For our planned experiments we needed to establish a fast and efficient PCR-based genotyping protocol.

First I tried to do a multiplex genotyping on the DNA samples from the F2 generation animals. During multiplex PCRs one can use more than one sets of primer pairs, with similar melting temperature to multiply more targets. In the case of *recql4* mutant fish my idea was to use four primers which I designed using NCBI Primer-Blast and the “Primer Wizard” function of the Benchling (<http://benchling.com>). The four primers were: *recql4*-outer-F, *recql4*-8bpdel-inner-

F, *recql4*-WT-inner-R *recql4*-outer-R (see their sequence in Table 1.) The two outer primers would always anneal in all of the 3 three genotypes (+/+, +/*elu16* and *elu16/elu16*) and would give an 809 bp long control product. The other two primers were different: *recql4*-8bpdel-inner-F could only anneal to a mutant allele and form a 633 bp long product with *recql4*-outer-R while *recql4*-WT-inner-R could only anneal to a wild type and form a 378 bp long product with *recql4*-outer-F. So in theory, a single PCR reaction would let us easily identify the genotype of any fish from a *recql4*<sup>+/elu16</sup> incross: in the case of wild type animals we would see 809 bp and 378 bp long amplicons, in the case of heterozygous animals 809 bp, 633 bp and 378 bp products, whereas in homozygous mutants only the 809 bp and 633 bp PCR products would be present. Unfortunately, despite of careful primer design the reactions proved to be aspecific and could not see the three expected bands after the gel electrophoresis.

Next, I decided to forgo multiplexing and use my two sets of primers (for the mutant allele and for the wild type one) in separate reactions. I tested several conditions, but the reactions were always aspecific. So in the end we decided to do the genotyping of the *elu16* allele by Sanger sequencing using *recql4*-outer-F and *recql4*-outer-R.

In the future I will try to genotype the fish using endonucleases to digest a larger DNA sequence, because in theory the mutation affected the cutting site of the *NlaIV* enzyme, so the resulting fragments will differ in the wild type, heterozygous and homozygous animals. The difference would enable us to genotype the animals in a quicker and more cost-effective way.

(For the newer allele I am isolating now mosaic founders, so we could not test different genotyping methods.)

Two of the PCR attempts can be seen below (Figure 5). One of them shows an optimisation test for the mutant allele and the other one shows an attempt for the multiplex PCR

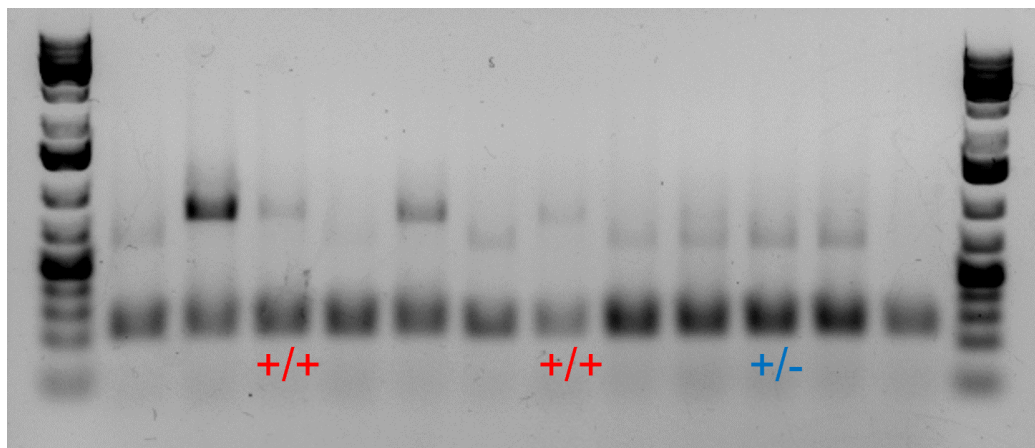
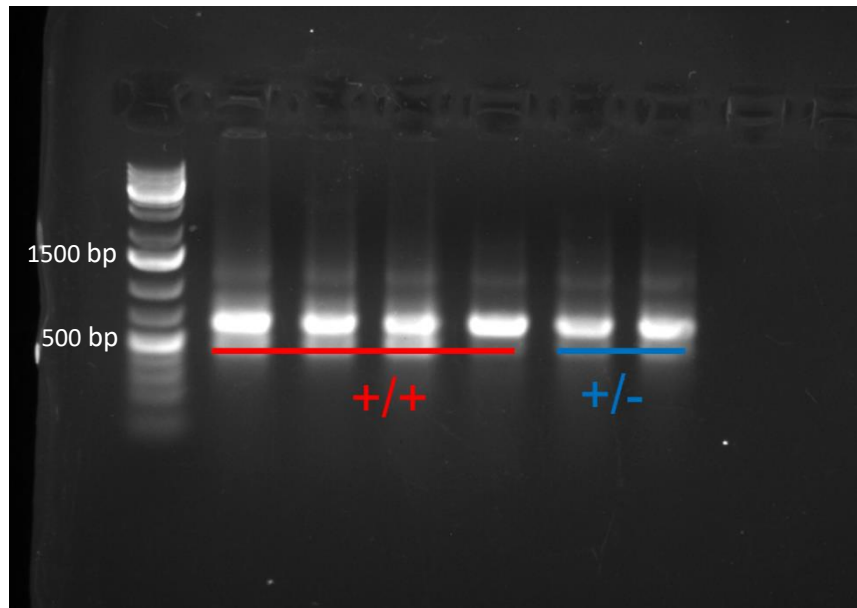


Figure 5: PCR optimisation attempts. On the upper picture we can see an optimisation attempt for the mutant allele. The lower picture shows the results for an attempted multiplex PCR. The genotype of those individuals where Sanger sequencing was performed later is shown in red and blue, respectively.

## Phenotyping

As mentioned above, zebrafish larvae born from *recql4*<sup>+/*elu16*</sup> incrosses displayed a distinctive phenotype. They had smaller eyes, kidney cysts and cartilage deformities (Figure 6A) and impaired movement capabilities also they died around 5 days post fertilization (dpf). I collected DNA samples from these animals and Sanger sequencing showed that these were *recql4*<sup>*elu16/elu16*</sup> homozygotes.

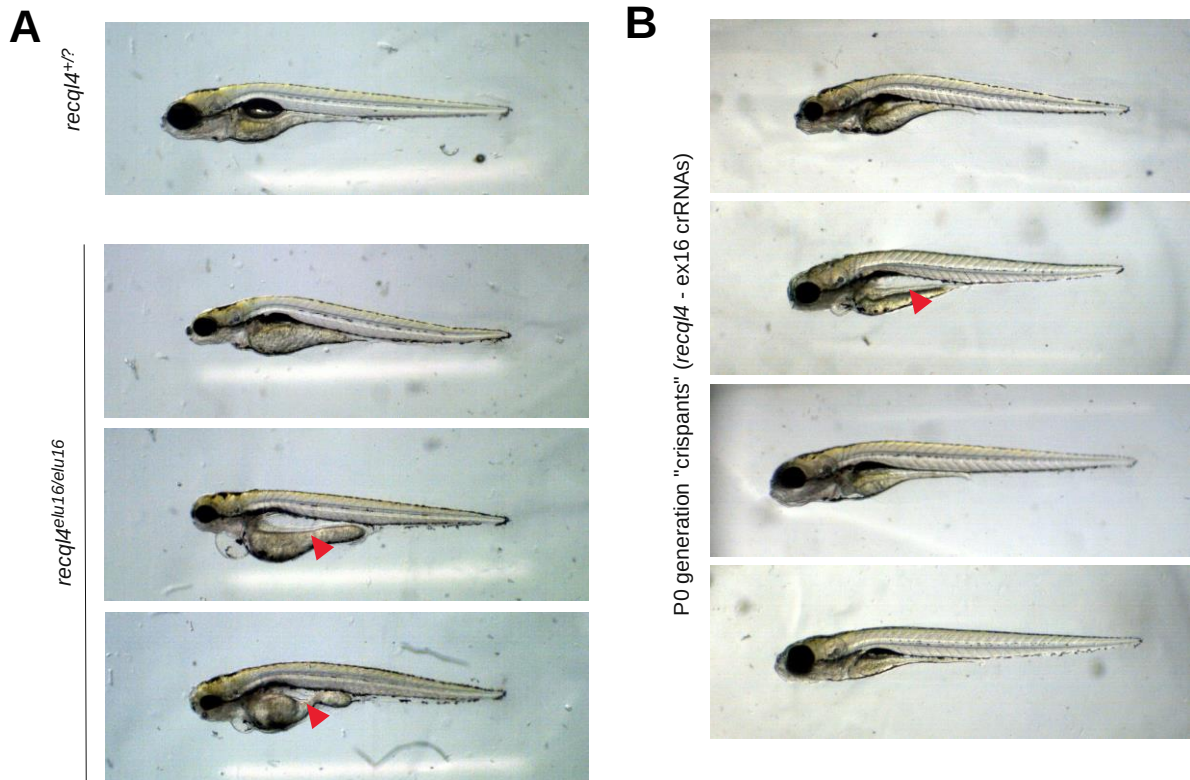


Figure 6: Phenotype of *recql4* mutant larvae (A) On the top we can see a healthy *recql4*<sup>+/?</sup> animal. On the lower three pictures we can see the homozygous *recql4*<sup>elu16/elu16</sup> mutant fish with their characteristic phenotype: smaller eyes and kidney cysts (marked with red arrowheads). (B) The *recql4*-exon16 “crispants” show similar phenotypes as observed in the *elu16* mutants.

Of note, *recql4*-exon16 “crispant” larvae (animals that were injected with Cas9 RNPs targeting exon 16 of *recql4*) also showed a similar phenotype at the comparable stages of development (Figure 6B).

### Touch-evoked escape response assay

During phenotyping it was clear that the movement capabilities of the homozygous mutants are impaired. To test this, I recorded their movement after giving them a tactile stimulus by touching them with the head of a pipette. We can see the results of this on Figure 7. On the pictures we can see how much the fish travels after given milliseconds. The healthy animals moved away quickly while the mutants stayed in one place for a long time.

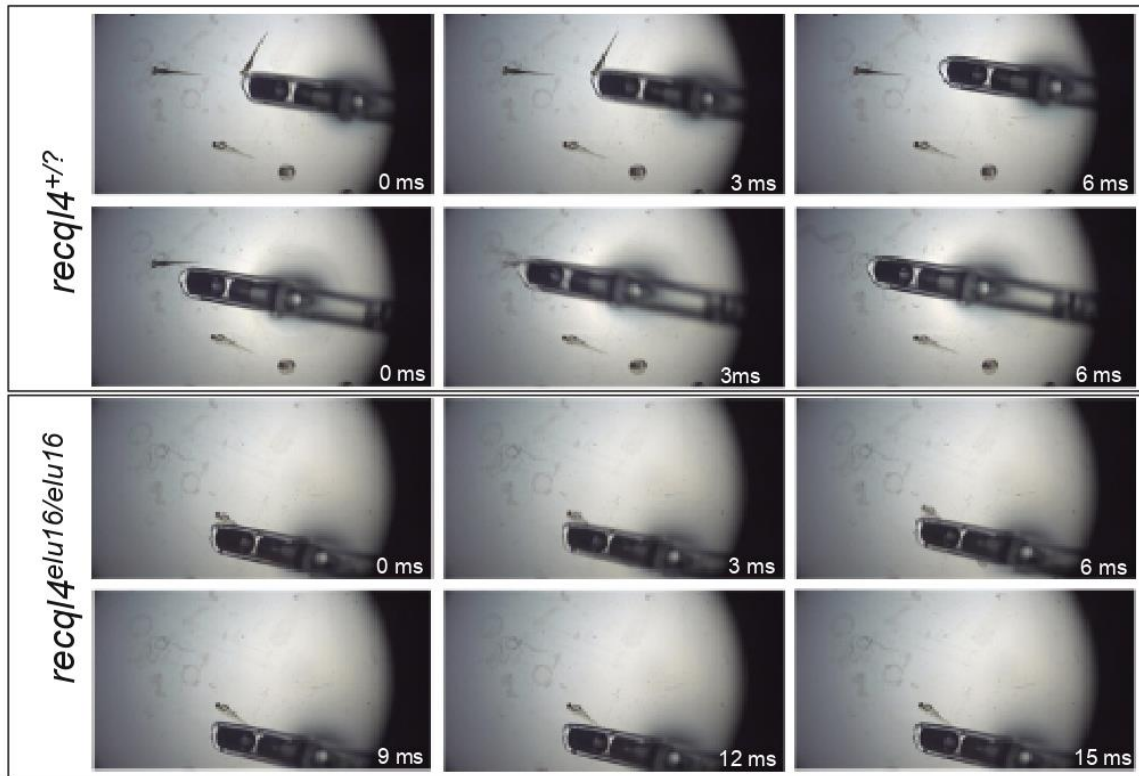


Figure 7: In upper panel we can see how fast 2 wild type larvae swam away from the stimuli. There are 3 milliseconds between the frames. In the bottom panel we can see a homozygous mutant fish with its characteristic small eye. It didn't move to the tactile stimuli only after a lot of stimulation.

## Cartilage staining

Since mutant fish had small heads, I decided to examine the head cartilage of these mutant fish with Alcian Blue staining, according to the protocols by MB Walker and CB Kimmel (Walker & Kimmel, 2007). This was also important because all diseases related to the mutations of *RECQL4* come with congenital developmental defects (Luong & Bernstein, 2021).

I used 5 dpf larvae ( $n_{\text{Sib}} = 40$ ,  $n_{\text{Mut}} = 12$ ) in these experiments and following the staining measured three characteristics of the jaw cartilage according to the protocols by Aron C. Petrey. (Petrey et al., 2012). Firstly, I checked if the end of the Meckel's cartilage meets with the end of the Palatum. Secondly, I measured the ratio between the distances of the Palatoquadratus and the distances between the Ceratohyalums and the Meckel's cartilages. Finally, I measured the angle of the Ceratohyalums. I performed a statistical analysis on the proportions of “trues” and “falses” from the first experiment using the *prop.test* command in R. As a result, I got a  $p$ -value of  $1,395 \cdot 10^{-10}$ . On the other hand my results shows in the case of the measured distances, that the homozygous mutants and their healthy siblings differed significantly (Figure 8).

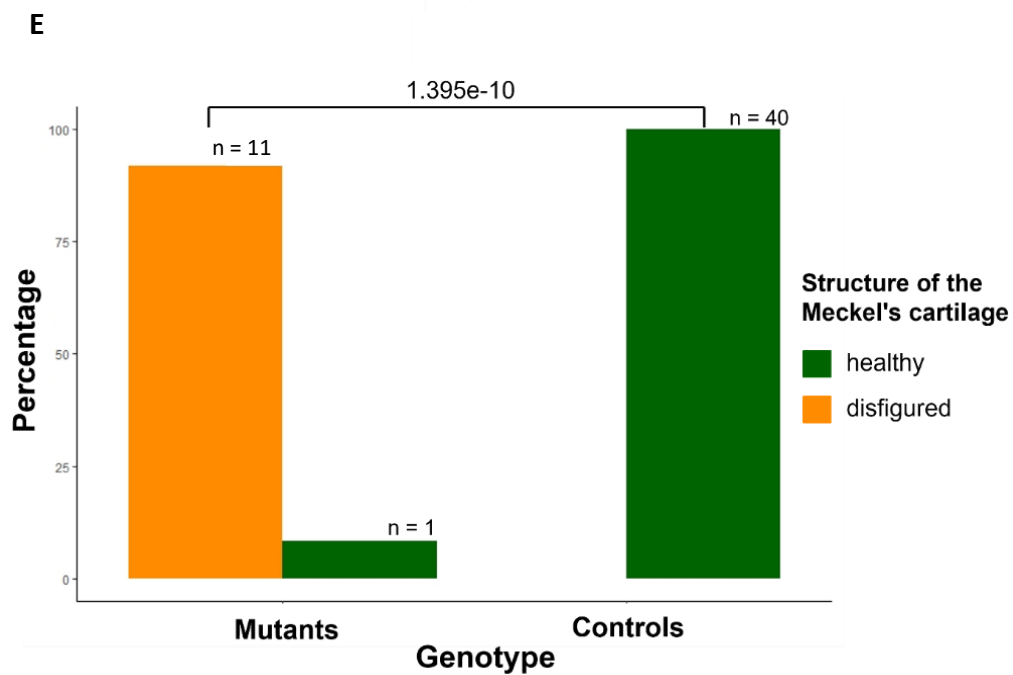
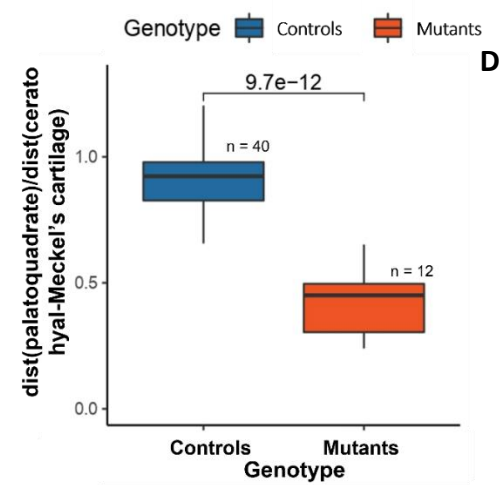
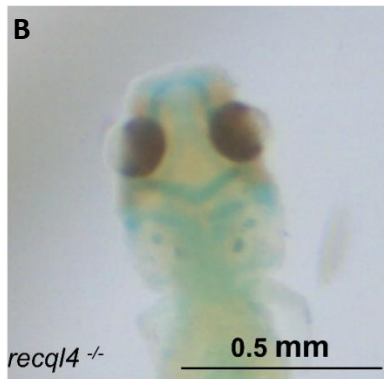
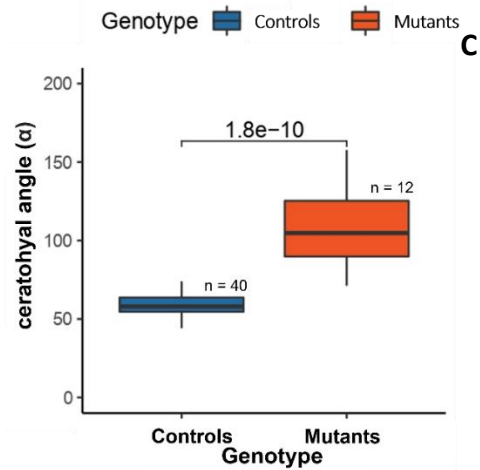
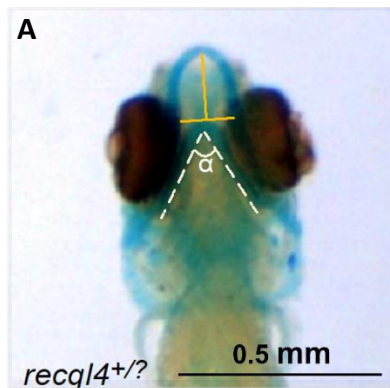


Figure 8: On picture A and B we can see a healthy and a homozygous mutant fish, with the measured distances represented on picture A. On picture C and D we can see the differences between the measured distances. On picture E we can see how many fishes Meckel's cartilage meets with the end of the Palatum in the case of the healthy (n=40) and mutant animals (n=12).

## Online databases searches

In order to gather *in silico* information about the expression of *recql4* in zebrafish I checked numerous online databases which contain single-cell RNA sequencing (scRNA-Seq) timecourse datasets.

Examining the data found in the Zebrafish Cell Landscape database (<https://bis.zju.edu.cn/ZCL/>) I found that primordial germ cells (PGCS), mesenchymal cells, neural progenitor cells, immune progenitor cells, neural progenitor cells, spermatocytes and neural cells are the most likely to express *recql4* (Figure 9). Using the ZebraHub (<https://zebrahub.ds.czbiohub.org>) datasets I was also able to tell that there is a big difference in *recql4* expression between the different developmental stages. Until the 30 somites stage the expression level is low, but a larger amount of cells express *recql4* compared to later stages. After 2 days post fertilization the expression levels are higher, but the number of expressing cells are lower. On the B and C picture of Figure 6 we can see the expression rate at 5 somite stage and 10 dpf. (Of note, while these are relatively significant differences, the absolute expression of *recql4*, compared to other, developmentally relevant genes, is still low.)

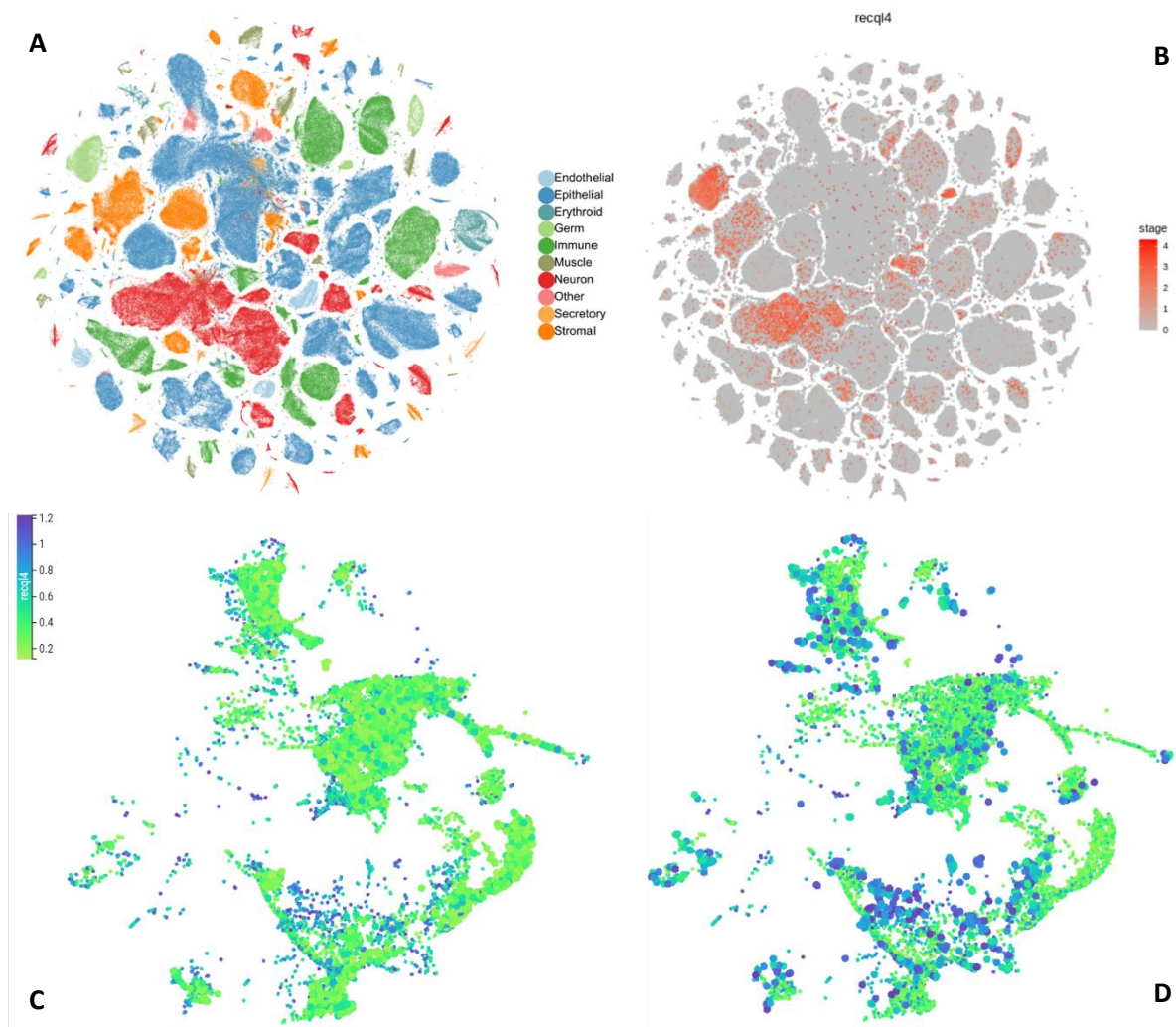


Figure 9: In silico analysis of *recql4* expression in online scRNA-Seq databases. (A) Cell clusters of the Zebrafish Cell Landscape database, coloured according to their type. (B) Cell clusters expressing *recql4*. (C, D) Zebrahub expression data on the expression of *recql4* at 5 somite stage (C) and 10 dpf (D).

## Whole mount *in situ* hybridisation experiments

I performed *in situ* hybridisation experiments to check if there's a unique expression pattern of *recql4*. I used wild type fish from 6 different stages which I have mentioned earlier. I was never able to see a distinctive pattern, even after repeating the experiments several times. These results (Figure 10) didn't come as a surprise for us because by looking at data from the online databases I was able to tell, that relatively few cells express the mRNA of *recql4* in higher levels, so its detection using *in situ* hybridisation is problematic.

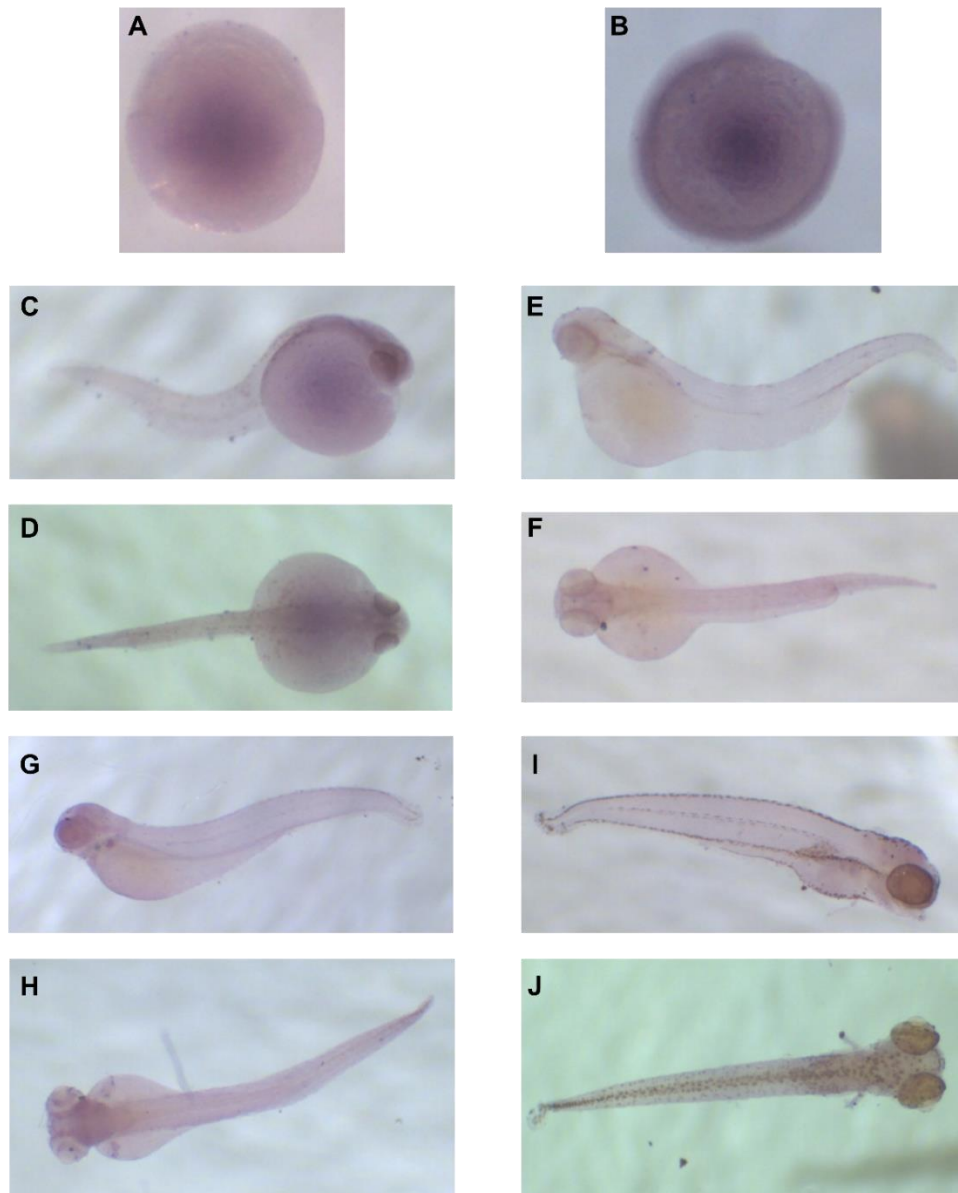


Figure 10: The results of the in situ hybridisation experiments. 6 developmental stages can be seen. 50% epiboly (A), 15 somite stage (B), 1 day after fertilisation (C, D), 2 days after fertilisation (E,F), 3 days after fertilisation (G,H), 5 days after fertilisation (I,J). In the 5 days old animal, the darker spots are pigment cells, not the detection of the mRNA

## Lifespan analysis

In the case of Bloom syndrome one of the most striking consequences of loss of BLM function is reduced lifespan in humans. Our group was able to document a similar phenotype in zebrafish *blm* mutants as well (Annus et al., 2022). Although the diseases that are linked to the mutation of *RECQL4* do not come with a shortened lifespan, I was still curious if we can observe and difference between the life expectancy of the homozygous mutants, the heterozygous and the wild type fish. As mentioned earlier, homozygous mutant fish die around 5 dpf, so I continued to monitor their heterozygous and wild type fish siblings to check for possible

haploinsufficiency phenotypes. Interestingly we did start to observe a higher incidence of death amongst heterozygotes (Figure 11). Currently our results show that there is a significant difference between the lifespans, but there are still remaining wild type fish, so we will have to wait to able to say a definite conclusion.

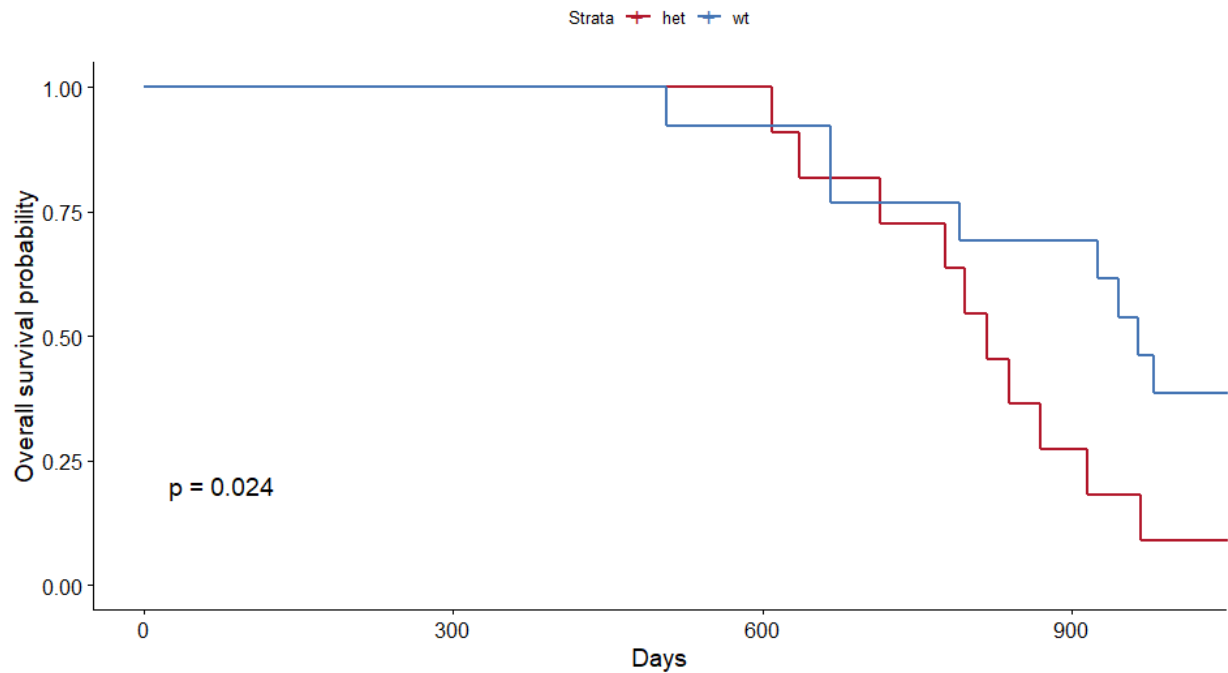


Figure 11: The overall survival probability of the heterozygous (represented with a red line) and the wild type fish (represented with blue line).

## Discussion

In conclusion we can say that we have successfully established a novel *recql4* mutant line and we made significant progress in creating a second, independent mutant allele. In the case of the mutant line that was already established the mutation was located on the fifth exon, and for the second line we have targeted the sixteenth exon using CRISPR/Cas9. The mutant animals show characteristic similarities with the human patients; of these perhaps the most striking are the skeletal deformities, which I characterized using cartilage staining. The movement of these mutants is also impaired – we hypothesize this is perhaps due to faulty mitochondria.

I performed *in situ* hybridisation experiments, to characterize the expression of the gene during early development, but I could not observe a distinctive pattern in the animals during the first five days of development. Analysing data in online single-cell RNASeq databases, I have found that the most likely reason for this is the general low expression rate of *recql4*.

Finally, I observed a potential impairment in the lifespan of the heterozygous animals. While homozygous *recql4* animals die during early larval stages, heterozygotes do not show early phenotypes. Therefore, the impairment in the lifespan could be indicating a case of haploinsufficiency.

In the future I intend to repeat some of these experiments to acquire bigger sample sizes. Also, we have planned several experiments that will test the integrity of the mitochondria in mutant animals, to test our hypothesis about the possible mitochondrial dysfunction.

## Acknowledgement

Foremost I would like to thank my supervisor Dr. Máté Varga who gave me tremendous amount of help during the planning of the experiments and the writing of this work. Also, I would like to thank Tamás Annus who helped me with his tips and advices since I joined the Fish genetics laboratory. Finally, I would like to thank every member of the lab and my friends who helped in any way to complete this research.

# Tételes szerzői hozzájárulás

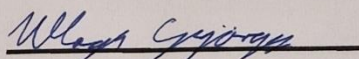
## A 36. OTDK BIOLÓGIA SZEKCIÓ FELHÍVÁSÁNAK 2. SZÁMÚ MELLÉKLETE

### TÉTELES SZERZŐI HOZZÁJÁRULÁS

Alulírott **Ullaga György** nyilatkozom, hogy a 36. OTDK-ra benyújtott **The modelling of RECQL4-related human diseases on zebrafish** című pályamunkámban leírtak esetében az alábbi munkafolyamatok elvégzésében vettem részt:

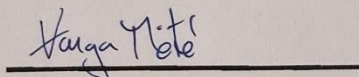
| Részfeladat <sup>1*</sup>                                                                  | A részvétel mértéke<br>(öt fokozatú skálán <sup>#</sup> jellemezve) |
|--------------------------------------------------------------------------------------------|---------------------------------------------------------------------|
| <i>A mutáns állatok létrehozása és új generációk fenntartása</i>                           | 4                                                                   |
| <i>Állatok genotipizálása Sanger szekvenálás segítségével</i>                              | 5                                                                   |
| <i>A mutáns állatok fenotipizálása, mely magába foglalja a porcfestést</i>                 | 5                                                                   |
| <i>In silico adatgyűjtés</i>                                                               | 5                                                                   |
| <i>Próbaszintézis az in situ kísérletekhez</i>                                             | 5                                                                   |
| <i>In situ hibridizációs kísérletek</i>                                                    | 5                                                                   |
| <i>A felnőtt állatok folyamatos ellenőrzése az élethosszos adatok begyűjtése érdekében</i> | 5                                                                   |
| <i>Az adatok statisztikai feldolgozása</i>                                                 | 3                                                                   |

Kelt: 2023.01.22



Ullaga György

A nyilatkozatban foglaltak helyességét megerősítem:



Dr. Varga Máté  
(ELTE TTK Genetika tanszék)

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