

Differential Gene Expression in Zebrafish *recql4* Mutants: A Transcriptomic Analysis

TDK Study

Master of Biology

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List of Abbreviations

AO	-	Acridine Orange staining
BGS	-	Baller-Gerold Syndrome
Cas9	-	CRISPR associated protein 9
CRISPR	-	clustered regularly interspaced short palindromic repeats
ddH ₂ O	-	double-distilled water
DEGs	-	differentially expressed genes
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxynucleotide triphosphate
dpf	-	days post-fertilization
DSB	-	Double-strand break
dsDNA	-	Double-stranded DNA
gDNA	-	Genomic DNA
gtPCR	-	Genotyping PCR
hpf	-	hours post-fertilization
HR	-	Homologous recombination
HRDC	-	Helicase- and RNaseD-like C-terminal domain
NHEJ	-	Non-homologous end joining
PCR	-	Polymerase chain reaction
PTU	-	1-phenyl-2-thiourea
RNA	-	Ribonucleic acid
RT	-	Room temperature
RTS	-	Rothmund-Thomson Syndrome
ssDNA	-	Single-stranded DNA

Summary

RecQ family helicases (WRN, BLM, RECQL1, RECQL4, and RECQL5) are crucial proteins responsible for maintaining the genomic stability of eukaryotic cells. Mutations in three of the genes encoding these helicases (*WRN*, *BLM*, and *RECQL4*) are associated with severe syndromes, including Werner syndrome, Bloom syndrome, and Rothmund-Thomson syndrome (RTS), respectively. The *RECQL4* gene is particularly significant, as its mutations can lead to three clinical phenotypes, RTS, RAPADILINO, and Baller-Gerold syndrome (BGS), each characterized by skeletal abnormalities, juvenile cataracts, and a predisposition to osteosarcoma.

Using zebrafish (*Danio rerio*) to model *RECQL4*-related diseases we generated homozygous *recql4* mutants that displayed severe and lethal phenotypes, including skeletal deformities, heart edema, and poor eye development, leading to death by 5-7 days post-fertilization (dpf). As the earliest phenotype observable in mutants is the presence of extended apoptosis in the brain, at 2 dpf, we decided to assess the molecular effects of *recql4* mutations using this timepoint. We extracted and sequenced the transcriptome from mutant and wild type embryos to identify differentially expressed genes (DEGs) and disrupted pathways. Our results showed upregulation of genes associated with apoptosis and cell-cycle regulation, and downregulation of genes related to DNA and chromatin integrity.

1. Introduction

DNA, the carrier of genetic information, requires precise maintenance to ensure cellular health and function. Disruptions in DNA repair mechanisms can lead to genomic instability and various diseases such as cancer and aging (Martins et al., 2023). RecQ helicases are crucial enzymes involved in maintaining genomic integrity by participating in DNA repair, replication, and recombination. They act in the 3'→5' direction, binding single-stranded DNA (ssDNA) which in turn activates their ability to unwind double-stranded DNA (dsDNA). In humans, the five RecQ helicases (WRN, BLM, RECQL1, RECQL4, and RECQL5) share a structurally conserved core helicase domain that is retained across a range of species, from prokaryotes to higher eukaryotes (Fig. 1). RecQ helicases participate in multiple DNA double-strand break (DSB) repair pathways, including non-homologous end joining (NHEJ) and homologous recombination (HR). Additionally, they play a critical role in telomere maintenance and repair (Fig. 2) (Croteau et al., 2014; Lu & Davis, 2021).

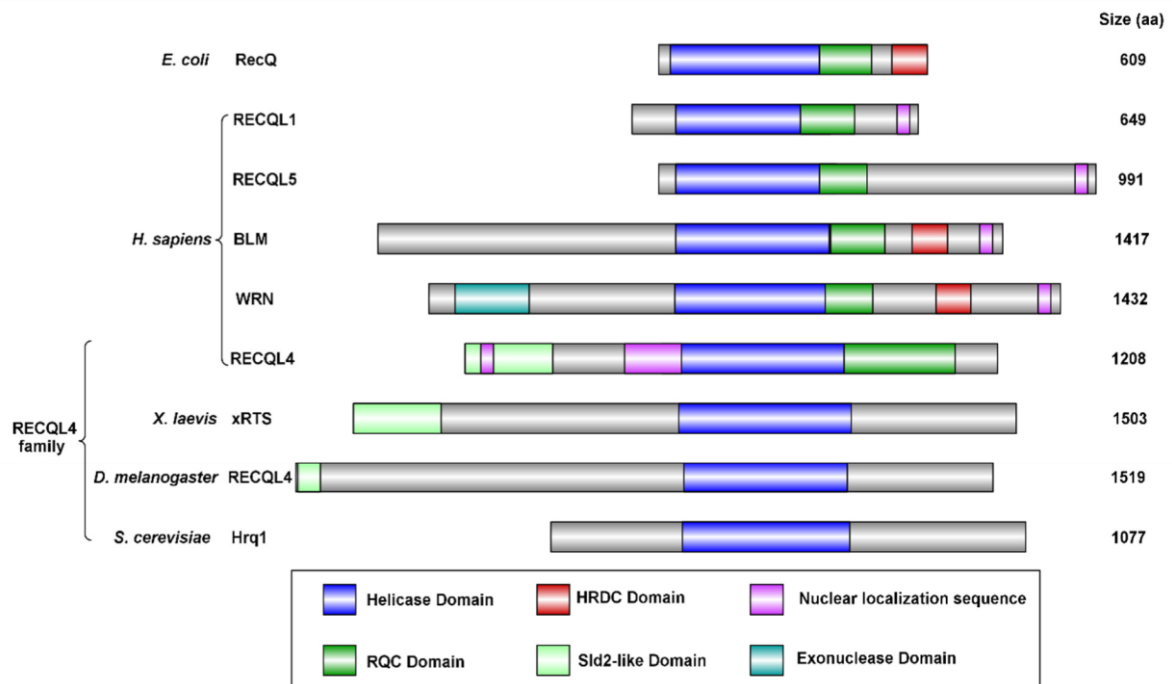


Figure 1: Domain structure and size of the human (*H. sapiens*) RecQ helicases and their conservation across species from prokaryotes (*E.coli*) to other eukaryotes (*Xenopus*, *Drosophila*, and *Saccharomyces*) (Luong & Bernstein, 2021).

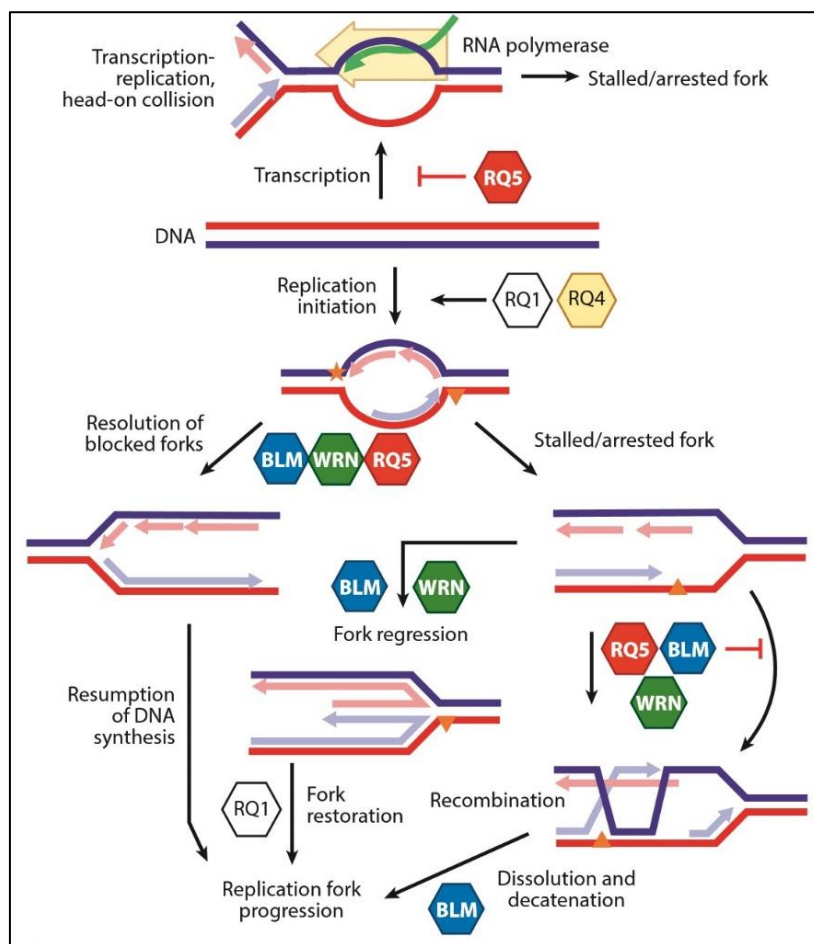


Figure 2: Roles of RecQ helicases in DNA replication and repair processes. The illustration highlights various functions of RecQ helicases, such as handling transcription-replication head-on collisions, initiation of replication, replication fork progression, fork restoration, and fork regression. (Croteau et al., 2014).

Among the human genes encode for these helicases, *RECQL4* plays a particularly critical role, as disruption of this gene can result in three different clinical phenotypes: Rothmund-Thomson Syndrome, type II (RTS), RAPADILINO, and Baller-Gerold Syndrome (BGS) (Luong et al., 2022), each characterized by skeletal deformities, facial skin abnormalities (Poikiloderma) (Fig. 3), juvenile cataracts, and predisposition to malignancy, especially osteosarcoma (Larizza et al., 2010; Zhang et al., 2021). Of note, it is hard to find clear genotype-phenotype association for specific mutations, but is noteworthy that patients with two truncating mutations (thus likely no *RECQL4* function at all) are usually diagnosed with RTS or BGS (Siitonen et al., 2009).

The molecular basis behind the varied phenotypes of *RECQL4* mutations remains incompletely understood. However, these syndromes share overlapping clinical features and are linked to different mutations in the different domains of the protein (Fig. 4) (Schmit & Bielinsky, 2021).



Figure 3: Clinical features of Rothmund-Thomson Syndrome (RTS). (A–D) Various presentations of poikiloderma. (E) Poikiloderma with knee valgus deformity. (F) Thumb aplasia. (G) X-ray showing cystic-like lesion on the humerus without cortical disruption. (Larizza et al., 2010)

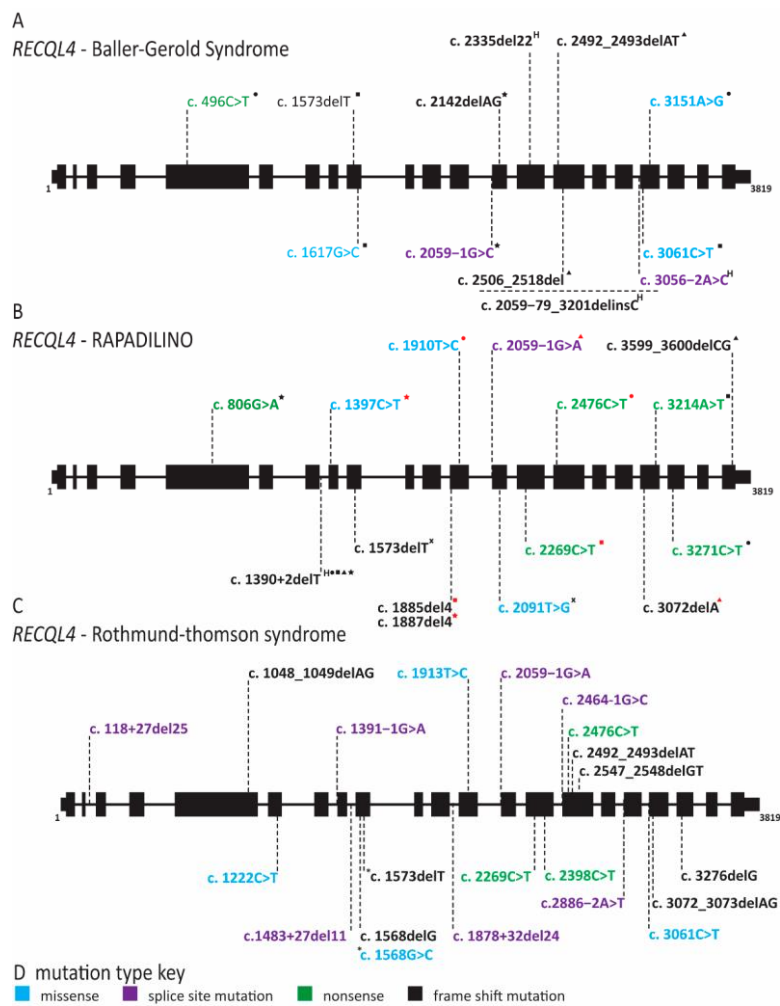


Figure 4: Types and Locations of Mutations in Clinical Syndromes Associated with RECQL4 Mutations.

Over the past decades zebrafish (*Danio rerio*) has become a powerful model organism in developmental genetics and disease modeling. Its genome is highly similar to the human genome, and it can also develop various human-related diseases, such as diabetes, cancers, cardiovascular and neurological disorders, as well as infectious diseases and many other complex conditions. (Longkumer et al., 2024).

Zebrafish are particularly valuable for investigating genes involved in DNA repair, as their transparent embryos and rapid development allow for easy observation of the phenotypic effects associated with genomic instability. For instance, mutants with impaired DNA repair pathways, such as those with mutations in the tumor suppressor gene *tp53*, which is a master regulator of the cell cycle, show increased susceptibility to various cancers. These mutants can develop a spectrum of tumors, including malignant peripheral nerve sheath tumors (MPNSTs), angiosarcomas, germ cell tumors, and aggressive natural killer cell-like leukaemia (Chen et al., 2023). Another example highlights the advantages of zebrafish's large sample size and rapid development is a recent study showing that mutations in one of the helicase genes, *blm*, which is also associated with DNA double-strand break (DSB) repair pathways, can lead to reproductive issues in this species as well, such as male-biased sex ratios due to increased apoptosis in developing germ cells (oocytes) in females (Annus et al., 2022).

Our group has already successfully generated a zebrafish *recql4* mutant allele (*elu16*) using targeted genome editing based on the CRISPR/Cas9 method (Kroll et al., 2021). Homozygous *recql4*^{*elu16/elu16*} mutants contain an allele with a specific mutation in exon 5 of the coding region, characterized by an 8 bp deletion (c.807_814delTGGCTCCC), which affects the helicase ATP-binding domain (Fig. 5), verified by Sanger sequencing. This deletion causes a frameshift, resulting in an early stop codon.

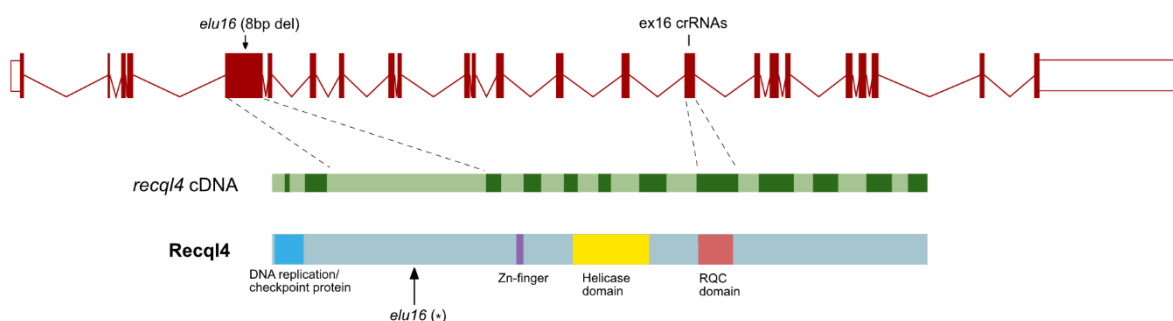


Figure 5: Schematic representation of the *recql4* gene (top) and *Recql4* protein (bottom) showing the positions of the mutation in exon 5 (*elu16*) and the targeted region in exon 16.

In the *recql4* mutants, severe phenotypes begin to manifest between 4–5 dpf, including skeletal deformities, particularly in the spine and jaws, reduced eye size, heart edema, kidney cysts, and impaired response to physical stimuli.

Building on our previous work, I initiated the generation of a second mutant allele targeting exon 16 of the *recql4* gene also using CRISPR/Cas9 construct, which affect the C-terminal domain of the functional protein (**Fig. 5**). This new variant would enable us to explore the effects of the mutation within different domains of Recql4, providing more understanding of the protein's functional roles in maintaining genome integrity and stability.

2. Aims

My work mainly aims to elucidate the molecular effects of *recql4* mutations in zebrafish (*Danio rerio*) as a model for RECQL4-associated diseases in human, using transcriptomic approach to advance our understanding of the gene's role in genomic stability. The specific aims can be summarized as follows:

- **Characterization of molecular alterations in *recql4* mutants:** my goal is to identify differentially expressed genes in zebrafish *recql4* mutants that are either up- or downregulated compared to their wildtype siblings at 2 dpf stage, which is the earliest observable stage of mutant phenotypes, using transcriptomic analysis. This would identify the main factors contributing to the onset of the syndrome.
- **Investigation of the disrupted molecular pathways in mutants:** I want to explore the involvement of Recql4 helicase in vital pathways related to DNA repair, chromatin organization, apoptosis, and cell cycle regulation, which can give insights to understand the helicase-related syndromes.
- **Generation and validation of a novel *recql4* mutant allele:** to support our findings and confirm the specificity of the phenotypes resulted from non-functional Recql4 protein due to null mutation in exon 5, I started to generate a second zebrafish *recql4* mutant allele targeting exon 16 using CRISPR/Cas9 technology, enabling the dissection of the gene's functional domains and their impact on the observed phenotypes.

3. Materials and Methods

3.1. Fish husbandry

All of our fish lines are maintained in the fish facility of ELTE Eötvös Loránd University following standard protocols (Aleström et al., 2020; Westerfield, 2000). We used wildtype and *recql4* heterozygous mutant fish in this study. Larvae were raised in E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂ and 0.33 mM MgSO₄ in distilled water) with methylene blue to prevent fungal infection until 5 dpf at 28.5°C. 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, PEI/001/1458-10/2015, PE/EA/2483-6/2016). Further recommendations were used in breeding (Longkumer et al., 2024) to increase fertilization efficiency.

3.2. Genotyping

For identification of the genotype of the mutants and their siblings, two pairs of primers were used (Table 1). The first pair is specific for the wild type allele resulting in 642 bp amplicon, while the second pair of primers is specific for the mutant allele (*elu16*) resulting in 193 bp amplicon.

Table 1: Primer Sequences and Expected Amplicon Sizes for Genotype Identification of *recql4* Mutants and Siblings.

Primer name	Sequence (5'-3')	Type	Specificity	Amplicon size (bp)
recql4-WT-F2	GCTGTTGGAAGCAAATCTGGCTC	Forward	WT allele	642
recql4-ex5-R2	GACCCTGACCGTGTCTTCAT	Reverse		
recql4-ex5-F2	TCCAGTTCCTGAGGCAGTCC	Forward	Mutant allele (<i>elu16</i>)	193
recql4-8bpdel-R3	TGCTGTTGGAAGCAAATCCG	Reverse		

For genotyping, genomic DNA (gDNA) was isolated from individual 2 dpf embryos using hotshot method (Meeker et al., 2007). Single larvae were placed in microcentrifuge tubes containing 50 µl of 50 mM NaOH and incubated in thermocycler at 95°C for 15 minutes. Then the tubes were cooled down to 4°C and 5 µl of 1 M Tris-HCl pH 8.0 was added to each tube to neutralize the basic solution. Finally, the tubes were centrifuged for one minute to pellet the debris (if any) and the gDNA is ready for use in PCR reaction.

Random fish were selected to test the accuracy of the primers in determining the genotype. Each fish's DNA served as a template for both primer pairs in a 12.5 μ l PCR reaction, with the following components: 9.35 μ l ddH₂O, 1.25 μ l DreamTaq Green buffer, 0.25 μ l dNTPs, 0.25 μ l primer pair mixture (10 mM), 0.15 μ l DreamTaq DNA polymerase (Thermo Scientific, EP0703), and 1.25 μ l DNA template. The PCR program used for amplification is outlined in (Table 2).

Table 2: Primer Sequences and Expected Amplicon Sizes for Genotype Identification of *recql4* Mutants and Siblings.

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	3:00	1
Denaturation	95	0:30	} 30
Annealing	67	0:30	
Extension	72	0:40	
Final extension	72	3:00	1
Hold (cooling)	10	∞ (infinite)	

3.3. Apoptosis assay

We used Acridine Orange stain according to the protocol (Tucker & Lardelli, 2007). Embryos were maintained in E3 medium containing 0.2 mM 1-phenyl-2-thiourea (PTU; Sigma-Aldrich, P7629-10G) from 10 hours post-fertilization (hpf) until staining. For dechoriation, embryos were placed in a 5 mg/ml Pronase solution (Sigma-Aldrich, 10165921001) and incubated in petri dishes, with periodic aspiration using a Pasteur pipette to aid chorion detachment. After complete dechoriation (approximately 10 minutes), embryos were rinsed three times with fresh E3 medium. For staining, embryos were incubated in a 5 μ g/ml Acridine Orange (AO; Sigma-Aldrich, A6014-25G) solution for 30 minutes, with the container wrapped in aluminum foil to protect the photosensitive AO from light. Following staining, embryos were rinsed three times with E3 medium and anesthetized in E3 medium containing 168 mg/l Tricaine (Sigma-Aldrich, E10521). The embryos were then imaged.

3.4. Total RNA isolation

Total RNA was isolated from 2 dpf embryos of a *recql4*^{+/*elu16*} heterozygous mutant incross, which were maintained in E3 medium with 0.2 mM PTU from 10 hpf to inhibit pigmentation, facilitating mutant identification by increased apoptosis in the brain compared to their wild-type siblings.

Three biological replicate sample pairs (one mutant and one control) were isolated, each from different breeding events using the same male and a different single female at a time. Total RNA was isolated from the mutants and their siblings separately and in parallel from each breeding using TRI Reagent (Zymo Research, R2050-1-50) following the manufacturer's protocol.

I placed 12 embryos in Eppendorf tubes, added 500 µl of TRI Reagent, and homogenized. After incubating at room temperature for 5 minutes, I added 100 µl of chloroform, shook gently, and incubated for 3 minutes. I then centrifuged the sample at 4°C and 11,000 g for 15 minutes, transferred the supernatant to a new tube, and added 250 µl of isopropanol. After a 10-minute incubation at room temperature, I centrifuged at 4°C and 9,500 g for 30 minutes and discarded the supernatant.

To wash the RNA pellet, I added 500 µl of cold 75% ethanol, centrifuged at 4°C and 6,000 g for 5 minutes, and discarded the supernatant. The pellet was air-dried for 10 minutes and dissolved in 25 µl of nuclease-free water at 55°C. I assessed RNA quality by running 2 µl on a 1% agarose gel.

For purification, I added 77 µl of nuclease-free water, 10 µl of 3M sodium acetate (pH 5.2), and 250 µl of ethanol, then incubated overnight at -80°C. After centrifuging and washing the pellet with 500 µl of cold 75% ethanol, I air-dried it and dissolved in 30 µl of nuclease-free water. I stored the purified RNA at -80°C.

3.5. Transcriptome analysis

Following RNA extraction, we sent six zebrafish total RNA samples for library preparation and sequencing to Biomarker Technologies (BMK) GmbH, Germany (www.bmkgene.com). The sequencing data were uploaded to the public Galaxy web server (<http://usegalaxy.eu>) for the analysis and extraction of the differentially expressed genes. Further analysis and visualization were performed using R Studio (R Core Team, 2023).

The RNA-Seq data analysis workflow on the Galaxy web server was performed following a tutorial based on (Batut et al., 2018; Hiltemann et al., 2023; Batut et al., 2024), after uploading the raw sequencing data in FASTQ format and the zebrafish (*Danio rerio*) reference genome (assembly GRCz11) from the NCBI database, as shown in the following table (Table 3):

Table 3: RNA-Seq data analysis workflow showing the tools used, input/output files, and the function of each tool.

Tool	Input file(s)	Output file(s)	Function
Quality Control (FastQC)	Raw FASTQ files	report	Assess the quality of raw sequenced reads.
Trimming (Cutadapt)	Raw FASTQ files	Trimmed FASTQ files	Remove low-quality bases and adapters to improve mapping accuracy.
Mapping (RNA STAR)	Trimmed FASTQ files, reference genome	BAM file	Align reads to the reference genome, determining each sequence origin.
Counting (FeatureCounts)	BAM file, gene annotation (GTF file)	Count matrix	Count the number of reads per gene for expression level estimation.
DE Analysis (DESeq2)	Count matrix	Tables of DE genes, plots	Identify genes with significant changes in expression between sample groups.

The output table file of *DESeq2* tool containing the differentially expressed genes was later imported to R Studio for visualization as shown in Results section.

3.6. Generating a new mutant allele

The mutagenesis was done using targeted genome editing based on CRISPR/Cas9 method targeting exon 16 in *recql4* gene. To generate the mutant allele, we used the two-part Alt-R™ crRNA and tracrRNA system provided by Integrated DNA technologies™ (IDT™), which has demonstrated high efficiency, RNase resistance, and no toxicity in injected embryos (Miles et al., 2024). The microinjection of the ribonucleoprotein (RNP) construct was carried out on embryos of wild-type (AB) background. I targeted two distinct sites within exon 16 using two different crRNAs. The crRNA sequences (written in the 5'–3' direction, with the PAM sequences in bold) were as follows: crRNA-1 (5'-GATAGCGGAGGCGTATCACG **CGG**-3') and crRNA-2 (5'-TTTATCCAGACCCATCCCGA **AGG**-3'). These two crRNAs were mixed in a 1:1 ratio during the RNP preparation.

To prepare the ribonucleoprotein construct (RNP), first I annealed 1 µl of crRNA (200 µM) with 2 µl of tracrRNA (100 µM) in ddH₂O for a total volume of 3.5 µl. Then I incubated the mixture at 95°C for 5 minutes. This will create 57 µM crRNA:tracrRNA hybrid which was stored at -80°C. For RNP assembly, 1 µl of Cas9 protein (10 µM) was combined with 0.7 µl of the RNA hybrid with ddH₂O added to reach a final volume of 3 µl. Then I incubated the mixture at 37°C for 5 minutes. The RNP construct was prepared fresh just before each injection process or the night before and stored at -80°C.

The injected embryos were maintained in E3 medium at 28.5°C and monitored from time to time to inspect any abnormal phenotypes. The targeted gene editing efficiency was assessed by PCR amplification of the flanking region of the targeted regions followed by Sanger sequencing.

4. Results

4.1 Genotyping and phenotypes characterization

We monitored the *recql4* mutants and their siblings to identify the defective phenotypes. We found that the earliest observable phenotype at 2 dpf was a significant increase in apoptosis, notably in the anterior head and eye regions (**Fig. 6B**), as confirmed by Acridine Orange (AO) staining (see 3.2). These tissues typically exhibit high cell proliferation rates, suggesting that the elevated apoptosis may result from disruptions in cell cycle regulation and DNA repair mechanisms. Later, the mutants began to show severe symptoms at 4–5 dpf (**Fig. 6D**) mainly affecting the skeletal and vascular tissues.

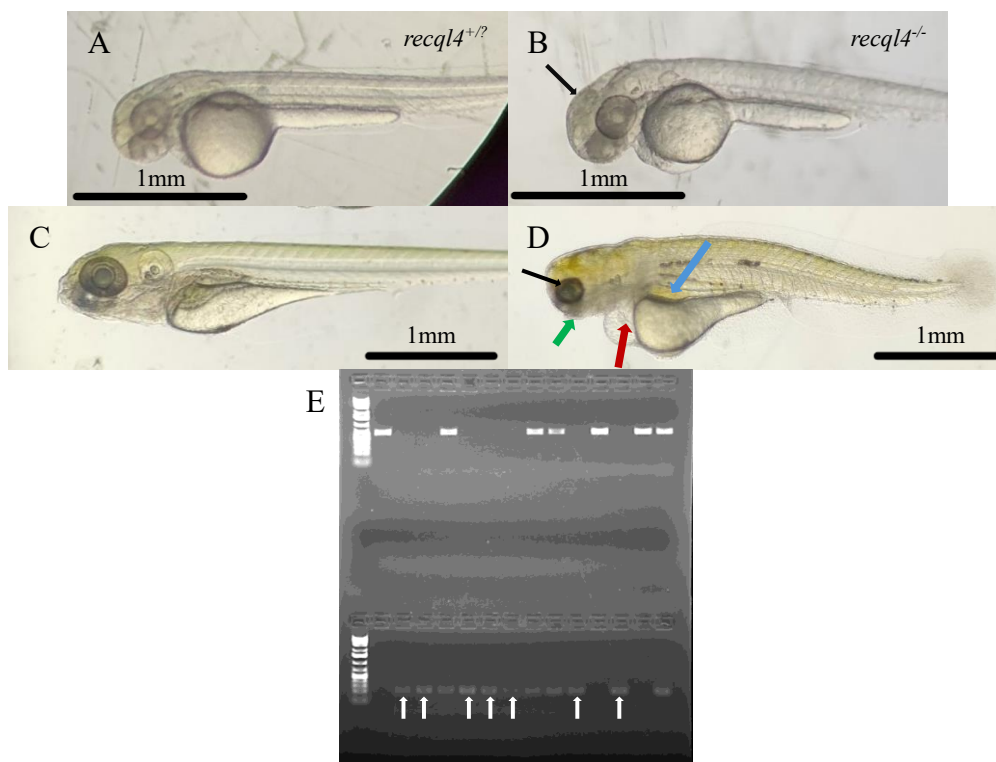


Figure 6 : **A-B)** Comparison of a *recql4* wild-type sibling (**A**) and a homozygous mutant (**B**) at 2 dpf, showing early phenotypic signs in the mutant. A significant increase in apoptosis is indicated by darkened regions (black arrow), particularly in the anterior head and eye areas of the mutant. **C)** A *recql4* WT phenotype sibling larva at 5 dpf. **D)** A *recql4* homozygous mutant larva at 5 dpf, exhibiting a severe phenotype with an abnormally curved body plan. Specific features include smaller eyes (black arrow), malformed jaws (green arrow), heart edema (red arrow), and a kidney cyst (blue arrow). **E)** Gel electrophoresis of genotyping PCR products from 4 dpf progeny of a *recql4* heterozygous mutant incross. The top row shows amplification with WT-specific primers, while the bottom row shows amplification with mutant allele (elu16)-specific primers. Each lane represents a single embryo. Bands indicating mutants are marked with white arrows. DNA ladder GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific, EP0703).

To further confirm our findings, we performed half-blind genotyping PCR on selected 4 dpf progeny from a *recql4* heterozygous mutant incross, including a 1:1 ratio of mutants to siblings based on phenotype. Primers specific to the WT allele and the mutant allele (*elu16*) were used separately (**Fig. 6E**).

4.2 Apoptosis assay using Acridine Orange (AO) staining

Upon staining with Acridine Orange, we found that there is a significant increase in apoptosis in the *recql4* mutants compared to their wild-type siblings (**Fig. 7**), particularly in the anterior head and eye regions of the mutants (indicated by arrows), suggesting disruptions in cell cycle regulation and DNA repair mechanisms.

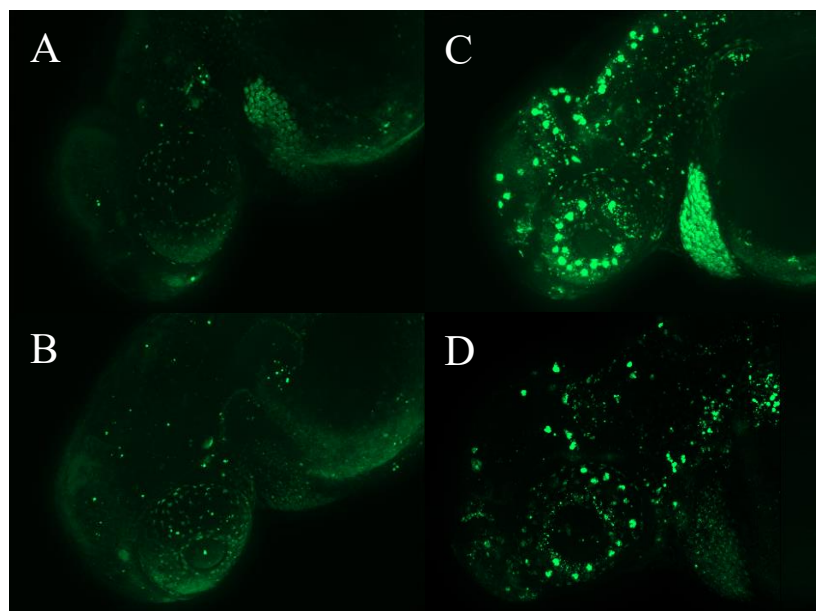


Figure 7 : Apoptosis in *recql4* heterozygous mutant incross progeny visualized by Acridine Orange staining. A is a wild-type sibling, B is a heterozygous, C and D are homozygous mutant embryos (confirmed by gtPCR after staining).

4.3. Total RNA isolation and transcriptome analysis

Total RNA was successfully isolated from 2 dpf zebrafish embryos from three separate breeding events using the same male and a different female at a time. RNA yield and quality were assessed, with a high purity of A260/A280 ratios ≥ 1.8 and A260/A230 ratios ≥ 2.0 , indicating minimal contamination. Quality checks through gel electrophoresis confirmed intact RNA, suitable for downstream transcriptomic analyses.

Differential gene expression analysis was conducted using the Galaxy platform for data processing and R Studio for visualization. Off note is the close position of the sample pairs in the PCA plot (**Fig. 8a**) suggesting that relatively few transcriptomic changes lead to the clear

phenotypic differences. The analysis of the three sample pairs also confirmed the downregulation of *recql4* in homozygous mutants, probably due to effects of the non-sense mediated decay (NMD) pathway. We found that 35 genes were significantly either up- or downregulated including members that participate in key cellular processes, particularly apoptosis, as shown in (Fig. 8b).

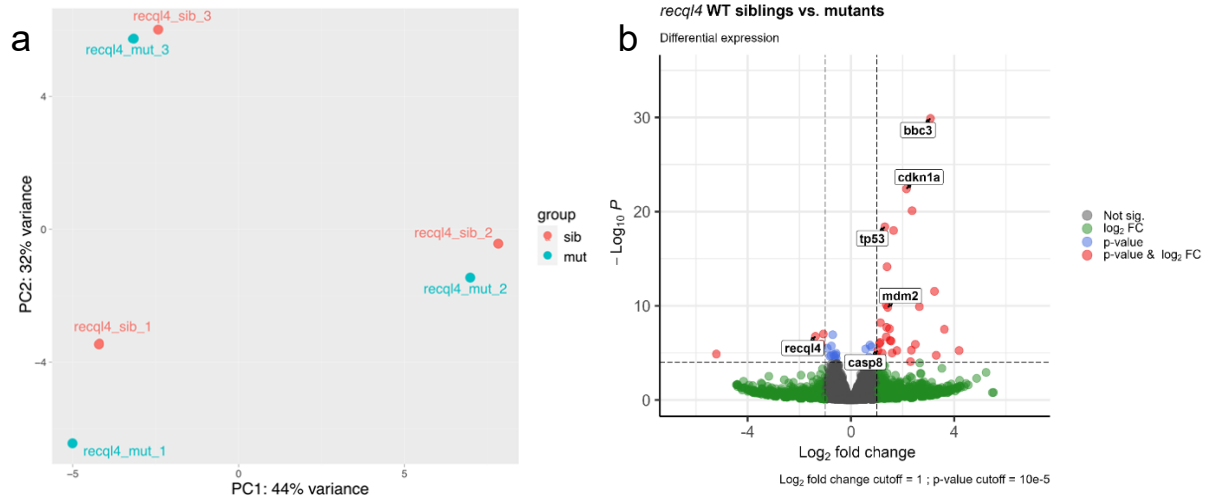


Figure 8 : **a)** Principal Component Analysis (PCA) plot (ggplot2 R package) shows the clustering of the 3 sample pairs based on differentially expressed genes. Mutants in each pair are consistently biased toward the same side compared to their WT siblings. **b)** Volcano plot showing the differentially expressed genes in *recql4* mutants compared to their siblings. Genes involved in apoptosis are labeled with white boxes, as it is *recql4* itself. Generated using EnhancedVolcano R package version 1.24.0 (Blighe et al., 2024).

To explore the disrupted cellular functions and pathways we applied functional enrichment analysis using the *clusterProfiler* R package (Wu et al., 2021). We used different sources of gene functional annotation to extract the maximum amount of information. As we can see in (Fig. 9A), several affected processes align with the observed phenotypes in *recql4* mutants including skeletal deformation and defected response to mechanical stimuli.

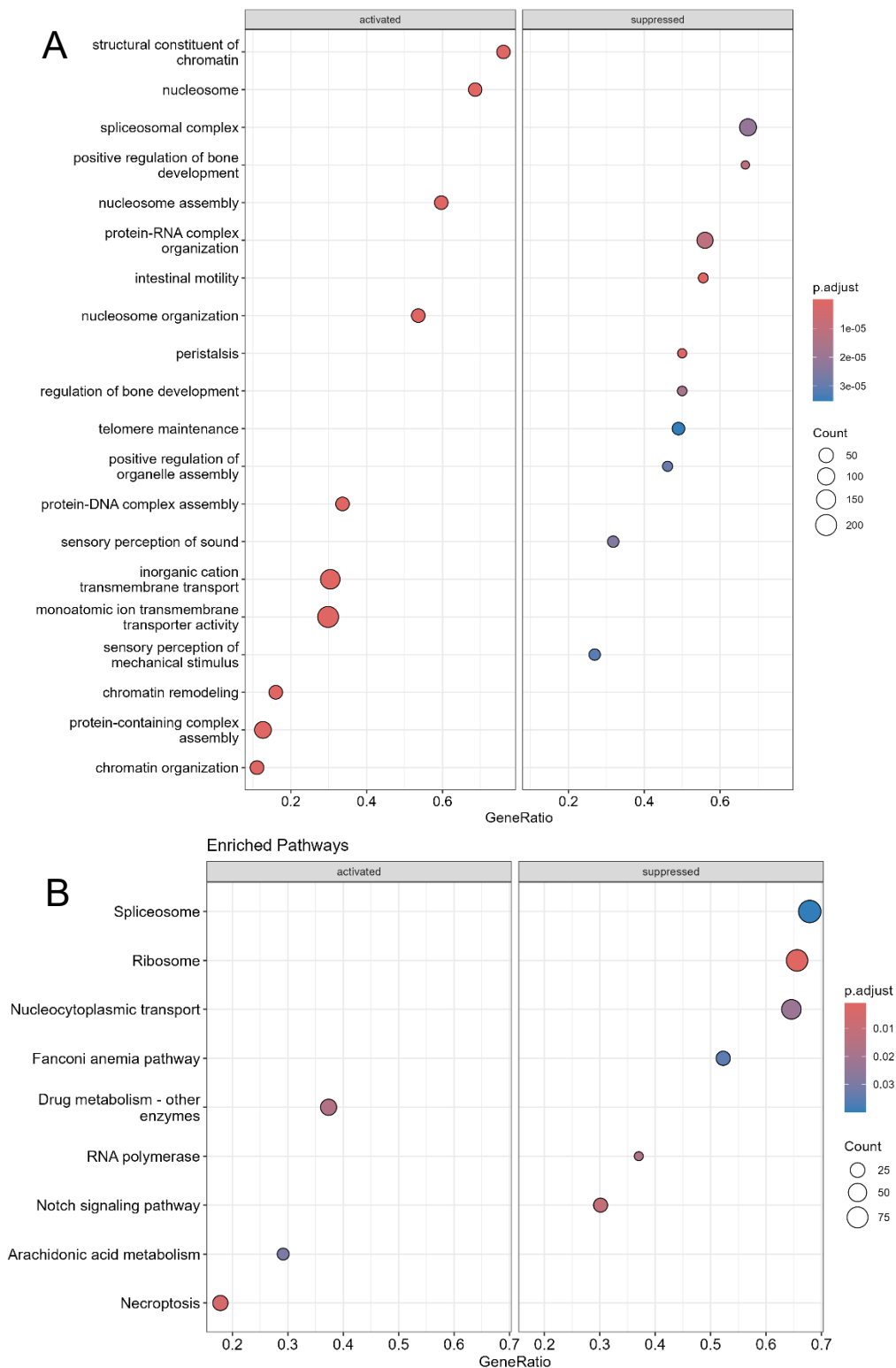


Figure 9 : A) Dot plot illustrating the first 10 enriched Gene Ontology (GO) categories based on the Ensembl database. Each dot represents a gene set, with the size of the dot indicating the number of genes in that set and the color reflecting the level of significance. **B)** Dot plot for the top 10 enriched pathways based on the KEGG database. Each dot corresponds to a pathway, where the size indicates the number of genes involved and the color represents the level of significance.

4.4. Generation of a new *recql4* mutant allele targeting exon 16

Finally, I initiated the generation of a new mutant allele for the *recql4* gene using CRISPR/Cas9-mediated genome editing, targeting exon 16 of the gene. Injection of the ribonucleoprotein (RNP) construct were performed into wild-type (AB) embryos, with crRNA and tracrRNA annealed and Cas9 protein incorporated as detailed in the Methods section. To enhance targeting precision, two distinct crRNAs were designed for different sites within exon 16, which were mixed in a 1:1 ratio during RNP preparation.

Analyzing the phenotypes, many of the injected (P_0) “crispant” embryos showed a quite severe phenotype that is similar to *recql4*^{elu16/elu16} mutants, including smaller eyes, skeletal deformities, and kidney cysts (**Fig. 10**).

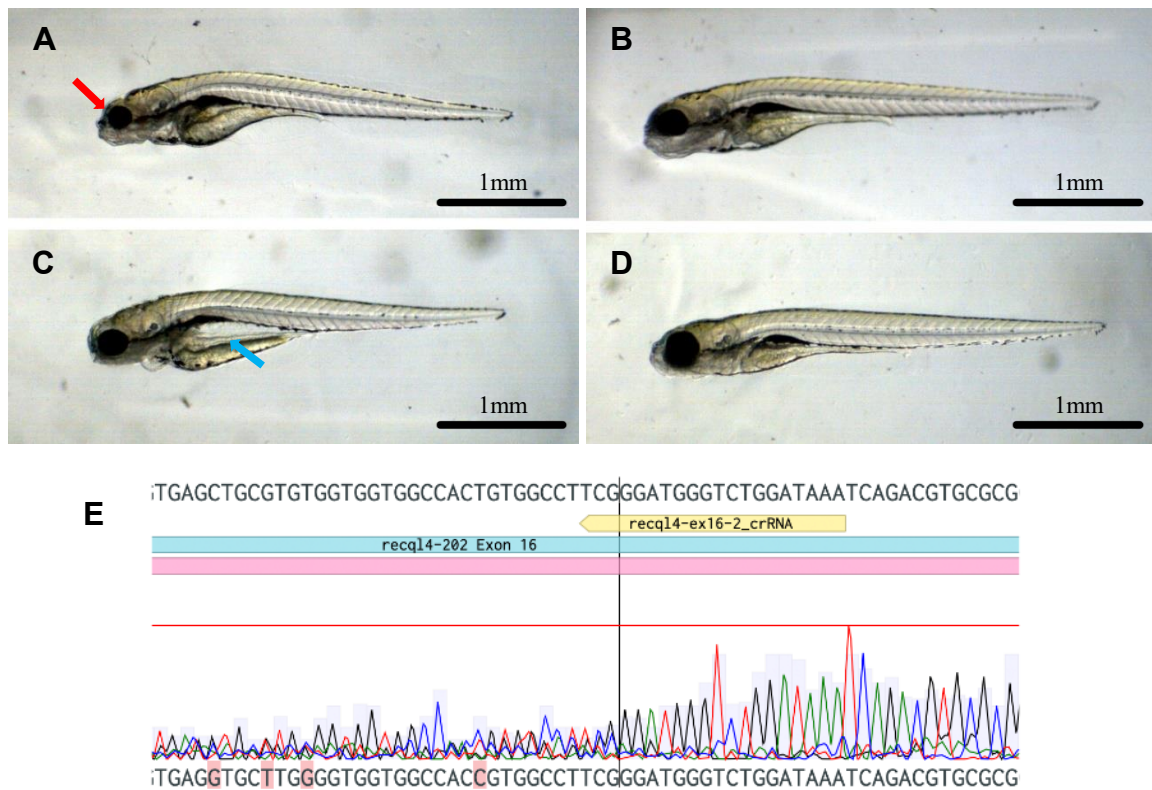


Figure 10 : A-D) P_0 generation larvae of *recql4*-exon16 crispants showing phenotypes similar to those seen in *recql4*^{elu16/elu16} including smaller eyes (red arrow) and kidney cysts (blue arrow). E) Sequence chromatogram via Sanger Sequencing showing a collapse at the targeted region which can be indicative of the presence for multiple indels suggesting successful editing.

5. Discussion

In my study, I have successfully characterized a zebrafish model for human diseases related to the dysfunction of the *RCEQL4* gene. This model will allow us to investigate the role of Recql4 in genome integrity and stability. Work in our lab already identified several phenotypic abnormalities including skeletal deformities and heart problems which are more severe and lethal in zebrafish. I have concentrated on one of the earliest phenotypes, an extended apoptosis observable in the highly proliferative regions of the retina and the brain of 2 dpf embryos.

Adhering to the domain structure of *recql4* which contains N-terminal Sld2-like domain that is crucial for initiation of replication, and the unique localisation of *recql4* within the mitochondria, we hypothesize that its mutation leads to the trigger of apoptosis pathway somehow due to the leakage of mitochondrial membranes and failure in proper DNA replication during the cell division.

I performed transcriptomic analysis to compare the gene expression profile of mutant and control animals and was able to show that relatively few genes contribute to the onset of phenotypic differences. My transcriptomic analysis also revealed the altered expression of genes and activation/suppression of molecular pathways (such as those related to chromatin organization, or protein synthesis) that could be the result of the cellular stress response resulting from Recql4 impairment.

By targeting a second exon within the *recql4* gene (exon 16) I found that P0 crispants show similar phenotypes, confirming the specificity of the phenotypes observed in our previous *recql4* mutant allele.

For future work, to test these potential roles of Recql4 in zebrafish, I plan to perform a specialized form of quantitative PCR (qPCR) targeting specific mitochondrial and nuclear DNA regions. This will allow us to measure the amount of DNA damage in the mutant genome (lesion frequency) and assess the mtDNA copy numbers which might explain the triggering of the apoptotic pathways due to malfunctioning mitochondria. I will also outcross the newly generated *recql4-exon 16* crispants with wild-type zebrafish to identify mutant carriers through offspring sequencing, ultimately establishing a definitive mutant allele for exon 16 as we did with exon 5.

Author Contribution

- I initiated the generation of a second mutant allele in *recql4* gene targeting exon 16 and optimized a PCR program using specific primers for genotyping to differentiate between mutants and wild-type for *exon5*.
- I Characterized observable phenotypes in *recql4* mutants, identifying apoptosis in specific regions at 2 dpf and further skeletal and vascular deformities at 5 dpf.
- I Performed apoptosis assays using Acridine Orange staining to confirm increased apoptosis in *recql4* mutants compared to wild-type controls.
- I isolated total RNA from zebrafish embryos at 2 dpf and conducted transcriptomic analysis using the Galaxy platform and visualized the data with R Studio.

Hereby I declare that I have not been awarded any prize at previous TDK conferences in Hungary. Should I be awarded a prize at another TDK conference before the current BTDK event, I will inform the BTDK and send my award-winning essay to them.

Statement

I summarized the detailed RNA isolation protocol steps in the Methods section using *ChatGPT* to adhere to character limits.

I reviewed and ensured the citation styles consistency using *ChatGPT*, especially for non-journal articles, after initially generating the bibliography with the reference manager *Zotero*.

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First and foremost, I would like to thank my dear supervisor, Dr. Máté Varga, for his invaluable guidance, encouragement, and unlimited support. His comments and feedback have been crucial in shaping my work and enhancing my understanding of the research objectives. I am also grateful for the opportunity to work in his lab.

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Lastly, I would like to thank my dear family for their endless support and belief in me which is always the fuel and motivation to overcome challenges and pursue my goals.

References

- Aleström, P., D'Angelo, L., Midtlyng, P. J., Schorderet, D. F., Schulte-Merker, S., Sohm, F., & Warner, S. (2020). Zebrafish: Housing and husbandry recommendations. *Laboratory Animals*, 54(3), 213–224. <https://doi.org/10.1177/0023677219869037>
- Annus, T., Müller, D., Jezsó, B., Ullaga, G., Németh, B., Harami, G. M., Orbán, L., Kovács, M., & Varga, M. (2022). Bloom syndrome helicase contributes to germ line development and longevity in zebrafish. *Cell Death & Disease*, 13(4), 363. <https://doi.org/10.1038/s41419-022-04815-8>
- Batut, B., Freeberg, M., Heydarian, M., Erxleben, A., Videm, P., Blank, C., Doyle, M., Soranzo, N., van Heusden, P., & Delisle, L. (2024). Reference-based RNA-Seq data analysis (Galaxy Training Materials). <https://training.galaxyproject.org/training-material/topics/transcriptomics/tutorials/ref-based/tutorial.html>. Online; accessed Jul 17, 2024.
- Batut, B., Hiltemann, S., Bagnacani, A., Baker, D., Bhardwaj, V., Blank, C., Bretaudeau, A., Brillet-Guéguen, L., Čech, M., Chilton, J., Clements, D., Doppelt-Azeroual, O., Erxleben, A., Freeberg, M. A., Gladman, S., Hoogstrate, Y., Hotz, H.-R., Houwaart, T., Jagtap, P., ... Gruning, B. (2018). Community-Driven Data Analysis Training for Biology. *Cell Systems*, 6(6), 752-758.e1. <https://doi.org/10.1016/j.cels.2018.05.012>
- Blighe, K., Rana, S., & Lewis, M. (2024). *EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling* (R package version 1.24.0). <https://github.com/kevinblighe/EnhancedVolcano>
- Chen, J., Baxi, K., Lipsitt, A. E., Hensch, N. R., Wang, L., Sreenivas, P., Modi, P., Zhao, X. R., Baudin, A., Robledo, D. G., Bandyopadhyay, A., Sugalski, A., Challa, A. K., Kurmashev, D., Gilbert, A. R., Tomlinson, G. E., Houghton, P., Chen, Y., Hayes, M. N., Chen, E. Y., Libich, D. S., & Ignatius, M. S. (2023). Defining the function of wild-type and three patient-specific TP53 mutations in a zebrafish model of embryonal rhabdomyosarcoma. *eLife*, 12, Article e68221. <https://doi.org/10.7554/eLife.68221>
- Croteau, D. L., Popuri, V., Opresko, P. L., & Bohr, V. A. (2014). Human RecQ Helicases in DNA Repair, Recombination, and Replication. *Annual Review of Biochemistry*, 83(1), 519–552. <https://doi.org/10.1146/annurev-biochem-060713-035428>

- Hiltemann, S., Rasche, H., Gladman, S., Hotz, H.-R., Larivière, D., Blankenberg, D., Jagtap, P. D., Wollmann, T., Bretaudeau, A., Goué, N., Griffin, T. J., Royaux, C., Le Bras, Y., Mehta, S., Syme, A., Coppens, F., Driesbeke, B., Soranzo, N., Bacon, W., ... Batut, B. (2023). Galaxy Training: A powerful framework for teaching! *PLOS Computational Biology*, 19(1), e1010752. <https://doi.org/10.1371/journal.pcbi.1010752>
- Kroll, F., Powell, G. T., Ghosh, M., Gestri, G., Antinucci, P., Hearn, T. J., Tunbak, H., Lim, S., Dennis, H. W., Fernandez, J. M., Whitmore, D., Dreosti, E., Wilson, S. W., Hoffman, E. J., & Rihel, J. (2021). A simple and effective F0 knockout method for rapid screening of behaviour and other complex phenotypes. *eLife*, 10, e59683. <https://doi.org/10.7554/eLife.59683>
- Larizza, L., Roversi, G., & Volpi, L. (2010). Rothmund-Thomson syndrome. *Orphanet Journal of Rare Diseases*, 5, Article 2. <https://doi.org/10.1186/1750-1172-5-2>
- Longkumer, S., Jamir, A., & Pankaj, P. P. (2024). Maintenance and breeding of zebrafish under laboratory conditions for animal research. *Agricultural Science Digest*, 44(3), 551–555. <https://doi.org/10.18805/ag.D-5599>
- Lu, H., & Davis, A. J. (2021). Human RecQ Helicases in DNA Double-Strand Break Repair. *Frontiers in Cell and Developmental Biology*, 9, 640755. <https://doi.org/10.3389/fcell.2021.640755>
- Luong, T. T., & Bernstein, K. A. (2021). Role and Regulation of the RECQL4 Family during Genomic Integrity Maintenance. *Genes*, 12(12), 1919. <https://doi.org/10.3390/genes12121919>
- Luong, T. T., Li, Z., Friedigkeit, N., Parker, P. S., Böhm, S., Rapchak, K., Lee, A. V., & Bernstein, K. A. (2022). Hrq1/RECQL4 regulation is critical for preventing aberrant recombination during DNA intrastrand crosslink repair and is upregulated in breast cancer. *PLOS Genetics*, 18(9), e1010122. <https://doi.org/10.1371/journal.pgen.1010122>
- Martins, D. J., Di Lazzaro Filho, R., Bertola, D. R., & Hoch, N. C. (2023). Rothmund-Thomson syndrome, a disorder far from solved. *Frontiers in Aging*, 4, 1296409. <https://doi.org/10.3389/fragi.2023.1296409>

- Meeker, N. D., Hutchinson, S. A., Ho, L., & Trede, N. S. (2007). Method for Isolation of PCR-Ready Genomic DNA from Zebrafish Tissues. *BioTechniques*, 43(5), 610–614. <https://doi.org/10.2144/000112619>
- Miles, L. B., Calcinotto, V., Oveissi, S., Serrano, R. J., Sonntag, C., Mulia, O., Lee, C., & Bryson-Richardson, R. J. (2024). CRIMP: A CRISPR/Cas9 insertional mutagenesis protocol and toolkit. *Nature Communications*, 15(1), 5011. <https://doi.org/10.1038/s41467-024-49341-7>
- R Core Team. (2023). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>
- Schmit, M., & Bielinsky, A.-K. (2021). Congenital Diseases of DNA Replication: Clinical Phenotypes and Molecular Mechanisms. *International Journal of Molecular Sciences*, 22(2), 911. <https://doi.org/10.3390/ijms22020911>
- Siitonen, H. A., Sotkasiira, J., Biervliet, M., Benmansour, A., Capri, Y., Cormier-Daire, V., Crandall, B., Hannula-Jouppi, K., Hennekam, R., Herzog, D., Keymolen, K., Lipsanen-Nyman, M., Miny, P., Plon, S. E., Riedl, S., Sarkar, A., Vargas, F. R., Verloes, A., Wang, L. L., ... Kestilä, M. (2009). The mutation spectrum in RECQL4 diseases. *European Journal of Human Genetics*, 17(2), 151–158. <https://doi.org/10.1038/ejhg.2008.154>
- Tucker, B., & Lardelli, M. (2007). A Rapid Apoptosis Assay Measuring Relative Acridine Orange Fluorescence in Zebrafish Embryos. *Zebrafish*, 4(2), 113–116. <https://doi.org/10.1089/zeb.2007.0508>
- Westerfield, M. (2000). *The zebrafish book: A guide for the laboratory use of zebrafish (Danio rerio) (4th ed.)*. University of Oregon Press. https://zfin.org/zf_info/zfbook/zfbk.html
- Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer-Verlag. <https://ggplot2.tidyverse.org>
- Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., Fu, X., Liu, S., Bo, X., & Yu, G. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *The Innovation*, 2(3), 100141. <https://doi.org/10.1016/j.xinn.2021.100141>

Zhang, Y., Qin, W., Wang, H., Lin, Z., Tang, Z., & Xu, Z. (2021). Novel pathogenic variants in the *RECQL4* gene causing Rothmund-Thomson syndrome in three Chinese patients. *The Journal of Dermatology*, 48(10), 1511–1517. <https://doi.org/10.1111/1346-8138.16015>