Ekspresija gena uključenih u popravak križnih veza DNA i proteina u zebricama (Danio rerio) koje nose mutaciju u genu tirozil-DNA fosfodiesteraza 2b

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Expression of genes involved in DNA-protein crosslink repair in zebrafish *(Danio rerio)* carrying a mutation in the *tyrosyl-DNA phosphodiesterase 2b* gene

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Sveučilište u Zagrebu
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Križne veze DNA i proteina (DPC) su oštećenja DNA u kojima su proteini nepovratno križno vezani s DNA. Zbog njihove veličine i kemijske stabilnosti, potrebno ih je ukloniti kako bi se omogućio nastavak bitnih procesa, uključujući replikaciju i transkripciju. Specifični put popravka nazvan DPC popravkom (DPCR) nedavno je otkriven, a način djelovanja nekoliko ključnih enzima proučavan je u uvjetima *in vitro* i u staničnoj kulturi. Jedan takav enzim je tirozil-DNA fosfodiesteraza 2 (Tdp2), koja sudjeluje u uklanjanju umrežene topoizomeraze 2 iz DNA. Osim proteaza i nukleaza, za potpuni popravak DPC oštećenja vjerojatno su potrebni i drugi pomoćni proteini. Za proučavanje potencijalnih interakcija između produkta gena *tdp2* i popravka DPCR *in vivo*, upotrijebila sam zebricu *Danio rerio*, koja je dobro okarakteriziran mali kralježnjak koji se rutinski koristi kao životinjski model. U ovom radu istražila sam promjene ekspresije gena u mutantu *tdp2b* zebrice, dominantnog ortologa odgovornog za većinu aktivnosti Tdp2 u embrijima zebrice. Od svih analiziranih gena, analiza ekspresije otkrila je značajno smanjenu ekspresiju *znf451* i *mre11a*, te pojačanu regulaciju gena *acrc* što bi moglo ukazivati na potencijalnu međuovisnost s genom *tdp2b* kod zebrica.

Ključne riječi: popravak DNA, DPCR, genska promjena
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Master thesis

Expression of genes involved in DNA-protein crosslink repair in zebrafish (*Danio rerio*) carrying a mutation in the *tyrosyl-DNA phosphodiesterase 2b gene*

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DNA-Protein Crosslinks (DPCs) are DNA lesions in which proteins are irreversibly cross-linked to DNA. Due to their size and chemical stability, they need to be removed to enable essential processes including replication and transcription to proceed. A specific repair pathway termed DPC repair (DPCR) has recently been uncovered and the mode of action of several key enzymes has been studied *in vitro* and in cell culture. One such enzyme is the tyrosyl-DNA phosphodiesterase 2 (Tdp2), which is involved in the removal of cross-linked Topoisomerase 2 from DNA. However, proteases, nucleases and other accessory proteins are likely required for the complete repair of DPC lesions. To study the potential interactions between *tdp2* gene product and DPCR *in vivo*, I used the zebrafish *Danio rerio*, which is a well-characterized small vertebrate that is routinely used as an animal model. In this work, I investigated gene expression changes in the *tdp2b* zebrafish mutant, of the more dominantly expressed orthologue that accounts for the majority of Tdp2 activity in zebrafish embryos. Out of all analysed genes, expression analysis revealed significantly reduced expression of *znf451* and *mre11a*, and upregulation of *acrc* gene that could indicate potential interdependency with *tdp2b* gene in zebrafish.

Keywords: DNA repair, DPCR, gene modification
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Sadržaj

Abbreviations

Acrc – acidic-repeat containing protein

AP site – apurinic/apyrimidic site

Apex 1 – Apurinic/Apyrimidinic Endodeoxyribonuclease 1

Apex 2 – Apurinic/Apyrimidinic Endodeoxyribonuclease 2

BER – base excision repair

BRCA – BReast CAncer gene

bp – base pairs

Ct – cycle threshold

DDR –DNA damage response

DPC – DNA-protein crosslink

dpf – days post fertilisation

DSB – double-strand break

ETP – etoposide

GG-NER – global genome NER

HR – homologous recombination

MMEJ – microhomology-mediated end joining

MMR – mismatch repair

MNE – Mean Normalized Expression

Mre11a – Meiotic recombination 11 a

Mus81 – crossover junction endonuclease

NER – nucleotide excision repair

NHEJ – non-homologous end joining

P97a/vcp – valosin-containing protein

PARP1 – poly(ADP-ribose) polymerase 1

PCNA – proliferating cell nuclear antigen

PCR – polymerase chain reaction

Pol – polymerase

SD – standard deviation

SSB – single-strand breaks

SUMO -small ubiquitin-like modifiers

TC-NER – transcription-coupled NER

Tdp1 – tyrosyl-DNA phosphodiesterase 1

Tdp2 – tyrosyl-DNA phosphodiesterase 2

Tex264 – Testis Expressed 264

Top1 – topoisomerase 1

Top2 – topoisomerase 2

TOP-cc – topoisomerase DNA covalent complex

1. Introduction

1.1. DNA damage and repair

DNA is a fundamental molecule, and its integrity is crucial for the proper existence and functioning of all organisms (Rastogi et al., 2010). DNA damage is the result of cellular injury or stress, and plays a role in the mechanisms of disease and aging, and it is widely associated with cancer, neurologic disorders, birth defects and premature aging. Most carcinogens function by inducing DNA damage and causing genetic mutations (Martin, 2008). Genome instability arises when pathways responsible for maintaining genome integrity fail to function properly. This can occur due to inherited defects or to environmental agents. Genome instability increases chances for permanent alterations and mutations of the DNA (Langie et al., 2015). Radiation and genotoxic chemicals can negatively impact the stability of the genome. Ultraviolet radiation (UVR), particularly UV-B (280-315 nm), is a potent agent that can disrupt normal biological processes by causing various mutagenic and cytotoxic DNA lesions, including cyclobutane-pyrimidine dimers (CPDs), 6-4 photoproducts (6-4PPs), their Dewar valence isomers, and DNA strand breaks, all of which compromise genome integrity (Rastogi et al., 2010).

In response to these lesions, organisms have evolved several repair mechanisms, such as nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR). Moreover, double-strand break (DSB) repair, homologous recombination (HR) and nonhomologous end joining (NHEJ), programmed cell death (apoptosis) and cell-cycle checkpoints are also active in various organisms, involving specific proteins and pathways (Rastogi et al., 2010). The DNA damage response (DDR) pathway is also crucial for the accurate transmission of genetic information and for the survival of individual organisms (Ciccia & Elledge, 2010).

Even under normal physiological conditions, a diverse array of endogenous electrophilic molecules within our cells persistently damages our DNA. Various methods have been used to measure the steady-state levels of endogenous DNA damage in mammalian cells, with reports from the Swenberg and Nakamura groups indicating that the total number of endogenous DNA lesions exceeds 37,000 per genome (Nakamura et al., 2014).

1.1.1. Base excision repair

Base excision repair (BER) addresses damage of the DNA resulting from alkylation, oxidation and deamination. BER begins when a DNA glycosylase identifies and excises the impaired base, creating an apurinic/apyrimidic (AP) site. Then, an AP endonuclease, such as Apex 1, cleaves the AP site, resulting in a single-strand break (SSB), which can then be processed through either long-patch repair or short-patch repair pathways, that predominantly utilize different sets of proteins. Short-patch follows when 5' deoxyribose phosphate (5'dRP) is in hemiacetal form, with unaltered AP sites, while long-patch happens when 5'dRP is in 3'α, β non-saturated aldehyde form and the AP site is oxidised or reduced. Lastly, ligation occurs by ligase I, proliferating cell nuclear antigen (PCNA) and the polymerase Polβ in long-patch repair, while in short-patch repair, ligase III interacts with X-ray repair cross-complementing protein 1 (XRCC1), Polβ and poly(ADP- ribose)polymerase-1 (PARP) for ligation. BER helps protect against cancer, aging, and neurodegenerative diseases, and occurs both in the nucleus and in mitochondria (Krokan & Bjørås, 2013;Leyns & Gonzalez, 2012). BER repair intermediates are single-strand DNA breaks (SSBs), which can also result from exposure to ionizing radiation, oxidative stress, or the abnormal activity of cellular enzymes such as DNA topoisomerase 1. Accurate BER is crucial to prevent these breaks from evolving into the more harmful double-strand breaks (DSBs) (Hossain et al., 2018). The BER repair scheme is shown in Figure 1.

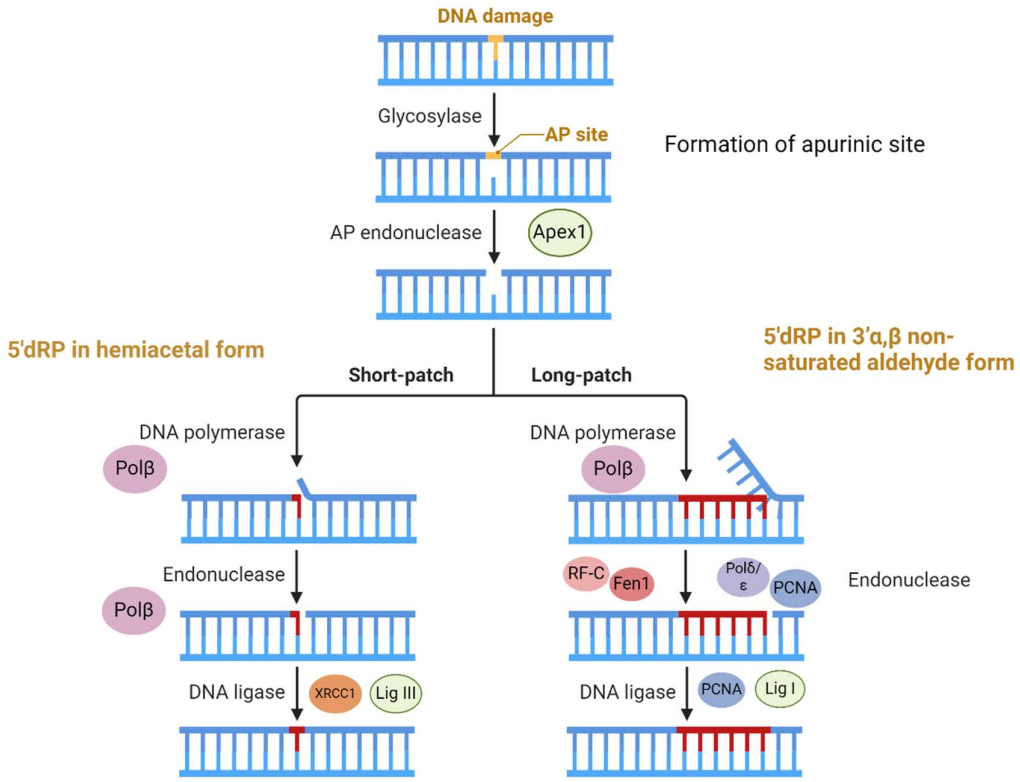

Figure 1. Schematic representation of the base excision repair pathways. The first, common step is damage recognition, then base removal and lastly incision. Short-patch follows when 5' deoxyribose phosphate (5'dRP) is in hemiacetal form, with unaltered AP sites and long-patch happens when 5'dRP is in 3'α, β non-saturated aldehyde form and AP site is oxidised or reduced (adapted from Leyns & Gonzalez, 2012). Created with BioRender.

1.1.2. Mismatch repair (MMR)

Mismatch repair (MMR) is a cellular post-replication mechanism that preserves DNA homeostasis and emerged during evolution to safeguard genomic stability (Kunkel, 2009). The primary function of the MMR system is to correct small insertion-deletion loops (indels) and spontaneous base-base mispairs that predominantly arise during DNA replication. Mismatch repair begins when heterodimers MutS homolog 2/MutS homolog 3 (MSH2/MSH3) or MutS homolog 2/MutS homolog 6 (MSH2/MSH6) recognise DNA lesions. The MSH2/MSH3

heterodimer efficiently repairs sequences with no more than ten unpaired nucleotides but it is less effective in recognising single nucleotide mismatches, and the MSH2/MSH6 heterodimer is specialized for repairing one or two unpaired nucleotides. Upon recognising a DNA lesion, MutL homolog 1 (MLH1) dimerizes with postmeiotic segregation increased 1 (PMS1), postmeiotic segregation increased 2 (PMS2) or MLH3 to form a complex with MSH2/MSH3 or MSH2/MSH6 on the lesion. ATP binding to the MSH2/MSH3 or MSH2/MSH6 complex attracts MLH1 and triggers the repair processes. Essential proteins involved in MMR are proliferating cell nuclear antigen (PCNA), DNA polymerase δ/ε (Pol δ/ε), exonuclease (EXO-1), replication protein A (RPA), replication factor C (RPC) and high mobility group box 1 protein (HMGB-1). Lastly DNA ligase I (LIG I) performs the ligation (Middel & Blattner, 2011). Figure 2. shows a scheme of the essential proteins involved in MMR (Schmidt & Pearson, 2016). MMR plays a crucial role in cancer protection and affects its biological behavior (Pećina-Šlaus et al., 2020).

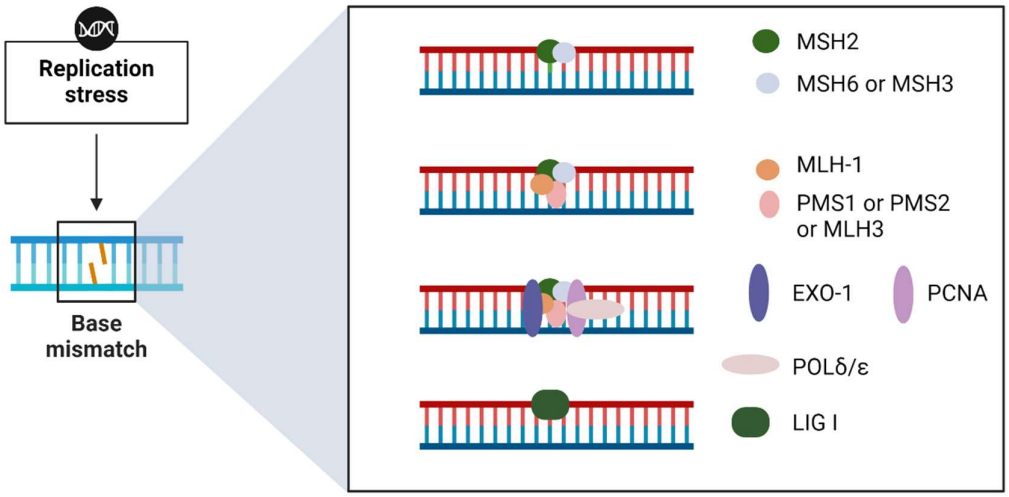

Figure 2. Mismatch repair (MMR) mechanism scheme with essential proteins (adapted from Middel & Blattner, 2011). Created with BioRender.

1.1.3. Nucleotide excision repair (NER)

Nucleotide excision repair (NER) is the primary pathway in mammals for removing bulky DNA lesions caused by environmental mutagens, UV light and some chemotherapeutic agents.

Deficiencies in NER are linked to xeroderma pigmentosum, an inherited disorder characterized by an extreme susceptibility to skin cancer (Schärer, 2013). NER can be initiated through two subpathways: global genome NER (GG-NER) and transcription-coupled NER (TC-NER) (Giglia-Mari et al., 2006). GG-NER operates throughout the genome, and TC-NER prioritizes the rapid repair of lesions on the transcribed strand of active genes. The GG-NER pathway starts with the damage recognition by xeroderma pigmentosum group C protein complex (XPC-RAD23B), assisted by UV-damaged DNA-binding protein (UV-DDB). TC-NER begins when the RNA polymerase stalls at a lesion, aided by Cockayne syndrome A (CSA), Cockayne syndrome B (CSB), and xeroderma pigmentosum A-binding protein 2 (XAB2). During the next steps of repair, both GG-NER and TC-NER use the same pathway. The DNA region with the lesion is unwound by the transcription factor IIH (TFIIH), which includes xeroderma pigmentosum, complementation group B (XPB), xeroderma pigmentosum, complementation group D (XPD), xeroderma pigmentosum, complementation group A (XPA) and replication protein A (RPA). Excision follows when endonuclease xeroderma pigmentosum, complementation group F/excision repair complementing defective repair in Chinese hamster 1 (XPF/ERCC1) and xeroderma pigmentosum, complementation group G (XPG) bind to DNA region containing damage. DNA polymerase δ/ε with PCNA or replication factor C (RFC) fills the gap, and DNA ligase I (LIG I) performs the ligation (OLSEN et al., 2005). Both pathways depend on essential NER factors to finalize the excision process (Tubbs et al., 2009). Figure 3. shows the essential proteins involved in NER.

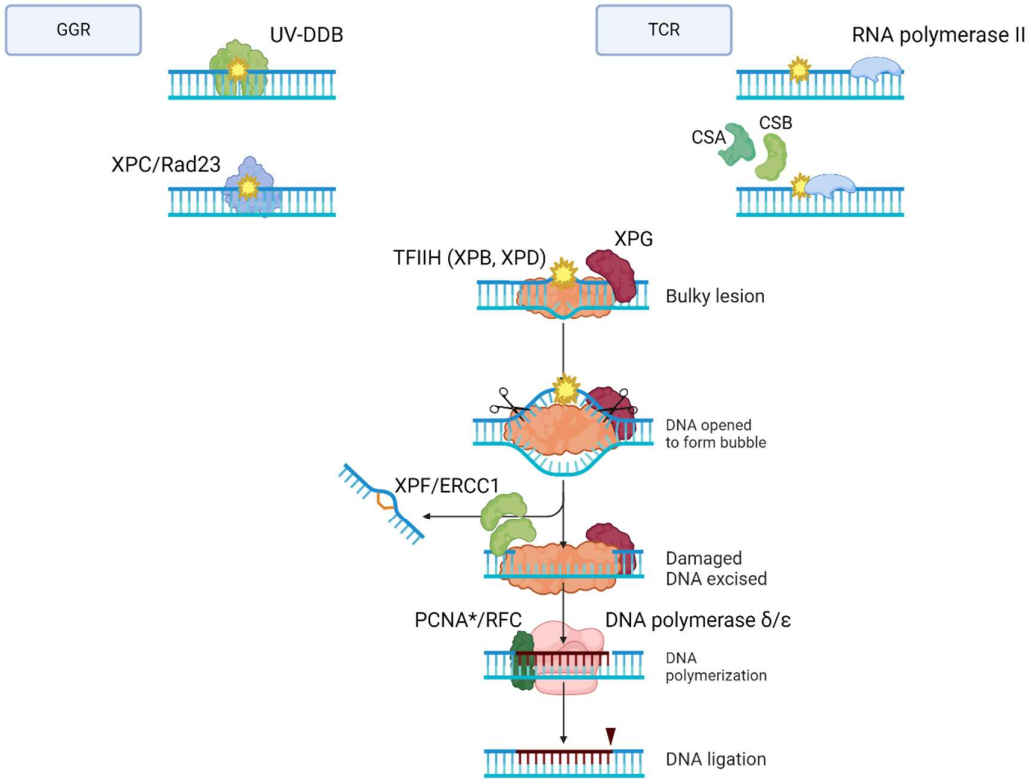

Figure 3. Nucleotide excision repair (NER). Scheme shows global genome repair (GG-NER) and transcription-coupled repair (TC-NER). NER can be divided into five steps: damage recognition, unwinding DNA to form a bubble, excision, DNA polymerisation and DNA ligation (adapted from Olsen et al., 2005). Created with BioRender.

1.1.4. Double-strand breaks (DSBs)

The most serious type of DNA damage is double-strand breaks (DSBs), which can lead to the loss of genetic information or chromosomal rearrangements if not repaired correctly. DSBs can be repaired through various pathways: microhomology-mediated end joining (MMEJ), non-homologous end joining (NHEJ) or homologous recombination (HR) (Povirk, 2012).

In NHEJ, the DSB is initially recognized by the Ku70–Ku80 heterodimer (Ku). This heterodimer functions as a 'tool belt' or loading platform, allowing other NHEJ proteins to be recruited to facilitate the joining of DNA ends. The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) exhibits a strong affinity for Ku-bound DNA ends and, in combination with Ku, forms the DNA-PK complex (Meek et al., 2008). Nucleases, for example Artemis,

can cut the DNA ends in case of blunt ends, thereby creating sticky ends, and base-pairing can join the DNA molecule (Brown TA, 2002). To secure the ends, the XRCC4-DNA Ligase IV-XLF complex seals the break (Brandsma & Gent, 2012). Defects in the NHEJ pathway leads to increased sensitivity to ionizing radiation and the loss of lymphocytes (Sishc & Davis, 2017). The NHEJ mechanism involves proteins that recognize, trim, polymerize, and ligate DNA ends in a versatile manner. This flexibility allows NHEJ to operate on various DNA-end configurations, but the repaired DNA junctions frequently contain mutations (Chang et al., 2017). Despite this, NHEJ is essential for maintaining genome integrity in resting and non-dividing cells. It is particularly vital in cells that never divide, such as neurons and muscle cells (McKinnon, 2013). Figure 4. shows essential proteins involved in the NHEJ mechanism.

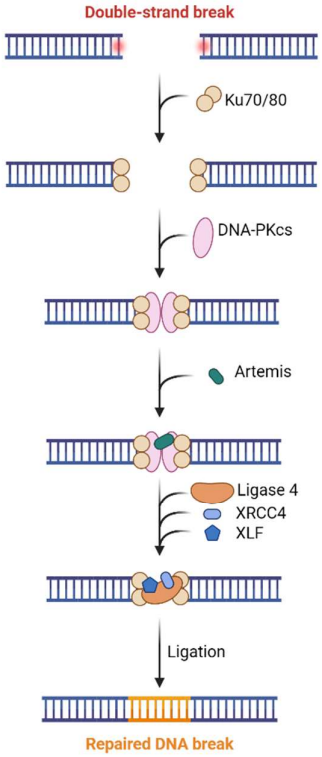

Figure 4. Non-homologous end joining (NHEJ) pathway mechanism (adapted from Brandsma & Gent, 2012). Created with BioRender.

Microhomology-mediated end joining (MMEJ) is a mutagenic mechanism for repairing double-strand breaks (DSBs) which are consistently associated with deletions near the break sites and contributing to chromosome translocations and rearrangements (Truong et al., 2013). MMEJ

depends on exposed microhomologous sequences adjacent to the broken junctions for repair (Wang & Xu, 2017). MMEJ appears to be a critical double-strand break (DSB) repair mechanism in tumors deficient in homologous recombination (HR) as loss of both repair mechanisms leads to genomic instability (Ceccaldi et al., 2015). However, recent studies have shown that MMEJ is frequently utilized even when homologous recombination (HR) is available (Truong et al., 2013). MMEJ requires the activity of PARP and DNA polymerase (Polθ). Cancers associated with loss of BRCA1/2 are extremely sensitive to PARP and Polθ inactivity and are reliant on MMEJ repair. Even in cells with functional HR repair mechanism, PARP inhibitors can enhance sensitivity to chemotherapeutic drugs, such as camptothecin (Yoshioka et al., 2021).

Homologous recombination (HR) involves several pathways that repair DNA double-strand breaks (DSBs) and interstrand crosslinks (ICLs). ICLs are DNA lesions where covalent bonds form between two nucleotides on opposite DNA strands (McVey & Lee, 2008). HR also supports DNA replication by helping to recover stalled or broken replication forks, and is central to DNA damage tolerance. The key reactions in HR, such as homology search and DNA strand invasion, are catalysed by core proteins, such as the RecA homolog Rad51. The various functions of HR require additional, context-specific factors that work with these core proteins (Li & Heyer, 2008). The homologous recombination (HR) pathway starts with recognition of the DSB and resection at the DNA break sites by the Mre11-Rad50-Nbs1 (MRN) complex together with exonucleases including CtBP-interacting protein (CtIP) which generates single-stranded DNA (ssDNA). Once the resection occurs, the break cannot be repaired by NHEJ. The single-stranded DNA tail is then coated with RPA, in order to prevent secondary structure formation, which later replaced by Rad51 with assistance from BRCA2. Rad51 forms a nucleoprotein filament that works as an intermediate in strand invasion by seeking the sister chromatid and homologous sequence. D-loop forms and extends on the sister chromatid and it results in successful repair (Brandsma & Gent, 2012). D-loop is a complex that forms when one strand of dsDNA separates from the other strand and creates a loop (Shibata et al., 2020). DNA polymerase δ is the main polymerase involved in DNA synthesis, but several other DNA polymerases (ε, η, λ, ζ) are also involved in HR but their exact role is yet to be discovered (McVey et al., 2016). Failure to repair complex DNA damage or resolve replication stress properly can lead to genomic instability and contribute to cancer development (Li & Heyer, 2008). A scheme of the mechanism of homologous repair is shown in Figure 5.

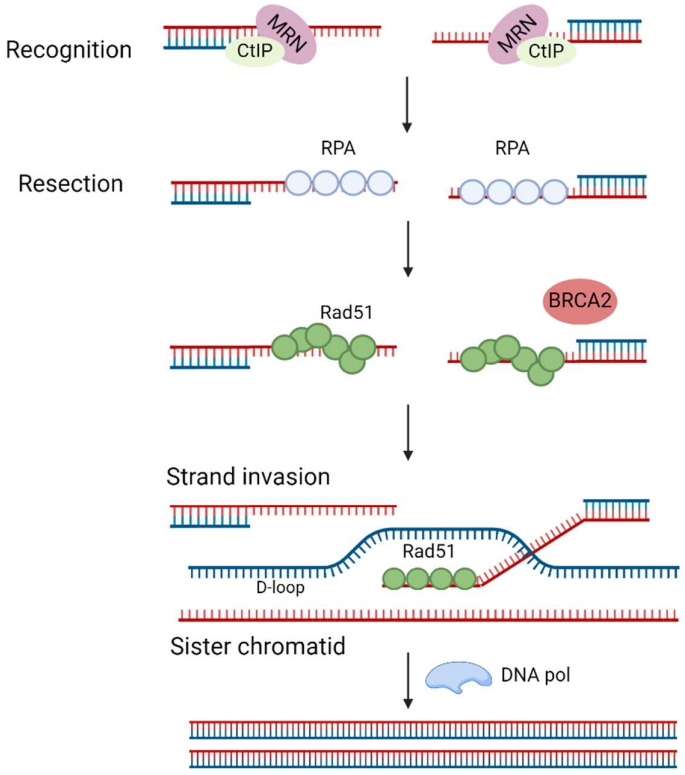

Figure 5. Homologous recombination (HR) pathway mechanism (adapted from Brandsma & Gent, 2012). Created with BioRender.

1.1.5. DNA damage response (DDR)

The DNA damage response (DDR) pathway is a network consisting of intricate signal transduction pathways that detects DNA damage and conveys this information across the cell, thereby influencing cellular responses to the damage: depending on the degree or type of DNA damage, the cell cycle can be arrested until the damage is repaired, or the cell can undergo apoptosis or senescence (Ciccia & Elledge, 2010). DDR orchestrates the activation of DNA damage checkpoints and facilitates the repair of DNA lesions. DNA checkpoints that can be activated are G1/S, S and G2/M (Visconti et al., 2016). Key proteins involved in DDR pathway include MDM2 (Mouse double minute 2), p53 (Tumour protein p53), ATM (Ataxia-telangiectasia mutated), ATR (Ataxia–telangiectasia and Rad3-related), p21 (Cyclin-dependent kinase inhibitor), CDK2/Cyclin E (Cyclin-dependent kinase 2) (Stewart et al., 1999; Liu et al., 2013; Subhasree et al., 2013; Tiwari & Wilson, 2019).

Recent studies have highlighted a significant interplay between the DDR and the immune system, demonstrating a crucial connection between these two systems (Nastasi et al., 2020). In the event of enduring DNA damage, cellular pathways leading to senescence (loss of cellular proliferation capacity) or even apoptosis (programmed cell death) can be activated. Apoptosis, senescence, inflammation and the immune response have primarily overall protective roles, but can have detrimental effect if misregulated or persistent. Accumulation of senescent cells and persistent inflammatory environment contribute to higher risk of mortality, tissue dysfunction, increases susceptibility to mutations and drives cancer and aging progression (Nastasi et al., 2020).

A dysfunctional DNA damage repair often leads to a predisposition for cancer, contributes to the "mutator phenotype" seen in many cancers, and enables tumour cells to survive and proliferate despite increased mutation rates and genomic instability. In contrast, the nervous system in mammals is extremely vulnerable to DNA damage and mutations, as neurons do not proliferate and damaged cells are not replaced (Jackson & Bartek, 2009). NER deficiencies can cause a variety of neurological syndromes such as Xeroderma pigmentosum, Cockayne syndrome, Trichothiodystrophy and Cerebro-oculo-facio-skeletal (COFS) syndrome. NHEJ deficiency due to inactivation of Ku70 or Ku80 in mouse models causes cause premature aging, cancer predisposition and lymphomas, and in humans, it is associated with immunodeficiency and microcephaly. Deficiencies in HR, e.g. due to mutations in the BRCA1 (BReast CAncer gene 1) and BRCA2 (BReast CAncer gene 2) genes can cause breast and ovarian cancer. DNA cross-link repair deficiency can lead to Fanconi anaemia. Neurodegenerative disorders such as Down syndrome, Alzheimer’s disease, Parkinson's disease, Huntington's disease, Friedreich's ataxia can be the result of DNA-damage-response impairment and defective DNA repair (Jackson & Bartek, 2009). These disorders highlight the importance of DNA repair mechanisms, especially in neurons which lack the ability to adequately repair their damaged DNA or to be replaced (Madabhushi et al., 2014).

Aging is a complex, multifaceted process that results in a widespread functional decline, impacting every organ and tissue. Surprisingly, it remains unclear whether aging stems from a singular causal mechanism or originates from multiple factors. Phenotypically, aging is linked to a diverse array of characteristics across molecular, cellular, and physiological levels, such as genomic and epigenomic changes, impaired proteostasis, reduced overall cellular and sub-cellular function, and disrupted signalling pathways. The DNA damage response (DDR) influences various age-related changes in both local and systemic communication mechanisms

by affecting inflammatory responses and crucial endocrine signalling components that contribute to the aging process (Schumacher et al., 2021).

In conclusion, DNA damage is very common and needs to be addressed to ensure the maintenance of genomic integrity. Better understanding DDR and generally how the cells cope with DNA damage will help improve life quality and life span, as well as to develop potential treatment of neurodegenerative diseases and cancers. One such type of DNA damage is DNA-protein crosslinks (DPCs); the mechanisms of DPC repair are less understood compared to that of other types of DNA lesions (Wei et al., 2021).

1.2. DNA-protein crosslinks

Covalent interactions between proteins and DNA strands result in DNA-protein crosslinks (DPCs), which are among the most harmful types of DNA damage and are believed to cause significant toxicity (Nakamura & Nakamura, 2020). Proteins can be crosslinked to intact or to disrupted DNA via single-strand breaks (SSB) or double-strand breaks (DSB). DPCs are highly diverse due to differences in protein sizes and to different types of chemical bonds between proteins and DNA. If left unrepaired, DPCs physically obstruct DNA transcription, replication and damage repair due to their large size, leading to compromised genomic stability and to tumorigenesis (Wei et al., 2021).

Based on the properties of the crosslinked proteins, DPCs can be divided into two groups: enzymatic and nonenzymatic DPCs (Nakamura & Nakamura, 2020).

1.2.2. Non-enzymatic DPCs

Non-enzymatic DPCs typically involve proteins attached to intact DNA strands and thus differ significantly from enzymatic DPC formations (Zhang et al., 2020). Enzymes are not the only proteins in the vicinity of DNA, thus many other proteins near DNA can form DPCs under certain circumstances (Zhang et al., 2020). DNA normally interacts with structural proteins. For example: around 145 DNA base pairs wind around a histone octamer, composed of two copies of H2A, H2B, H3 and H4 forming a nucleosome core particle (NCP), the fundamental chromatin unit. These interactions create opportunities for endogenous and environmental agents to irreversibly covalently link DNA to histones, resulting in DPCs (Wei et al., 2021).

Besides histones, DNA helical modifications like AP sites can covalently bind to, for example, DNA glycosylases (Prasad et al., 2020). Non-enzymatic DPCs are more common than enzymatic, and they are formed in the presence of the chemical agents such as ionising and UV radiations, aldehydes, chromates (Nakano et al., 2007).

1.2.1. Enzymatic DPCs

Numerous enzymatic processes involving DNA result in transient covalent attachments between DNA and enzymes. These enzymes typically include DNA topoisomerases (TOPs), DNA polymerases, DNA methyltransferases, DNA glycosylases, and apurinic or apyrimidinic lyases. Normally, these intermediates are unstable, and the covalent bonds can quickly revert. However, under specific conditions, these fleeting complexes can become trapped, leading to the formation of stable DPCs (Zhang et al., 2020). These events are triggered by different agents like radiation, aldehydes and metal ions. Formaldehyde (FA), the most prevalent endogenous carcinogen in humans, is a type of aldehyde naturally produced during the process of histone demethylation. Formaldehyde leads to the formation of DPCs by creating a methylene bridge between nucleophilic amino acid residues and DNA bases. To our current understanding, ionizing radiation causes the breakdown of water molecules through radiolysis, leading to the production of elevated levels of free radicals and reactive oxygen species within a localized area. These highly reactive substances induce various types of damage to DNA, including DPCs (Nakamura & Nakamura, 2020; Zhang et al., 2020).

Enzymatic DPCs can be divided into three classes according to type of bond proteins form (Figure 6): class (I) where, for example, Topo I is trapped at 3' end of SSB, class (II) in which, for example, Topo II is trapped at 5' end of DSB (double strand break), class (III) where, for example, DNA polymerase β is trapped at 5' ends of SSB. Class (I) DPCs form when Topo I creates a covalent reaction intermediate with a tyrosine-phosphodiester linkage to DNA, typically induced by Topo drugs or adjacent DNA damage. Class (II) DPCs are thought to arise through a similar process. Class (III) DPCs occur when an amide bond forms between polymerase β and deoxyribonolactone, an oxidized sugar moiety, during base excision repair attempts.

Another example of a protein that can form enzymatic DPCs is poly (ADP-ribose) polymerase 1 (PARP1), which plays a crucial role in multiple repair mechanisms, detecting DNA damage and in preserving genomic integrity. PARP1 binds to damaged DNA with its DNA-binding

domain (DBD) through N-terminal zinc finger motifs which activates the catalytic C-terminal domain for hydrolyzation of NAD+ and production of many PAR (poly (ADP-ribose)) chains (Chaudhuri & Nussenzweig, 2017). If PARP1 is trapped to the DNA, it can lead to cell death (Murai et al., 2012). Indeed, the trapping of PARP1 is induced by certain chemicals such as talazoparib, which is used in clinical settings to treat various cancers particularly ones with deficiencies in DNA repair mechanisms, such as ovarian and prostate cancers (Chaudhuri & Nussenzweig, 2017).

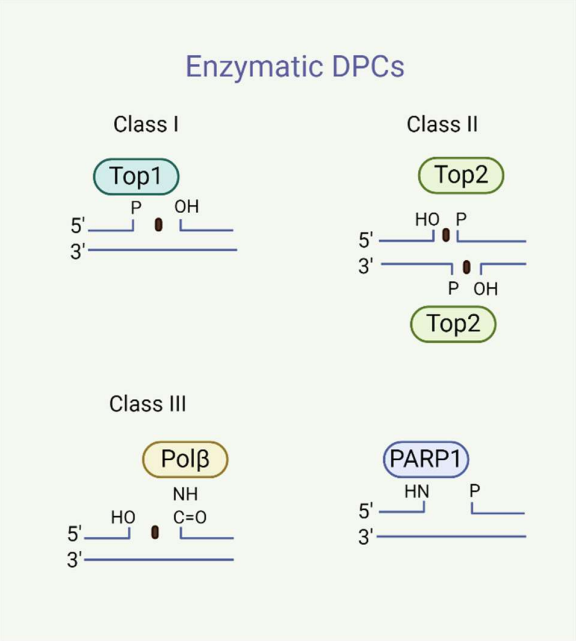

Figure 6. Examples of enzymatic DPCs which are formed when enzymes, such as DNA TOPs, DNA methyltransferase or polymerase covalently bind to DNA (adapted from Zhang et al., 2020). Created in BioRender.

To conclude, DPCs are harmful and complex DNA lesions that form from different exogenous and endogenous sources. They can be divided into two groups based on protein type: enzymatic and non-enzymatic. Understanding DPC formation and repair will be crucial for developing and improving cancer therapies associated with defective DPC repair.

1.3. DNA-protein crosslink repair

Because DPCs can greatly vary in size and chemically, cells are probably not able to recognise DPCs using specific sensors. Three specific aspects of DPC repair (DPCR) are: nuclease-dependent DPCR, proteolysis-dependent DPCR and direct cross-link hydrolysis (Figure 7) (Zhang et al., 2020).

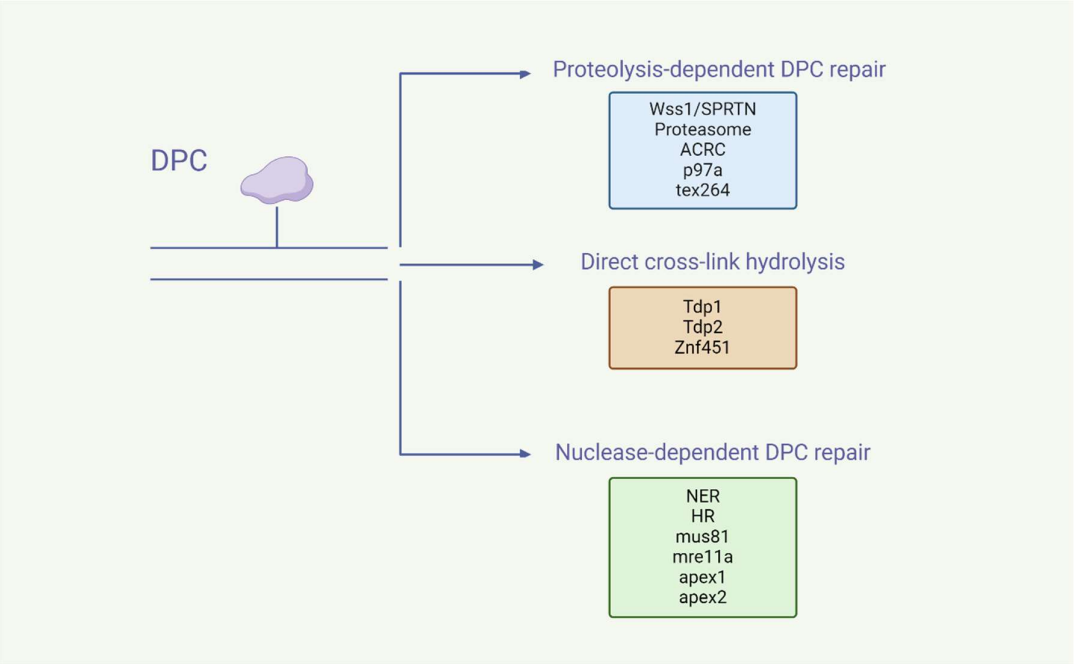

Figure 7. Categorization of DPC repair aspects based on targeted chemical bonds (adapted from Zhang et al., 2020). Created with BioRender.

1.3.1. Nuclease-dependent repair mechanisms

Nuclease-dependent repair mechanisms include NER and HR. NER and HR are known to cooperate but engage differently in DPCR. NER repairs smaller cross-linked proteins, smaller than 12–14 kDa and HR exclusively works on larger DPCs. In unusually bulky lesions, class IV DPCs, where proteins bind to an intact DNA, NER and HR work together (Nakano et al., 2007).

Meiotic recombination 11 a (*MRE11A*) is an endonuclease which binds to RAD50 Double Strand Break Repair Protein (RAD50) and Nijmegen breakage syndrome protein 1, Nibrin (NBS1) creating a Mre11-Rad50-Nbs1 (MRN) complex. This complex recognises DSBs induced by TOP2-like enzyme SPO11 (Meiosis-specific protein SPO11) and is important in

DNA replication fork resolution, meiosis, DNA homologous recombination. *MRE11A* also participates in break-induced repair (BIR) and interstrand crosslink repair (ICL) and has a possible role in MMR (Stracker & Petrini, 2011; Du et al., 2024). *MRE11A* can repair Top2cc independently of HR, works together with Ku in NHEJ in removal of Top2ccs. It removes TOP2ccs with high efficiency and removes entire DPCs during DNA resection (Hoa et al., 2016).

Apurinic/Apyrimidinic Endodeoxyribonuclease 1 (*APEX1*) and Apurinic/Apyrimidinic Endodeoxyribonuclease 2 (*APEX2*) are endonucleases involved in BER and repair of AP sites in human cells. AP sites are among the most common occurring mutagenic DNA lesions. AP sites cause DSB and covalent sequestration of histones and topoisomerases. In yeast cells, complete loss of AP repair is lethal (Kim et al., 2021). Moreover, high expression levels of *APEX1* are associated with a risk of breast, colon, neck, prostate and more cancers (Kim et al., 2013; Pei et al., 2019). Loss of *apex1* leads to buildup of oxidative DNA damage in zebrafish embryos, deformity in neuronal development and brain ventricle abnormalities (Pei et al., 2019).

APEX2 deficiency is lethal in cells with *BRCA1* and *BRCA2* mutations (Álvarez-Quilón et al., 2020). It is demonstrated that *APEX2* interacts with PCNA and is crucial for SSB repair. *APEX1* is crucial for *APEX2* activation and for SSB signalling and repair (Yan, 2024). Moreover, *APEX1* and *APEX2* deficiency leads to lethality when combined with TDP1 deficiency, both in untreated and in camptothecin (CPT) treated cells. This indicates that *APEX2* and *TDP1* have redundant roles in TOP1ccs repair (Zhang et al., 2020; Zhang et al., 2022).

Crossover junction endonuclease (*MUS81*) is crucial for resolving recombination intermediates, is involved in repairing replicative stress and regulates DNA replication fork progression. In the absence of *MUS81*, DNA synthesis slows down and cells rely on the xeroderma pigmentosum F (XPF) endonuclease for survival (Fu et al., 2015). *MUS81* is also involved in generating TOP1 induced DSBs since it cleaves stalled replication forks caused by TOP1ccs. Furthermore, TOP1 poisons are known to induce PARP-mediated replication fork reversal, which *MUS81* can also process (Zhang et al., 2022). *MUS81* was recently discovered to be play a role in TOP1cc repair in parallel to the TDP1 pathway (Marini et al., 2023).

In this thesis I investigated the potential link between *tdp2b* and the nucleases mentioned above, as we hypothesize that DPCs must first be rendered accessible, either through the action of

nucleases, or through protein degradation, before Tdp enzymes can process the peptide remnants.

1.3.2. Proteolysis-dependent repair

Proteolysis of covalently bound proteins during DPC repair was long observed and initially attributed to the function of proteasomes. The 26S proteasome, the primary protein degradation machine in eukaryotic cells, targets proteins marked by polyubiquitin chains for degradation. Interestingly, DPCs were found to be ubiquitinated and SUMOylated, but the precise timepoint at which the proteasome might play a role in DPCR, as well as the orchestration of the DPCR events still need to be further explored (Sun, Miller Jenkins et al., 2020; Essawy et al., 2023). Proteolysis-dependent repair was recently identified in a specific pathway involving Wss1 in yeast and SPRTN in mammalian cells. Wss1 and SPRTN cleave most of the protein part of DPC, but additional repair mechanism like NER or HR are then further required for complete resolution of the DPC (Zhang et al., 2020).

Valosin-containing protein (VCP or p97) is a key component of the ubiquitin system and belongs to a family of hexameric ATPases associated with diverse cellular activities (AAA) that harness energy from ATP to unfold protein substrates, aiding in their degradation by the proteasome. VCP extracts target proteins from cellular structures such as chromatin and assists in their translocation into the proteolytic core of the proteasome for degradation. It is involved in converting TOP2-DNA into protein-free double-strand breaks (Meyer & Weihl, 2014; Swan et al., 2021). The yeast ortholog of VCP, Cdc48, has been shown to be involved in repairing TOP1ccs. To accommodate this variety of functions, VCP/Cdc48 needs cofactors to engage the various substrates. Testis Expressed 264 (*TEX264*) is known as a cofactor of VCP/p97 ATPase and was shown to be required for TOP1-DNA crosslink repair by directing VCP to TOP1. Cells lacking TEX264 accumulate TOPccs, experience replication stress, DNA damage, and are highly sensitive to drugs that stabilise TOP1ccs (Fielden et al., 2022).

In contrast to these general proteolysis machineries, proteases have also been shown to be directly involved in DPCR. The first to be discovered, *SPRTN* is protease that possesses a ubiquitin interaction domain and a proliferating cell nuclear antigen PCNA-interacting motif, or PIP box. SPRTN mutations can cause Ruijs-Aalfs syndrome, a human disorder that is characterised by hepatocellular cancer, premature aging and chromosomal instability. *In vivo* studies revealed that cells lacking SPRTN are highly sensitive DPC-inducing agents, and

accumulate non-specific and Topoisomerase-related DPCs due to impaired protease activity. SPRTN can degrade DNA-bound Topoisomerase, histones and itself in DNA-dependent manner. It can also travel with the replication fork, removing DPCs as DNA replication occurs. Its protease activity is tightly regulated and depends on its DNA binding, autocleavage and ubiquitination. Both single stranded and double stranded DNA can activate SPRTN (Lopez-Mosqueda et al., 2016; Stingele et al., 2016; Vaz et al., 2016; Zhang et al., 2020).

Acidic-repeat containing protein or germ cell nuclear antigen (ACRC/GCNA) is an evolutionary highly conserved protease that regulates genome stability in humans, zebrafish, flies and worms. It consists of an intrinsically disordered region (IDR) and a protease-like SprT domain. ACRC interacts with TOP2 and minichromosome maintenance (MCM), which are involved in replication. In *acrc* mutants, there are increased levels of TOP2 and MCM, which are likely trapped (Bhargava et al., 2020). In a recent paper published on bioRxiv, Otten et al. showed that in zebrafish, *acrc* is an active protease and that *acrc* mutants have increased levels of DPCs, thereby suggesting that Acrc is directly involved in DPCR (Otten et al., 2023).

SPRTN, ACRC and VCP/p97 are crucial for DPC repair but they do not completely remove DPCs and there is still a peptide attached to DNA. These proteins require cooperation with other repair mechanisms to fully resolve DPCs (van den Boom et al., 2016; Larsen et al., 2019; Dokshin et al., 2020). In this thesis I determined whether *p97a*, *tex264a* and *tex264b*, *sprtn* or *acrc* gene expression are changed in *tdp2b* mutant embryos, as an indication of their implication in *tdp2b*-mediated DPCR. These proteins could be necessary to remove the bulk of the protein part of the DPC, before Tdp2 can cleave the rest. It remains to be determined whether P97a, Sprtn and/or Acrc function in the same repair pathway as Tdp2b in zebrafish, and in which cellular context, such as germ cells, neurons, during replication and/or transcription etc.

1.4. Topoisomerases

DNA topoisomerases are essential in life, addressing many challenges posed by the extensive length of the human DNA double helix – approximately 3 billion base pairs. DNA must be intricately folded, bent and compacted, but still be accessible to RNA and DNA polymerases. Each human cell has between 100 and 1,000 copies of mitochondrial DNA (mtDNA), which is about 16,000 bp long. Furthermore, cells have folded RNA molecules that are even more prevalent than DNA (Pommier et al., 2022).

The family of human topoisomerases includes six enzymes: type IB (TOP1 and mitochondrial TOP1), type IIA (TOP2A and TOP2B) and type IA topoisomerases (TOP3A and TOP3B). These enzymes are used often redundantly, but also specifically, depending on the topological challenge, on the differentiation state of the cell and other nearby cellular structures (Pommier et al., 2022).

DNA and RNA undergo three-dimensional transformations mediated by topoisomerases during the opening of a double helix in transcription and replication. The genome is very long and attached to the nuclear scaffold, therefore the DNA is not able to freely rotate so during the opening of a double helix, which results in supercoiling (Figure 8). DNA can be over twisted which is then called positive supercoiling (Sc+) and it happens ahead of motor proteins, and under twisted, behind motor proteins, which is called negative supercoiling (Sc–). This extreme torsional twist causes the DNA or RNA duplex to wind around itself forming entanglements and crossovers. Type IB and type IIA topoisomerases remove both supercoilings until the strain is gone. Type IA topoisomerases relax hyper-negative supercoiling since they can create complexes around ssDNA or ssRNA (Pommier et al., 2022).

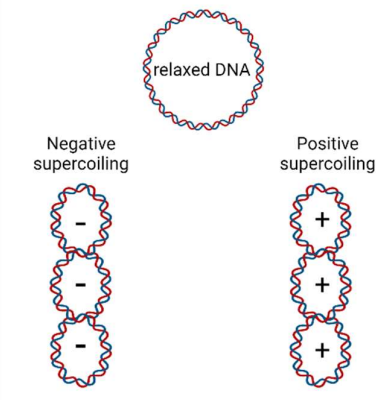

Figure 8. DNA supercoiling (adapted from Jian & Osheroff, 2023). Created in BioRender.

Topoisomerases are essential enzymes that manage the opening of the double helix by creating temporary breaks in the DNA. However, these DSBs pose a significant risk for mutations particularly when topoisomerases become trapped on DNA due to chemical inhibitors or DNA damage, leading to the formation of DPCs. Thus, it is important to monitor and repair these DPCs to prevent mutations and maintain genomic stability (Sun, Saha et al., 2020).

1.5. Repair mechanisms of Topoisomerase-1 and Topoisomerase-2 DNA-protein crosslinks

A TOPcc is a catalytic intermediate of Topoisomerase bound to DNA. It is self-repairing since the deoxyribose hydroxyl end of the cut DNA acts as nucleophile towards the tyrosyl-DNA phosphodiester bond. To efficiently reseal the damaged DNA, it needs to align within TOPcc with the topoisomerase. If resealing fails, TOPccs become DNA-protein crosslink (TOP-DPCs) which are stalled irreversibly and need DNA repair to resolve them. This trapping mechanism can be chemically induced (e.g. by camptothecin for TOP1 and etoposide for TOP2), and is used as a treatment in many cancers and bacterial diseases, since the cell cycle is then interrupted and the cell dies (Pommier et al., 2022). Both TOP1cc and TOP2cc have two-step repair mechanisms which involve first proteolytic degradation of TOPcc by SPRTN or other proteases, and then removal of the remaining crosslinked peptides by TDP enzymes (Antičević et al., 2023b). TDP1 and TDP2 repair TOP1cc and TOP2cc, respectively. Neither Tdp1 or Tdp2 can remove intact Top1 or Top2 proteins without prior proteasomal degradation (Zhang et al., 2020). Interestingly, other proteins have also been proposed to be involved in the repair of TOP2cc, and it is the focus of this work to investigate their relationship to *tdp2*, whether they act together or in parallel pathways.

1.5.1. TDP1

TDP1 excises the TOP1-derived peptide with its 3'-tyrosyl-DNA phosphodiesterase (3'-TDP) activity, which operates independently of divalent metal ions. TDP1 has a histidine which targets the 3'-phosphotyrosyl bond of DNA and creates a covalent bond with the 3'-phosphate and releases the TOP1-derived peptide. Another histidine in TDP1 activates a water molecule, which then cleaves the covalent bond between the histidine and the 3'-phosphate, freeing the DNA from TDP1 (Figure 9). TDP1 exhibits a wide array of DNA repair functions and is considered a promising target for anticancer drugs (Shimizu et al., 2023).

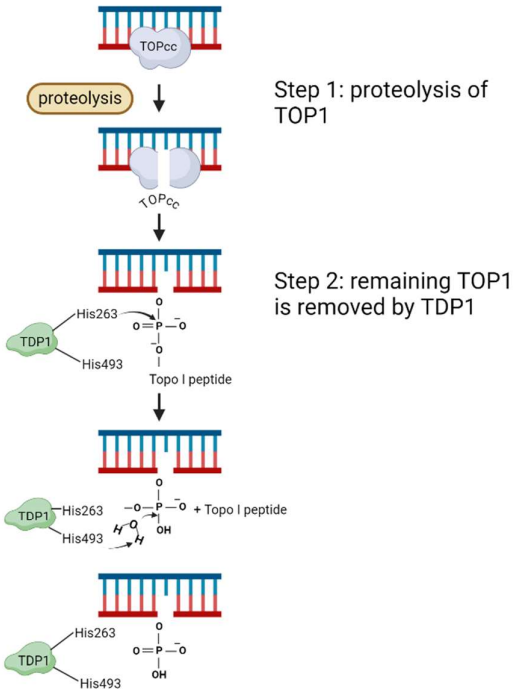

Figure 9. Tdp1 activity in TOP1cc removal (adapted from Kawale & Povirk, 2018). Created in BioRender.

1.5.2. TDP2

Tyrosyl-DNA phosphodiesterase 2 (Tdp2) is crucial for repairing TOP2ccs due to its strong 5'-tyrosyl-DNA phosphodiesterase (5'-TDP) activity thereby contributing to resistance against the chemotherapeutic chemical etoposide (Shimizu et al., 2023). For its catalytic function, TDP2 needs two divalent metals and creates 5'-phosphate ends, which ligases then process (Figure 10).

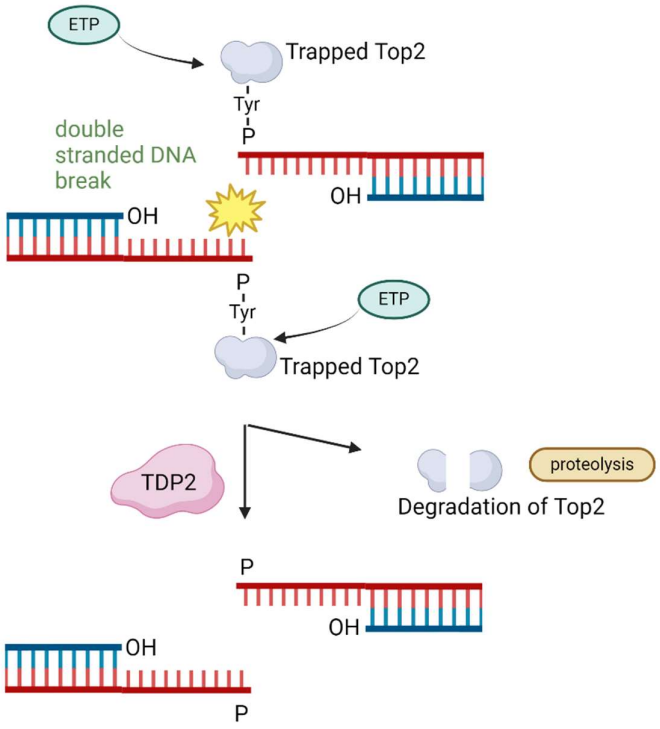

Figure 10. Tdp2 activity in TOP2cc removal (adapted from Zagnoli-Vieira & Caldecott, 2017) Created in BioRender.

TDP2 is an evolutionarily ancient protein and can be found across all forms of life, although it was lost in yeasts over time (Ledesma et al., 2009). Approximately 320 million years ago, a genome duplication event occurred in teleost resulting in two paralogs for genes usually only existing in one form. Because of that, zebrafish (*Danio rerio*) harbours two TDP2 paralogs: *tdp2a* and *tdp2b.* Sequence analysis indicates that Tdp2b is more similar to TDP2 in human and other vertebrates, but both Tdp2a and Tdp2b share the same domains as human TDP2 (Figure 11) (Antičević& Otten, 2024).

Both human TDP2 and its zebrafish orthologs have an N-terminal non-canonical ubiquitin-associated (UBA) domain and a C-terminal exonuclease/endonuclease/phosphodiesterase (EEP) domain, four catalytic motifs and four residues that create a magnesium coordination site (Schellenberg et al., 2012).

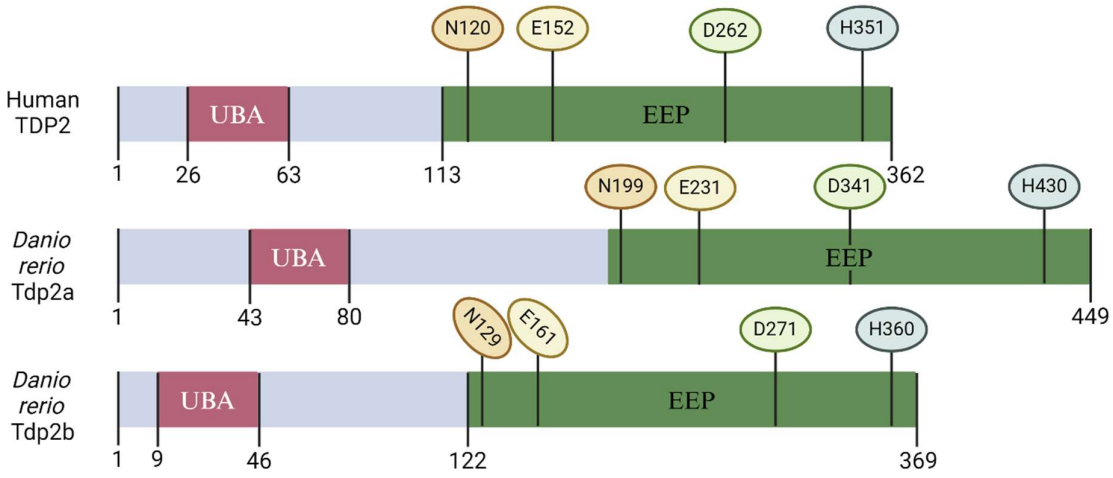

Figure 11. Human TDP2, *Danio rerio* Tdp2a and *Danio rerio* Tdp2b comparison (adapted from Antičević& Otten, 2024). Created with BioRender.

In research conducted in the Popović group, it was discovered that during zebrafish embryonic development, *tdp2b* exhibited higher expression than *tdp2a.* Moreover, silencing of *tdp2b* in zebrafish embryos was sufficient for a reduction in total TDP2 activity, whereas silencing of *tdp2a* did not have any measurable effect on TDP2 activity (Antičević& Otten, 2024).

In an older study using *TDP2* knock-out mouse models, it was shown that TDP2 could compensate for loss of TDP1 and vice versa, but these animals still had neurological defects, which indicates that TDP1 and TDP2 have overlapping but distinct functions (Takashima et al., 2002). This was confirmed in the zebrafish embryos, where overexpression of Tdp2b did not repair the *in vitro* model Top1cc (Antičević& Otten, 2024).

Nevertheless, due to the low level of expression of *tdp2a* in embryos, it could still play a role in DPCR, but on a small scale. The importance of Tdp2b in overall TDP2 activity was further verified in this work, where I investigated whether the *tdp2a* and *tdp1* genes were upregulated in *tdp2b* mutants, as part of a mechanism of adaptation to loss of *tdp2b* function.

Recently, the Popović group also showed that *tdp2* silencing leads to accumulation of DPCs ranging from 10 to 250 kDa, which is comparable to DPCs induced by formaldehyde (FA) treatment (Antičević& Otten, 2024). The broad range of sizes of DPCs (>150 kDa – 10 kDa) found in embryos with silenced *tdp2b* indicates that Tdp2b can repair DPCs other that just

Top2cc, which have a size of 176 kDa. This means Tdp2 has an important role in the repair of DPC beyond Top2 DPCs (Antičević& Otten, 2024).

In addition to its function in DNA crosslink repair, TDP2, also known as TTRAP or EAP II, is involved in Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling, mitogen activated protein kinase-extracellular-signal-regulated kinase (MAPK-ERK) signalling and Human Immunodeficiency Virus (HIV-1) integration (Pype et al., 2000; Zhang et al., 2009; C. Li et al., 2011).

In mouse, TDP2 deficiency resulted in substantial alterations in gene expression with over 100 downregulated genes in neurons lacking the TDP2 gene in comparison to wild-type neurons (Gómez-Herreros et al., 2014). Around 50 of these genes are linked to development of epilepsy and ataxia. In humans, mutations in *TDP2* can lead to the genetic disorder Spinocerebellar ataxia autosomal recessive type 23 (SCAR23) that results in intellectual disability, ataxia and epilepsy (Zagnoli-Vieira et al., 2018, Errichiello et al., 2020). Adult *Tdp2* knock-out mice exhibit phenotypes like weight loss because of intestinal damage and lymphoid toxicity following etoposide treatment (Gómez-Herreros et al., 2013).

Zinc Finger Protein 451 (*znf451*) is a small ubiquitin-like modifier (SUMO) ligase, and a versatile DNA repair factor also called Zinc Finger Protein Associated with TDP2 And TOP2 (ZATT). The SUMO ligase activity of ZNF451 enhances the interaction between TDP2 and SUMOylated TOP2, indicating a ZATT-TDP2 catalysed pathway in TOP2cc repair. Moreover, ZNF451 binds to TDP2 and enables its TOP2 hydrolase activity without any proteasome activity. ZNF451 also affects cellular response to TDP2-independent TOP2 repair. This ZATT-TDP2 regulated pathway may help tumours adapt during chemotherapy with TOP2 poisons, making it a promising target for improved chemotherapy (Schellenberg et al., 2012).

1.6. Zebrafish (*Danio rerio*) as an animal model

Zebrafish *(Danio rerio)* is valuable animal model for studying processes and mechanisms conserved between humans and other vertebrates. Introduced as an experimental model in the 1980ies, zebrafish research has since been supported by the development of numerous tools that facilitate the handling and manipulation of zebrafish, particularly during early developmental stages (Veldman & Lin, 2008).

Zebrafish has multiple advantages as a model organism over larger vertebrates like mice. For example, zebrafish can breed year-round, with females producing hundreds of eggs weekly. The embryos hatch within two days of fertilization and begin feeding at five days post fertilization (dpf). Within those first 5 days of development, most organs such as heart, brain or liver have formed, which allows for the study of many signalling pathways or cellular processes during embryonic development. Extra-uterine development and the transparency of zebrafish embryos are unique advantages compared to mouse, as it allows genetic or pharmacological manipulations (e.g. creation of mutant and transgenic lines), as well as direct observation of embryonic development (Veldman & Lin, 2008). With genome sizes ranging from 20% to 40% of those found in mammals, zebrafish are unique vertebrates for mutagenesis studies. Genetics techniques used in zebrafish research include the creation of mutant lines using CRISPR/Cas9, of transgenic lines, and knocking-down gene expression using morpholino antisense oligonucleotides (Lin et al., 2016). Morpholinos, typically 25 subunits in length, are composed of nucleic acid bases, but instead of ribose or deoxyribose rings, they have morpholine rings (Summerton & Weller, 1997).

Zebrafish share significant genomic, molecular and anatomical similarities with mammals, making them a useful model for comparative studies and they exhibit phenotypes that recapitulate aspects of human diseases, such as cardiovascular and neurological diseases (Shin & Fishman, 2002; Best & Alderton, 2008; Veldman & Lin, 2008; Chen et al., 2012).

Zebrafish are successfully used to study developmental biology, including heart (Walsh & Stainier, 2001) and muscle development (Chen et al., 2003), ocular and nerve development (Ma et al., 2001), microRNAs (miRNAs) (Djuranovic et al., 2011). However, they are missing specific anatomical structures (lungs, limbs, mammary glands) and their genome contains duplicated genes, which can occasionally complicate genetic studies (Liu et al., 2012; d'Amora & Giordani, 2018).

The *tdp2b*$^{rbi10/rbi10}$ mutant fish line used in this study was created in the Popović lab and described in (Antičević, 2023a). This fish line was created targeting the second exon of *tdp2b* using the guide RNA/CRISPR-associated protein 9 (gRNA/Cas9) system. One of the resulting alleles which was selected, *rbi10,* had a frameshift and a premature stop after 95 amino acids (Tdp2b normally has 383 amino acids), thereby effectively resulting in a loss of function mutant line (Figure 12) (Antičević, 2023a).

Mutant fish were monitored, they are viable and fertile and had no visible phenotype (Figure 13) (Antičević, 2023a).

Figure 12. Amino acid sequence of Tdp2b in WT and *tdp2b*$^{rbi10/rbi10}$ mutant zebrafish. X indicates a STOP codon (source Antičević, 2023a).

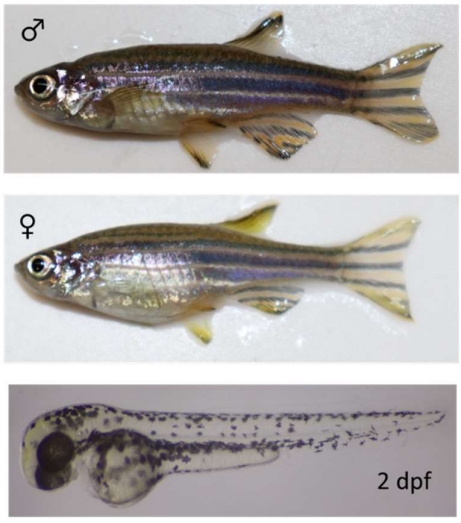

Figure 13. Zebrafish *(Danio rerio).* Adult male and female fish and embryo at 2 dpf (source Antičević, 2023a).

In conclusion, zebrafish as a model organism is a useful tool in genetic research in spite of some limitations. In this thesis I investigated the expression of genes involved in DNA-protein crosslink repair in zebrafish (*Danio rerio*) carrying a mutation in the *tyrosyl-DNA phosphodiesterase 2b* gene.

Aim

The aim of this study is to investigate the changes in expression levels of genes relevant for DPCR in *tdp2b*$^{rbi10/rbi10}$ zebrafish mutants: tyrosyl DNA phosphodiesterase 1 (*tdp1*), tyrosyl DNA phosphodiesterase 2 a (*tdp2a*), *sprtn* (sprt-like N-terminal domain), *acrc* (acidic-repeat containing protein), *tex264a* (Testis Expressed 264 a), *tex264b* (Testis Expressed 264 b), *mre11a* (meiotic recombination 11 a), *apex1* (Apurinic/Apyrimidinic Endodeoxyribonuclease 1), *apex2* (Apurinic/Apyrimidinic Endodeoxyribonuclease 2), *mus81* (Crossover junction endonuclease), *znf451* (Zinc Finger Protein 451) and *p97a* (or *vcp*, valosin-containing protein). The object of my study was the *tdp2b*$^{rbi10/rbi10}$ zebrafish mutant model previously created in the Popović lab and described by Ivan Antičević (Antičević, 2023a).

3. Materials and methods

3.1. Materials

3.1.1. Biological materials

I used zebrafish (*Danio rerio*) embryos derived from WT and *tdp2b*$^{rbi10/rbi10}$ mutant parents which were created in the Laboratory for Molecular Ecotoxicology at the Ruđer Bošković Institute for qRT-PCR analyses of DPCR gene expression.

3.1.2. Non – biological materials

Table 1. Standard chemicals used in this study

CHEMICAL	SOURCE	CAT. NO.
96% ethanol	Kemika, Croatia	505655
Agarose	Sigma-Aldrich, Germany	A9535
DNase/RNase-free water	Invitrogen, USA	10977035
Gel Loading Dye Purple (6X)	New England Biolabs, USA	B7024S
Quick-Load Purple 1kb Plus DNA Ladder	New England Biolabs, USA	N0550G

Table 2. Commercial Bio kits used in the study

MOLECULAR BIOLOGY KIT	SOURCE	CAT NO.
GoTaq qPCR Master Mix	Promega	A6001
Monarch PCR & DNA Cleanup Kit	New England Biolabs, USA	T3010S
Monarch Total RNA Miniprep kit	New England Biolabs, USA	T2010S-50

ProtoScript II First Strand cDNA Synthesis Kit	New England Biolabs, USA	E6560l
qScriber™ cDNA Synthesis Kit	HighQu	RTK0101

Table 3. Oligonucleotides used for qPCR in the study. The *tex264b* gene is only identified in ENSEMBL under the name ENSDARG00000087939.

OLIGONUCLEOTIDE NAME	**SEQUENCE**	**TARGETED GENE ID (NCBI)**
DrAcrc-F-qPCR	5'ACATTGTTAAAGAGGATCGGTAGT3'	541448
DrAcrc-R-qPCR	5'TCTGGTCTTCTTCAGGCTCT3'	541448
DrApex1-F-qPCR	5'AAGAACGGACTTGATTGGGT3'	406730
DrApex1-R-qPCR	5'AGAGCTTTCTCAGCACACTT3'	406730
DrApex2-F-qPCR	5'TCCTGGATTCATTTGATGCGG3'	393115
DrApex2-R-qPCR	5'CAGGTCGCGGGTAACTTTG3'	393115
DrAtp5po-F-qPCR	5′CTTGCAGAGCTGAAAGTGGC3′	335191
DrAtp5po-R-qPCR	5′ACCACCAAGGATTGAGGCAT3′	335191
DrMre11a-F-qPCR	5′ATAAACCGTCCCGCAAGACC3′	406637
DrMre11-R-qPCR	5′CATGCACACTGAAGATAGGGAT3′	406637
DrMus81-F-qPCR	5′GAGAGAGTGACACCTGTTCC3′	406839
DrMus81-R-qPCR	5′GAAGCGTCCGTCTATAATGCT3′	406839
Drp97a-F-qPCR	5'TTGCACCCAAACGAGAGAAG3'	327197
Drp97a-R-qPCR	5'CATGAGCCCTTTGCTTGAGT3'	327197
DrSprtn-F-qPCR	5'AATGACAAGTTCTTCTGGGGG3'	101886162
DrSprtn-R-qPCR	5'AAACACCAGCACATAGCGTCA3'	101886162

DrTdp1-F-qPCR	5'ACAGATGCTCCTGATTTACCCA3'	571485
DrTdp1-R-qPCR	5'TGTGCCGTCTGTATGCTGTA3'	571485
DrTdp2a-F-qPCR	5′CAGAGTCTCTCCAATGTCAATCCA3′	101887157
DrTdp2a-R-qPCR	5’TGGGTGCACTTGGTTTCTGT3′	101887157
DrTex264a-F-qPCR	5′ACCGAAGCTCTCGTCTGTC3′	402960
DrTex264a-R-qPCR	5′GGAGTTCTTCGTCGGGTTTC3′	402960
DrTex264b-F-qPCR	5′GAGTGCAGAGGGTTTATCCA3′	N/A
DrTex264b-R-qPCR	5′GATCGTTCCCCCTCGATAGAT3′	N/A
DrZnf451-F-qPCR	5′GATAATAGTGCAAGCGGCGA3′	557758
DrZnf 451-R-qPCR	5′AGCCTGAATAAATGGAGCCC3′	557758

Table 4. Instruments used in this study

INSTRUMENT	PRODUCER	PURPOSE
Biometra Compact XS/S	Analytik Jena	Gel electrophoresis
BioSpec-nano	Shimadzu	spectrophotometer
Bio Vortex V1	Biosan	vortex
BP6100 Basic Plus Balance	Heraeus	centrifuge
Centrifuge Mikro 120	Hettich, Germany	Sample preparation
Centrifuge Universal 32R	Hettich, Germany	Sample preparation
ChemiDoc XRS+ System	Bio-Rad	Image capture and gel analysis
EMBN 200-2	Kern	Analytical scale
LaminAir HB 2448	Heraeus	Safety workbench
LSE Mini Microcentrifuge	Corning	centrifuge
Real-Time PCR System	Applied Biosystems, USA	qPCR analysis

SoniPrep 150	MSE, UK	sonication
T100 Thermal Cycler	Bio-Rad	incubation
Thermomixer 5436	Eppendorf	termoblock

3.2. Methods

3.2.1. Zebrafish *(Danio rerio)*

Zebrafish (*Danio rerio*), of the AB strain, were originally purchased from the European Zebrafish Resource Centre (EZRC) from Germany. The adult fish were kept at a stable temperature of 28°C with the light cycle of 14 hours and 10 hours of dark cycle. Water quality, temperature, pH and conductivity were monitored daily. The following lines were used: WT and the *tdp2b*$^{rbi10/rbi10}$ mutant line which was created in in the Laboratory for Molecular Ecotoxicology at the Ruđer Bošković Institute (Antičević, 2023a), which harbours a mutation resulting in a premature stop, thereby leading to a *tdp2b* loss of function. Nevertheless, the adults *tdp2b*$^{rbi10/rbi10}$ mutants are viable and were incrossed to obtain the samples used in this study. In the pools of adult fish, I picked the most representative individuals and set up five pools of WT and five pools of *tdp2b*$^{rbi10/rbi10}$ mutants with at least one female and one male fish in each pool. I put up a partition between male and female fish until the next morning to let the fish acclimatise to the new environment. I removed the partition so the fish could mate. After 2 to 4 hours, I collected the eggs and put them in E3 media (composed of 5mM sodium chloride (NaCl), 0.17 mM potassium chloride (KCl), 0.33 mM calcium chloride ($CaCl_2$), and 0.33 mM magnesium sulphate ($MgSO_4$)) in petri dishes at 28°C until they reached 2 days post-fertilisation (2 dpf). All procedures and experiments adhered to the EU Guide for the Care and Use of Laboratory Animals, Council Directive (86/609/EEC), and the Croatian Federal Act on the Protection of Animals (NN 135/06 and 37/13), under the project license HR-POK-023.

3.2.2. Zebrafish tissue RNA extraction and reverse transcription

To create cDNA that I used in quantitative real-time polymerase chain reaction (qPCR), I reverse transcribed RNA extracted from zebrafish tissue. Zebrafish tissues were pooled together for the qPCR analysis: at 2 dpf I collected 10 embryos per sample, obtaining three WT samples

and five mutant *tdp2b*$^{rbi10/rbi10}$ samples. RNA isolation was performed using Monarch Total RNA Miniprep Kit (NEB, T2010L). In each sample I added protect reagent prepared according to the manufacturer's instructions. I did sonication on tissue samples using SoniPrep150 at high intensity, applying it for 5 seconds on and then putting sample on ice for 10 seconds, repeated once. Afterwards, I added Proteinase K (Prot K), incubated the samples for 5 minutes on 55°C and then proceeded according to manufacturer's instructions, including DNase I treatment for further removal of residual gDNA. Lastly, I eluted the purified RNA samples with 30 µl water each. I checked the RNA concentrations using a Nanodrop spectrophotometer and stored them at -20°C.

The RNA samples were reverse transcribed using the ProtoScript II First Strand cDNA Synthesis Kit (NEB, E6560L). Following manufacturer's instructions, I reverse transcribed total RNA using random hexamer primers (RH primers) mixed 1:1 with oligodT on 500 ng RNA each. Since we ran out of protoscript, I used qScriber™ cDNA Synthesis Kit (HighQu, RTK0101) following manufacturer's instructions on three sets of WT and mutants using 1000 ng RNA each.

3.2.3. Primer design for *mus81*

To analyse the expression of genes of interest, it is necessary to design primers that enable their specific amplification. For this project, I designed primers for the zebrafish gene *mus81*, since they were not yet available in the Laboratory of Molecular Ecotoxicology. Therefore, I used the gene sequences from zebrafish available in the Ensembl database and the online tool Primer-BLAST (NCBI), which allows the input of parameters to precisely define the properties of the primers. First, I found the gene of interest, *mus81,* in the Ensembl database. Then I chose the most conserved transcript, which has the longest coding sequence (2071 base pairs) and is highly expressed. Using the online tool Primer-BLAST (NCBI), I defined primer parameters: size of the amplicon: 70-250bp, melting temperature: 55-58-63°C, exon junction match: 7-4-8, intron length range: 1000-10000, primer pair specificity database Refseq mRNA and exclusion organism: *Danio rerio*. To prevent accidental amplification from genomic DNA that may remain in the sample after RNA isolation, I chose primers overlapping with two exons or encompassing an exon-intron boundary. Alternatively, exons to which the primers bind should be separated by intronic sequences larger than 1kb. Finally, I checked for potential self-complementarity and dimer formation using the online tool Oligo Calc (Kibbe, 2007).

3.2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative PCR (qPCR) is a technique that integrates the amplification of a target DNA sequence with the measurement of its concentration during the reaction (Dymond, 2013). A real-time PCR readout is expressed as the number of PCR cycles, known as the "cycle threshold" (Ct), required to reach a specified fluorescence level (Ponchel et al., 2003). I performed qRT-PCR experiments using the *GoTaq qPCR mix (PROMEGA, A6001)* kit and the AB73OO Real-Time PCR System (Applied Biosystems). The primers used (see Table 3) were designed in the Laboratory for Molecular Ecotoxicology in the group of dr. sc. Popović. I determined the expression of the following genes: *sprtn* (sprt-like N-terminal domain) (Antičević et al., 2023b), *acrc* (acidic-repeat containing protein), *tdp1* (tyrosyl-DNA phosphodiesterase 1) (Antičević et al., 2023), *tdp2a* (tyrosyl-DNA phosphodiesterase 2a) (Antičević et al., 2023b), *tex264a* (Testis Expressed 264 a), *tex264b* (Testis Expressed 264 b), *mre11a* (meiotic recombination 11 a), *apex1* (Apurinic/Apyrimidinic Endodeoxyribonuclease 1), *apex2* (Apurinic/Apyrimidinic Endodeoxyribonuclease 2), *mus81* (Crossover junction endonuclease), *znf451* (Zinc Finger Protein 451), *p97a* (or *vcp*, valosin-containing protein). The housekeeping gene *atp5po* (ATP Synthase Peripheral Stalk Subunit OSCP) was used for normalisation as previously described (Antičević et al., 2023b). All primers, including the newly designed primers for *mus81*, were designed to amplify all transcript isoforms of the gene. I designed and tested primers for the *mus81* gene, and tested the *znf451* and *mre11a* primer pairs that were previously designed (see Table 3). The expression of the 12 DPCR genes was determined in technical triplicates for each sample.

3.2.5. Testing the primers

After designing and ordering primer pairs for *mus81* and ordering the primer pairs for *mre11a* and *znf451* I set up the qRT-PCR experiment in which I determined the efficiency of chosen primer pairs. I used cDNA from previously extracted and reverse transcribed RNA from WT fish bred in the Popović lab. Then I prepared a series of dilution standards to establish a standard curve, ensuring that primers work effectively across a range of concentrations without reaching a plateau too early. The first concentration I used in a serial dilution was 40 ng/µL (20 ng/reaction), following with 8 ng/µL, 4 ng/µL, 2 ng/µL, 1 ng/µL, 0,6 ng/µL, 0,4 ng/µL and no-

template control (NTC). I ran technical triplicates to ensure the reproducibility of the measurements. I used Ct values from the qRT-PCR analysis and created standard curve of the regression. Then, I defined the slope of the standard curve and using the equation $Eff = (10^{-1 \div slope} - 1) \times 100$ defined the efficiency of primer pairs.

3.2.6. Statistical Analysis

After completion of the qRT-PCR reactions I defined the thresholds and determined Ct values for each reaction using the 7500 Fast System Software (Applied Biosystems) and exported the data in Microsoft excel (Microsoft Corporation, 2018). For each gene, I calculated the average for the Ct values of the technical triplicates for each of the three biological triplicates for both WT and *tdp2b*$^{rbi10/rbi10}$ mutants. Then, I analysed the relative gene expression levels for each gene using the corresponding Ct values determined for the housekeeping gene *atp5po* for those samples. Then, I determined the average of the three biological triplicates for WT and *tdp2b*$^{rbi10/rbi10}$ mutants. I used the Q-Gene method (Simon, 2003) to perform quantification and calculated mean normalized error (MNE) based on the primer efficiencies (E) and mean Ct values for both the housekeeping gene (Ct(HKG)) and the target gene (Ct(gene)), using the equation: $\boldsymbol{MNE = E(HKG)^{Ct(HKG)} / E(gene)^{Ct(gene)} \times 10^6}$ as previously described (Popovic et al., 2012). For calculations I used Microsoft excel (Microsoft Corporation, 2018). I further analysed fold change of gene expression levels relative to wild-type samples using the GraphPad (Prism) software, with 2-3 biological replicates per condition. Significance was established when the differences between two independent variables had a p-value of less than 0.05. I presented data as mean values ± standard deviation (SD). I performed Student's t-tests to determine gene expression changes in *tdp2b*$^{rbi10/rbi10}$ mutant embryos at 2 dpf.

4. Results

4.1. The newly designed *mre11a, znf451 and mus81* primer pairs are suitable for qRT-PCR analysis

After designing the primers for *mus81* as described in materials and methods (chapter 3.2.3.), I tested their efficiency by performing qRT-PCR reactions on a serial dilution of 2 dpf wild-type zebrafish cDNA, starting with 20 ng per reaction in technical triplicates. Subsequently, I determined the Ct values and generated standard curves, from which I determined the primer efficiencies. Mean Ct values, sample quantity and logarithmic value of sample quantity are shown for the primers amplifying *mre11a, mus81* and *znf451* in Tables 5, 6 and 7, respectively. Standard curves are shown in Figures 14, 15 and 16.

Table 5. Triplicate logarithmic values of sample quantity, sample quantity, Ct values, mean Ct values from qRT-PCR reactions using a primer pair amplifying *mre11a*. Values in *italics* are excluded from the efficiency calculation because they are technical pipetting mistakes

log (sample quantity)	1.301	1.000	0.699	0.398	0.176
Sample quantity (ng/well)	20	10	5	2.5	1.5
Ct value	26.5534	27.955	28.6684	29.4997	30.8673
	26.35	27.3613	28.86	29.4104	*30.0118*
	26.4114	27.4479	28.7545	29.1857	30.6042
Mean (Ct value)	26.44	27.59	28.76	29.37	30.74

Table 6. Triplicate logarithmic values of sample quantity, sample quantity, Ct values, mean Ct values from qRT-PCR reactions using a primer pair amplifying *mus81*. Values in *italics* are excluded from the efficiency calculation because they are technical pipetting mistakes

log (sample quantity)	1.301	1.000	0.699	0.398	0.176	0
Sample quantity (ng/well)	20	10	5	2.5	1.5	1
Ct value	24.0683	*25.3774*	26.4418	*27.7354*	*27.7257*	29.3337
	24.2081	25.0541	26.2873	27.2566	28.1392	29.2038
	24.1962	25.1305	26.2792	27.2314	28.0787	*28.5785*
Mean (Ct value)	24.16	25.09	26.34	27.24	28.11	29.27

Table 7. Triplicate logarithmic values of sample quantity, sample quantity, Ct values, mean Ct values from qRT-PCR reactions using a primer pair amplifying *znf451*. Values in *italics* are excluded from the efficiency calculation because they are technical pipetting mistakes

log (sample quantity)	1.301	1.000	0.699	0.398	0.176
Sample quantity (ng/well)	20	10	5	2.5	1.5
Ct value	24.2207	24.9601	26.1167	27.2209	*27.9771*
	24.2173	25.0185	26.2641	27.4062	27.6114
	24.762	*26.4173*	26.6511	27.5693	28.1617
Mean (Ct value)	24.22	24.99	26.34	27.40	27.89

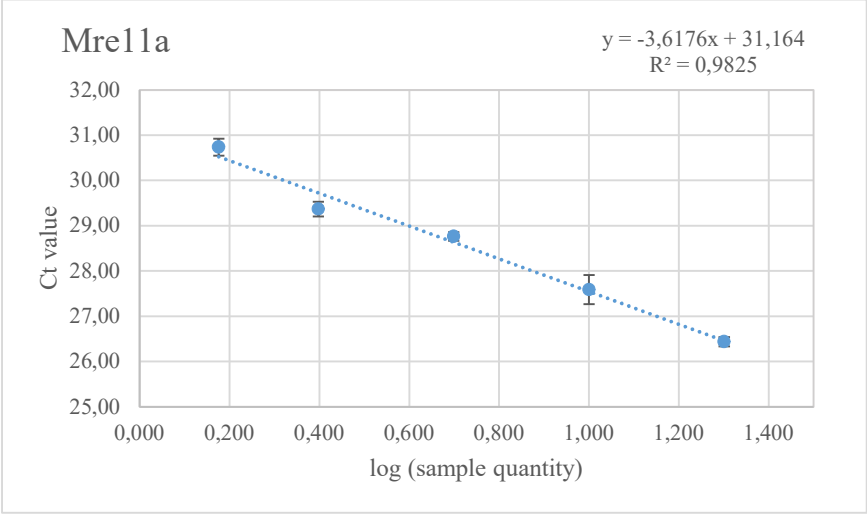

Figure 14. Standard curve of the regression between the average Ct values of triplicates and logarithmic values of cDNA amount for primer pairs of gene *mre11a*.

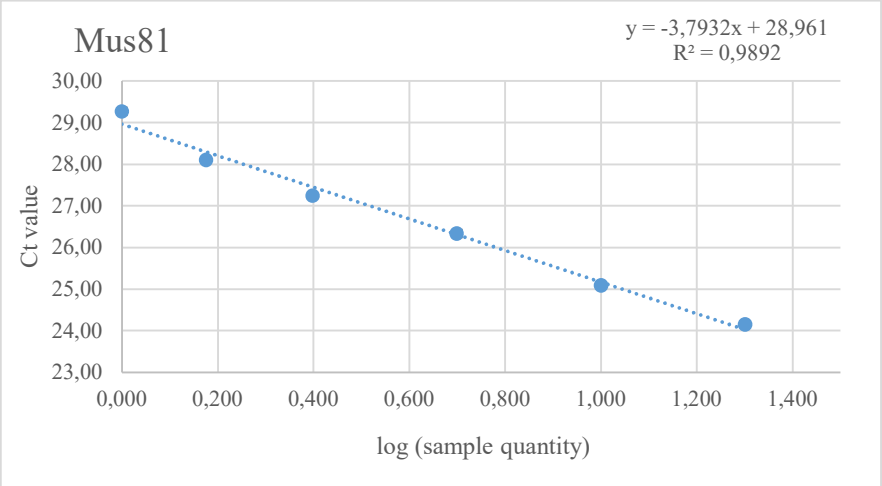

Figure 15. Standard curve of the regression between the average Ct values of triplicates and logarithmic values of cDNA amount for primer pairs of gene *mus81*.

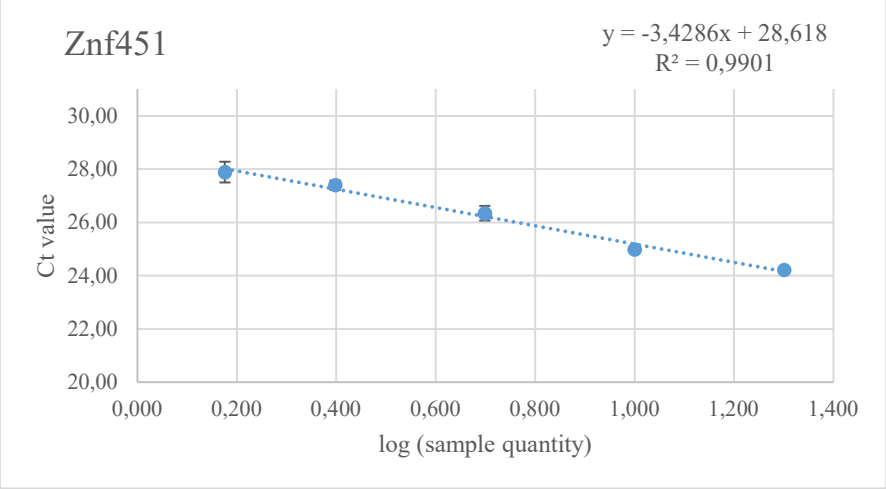

Figure 16. Standard curve of the regression between the average Ct values of triplicates and logarithmic values of cDNA amount for primer pairs of gene *znf451*.

To calculate the primer efficiency, I used the slope value and the equation $Eff = (10^{-1 \div slope} - 1) \times 100$. The primer pair efficiency is 89% for *mre11a*, 83% for *mus81*, and 90% for *znf451* primer pairs. All primer pairs had satisfying values and were used for further analysis. For all three primer pairs, within the chosen range of cDNA quantities (20–1,5 ng/reaction for *mre11a* and *znf451*; 20–1 ng/reaction for *mus81*), there was a linear relationship

between the logarithmic value of the amount of cDNA and the Ct value, which means that gene expression could be quantified.

4.2. The extracted RNA is of suitable quality for subsequent qRT-PCR analyses

To compare gene expression levels between WT and *tdp2b*$^{rbi10/rbi10}$ mutant zebrafish embryos, I set up adult fish for breeding and collected pools of ten eggs each from three different WT clutches and five different *tdp2b*$^{rbi10/rbi10}$ mutant clutches at 2 dpf. I extracted RNA as previously described in materials and methods (chapter 3.2.2.), in order to reverse transcribe it to cDNA, which I used in qRT-PCRs.

To determine the concentration and purity of the extracted RNA, I analysed RNA samples using a spectrophotometer. Results are shown in the Table 8 below.

Table 8. Extracted RNA from WT and *tdp2b*$^{rbi10/rbi10}$ mutant zebrafish samples #1-3 and #1-5, respectively. Shown are concentrations in ng/µl, absorbance ratios at 260 nm / 280 nm and at 260 nm / 230 nm. I repeated the measurements a second time to ensure accuracy and calculated the average concentration of the RNAs.

	1st measurement			2nd measurement			
RNA	Conc. (ng/µl)	260/280	260/230	Conc. (ng/µl)	260/280	260/230	average conc. (ng/µl)
WT 1	170.38	2.12	2.12	167.65	2.12	2.15	169.02
WT 2	187.34	2.13	2.07	187.63	2.07	2.3	187.49
WT 3	191.05	2.14	2.09	191.2	2.13	2.13	191.13
mut 1	150.07	2.17	1.82	156.56	2.15	1.86	153.32
mut 2	193.82	2.15	2.13	193.5	2.16	2.15	193.66
mut 3	117.65	2.14	2.08	123.34	2.14	1.98	120.50
mut 4	168.34	2.3	1.68	176.93	2.3	1.64	172.64
mut 5	168.12	2.27	1.61	168.05	2.3	1.61	168.09

Absorbance ratios are used to assess the purity of nucleic acid samples, with a low 260/280 ratio indicating protein contamination (Fleige & Pfaffl, 2006). All 260/280 ratio measurements shown in Table 8 have values greater than 1.8, which indicates good RNA purity (Sambrook J, 2001). A low A260/230 ratio indicates the presence of organic compounds like phenol or guanidine, which are characteristic of methods utilizing chaotropic lysis buffers. However, a

concentration of up to 100 mM guanidine thiocyanate in RNA samples has been shown not to interfere with downstream molecular applications. Many applications, including cDNA synthesis are not affected by low 260/230 ratio (Nouvel et al., 2021). Therefore, these RNA samples were further used for cDNA synthesis and subsequent qRT-PCR reactions. The cDNA synthesis protocol is described above in chapter 3.2.2.

4.3. Analysis of the expression levels of the following genes in *tdp2b*$^{rbi10/rbi10}$ zebrafish mutants: *sprtn, acrc, tdp1, tdp2a, tex264b, apex1, apex2, p97a, mre11a, mus81* and *znf451*

I determined whether the expression of the following genes was changed in the *tdp2b*$^{rbi10/rbi10}$ embryos samples compared to WT by performing qRT-PCRs using the freshly synthesized cDNA described above and primers specific for the genes *sprtn, acrc, tdp1, tdp2a, tex264a, tex264b, apex1, apex2, p97a, mre11a, mus81* and *znf451*, as well as for the housekeeping gene *atp5po* for normalization, as described in chapter 3.2.4. (materials and methods). I then determined the Ct values for each reaction and calculated the mean normalised expression (MNE) for three biological replicates for each genotype using the Q-Gene method as described in chapter 3.2.6. The MNE values of each biological replicate were analysed statistically using GraphPad Prism 8 as described in chapter 3.2.6, and are shown in the tables 9-20 and in Figures 17, 18 and 19, together with their standard deviation (SD) to show the variation within the samples.

4.3.1. *Tdp2a* and *tdp1* show lower expression in *tdp2b*$^{rbi10/rbi10}$ mutant zebrafish embryos than in WT

First, I investigated whether *tdp2a* (the paralog of *tdp2b)* and/or *tdp1* (another tyrosyl-phosphodiesterase enzyme with partially overlapping function) might be upregulated to compensate for loss of Tdp2b in mutant embryos. Indeed, these two enzymes are functionally the most closely related to *tdp2b* and therefore the most likely candidates for compensation. To determine whether the expression levels of *tdp1* and *tdp2a* were significantly changed in *tdp2b*$^{rbi10/rbi10}$ mutant versus WT embryos, an unpaired t-test was performed for each gene.

Surprisingly, the expression of *tdp2a* was tendentially lower in *tdp2b*$^{rbi10/rbi10}$ mutants than in WT, although not statistically significantly (P = 0.0685) (Table 9, Figure 17). Similarly, the expression of *tdp1* was tendentially lower in *tdp2b*$^{rbi10/rbi10}$ mutants than in WT (Table 10, Figure 17), but the difference was not statistically significant, with a P value of 0.0798. The differences in expression levels are large: *tdp2a* and *tdp1* expressions are 2 fold and 1.5 fold smaller than in WT, respectively, but not significant, likely due to the small number of samples analysed in this study (between n=2 and n=3). Unfortunately, some replicate values had to be excluded from this study for technical reasons. Generally, as can also be seen in the following chapters, there was a large degree of variation between samples of one genotype, so it would be interesting to further extend this analysis with more samples.

Table 9. Mean Normalized Expression (MNE) values for each biological replicate for *tdp2a* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	33315	34082 (SD 1085)
WT #2	34849	
tdp2b$^{rbi10/rbi10}$ #1	19226	17621 (SD 7885)
tdp2b$^{rbi10/rbi10}$ #2	24580	
tdp2b$^{rbi10/rbi10}$ #3	9057	

Table 10. Mean Normalized Expression (MNE) values for each biological replicate for *tdp1* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	20653	17447 (SD 2807)
WT #2	16262	
WT #3	15427	
tdp2b$^{rbi10/rbi10}$ #1	8776	11431 (SD 3469)
tdp2b$^{rbi10/rbi10}$ #2	15356	
tdp2b$^{rbi10/rbi10}$ #3	10162	

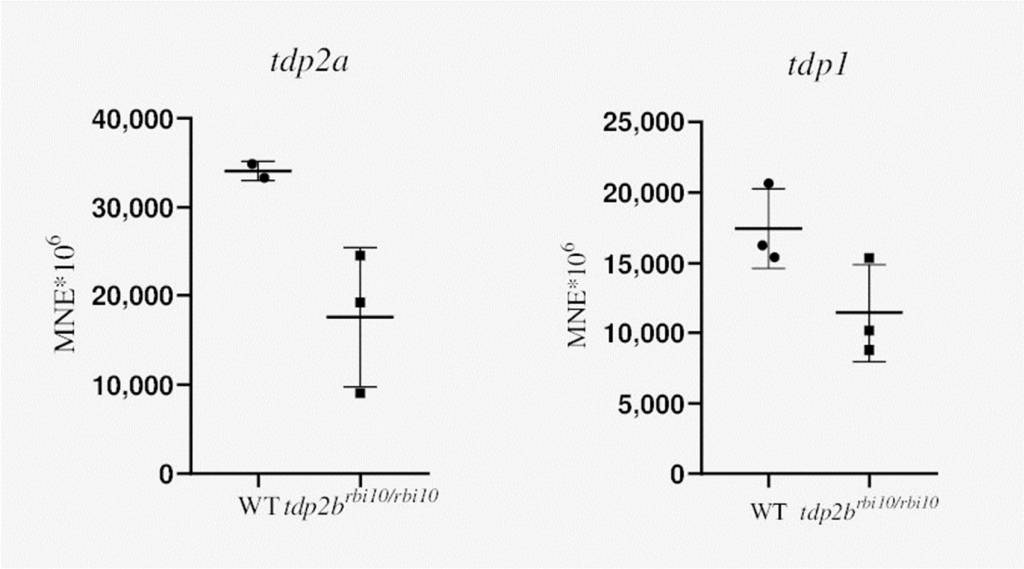

Figure 17. Graphic representation of the level of *tdp2a* and *tdp1* expression in 2 dpf WT and *tdp2b*$^{rbi10/rbi10}$ mutant zebrafish embryos expressed as MNE*10^6. Each graph represents mean normalised expression of 3 biological replicates +/- standard deviation (SD). Statistically significant differences were determined by unpaired t-test (*P < 0.05).

4.3.2. *Znf451* and *mre11a* show lower expression, *acrc* shows higher expression, and *sprtn* shows no change in expression in *tdp2b*$^{rbi10/rbi10}$ mutant zebrafish embryos compared to WT

Next, I performed qRT-PCR analyses to determine the expression levels of the nucleases *znf451* and *mre11a* and of the proteases *sprtn* and *acrc*, which might act together with *tdp2b* in DPC repair (Figure 18). As described above for the *tdp2a* and *tdp1* genes, an unpaired t-test was performed to determine whether their expression levels were changed in *tdp2b*$^{rbi10/rbi10}$ mutant zebrafish compared to WT.

Analysis of MNE values revealed a statistically significant downregulation by more than 2.5-fold of *znf451* expression in *tdp2b*$^{rbi10/rbi10}$ mutants compared to WT, with a P value of 0.0267 (P < 0.05) (Table 11). Similarly, *mre11a* expression was also statistically significantly downregulated by 1.7-fold in *tdp2b*$^{rbi10/rbi10}$ mutants with a P value of 0.0257 (P < 0.05) (Table 12). However, whether this change is functionally significant remains to be discovered, as *mre11a* is still extremely highly expressed in mutants, as compared to the other genes analysed

in this study, with the lowest MNE value of over 76000. *Acrc,* on the other hand, with its very low expression in WT, showed statistically significantly higher expression by 2-fold in *tdp2b*$^{rbi10/rbi10}$ mutants vs. WT, with a P value of 0.0357 ($P < 0.05$) (Table 13). In contrast, the moderate levels of *sprtn* expression were comparable between WT and *tdp2b*$^{rbi10/rbi10}$ mutants, with a P value of 0.6966 ($P > 0.05$) (Table 14). As mentioned above, repeating the measurements with additional samples would increase the robustness of the observed changes, especially as some values vary for example two-fold within genotypes (e.g. *sprtn* in *tdp2b* mutants).

Table 11. Mean Normalized Expression (MNE) values for each biological replicate for *znf451* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	18971	18789 (SD 5496)
WT #2	24192	
WT #3	13205	
tdp2b$^{rbi10/rbi10}$ #1	8833	7345 (SD 1827)
tdp2b$^{rbi10/rbi10}$ #2	7896	
tdp2b$^{rbi10/rbi10}$ #3	5306	

Table 12. Mean Normalized Expression (MNE) values for each biological replicate for *mre11a* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	168646	178133 (SD 11823)
WT #2	174374	
WT #3	191378	
tdp2b$^{rbi10/rbi10}$ #1	76457	100639 (SD 36885)
tdp2b$^{rbi10/rbi10}$ #2	143094	
tdp2b$^{rbi10/rbi10}$ #3	82367	

Table 13. Mean Normalized Expression (MNE) values for each biological replicate for *acrc* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	2307	1792 (SD 476)
WT #2	1368	
WT #3	1700	
tdp2b$^{rbi10/rbi10}$ #1	4286	3570 (SD 866)
tdp2b$^{rbi10/rbi10}$ #2	3816	
tdp2b$^{rbi10/rbi10}$ #3	2607	

Table 14. Mean Normalized Expression (MNE) values for each biological replicate for *sprtn* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	7870	6638 (SD 1742)
WT #2	5406	
tdp2b$^{rbi10/rbi10}$ #1	11501	7763 (SD 3292)
tdp2b$^{rbi10/rbi10}$ #2	6493	
tdp2b$^{rbi10/rbi10}$ #3	5295	

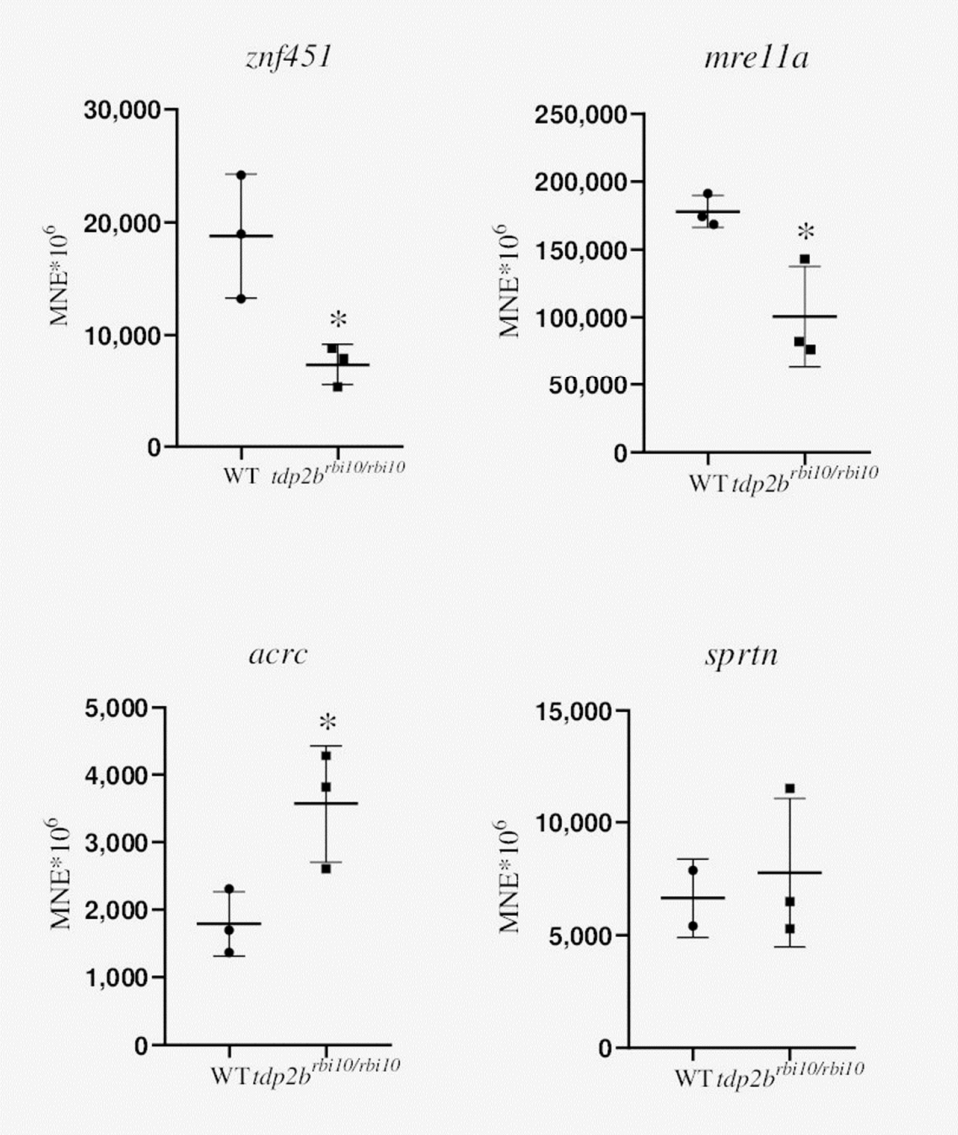

Figure 18. Graphic representation of gene expression in WT and *tdp2b*$^{rbi10/rbi10}$ mutant zebrafish embryos expressed as MNE*10^6. Each graph represents mean normalised expression of 2-3 biological replicates +/- standard deviation (SD). Statistically significant differences were determined by unpaired t-test (*$P < 0.05$).

4.3.3. The expression levels of *tex264a, tex264b, apex1, apex2, p97a* and *mus81* are not significantly changed in *tdp2b*$^{rbi10/rbi10}$ mutant zebrafish embryos compared to WT

Finally, I determined whether the expression levels of other enzymes involved in DPC repair were statistically significantly changed in *tdp2b*$^{rbi10/rbi10}$ mutant embryos compared to WT by performing an unpaired t-test.

The P97/VCP ATPase and one of its cofactors, TEX264, were shown to be involved in the repair of particular DPCs by unfolding them, which allows further degradation of the DPCs by proteases and/or direct resolution of DPCs by TDPs or nucleases (Fielden et al., 2022). As for *TDP2*, Zebrafish also harbours two paralogs of mammalian *TEX264*: *tex264a* and *tex264b*; based on expression levels, it appears that *tex264a* is the most dominantly expressed gene (Figure 19). In this study, *p97a* was very highly expressed, and average expression levels in WT and *tdp2b*$^{rbi10/rbi10}$ mutants were comparable, with a P value of 0,7943. The expression levels of the *tex264a* and *tex264b* genes in WT and mutant *tdp2b*$^{rbi10/rbi10}$ zebrafish were also not significantly different (p-value for *tex264a* = 0.1385; p-value for *tex264b* = 0.3952), but as for *p97a*, there was high variability between biological replicates (Tables 15-17, Figure 19).

Table 15. Mean Normalized Expression (MNE) values for each biological replicate for *p97a* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	165810	120276 (SD 39998)
WT #2	104207	
WT #3	90811	
tdp2b$^{rbi10/rbi10}$ #1	160176	128591 (SD 32713)
tdp2b$^{rbi10/rbi10}$ #2	130740	
tdp2b$^{rbi10/rbi10}$ #3	94856	

Table 16. Mean Normalized Expression (MNE) values for each biological replicate for *tex264a* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	67200	53057 (SD 12396)
WT #2	47895	
WT #3	44076	
tdp2b$^{rbi10/rbi10}$ #1	79347	72087 (SD 12844)
tdp2b$^{rbi10/rbi10}$ #2	57257	
tdp2b$^{rbi10/rbi10}$ #3	79656	

Table 17. Mean Normalized Expression (MNE) values for each biological replicate for *tex264b* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	19389	17414 (SD 1736)
WT #2	16725	
WT #3	16129	
tdp2b$^{rbi10/rbi10}$ #1	20282	19849 (SD 4078)
tdp2b$^{rbi10/rbi10}$ #2	23693	
tdp2b$^{rbi10/rbi10}$ #3	15572	

The endonucleases APEX1, APEX2 and MUS81 have been described to have specific functions in DNA damage repair, as well as in the repair of TOP1cc (H. Zhang et al., 2022) but it is not yet known whether they also have a role in TOP2cc repair. In this study, I found that *mus81*, *apex1* and *apex2* expression levels were not significantly different between WT and *tdp2b*$^{rbi10/rbi10}$ mutants (p-value for *mus81* = 0.7421, p-value for *apex1* = 0.5454; p-value for *apex2* = 0.2262), but that there was high variability between biological replicates.

Table 18. Mean Normalized Expression (MNE) values for each biological replicate for *apex1* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	199794	139370 (SD 52369)
WT #2	107087	
WT #3	111230	
tdp2b$^{rbi10/rbi10}$ #1	118763	118453 (SD 16512)
tdp2b$^{rbi10/rbi10}$ #2	101788	
tdp2b$^{rbi10/rbi10}$ #3	134808	

Table 19. Mean Normalized Expression (MNE) values for each biological replicate for *apex2* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	4759	3855 (SD 782)
WT #2	3407	
WT #3	3400	
tdp2b$^{rbi10/rbi10}$ #1	5448	4714 (SD 685)
tdp2b$^{rbi10/rbi10}$ #2	4091	
tdp2b$^{rbi10/rbi10}$ #3	4602	

Table 20. Mean Normalized Expression (MNE) values for each biological replicate for *mus81* expression, their average and standard deviation (SD).

sample	MNE * 10^6	Average MNE * 10^6
WT #1	144904	121397 (SD 30101)
WT #2	131817	
WT #3	87471	
tdp2b$^{rbi10/rbi10}$ #1	143423	113272 (SD 26185)
tdp2b$^{rbi10/rbi10}$ #2	100152	
tdp2b$^{rbi10/rbi10}$ #3	96240	

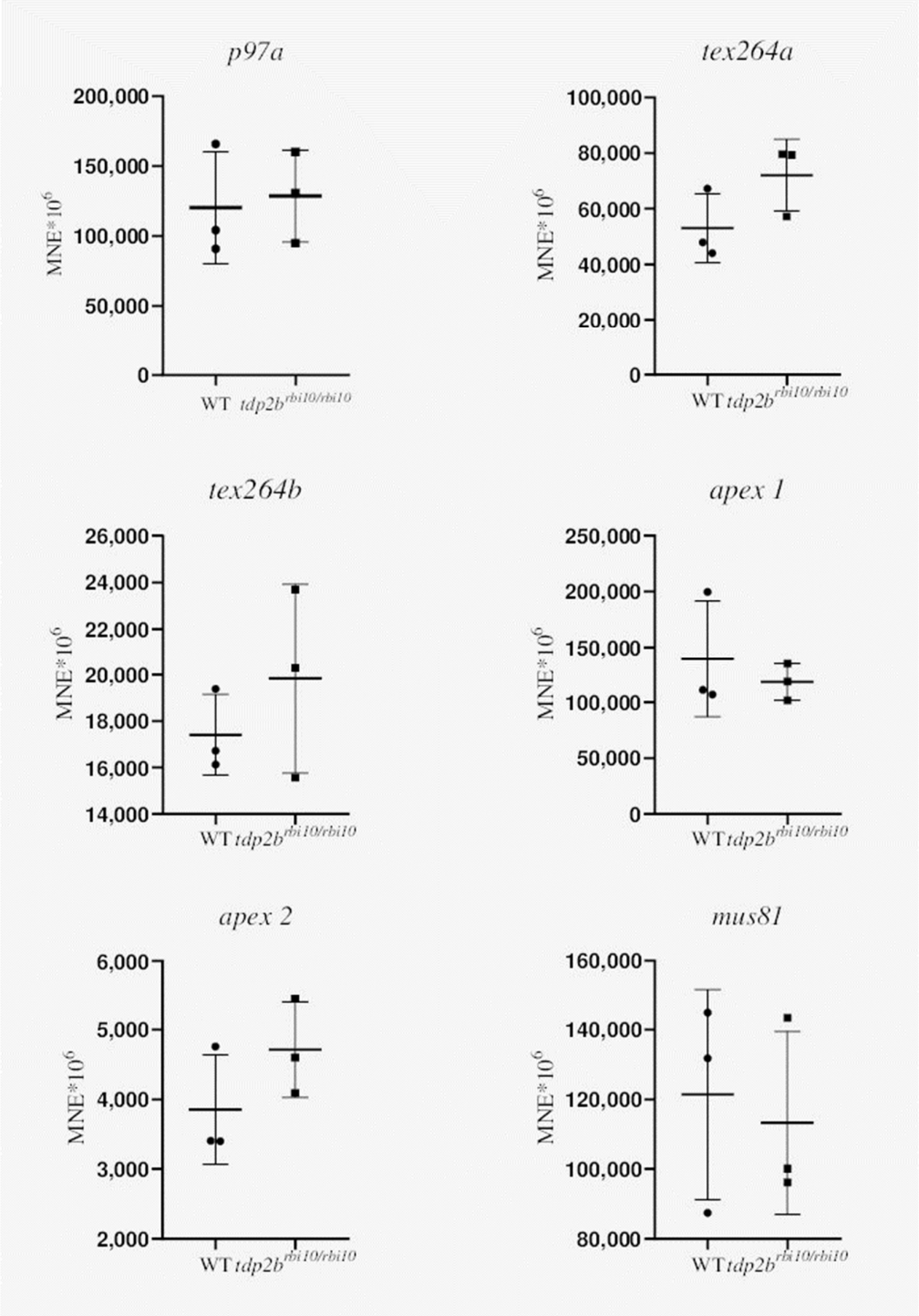

Figure 19. Graphic representation of the level of gene expression in zebrafish embryos expressed as MNE*10^6. Each graph represents mean normalised expression of 3 biological replicates +/- standard deviation (SD).

5. Discussion

DNA repair mechanisms play a crucial role in maintaining genome stability and DPCR is one of the crucial cellular responses to genetic damage. Tyrosyl-DNA phosphodiesterase 2 (TDP2) is an enzyme involved in the resolution of specific DPCs, in particular TOP2-DPCs. The zebrafish (*Danio rerio*) offers unique advantages for studying DPCR, providing insights that complement findings from much simpler cell models. *Tdp2b*$^{rbi10/rbi10}$ zebrafish mutants that were created in the lab of dr. sc. Popović (IRB, Zagreb) using the CrispR/Cas9 technology are viable, undergo normal embryonic development, and no overt phenotypic changes were observed (Antičević, 2023a). In parallel, they created and optimized another zebrafish model in which *tdp2b* expression is silenced in embryos using a specific antisense morpholino oligonucleotide (Antičević& Otten, 2024). Using an *in vitro* activity assay mimicking Topo2cc, they found that Tdp2b, but not Tdp2a, contributed most to overall TDP2 function, using both the morpholino approach and the *tdp2b* mutant (Antičević, 2023a; Antičević et al., 2023b). So far, the Popović lab showed that *tdp2b* silenced zebrafish embryos had an accumulation of TOP2-DPCs resulting in DBS, but this has not yet been shown for the *tdp2b*$^{rbi10/rbi10}$ zebrafish mutants (Antičević& Otten, 2024). Since the *tdp2b*$^{rbi10/rbi10}$ zebrafish mutants are viable, it is possible that those zebrafish mutants have developed compensatory mechanisms that are involved in TOP2-DPC repair and/or in bypassing TOP2-DPC toxicity (Antičević, 2023a). Interestingly, the compensation mechanisms could be non-existent or different in the case of the *tdp2b*-silenced embryos. However, independently of the type of *tdp2* loss of function model, it is also clear that other parallel pathways exist to repair Topo2cc DPCs, such as those involving *mre11a* and *znf451* (Hoa et al. 2016, Schellenberg, 2017). Many other enzymes are involved in DPCR, and in this study, I investigated their expression in *tdp2b*$^{rbi10/rbi10}$ zebrafish mutants.

5.1. Expression analysis of *tdp1* and *tdp2a*

In *tdp2b*$^{rbi10/rbi10}$ zebrafish mutants, both *tdp1* and *tdp2a* exhibited a trend towards lower expression than in WT, but it was not statistically significant. However, findings collected in this experiment differ from previous findings from 2023, which suggest that *tdp2a* might compensate for loss of *tdp2b* in zebrafish embryos as shown by the 2.5-fold increased expression of the *tdp2a* ortholog in *tdp2b* mutants (Antičević, 2023a). In contrast, as seen in those previous findings, *tdp1* levels were not changed in *tdp2b* mutants (Antičević, 2023a). Whether this might be due to technical differences or whether *tdp2a* levels are really changed

will need to be further addressed. From another angle, the functional activity assay recently published using *tdp2a* and *tdp2b* morpholinos revealed that overall TDP2 activity was dependent on Tdp2b, thereby suggesting that Tdp2a cannot functionally compensate for Tdp2b loss of function, irrespectively of expression levels.

5.2. Expression analysis of *znf451*, *mre11a, acrc and sprtn*

In *tdp2b*$^{rbi10/rbi10}$ zebrafish mutants, *znf451* and *mre11a* showed significantly lower expression than in WT. It was discovered that *ZNF451* has a role in resolution of TOP2ccs since it directly binds to TOP2ccs and regulates the recruitment of TDP2, which then hydrolyse the peptide remnant (Schellenberg et al., 2017). MRE11A also has a role in TOP2-DPC removal: it was found that TOP2cc accumulation can cause mortality in *MRE11* mutant cells, and that *TDP2* overexpression in those mutant cells can restore genome integrity (Hoa et al., 2016). Lower *mre11a* expression in zebrafish mutants indicates a possible interdependency between *mre11a* and *tdp2b.* However, like the vast majority of the studies on DPCR, data on the role of ZNF451 and MRE11A in DPCR was obtained from work performed *in vitro* and using cell culture, so it will be interesting in the future to confirm it in an *in vivo* model such as the zebrafish. In the Popović lab, functional studies using morpholinos targeting *znf451* and *mre11a* are being currently performed, and RADAR assays will be performed to determine whether and which types of DPCs are repaired when *znf451* or *mre11a* are silenced.

Acrc is the only gene analysed in this thesis that was upregulated in *tdp2b*$^{rbi10/rbi10}$ zebrafish mutants. It has been suggested that *Acrc* might play a role in TOP2-DPC repair (Dokshin et al., 2020) and recently the Popović group showed that Acrc repairs DPCs, in particular Histone-3 DPCs, as published on bioRxiv (Otten et al., 2023). Since then, detection of zebrafish Top2-DPCs has been optimized and future work will show whether Acrc can also repair Top2-DPCs. The increased expression of *acrc* could be a compensatory mechanism triggered by the loss of *tdp2b* function in DPCR, but also by its role in other pathways.

In contrast to *acrc*, *sprtn* expression levels were similar between *tdp2b*$^{rbi10/rbi10}$ mutants and WT. *Sprtn* is a crucial protease for DPCR and knockdown of *sprtn* leads to DPC accumulation (Stingele et al., 2016; Vaz et al., 2016; Antičević et al., 2023b). Interestingly, it was observed that *sprtn* silencing in both *tdp1* zebrafish mutants and in WT resulted in significantly increased *tdp2b* expression, even in the absence of DPC inducers (Antičević, 2023a). As for *acrc*, future

work using *sprtn* morpholinos will uncover its role in Top2-DPC repair, and epistasis experiment with *sprtn* and *tdp2b* will clarify their possible functional interaction.

5.3. Expression analysis of *tex264a, tex264b, apex1, apex2, p97a* and *mus81*

In this study, I found that the expression levels of *p97a*, *tex264a* and *tex264b* were not significantly different between WT and *tdp2b*$^{rbi10/rbi10}$ mutants. The ATPase P97 has many functions across the cell and therefore required various co-factors, such as TEX264. P97 was shown to unfold DPC proteins before their degradation by SPRTN (Kröning et al., 2022) and to remove the Ku70-Ku80 heterodimer (which is involved in damage recognition in NHEJ) from chromatin at DSB site (van den Boom et al., 2016). Ku70/80 is one of the most common type of DPCs (Kiianitsa & Maizels, 2020) and Ku80-DPCs were found to be specifically induced in *tdp2b*-silenced embryos (Antičević & Otten, 2024). Recently, TEX264 was shown to promote the repair of TOP1-DPCss (Fielden et al., 2020). The exact role of *p97a* and its co-factors in Top2cc removal or its potential interaction with *tdp2b* has yet to be described. Furthermore, as there are two paralogs of *TEX264* in zebrafish, it is possible that they may play separate roles in distinct tissues.

APEX1 and APEX2 have key roles in BER, and it was recently discovered that they have a role in TOP1-DPCs removal as well. TOP1cc can be removed by either TDP1 or APEX1 and APEX2 and these complexes can be converted into DSB or repaired by HR. This redundancy explains why *TDP1* deficiency does not cause significant changes in rapidly replicating tissues, since APEX1 and APEX2 can compensate for the loss of TDP1 by removing TOP1ccs (H. Zhang et al., 2022). Moreover, MUS81 can also convert TOP1cc into DSBs in TDP1-KO cells, highlighting its involvement in the DNA damage repair (Zhang et al., 2022). The role of MUS81, APEX1 and APEX2 in TOP2cc removal has not yet been investigated, but results of this study found no significant change in the expression levels of *mus81*, *apex1* or *apex2* between WT and *tdp2b*$^{rbi10/rbi10}$ zebrafish embryos.

5.4. Outlook

As there was an unexpected high variability between biological replicates, it would be recommended to repeat these measurements with more biological replicates. The samples themselves were satisfactory, with 10 embryos per pool, since the RNA quantities and qualities

were good. Another improvement could be to perform the analysis on embryos of a different age, based on the expression of each individual gene. Indeed, some genes such *acrc* or *tdp2* are more highly expressed at early stages than at 2dpf, when the embryos were collected for this study, so the measurements at that stage might be less relevant (Otten et al., 2023; Antičević & Otten, 2024).

Next, the expression levels of other DPCR candidate genes could be investigated in the same manner: the endonuclease *fen1* (Saha et al., 2024), the glycosylase *neil3* (Semlow et al., 2016), the endopeptidases *ddi1*, *ddi2* (Serbyn et al., 2020; Yip et al., 2020), the peptidase *fam111a* (Kojima et al., 2020; Rios-Szwed et al., 2023; Palani et al., 2024), the helicase *fancj/brip1* (Yaneva et al., 2023), the DNA-binding ATPase *csb/ercc6* (van Sluis et al., 2024). All these genes belong to a growing list of enzymes that have been recently implicated in DPCR in various ways: experiments were conducted to determine general or specific DPC repair, together with other known factors or in new alternative pathways, in certain organisms (yeast s. mammalian cells), or just biochemically. Indeed, qRT-PCR provides a quick read-out for potential involvement of these many genes in Tdp2-mediated DPC repair, but negative results in this assay does not exclude potential functional interactions of Tdp2 with those enzymes on the protein level. In a long-term effort, the Popović lab is currently creating single and double loss-of-function zebrafish models to address these potential interactions functionally *in vivo*. It will be therefore very interesting to perform epistasis experiments and analyse DPC repair in *tdp2b* mutants in which other DPCR factors have been silenced or mutated.

6. Conclusion

DPCs are dangerous DNA lesions composed of proteins covalently bound to DNA. DNA repair mechanisms are crucial in preserving genomic stability and preventing DPCs accumulation. Topoisomerases are enzymes involved in opening DNA double helix in transcription and replication. They relax supercoilings that happen when DNA gets overtwisted. TOPccs are self-repairing catalytic intermediates that can turn into TOP-DPCs if resealing fails. Tyrosyl-DNA phosphodiesterase 2b (*tdp2b*) is one of two orthologs of the mammalian *TDP2* gene that is involved in TOP2-DPC removal. In this study, I analysed the expression of genes involved in DPC repair to determine their connection with *tdp2b*.

Expression analysis revealed that neither *tdp2a* nor *tdp1* expression levels were significantly lower in *tdp2b*$^{rbi10/rbi10}$ zebrafish mutants than in WT. Interestingly, the significantly reduced expression of *znf451* and *mre11a,* which are involved in the resolution of TOP2-DPCs, suggests a potential interdependency with *tdp2b.* The upregulation of *acrc* in *tdp2b* mutants could indicate a compensatory mechanism in response to *tdp2b* loss of function. No significant differences were observed in the expression of *sprtn, tex264a, tex264b, apex1, apex2* and *mus81* between wild-type and *tdp2b* mutants, suggesting that these genes operate independently of *tdp2b.* Overall, these findings highlight the complexity of DPCR and the potential interdependencies between various repair proteins in zebrafish, providing a foundation for further research into the mechanisms underlying DNA repair and genomic stability.

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8. Curriculum vitae

I was born in Dubrovnik, 29th August 1999. After completing my education at Elementary School Cavtat, I continued my studies at diocesan Classical Gymnasium "Ruđer Bošković". During my high school education, I was one of the first members of the Association for the Promotion of Natural Sciences in Dubrovnik (2016 - 2018) and I was their scholarship holder from beginning to the 4th year of study. In 2018, I enrolled in the Integrated Undergraduate and Graduate program for Biology and Chemistry Education at the Faculty of Science in Zagreb. During that time, I received a scholarship from the City of Zagreb for deficit occupations in academic year 2023/2024. In 2024. I volunteered at the Muzza Festival in Zagreb as part of the research team in the Laboratory for Molecular Ecotoxicology at the Ruđer Bošković Institute I joined while writing my master's thesis. During the event, I introduced zebrafish as a model organism, which I used in my research.