Roadblocks & bypasses : protection of genome stability by translesion DNA synthesis in C. elegans

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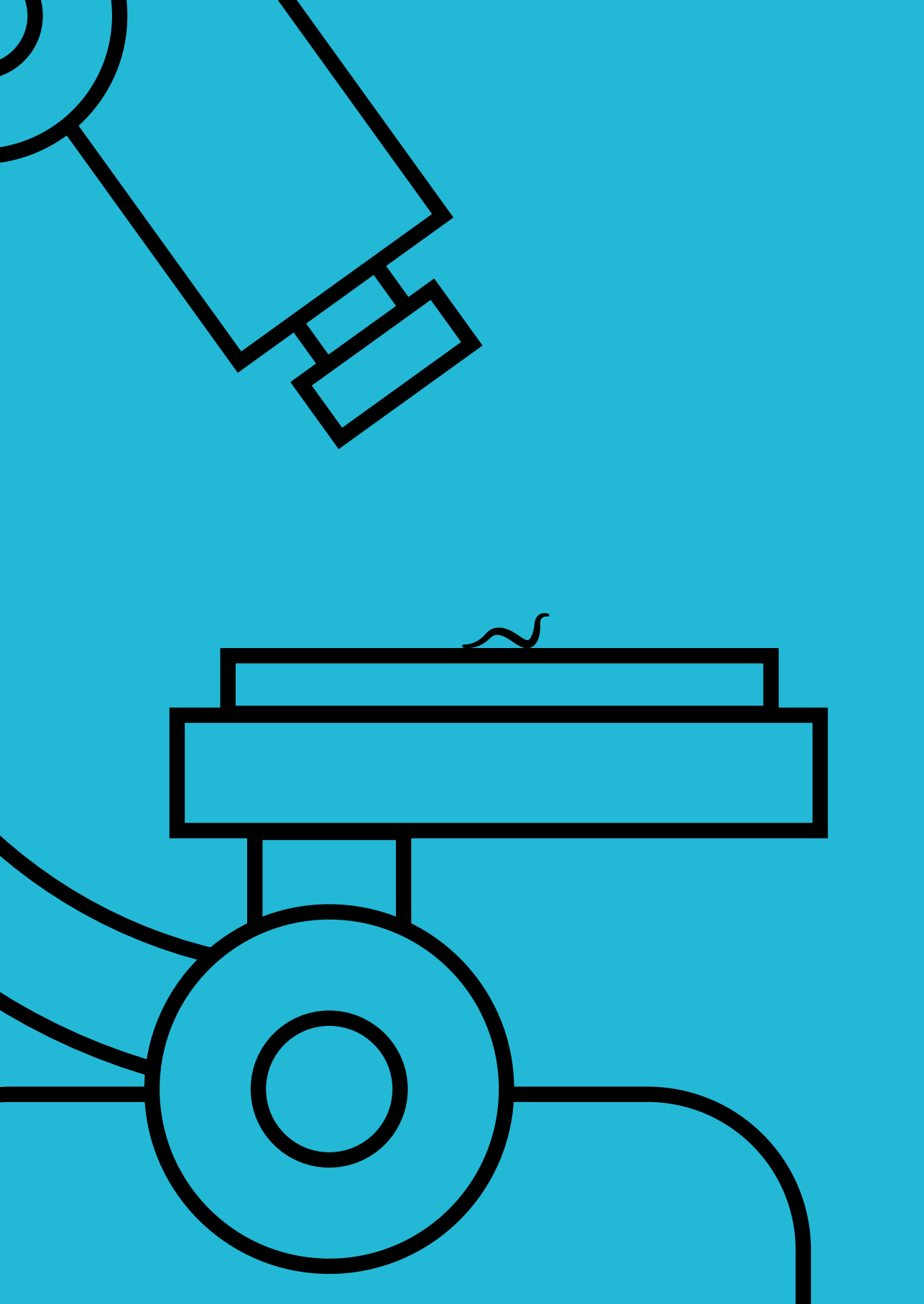

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General introduction

A fragile molecule central to life on earth

Deoxyribonucleic acid - DNA - is central to life on earth. It is literally at the core of each living cell and it is fundamental to all living organisms because it encodes the genetic instructions for life. Therefore, its fragility seems counterintuitive; DNA is chemically instable and this leads to decay over time. Spontaneous reactions such as hydrolysis and oxidation damage the DNA, and threats to the stability of DNA do not end there: reactive byproducts of the cells metabolism chemically alter the DNA, and environmental sources of DNA damage are numerous [1,2]. So, don't organisms need stable genomes to guarantee their survival and proliferation? Of course they do, because lesions in DNA interfere with the most essential biological processes: transcription and replication. Additionally, DNA damage is a major driver of mutation formation - a process that causes disease like cancer, ageing, and congenital disorders. While an instable genome might be a bad thing for the health of an individual organism, a certain level of mutagenesis is advantageous for species and for life on earth in general. Without genome instability there would not be any genomic diversity, and without genomic diversity there cannot be evolution. Intriguingly, it is also evolution that has spawned a multitude of bio-molecular pathways that combat genome instability caused by DNA damage, a network of pathways that is termed the DNA damage response.

Before I go into more detail about the pathways that govern the repair of DNA damage, I will briefly introduce the sources of DNA damage. The well-known double helix of the DNA molecule with bases adenine (A), cytosine (C), guanine (G) and thymine (T) paired between sugar (deoxyribose) and phosphate backbones has the potential to undergo many different chemical alterations. Spontaneous hydrolysis of the *N*-glycosyl bond between the base and sugar group, results in an abasic site. These lesions are by far the most abundant and it is estimated that a human cell has a steady-state level of 30.000 abasic sites. Additionally, hydrolysis can lead to deamination of bases, thereby altering the chemical structure of the base. Reactive molecules - often the result of the cells own metabolism - also contribute to endogenous DNA damage; oxidation, methylation and alkylation of nucleotides result in alterations to nucleotides and pose threats to genome stability [2]. Although DNA replication is not a source of DNA damage per se, it is an endogenous source of genome instability because occasional replication errors introduce mutations. The wrong base can be inserted, but also sequence specifics can lead to replications errors: small insertions and deletions can occur at repetitive stretches of DNA. On top of that, some sequences have the potential to form secondary structures, exemplified by G-quadruplexes [3]. The micro-environment in the nucleus of a living cell already seems a harsh place for DNA to be, but when we step outside, into the sunlight, other dangers present themselves. Sunlight contains ultra violet (UV) light that can induce dimerization of adjacent pyrimidines and oxidative damage as well. Another well-known example of an exogenous source of DNA damage is ionizing

radiation (IR), which induces a wide variety of damages, including, oxidized bases and breaks of the DNA backbone. Moreover, there are many natural and synthetic genotoxic chemicals. Alkylating agents like MMS and EMS for example, cause base adducts. Another striking example is cisplatin, a chemical that can form a covalent bond between bases of opposing DNA strands. This kind of lesion is called an interstrand crosslink (ICL), and is extremely toxic because it prevents strand separation of DNA, thereby forming an absolute block of transcription and replication. These examples of chemotoxins are familiar, and there are many more chemicals that damage DNA, too many to discuss them all here. It is clear that there is a plethora of threats to the integrity of DNA, with which cells have to deal continuously. This calls for efficient and faithful mechanisms that maintain the healthy state of the genetic material and thereby safeguard transcriptional and replicative potential.

The DNA damage response

The network of molecular pathways that governs the cells response to DNA damage is called the DNA damage response (DDR); it entails damage detection, downstream signaling and DNA repair actions. While DDR includes the promotion and execution of repair, it also initiates senescence and apoptosis when the damage load is too high. All these processes together suppress the accumulation of too much DNA damage and mutagenesis and prevents cells from becoming cancerous [4]. As I have described earlier, there are many types of DNA lesions and evolution has provided cells with recognition and repair pathways that are tailored to different classes of lesion. To keep within the scope of this thesis I will limit the discussion of the DNA damage response to the most important DNA repair pathways and damage tolerance mechanisms, with a focus on the central theme of this thesis: Translesion DNA synthesis.

Base excision repair (BER)

This pathway is highly conserved from *E. coli* until humans and deleterious mutations in BER genes cause increased mutagenesis and higher chance of developing cancer [5,6]. Simply put, BER removes damaged bases and replaces them with new nucleotides, specifically for lesions that do not cause much structural change to the normal helical organization of the DNA. These lesions have the potential to induce base substitutions and cause polymerases to stall. By repairing them, BER safeguards replicative and transcriptional potential and genomic stability. Substrates include the common abasic sites, but also oxidized and alkylated bases. These last two types are recognized and removed by a lesion specific glycosylase, which thus generates abasic sites. The DNA backbone is subsequently cut by an AP endonuclease, generating a DNA single-strand break (SSB) and a single nucleotide gap. Polymerase β then fills the gap. When only one new nucleotide is incorporated this is called short-patch BER and the downstream

processing and ligation is done by XRCC1 and LIG3, respectively. Long-patch BER incorporates up to 11 new nucleotides and processing and ligation is performed by FEN1 and LIG1 [7,8]. The enzymes involved in ligation during BER are also responsible for the repair of SSBs from other sources [9].

Nucleotide excision repair (NER)

Lesions that distort the helical structure of the DNA require a different repair mechanism. Most notably, and widely studied in the DNA repair field, are pyrimidine dimers that are induced by UV light. The covalent bonds between neighboring cytosines and thymines - in the forms of cyclobutane–pyrimidine dimers (CPDs) and 6–4-pyrimidine–pyrimidone photoproducts (6–4PPs) - induce tension on the DNA molecule and cause bending of the backbone. Additional lesions that are subject to NER include a wide variety of structures: cisplatin induced intrastrand crosslinks, bulky chemical adducts, products of reactions with reactive oxygen species (ROS) like cyclopuriones, and more. Transcription and replication are performed by high fidelity polymerases that cannot use such distorted templates; therefore, all these lesions block these essential processes. Detection of NER-specific lesions happens through two separate sensing mechanisms. Transcription-coupled NER (TC-NER) safeguards efficient transcription; it is activated when the RNA polymerase stalls on a lesion. This recruits the CSA and CSB proteins, among others, that then promote repair. The other sensing mechanism of NER is referred to as global genome NER (GG-NER). GG-NER continually scans the whole genome for distortions in the DNA helix and mainly employs DDB1 (also known as XPE) for lesion recognition and promotion of repair. After damage recognition the downstream steps of the repair pathway are identical; the TFIIH complex opens the DNA, XPF and XPG excise the damaged DNA, and then a polymerase and DNA ligase restore the DNA to the original state [10], (Fig. 1).

Many NER factors are named for their association to the disease *Xeroderma pigmentosum* (XP). Deleterious mutations in the XP genes result in a deficiency to deal with UV-induced DNA damage. Patients are extremely sensitive to sunlight and have ~1000-fold elevated risk for developing skin cancer. Cockayne syndrome (CS) and trichothiodystrophy (TTD) are other genetic disorders associated with NER and, among other symptoms, also display hypersensitivity of the skin to sunlight [10,11].

Mismatch repair (MMR)

Although it is debatable if mismatches fall in the category of DNA damage, the MMR pathway is essential for the maintenance of genome stability. The polymerases that are responsible for DNA replication have incredibly high fidelity; the main replicative polymerases in mammals Polδ and Polε generate just one error for up to 10^7 correct incorporation events. The rare errors are either mismatched bases or microsatellite

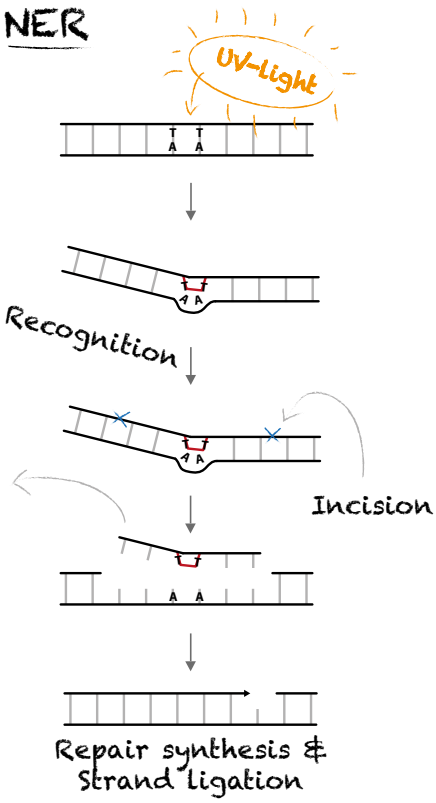

Figure 1. Nucleotide excision repair.
A schematic representation; for details see text.

mutations: small insertions or deletions (indels) in repetitive DNA sequences due to polymerase slippage. These polymerases achieve this high fidelity because their tight catalytic centers do not accommodate mismatched bases and they have proofreading activity that detects misaligned DNA after replication [12]. Nevertheless, this level of mutagenesis would still be substantial, especially if you consider the number of rounds of replication necessary to build and maintain a human being. The MMR pathway is able to recognize mismatches and small loops caused by slippage at microsatellite sequences, while also distinguishing the old and new DNA strands. In eukaryotes the MutS-homologs MSH2, MSH6 and MSH3 enable recognition, which is followed by nicking of the nascent strand by MutL-homologs MLH1 and PMS2, Then, EXO1 removes the nicked DNA, before a replicative polymerase and LIG1 take care of resynthesis and ligation, respectively. Thereby MMR reduces the error rate approximately 100-fold to an estimated 1 for every 10^9 correct base incorporations [13,14]. The importance of this pathway in the maintenance of genome stability is illustrated by the symptoms of Lynch syndrome patients. Lynch syndrome is caused by deleterious mutations in MMR genes and patients have an increased risk of developing colon cancer early in life.

A hallmark of Lynch syndrome is microsatellite instability: patients have increased genomic instability at mono- di- and tri-nucleotide repeats [15].

DNA damage tolerance mechanisms

Although BER and NER provide efficient DNA repair of lesions that stall replicative polymerases, the abundance of base damages has led to a strong evolutionary selection pressure on alternate systems that help to sustain replicative potential. These alternative pathways, together termed DNA Damage Tolerance (DDT), do not remove lesions but provide the potential to complete DNA replication in the presence of damage and thereby suppress the formation of replication associated DSBs. There are two well-known pathways described in literature: Template Switching (TS) & Translesion Synthesis (TLS). Template switching uses the undamaged strand as a template for replication past the lesion and in that way bypasses the damage without induction of mutations [16,17]. This mechanism shares intermediates with homologous recombination (HR, discussed later in more detail). It is employed in bacteria and yeast, but it is unclear whether such a pathway is also used extensively in higher eukaryotes [18-20]. Although TS is error free, it is dependent on complex HR-like reactions, which may be a time-consuming process for the cell especially when, during stages of embryogenesis, there is a demand for a quick fix to resolve replication blocks. Also, it is thought that TS can cause genomic rearrangements when the HR-like intermediates cannot be resolved properly [21,22]. The second DDR pathway - TLS - is conserved among all kingdoms of life and in multicellular eukaryotes it is arguably the most essential DDT pathway [23].

Translesion DNA synthesis (TLS)

Specialized TLS polymerases are employed to synthesize DNA opposite damaged templates. Their wider catalytic center and lack of proofreading capacity allow them to accommodate damaged bases and bulky adducts as templates, thereby permitting the continuation of DNA replication without actual repair [24]. This efficient process protects against the many deleterious effects of replication form collapse, but due to the intrinsic properties of TLS polymerases and their inability to read certain damaged templates correctly it is also mutagenic [25-27], (Fig. 2). In eukaryotes TLS is predominantly mediated by polymerases from the Y-family: Polη, Polκ, Polι, and REV1 and by B-family polymerase Polζ [28,29]. Structural differences of these enzymes define the functional specificities of TLS polymerases and bypass of more demanding lesions may require the combined efforts of more than one TLS polymerase [30]. TLS can happen directly at the replication fork by switching the replicative polymerase temporarily for a TLS polymerase or it can happen post-replication when the single strand DNA regions that are left opposite the DNA lesions are filled in by TLS polymerases [31]. Before I go into more detail about the regulation of TLS and the current models, I will first introduce the main players.

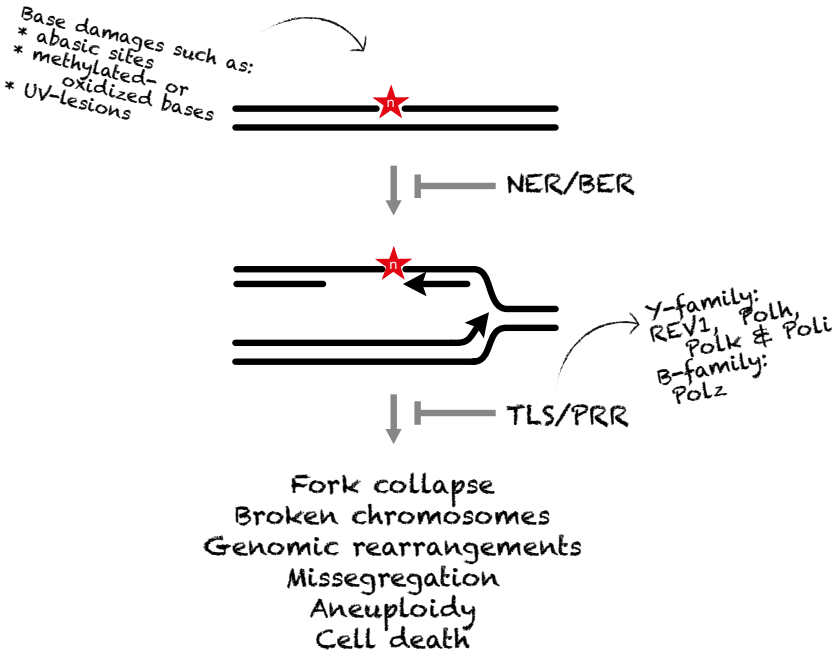

Figure 2. How translesion synthesis protect against deleterious consequences. *For details see text.*

Polymerase eta (Polη)

Polη is involved in the bypass of UV photolesions, especially CPDs and likely also 6-4PPs [32-34]. This is highlighted by the increased cancer risk and sensitivity to sunlight of *Xeroderma pigmentosum* variant (XP-V) patients that have a mutation in the Polη encoding gene [32,35,36]. In addition, Polη is important for the bypass of the most abundant oxidized base, 8oxoG, that is also indirectly induced by sunlight [37,38] and many others lesions: (+)-*trans*-*anti*-benzo[a]pyrene-N2-dG [39], acetylaminofluorene-adducted guanine [40], *O6*-methylguanine [41], thymine glycol [42], and adducts derived from cisplatin and oxaliplatin [43]. Bypass of CPDs and 8oxoG by Polη is remarkably error free, but on undamaged DNA Polη's fidelity is very low as compared to replicative polymerases. Despite this high mutagenic potential, knockdown in human cells and knockout in MEFs actually increases mutagenicity [44] and Polη loss in yeast does not change mutagenesis [32,45]. Due to redundancies between the TLS polymerases it is likely that other TLS polymerases, possibly Polι or Polκ are able to take over [46]. Also, the observations that overexpression of Polη in human cells and yeast has limited effect on the mutation rate point to very stringent regulation that restricts Polη activity only to

damaged DNA [47,48]. Different protein interactions contribute to this strict regulation: the sliding clamp PCNA can interact directly with Polη and this interaction is boosted by the Rad6-Rad18 dependent mono-ubiquitination of PCNA [49]. Mammalian Rev1 can also physically interact with Polη [50]. Work in DT40 chicken cells has shown that these two interactions regulate Polη in temporarily different ways: REV1 is needed for Polη dependent bypass at the replication fork, while PCNA mono-ubiquitination recruits Polη to post-replicative ssDNA gaps opposite lesions [51]. In *C. elegans* Polη is especially important during embryogenesis, likely because embryos are extremely sensitive to cell cycle delays and thus need a rapid solution to stalled replication [52-54].

Polymerase kappa (Polκ)

Polκ is the most conserved TLS polymerase and was identified via its homology with the *E. coli dinB* gene [55]. While the function in TLS of the *dinB* gene product, PolIV, has been extensively studied in *E. coli*, the role of Polκ in eukaryotes is less clear. This could be due to the lack of a Polκ homolog in the most abundantly studied eukaryote, *S. cerevisiae*, or because loss of Polκ does not cause profound changes in spontaneous or damage induces mutagenesis [56]. Also, Polκ has a relatively high fidelity on undamaged DNA when compared to other TLS polymerases. The catalytic activity of Polκ seems to be restricted to N^2-adducted dG lesions [57-60]. In addition, loss of Polκ sensitizes vertebrate cells and *C. elegans* to alkylating agents, suggesting Polκ also acts on these lesions, together or redundantly with Polη [52,61]. Similarly to Polη, Polκ also interacts with PCNA and REV1 [50,62]. When a lesion requires the concerted efforts of two TLS polymerase the insertion step of 1-2 nucleotides directly opposite the damaged nucleotide is performed by one polymerases and this is followed by extension of the aberrant primer terminus by another. As Polκ is able to extend mispaired primer termini and is relatively accurate on undamaged DNA it is a good candidate to fulfill the role of extender in addition to its specialized role in direct lesion bypass [24].

Polymerase iota (Polι)

Polι is the most recent addition to the Y-family and is not conserved in yeast and *C. elegans*. It is highly accurate when it uses dT templates, but very mutagenic when using dA templates. This is due to its specialized enzymatic core, which prefers *Hoogsteen* pairing [29]. This characteristic may also explain why Polι is able to bypass of 8oxoG in an error-free manner [63,64]. In addition, Polι may have a role in BER, possibly as a gap-filling polymerase [64].

REV1

The Rev1 gene was named for its reversion-less phenotype in yeast in knockout strains; Rev1 deficient mutants have reduced UV-induces mutagenesis [65-67].

REV1 is an a-typical polymerase because its catalytic activity is limited to incorporation of deoxycytidines (dC). REV1 can perform this opposite undamaged or damaged guanines but also across adenines, uracil and abasic sites *in vitro*, which can explain why it promotes mutagenesis [24,68-72]. During TLS, REV1 is important for bypass of lesions that are caused by lipid peroxidation and UV induced damage [67,73]. Although REV1 is structurally alike to the other Y-family members, its limited catalytic activity may suggest it has other roles. There are various studies showing that REV1 performs non-catalytic functions via interactions with other proteins: the BRCT domain of REV1 interacts with PCNA and is involved in the bypass of UV-C induced lesions in mouse ES cells [74-76]. Additionally, the C-terminal part of mammalian REV1 contains ubiquitin binding motifs (UBMs) that interact with ubiquitinated PCNA, a region able to interact with other Y-family polymerases, and a motif that interacts with the REV7 subunit of B-family TLS polymerase Polζ [24,50,77-79]. These interactions support a proposed role of REV1 as a master regulator of TLS. While the polymerase and BRCT-domain are conserved between mammalian REV1 and *C. elegans* REV-1, this is not obviously the case for other protein interaction domains. However, the functions may be conserved. In addition to its role in TLS, REV1 may play a role in maintenance of epigenetic stability at G4 sequences, possibly by facilitating replication across these hard-to-replicate sequences [80,81], and (together with Polζ) in homologous recombination (HR) break repair [82,83].

Polymerase zeta (Polζ)

The catalytic subunit and member of the B-family polymerases, REV3, and the accessory subunit REV7 make up the core of Polζ [28,84]. Like Rev1, these two genes were also discovered in screens for mutants that have a reduced UV-C induced mutation frequency in yeast [65,66,85]. More recently it was shown that accessory subunits Pol31 and Pol32 of Polδ are also part of the Polζ complex [86-89]. While Polη efficiently bypasses UV-C induced CPDs, Rev1 and Polζ are important for the bypass of 6-4PPs [68,84,90]. As Polζ is able to extend aberrant primer termini at a lesion but also at mismatched bases, and is relatively accurate on undamaged templates, it may also function as the extender polymerase after one of the Y-family polymerase has performed the insertion step of lesion bypass [24]. The mammalian homolog *REV3L* produces a protein twice the size of the yeast protein, which may indicate more complex or diverse functions compared to the yeast protein [28]. Rev3-/- mice are embryonic lethal due to high levels of apoptosis that is likely caused by the observed chromosomal instability. In humans, heterozygosity of deleterious mutations in *REV3L* cause a genetic developmental disorder called Möbius syndrome [91,92]. Together with REV1, Polζ may also play a yet ill-defined role in homologous recombination (HR) break repair [82,83].

Other polymerases involved in lesion bypass

The proteins I have just discussed are thought to perform the bulk of TLS. However, there are a few other polymerases that have shown bypass capacity, mainly in biochemical assays. Polβ has, next to its function in BER, the capacity to bypass certain cisplatin induced lesions [93], Polv and Polθ can bypass thymine glycols, and Polθ can efficiently synthesize DNA across abasic sites [94]. While Polκ and Polζ are able to extend from aberrant primer termini, making them suitable to perform the extension step of TLS, Polλ, Polμ and Polθ can also perform extension from mismatched primer termini [95-97].

Although Polθ was initially proposed to be involved in TLS, a genetic study in Drosophila showed that its function in tolerance to several kinds of DNA damage is due to a role in microhomology driven double strand break (DSB) repair [98]. Recently, the interest in Polθ has spiked and studies from our lab [99-103] and others [Reviewed in 104,105] have further characterized its biological roles. Because Polθ is of special interest for this thesis, I will discuss Polθ-mediated end joining (TMEJ) in more detail later, in the paragraph *Alternative end joining*.

Regulation of TLS

The high mutation rate and low processivity of TLS polymerases on undamaged DNA calls for a strict regulation that only allows access to the DNA when a lesion blocks replicative polymerases. Generally, this is not achieved by regulation of overall cellular concentration, but it appears that the localization of TLS polymerases is controlled. TLS polymerases are concentrated in replication factories dependent on PCNA ubiquitination [106,107]. Once the replication fork arrests at the site of a blocking lesion the eukaryotic sliding clamp PCNA is mono-ubiquitinated at lysine 164 by the E3 ubiquitin ligase RAD18 and the E2 conjugating enzyme RAD6 [108]. This reaction is promoted by the interaction of RAD18 with RPA coated ssDNA in close proximity to the lesion. Mono-ubiquitination of PCNA recruits Y-family polymerases via the ubiquitin binding motifs at the C-terminus of these polymerases [62]. Yeast cells that carry a K164R substitution have defects in UV-induced mutagenesis and mammalian cells with the same substitution are sensitive to genotoxic stress [109]. Additionally, in yeast PCNA can be poly-ubiquitinated at the same lysine residue, which depends on Rad5, and can also be SUMOylated. Both these signals are linked to recombinational DNA damage bypass but the crosstalk between regulation of TS and TLS is complex [110]. For example, Rad5 dependent poly-ubiquitination in yeast also seems to be important for efficient TLS [20]. Furthermore, the mammalian homologs of Rad5 - SHPRH and HLTF - have complex roles in DDT and seem to have functions in both TLS and other DDT mechanisms [18]. It is proposed that these proteins suppress mutagenesis after DNA damage by directing the most appropriate TLS polymerase or other bypass mechanisms to replication fork impediments [19].

In mammalian cells the PCNA-K164R substitution mutation strongly decreases TLS efficiency, but it is not completely abrogated [111]. In DT40 chicken cells the remaining TLS activity is largely dependent on the non-catalytic function of REV1, but whether this is also the case in other organisms is unclear [51]. Nevertheless, the many interactions of REV1 with different TLS factors support that REV1 also regulates TLS [75,76]. Additionally, post-translational modifications like phosphorylation and ubiquitination of TLS polymerases can provide further layers of regulation, but how this exactly works remains unclear; emerging evidence indicates that some Fanconi anemia (FA) factors also regulate TLS independent from their well-known role in interstrand crosslink (ICL) repair (this pathway will be discussed in detail later) [112]. Finally, TLS polymerases were shown to interact with the PCNA-like 9-1-1 clamp, which may be especially important for highly helix-distorting lesions [113], and lesion bypass also proved to be dependent on chromatin dynamics [114].

The lesion itself has a strong effect on the bypass mechanism and polymerase choice. For some lesions bypass can be quick and easy: for example UV-light-induced thymine-thymine cyclobutane pyrimidine dimers are efficiently bypassed by Polη [33]. Many other lesions may require more complex mechanisms and the collaborative efforts of more than one TLS polymerase [115]. TLS is also affected by the cell cycle, and specifically the progression through S-phase. This is illustrated by the identification of temporal distinct sub-pathways of TLS [51,116]. The development of novel techniques - especially high-resolution genetic assays - should help to determine the complex regulatory network of DDT and shed more light on possible TLS models, providing an interesting subject for future research [117]. The value of such future work is emphasized by the many and pleiotropic ways in which TLS is involved in health.

TLS in health and disease

As I have discussed earlier, TLS polymerases have low fidelity, especially when compared to the orders of magnitude higher fidelity of replicative polymerases. Interestingly, the mutagenicity of TLS polymerases is used in a way that is beneficial to human health. Somatic hypermutation is an essential part of our adaptive immune response; it requires induction of base substitutions in the variable immunoglobulin genes to produce a wide variety of antibodies. There is evidence that at least a subset of these base substitutions are generated through mutagenic TLS performed by Polζ, Polη, Polι and REV1 [118-120].

Although TLS may be mutagenic on occasion there is much evidence that TLS actually protects genome integrity by preventing replication fork collapse. Persistent replication fork stalling causes a very serious domino effect of problems linked to cancer and inborn disease: broken chromosomes, genomic rearrangements or missegregation and eventually aneuploidy and cell death (Fig. 2), [26]. Another positive effect of TLS

on human health is illustrated by the genetic disorder *Xeroderma pigmentosum* variant (XPV), caused by deleterious mutations in the gene that encodes Polη. Patients display hypersensitivity toward UV light and develop malignant skin neoplasia at early age due to increased mutagenesis [35,121-123]. Furthermore, mice with a hypomorphic allele of REV1 in a sensitized NER deficient background show increased UV induced skin carcinogenesis. It is proposed that this is caused by increased levels of replication stress that trigger DNA damage signaling, which in turn leads to induction of inflammation and senescence [124]. The recent development of increasingly sensitive and robust affordable sequencing techniques has also contributed to the identification of somatic mutations of TLS polymerase genes in cancers. Deleterious mutations in the genes coding for Polη and Polκ have been identified in melanomas, breast cancers, prostate tumors, ovarian- and kidney cancer [125]. Moreover, there is abundant evidence of misregulation of TLS in many different kinds of cancer. Given the fact that cancer cell rely on efficient DNA replication in the presence of high levels of replication stress it is perhaps not surprising that many cancers have upregulated TLS activity [126,127]. Additionally, TLS polymerases support chemoresistance of tumors, because many of cancer therapeutics function though the induction of replication blocking lesions that can be bypassed by TLS polymerases. The inhibition of TLS has thus been suggested as a promising approach in the treatment of cancer [127-129]. A TLS inhibitor may be used to sensitize cancer cells to existing chemotherapeutics or synthetic lethal interactions can be exploited, for example a tumor deficient in one of the DNA repair pathways may rely more on efficient TLS [130-132].

Interstrand crosslink repair

Interstrand crosslinks (ICLs) are one of the most toxic types of DNA damage and require a complex network of repair pathways and TLS. The crosslinking of paired DNA strands through a covalent bond between bases in opposing strands forms an absolute block for transcription and replication because it prevents strand separation. ICLs also have a high potential to induce base substitutions, indels and chromosomal rearrangements when mutagenic repair occurs. The high toxicity of ICLs is illustrated by the effective treatment of cancer with agents that induce ICLs; especially replicating cells have great difficulty with ICLs [133]. Synthetic chemicals such as nitrogen mustards and cisplatin can induce these lesions, but there are also natural sources of ICLs: aldehydes and natural psoralens for example [134].

Studies of the genetic disorder Fanconi anemia (FA) have provided great insight in the molecular mechanism of ICL repair. Patients with this syndrome have developmental aberrations, progressive bone marrow failure and greatly increased cancer risk, and cultured FA cells are hypersensitive to ICL-inducing drugs [135]. Currently, nineteen FA genes have been identified, and all gene products act in the same ICL repair pathway

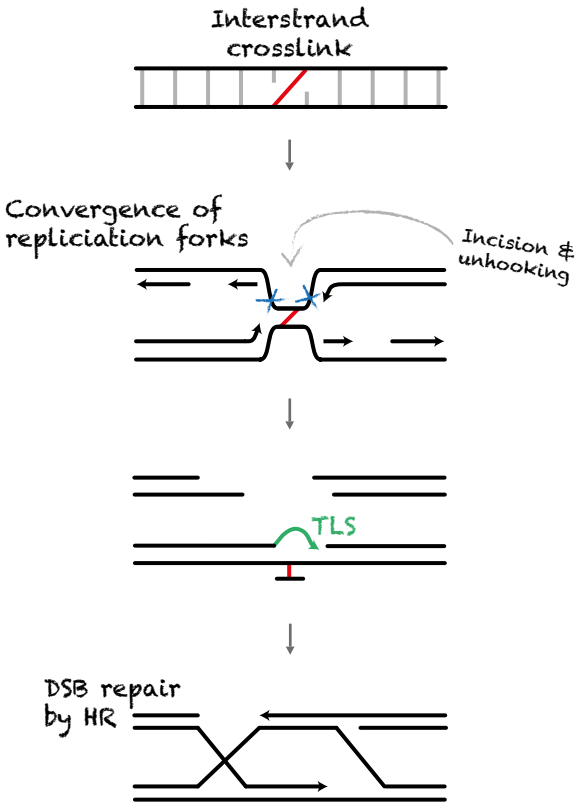

Figure 3. A schematic of interstrand crosslink repair. *For details see text.*

[136]. In the fifth chapter of this thesis I will discuss the FA pathway in more detail. Briefly, the best-known mechanism of ICL repair is as follows: upon two replication forks converging on an ICL the first step of the repair is unhooking of the crosslink by stimulating incisions in the backbone up- and downstream of the lesion through the action of by two different endonucleases. This process creates a flipped-out ICL-containing oligo still covalently attached to one of the strands. Translesion synthesis across the unhooked lesion restores the sister chromatid, before HR repair can use this as a template to repair the DSB on the other chromatid that resulted from the dual incisions (Fig. 3). TLS polymerases Polζ and REV1 are especially important for this TLS step but likely other TLS polymerases are involved too. The unhooked ICL that remains may be removed via NER or by hydrolysis, but not much is known about this step yet [137,138]. Much of what we currently know about this complex repair mechanism has come from studying the replication of plasmids that contain a single cisplatin crosslink in *Xenopus laevis* egg extracts [139-142]. While it was first thought that this was the predominant pathway for any ICL, recently it was shown that psoralen crosslinks are

repaired differently. For these lesions the DNA backbone is not incised but one of the two N-glycosyl bonds forming the crosslink is simply cleaved by a glycosylase. TLS is involved here too, but because no DSB is generated HR is not necessary for repair. This alternative pathway shows that the mechanisms of ICL repair are flexible and dependent on the chemical nature of the crosslink [143]. Both these mechanisms function in S-phase when replication forks run into ICLs. Outside the context of DNA replication the recognition and repair of ICLs is not fully understood but NER and mismatch repair factors seem to play important roles in recognition of ICLs. The efficiency of ICL repair outside S-phase is dependent on their level of distortion of the normal DNA helix structure; lesions that strongly disrupt the normal structure of the DNA helix, like cisplatin crosslinks, are more readily recognized and repaired [144]. TLS is important in replication-independent ICL repair also: Polκ and PCNA-K164 ubiquitination function in replication-independent repair in *Xenopus* egg extracts and similar a role for Polκ was confirmed in mammalian cells [145].

The sources and repair of double stranded breaks

Persistent stalling of replication - for instance when TLS is not functional - causes collapse of the replication fork eventually resulting in DNA double strand breaks (DSBs). These highly toxic and mutagenic lesions are also induced by exposure to ionizing radiation or by physical stress on the DNA molecule. Because DSBs represent physical breaks of the chromosome they can lead to loss of genomic information, but also when not properly repaired to genomic rearrangements such as translocations events that are causal to cancer development. Intriguingly, DSBs are also generated endogenously to promote meiotic crossover formation, and during V(D)J recombination. Here, DSBs are introduced to generate diversity in the antigen receptor repertoires. In all cases these breaks need to be repaired efficiently and – with exception of the breaks generated during V(D)J recombination – without aberrant loss of genetic information. The two most widely studied DSB repair pathways are homologous recombination (HR) and non-homologous end joining (NHEJ), and there are several alternative pathways [146].

Homologous recombination (HR)

This pathway uses homologous sequences from the sister chromatid or the homologous chromosome as a template to repair the chromosomal break and therefore it is *grosso modo* error free. Upon recognition of DSBs the cell cycle is halted and initiation of repair is induced via ATM signaling. Regulated processing and short-range resection of the DNA is then performed by the MRN complex (MRE11, RAD50 and NBS1), which creates small 3' single stranded overhangs. EXO1 and DNA2 can then further resect the DNA to generate long stretches of ssDNA, which become coated by the heterotrimeric RPA complex. Subsequently, RAD51 - the recombinase protein that facilitates strand invasion

- replaces RPA dependent on BRCA2. The RAD51-ssDNA filaments can then invade the sister chromatid or homologous chromosome to pair with complementary sequences, which is then used as a template for extension by a replicative polymerase. Next, the newly synthesized DNA can be displaced and anneal to the resected other end of the DSB. This reaction can use just one end of the DSB for invasion and extension, which is referred to as synthesis dependent strand annealing (SDSA). Simultaneous reactions from both sides of the DSB are also possible, leading to a complex intermediate: the double Holliday junction (dHJ). The dHJ can be resolved to form a crossover, which is essential during meiotic recombination, but non-crossovers are also a possible outcome [147]. Although HR is error free in most cases, it can lead to loss of heterozygosity when not the sister chromatid but the other homologous chromosome is used as a template in somatic cells. Also, gene conversion events are possible when another homologous sequence is used, for example a nonfunctional pseudogene [146].

HR is an essential pathway for human health; mutations in HR factors such as BRCA1, BRCA2, and RAD51 are lethal in mice and sexually reproducing organisms without functional HR cannot form meiotic crossovers and are infertile. In humans the vital function of error free repair by HR is also reflected in the various cancer predisposition syndromes associated with HR genes. Examples are: hereditary breast and ovarian cancer caused by *BRCA1* & *BRCA2* mutations, Bloom's syndrome cause by defective *BLM* (dHJ resolvase), and ataxia telangiectasia resulting from *ATM* mutations [4].

Non-homologous end joining (NHEJ)

A second major DSB repair pathway - non-homologous end joining (NHEJ) - has a much simpler mode of action: it does not use a homologous template but directly ligates the broken ends together. Arguably it is the go-to DSB repair mechanism in most somatic cells, also because the sister chromatid or homologous chromosome is not available. The major drawback of NHEJ is the potential loss of sequence at the break site, and therefore it is considered to be error-prone. NHEJ of a DSB is as follows: first, the ends of the break are bound by Ku70/Ku80 heterodimers, then DNA-PKcs tethers both ends together, and subsequently the ends are ligated by Ligase IV (Lig4) in complex with XRCC4. When the ends of the break require processing before repair this can be performed by a variety of biochemical activities, including those performed by Artemis, Polλ or Polμ [148]. Although the NHEJ pathway is conserved throughout all known eukaryotes, there are also notable differences, for instance, DNA-PKcs and Artemis, which are important NHEJ factors in mammals are not conserved in the lower eukaryotes yeast and *C. elegans* [149].

Because NHEJ is also employed to repair the programmed DSBs generated during V(D)J recombination, mutations in KU70, KU80 and DNA-PKcs can lead to severe combined immunodeficiency (SCID) [150]. In addition, in mice inactivation of NHEJ

factors XRCC4 and Lig4 is lethal, which underwrites the vital importance of this DNA repair pathway [148].

Alternative end joining

Keeping the importance of HR and NHEJ in mind and considering the tremendous toxicity of DSBs, it may not come as a surprise that there are additional alternative mechanisms a cell can employ to repair DSBs. Alternative end joining can be achieved when there is sequence homology in the flanks of the DSB, which can be used to promote end joining by annealing of the complementary sequences that are exposed after the break ends have been resected. This mode of repair results in sequence loss of the DNA between the homologous sequences. The homology in the flanks of the break can be minimal (*e.g.* up to 25 bases) in which case the repair mode is termed micro-homology mediated end joining (MMEJ). Alternatively, the term single strand annealing (SSA) is used when longer stretches of homology are used. After the ssDNA ends hybridize a polymerase can act to fill the remaining ssDNA and a ligase to ligate the DNA backbones [151].

In *C. elegans* it was found that even when HR, NHEJ and SSA were knocked out still DSB repair could be found [152]. It was subsequently found that this DSB repair route resulted a specific mutational footprint: small deletions with minimal micro-homology of 1 nt, and a subset of the deletion had inserts, which were argued to be templated from the flanks of the break. The templated inserts suggested the involvement of a polymerase in this DSB repair pathway. Following the finding that Polymerase theta (Polθ) was involved in break repair mediated by microhomology in Drosophila [98], this seemed a likely candidate. Indeed, multiple studies in recent years have shown that Polθ mediated end joining (TMEJ) plays an essential role in DSB repair in *C. elegans* [99-103]. TMEJ is especially key to repair DSBs that occur due to varying persistent replication blocks. Worms that lack DOG-1 have increased loss of sequences that can form quadruplex structures. These structures cause replication associated breaks that are repaired via TMEJ, which leaves behind a very characteristic mutagenic footprint [99,101]. The same genomic scar is found in worms that lack TLS polymerases Polη and Polκ [100]. Subsequently, it became clear that the Polθ is the key player of a very versatile repair mechanisms that is employed widely in *C. elegans*, also for DSBs from other sources [102,103]. Polθ has the ability to capture a 3' ssDNA and stabilizes minimal (1 nt) base pairing, before it can extend to stabilize joining of the break ends. When this directly leads to repair a deletion results that is characterized by single nucleotide homology at the repair junction. On occasions that the newly synthesized sequence tract detaches prior to another round of annealing and extension a templated inserts results (Fig. 4).

Parallel to the research in worms, the interest in Polθ in mammals has greatly increased over the last years [extensively reviewed in 104,105]. Importantly, POLQ is

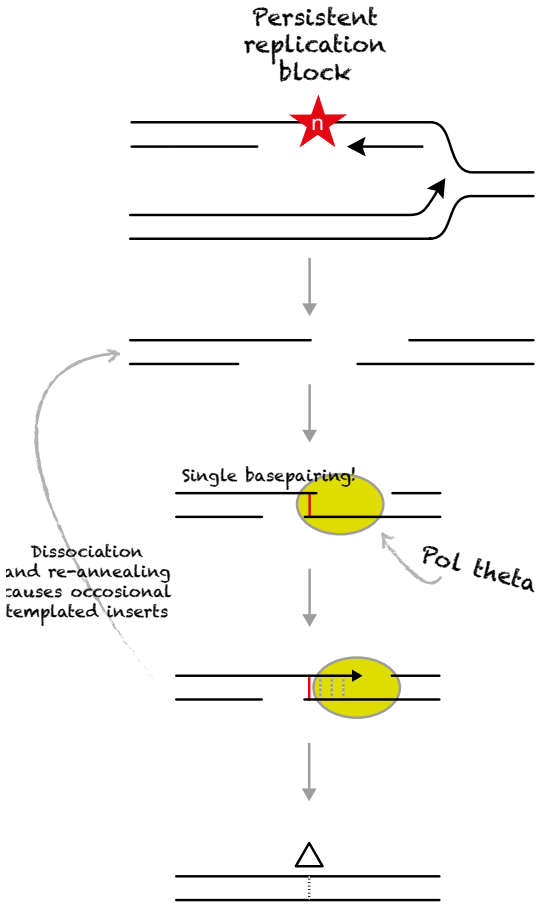

Figure 4. Simplified representation of polymerase theta mediated end joining. *For details see text.*

part of a group of upregulated genes correlated to reduced clinical outcome for a set of different cancers. More importantly HR defective tumor cells require Polθ for their growth [105]. Therefore, inhibition of Polθ is considered a promising approach to the treatment of (at least certain types of) cancer.

Methods that matter

Biological research has developed rapidly during recent history. There are several technological advances especially important for the work described in the experimental chapters that follow this introduction. I will emphasize a few that have been essential for my work, and will discuss their specific application. The discovery of DNA, its structure and function, starting almost 150 years ago, is at the base of these innovations. In the late 19th century, long before the link between chromosomes and heritability was proposed, Freidrich Miescher and Richard Altman isolated DNA and found that

it was part of chromosomes. In the early 20th century Phoebus Levene discovered the building blocks of DNA: A, T, G, and C [153]. Over the next decades the field of molecular biology leaped forward when the link between DNA and heritability became clear, when it was understood that DNA carried genes, the genetic code for the synthesis of proteins. The discovery of the double-helix structure of the DNA molecule by Watson and Crick is another iconic breakthrough of that era [154]. In the years that followed numerous advances have paved the way for modern genetic research and molecular biology. Among these are the development of techniques to determine DNA sequences and methods for genetic engineering. In addition, gaining a thorough understanding of the function and interactions of genes and their corresponding proteins would not have been possible without the use of model organisms, such as *C. elegans*.

Model organisms in genetics – Caenorhabditis elegans

In his book *Thus Spoke Zarathustra: A Book for All and None* from 1883, Friedrich Nietzsche wrote: "You have made your way from worm to man, but much within you is still worm." Although he was not discussing genetics or molecular biology, we now know that it also applies to these fields. Model organisms are widely used in biological and biomedical research, ranging from the humble *E. coli*, to plants like Arabidopsis, unicellular organisms such as yeast, multicellular animals such as *C. elegans*, fruit flies, zebra fish, up to complex mammalian models like rodents, human cell cultures and organoids. It is humbling to realize we are similar to these simple systems in so many ways, and these models have been, and will remain, incredibly valuable for genetic research.

In the sixties Sydney Brenner chose the nematode *C. elegans*, primarily to study neuronal development. Somewhat later in 1974, he established it as a genetic model with a much wider application [155]. There several characteristics that make it such a valuable model: an adult *C. elegans* has just 959 cells and an approximate length of only 1 mm; it grows and reproduces quickly and is inexpensive to maintain, within 3 days a fertilized egg grows into a fertile adult that can produce 200 to 300 offspring; and *C. elegans* can be grown easily on agar plates seeded with *E. coli* as food, at room temperature. When food becomes limited the worms go into a special survival stage (referred to as dauer stage) that can last for months. Also important: populations of worms can be frozen at -80°C for decades, if not longer. Typically, *C. elegans* exist as self-fertilizing hermaphrodites, but one in every thousand animals is born as a male. This provides an essential opportunity: males from one genetic background can be crossed to hermaphrodites from another, which allows researchers to combine mutations in different genes for example. In addition, the ability to self-fertilize allows the growth of large clonal populations. For many years, forward genetic screening and the generation of mutants have relied on random mutagenesis with DNA damaging agents. Most of

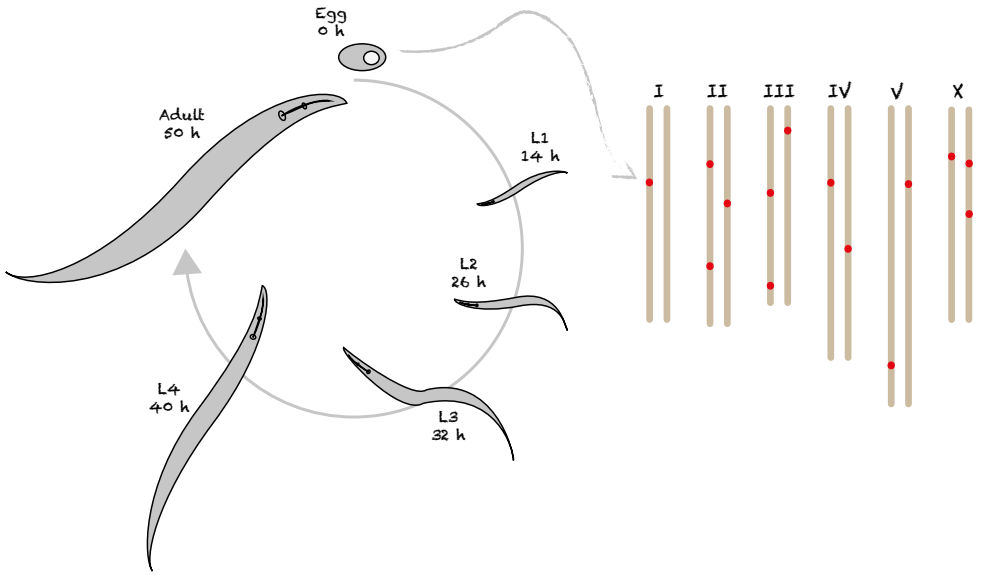

Figure 5. Mutation accumulation over multiple generations in C. elegans.

the generated strains are documented and available through the WormBase initiative and the number of available mutants exploded with the Million mutation project [156]. Alternatively, for reverse genetic screening genome wide RNAi libraries were widely used, and transgenic DNA can easily be introduced through microinjection. For many years targeted genome editing offered great difficulty, but recently this was overcome by the development of the CRISPR/Cas9 system and the application of it in *C. elegans* [157,158]. When in 1998 *C. elegans* became the first multi-cellular organism with a fully sequenced genome, it showed strong conservation of many genes and pathways. This includes the DNA repair pathways I discussed earlier and provides one of the many arguments why *C. elegans* is so well suited as a model to study DNA repair and the consequences of DNA damage [159-161].

All of the research in this thesis is performed in *C. elegans* and I exploited many of its benefits as a genetic model. For example, the high conservation of TLS, NER and POLQ and ease of crossing different genotypes served us well for chapter 2. In chapter 3 & 4 we made use of the quick life cycle and the possibility to grow clonal populations to investigate the accumulation of mutation over many generations (Fig. 5), while in chapter 5 we use microinjection of foreign DNA to study the repair of crosslinked DNA.

DNA sequencing: from 77 to 3.200.000.000 nucleotides and beyond

After it became clear that DNA holds the genetic code, the race was on to develop methods to determine the order of nucleotides in DNA and RNA molecules. Robert Holley was the first to do this and published the sequence of 77 ribonucleotides of alanine tRNA in 1965 [162]. Sequencing DNA proved to be more difficult, but in 1977 two scientists independently developed a chain termination technique to sequence DNA [163,164]. This first-generation sequencing technique, referred to as Sanger sequencing, is still widely used. For the research discussed in the following chapters Sanger sequencing has provided innumerable DNA repair footprints. Over the years that followed further advances in sequencing technique and computational power led to an iconic achievement; sequencing of the entire human genome of 3,200,000,000 bases was initiated in 1990, a first draft published in 2001, and completed in 2003 [165]. This project drove the development of next generation sequencing (NGS) techniques that are faster, cheaper and have increasingly higher throughput. The current NGS platforms can sequence up to hundreds of gigabases per run, and soon it will cost <$1000 to fully sequence an entire human genome. The dropping costs and increased sequencing speed and computational capacity have provided many new possibilities in genetic research and diagnostics and will continue to do so [166]. From the point of view of a researcher the identification of somatic mutations in tumor cells may lead to a deeper understanding of cancer, while for a patient it can be applied to assign personalized treatments that target the now identified weaknesses of the tumor. In chapter 3 & 4 I used NGS to identify novel mutations that have accumulated in the genome of *C. elegans* over a known number of generations, allowing me to identify mutational footprints and mutation frequency in a very unbiased way.

Genetic engineering

The development of our ability to read the genetic code goes hand in hand with advances to edit the genetic code. The first genetically modified viruses, bacteria and even the first transgenic mouse were already made in the seventies. Nowadays, transgenic plants are widely used as a food source, and applications of genome editing in medicine and research are countless. Therefore, I will limit myself to mention a few examples that are applied in *C. elegans* and relevant for the work described in this thesis.

Transgenic DNA can be introduced by microinjection into the gonadal syncytium of a young adult hermaphrodite. Multiple copies of the exogenous DNA then recombine to form an extrachromosomal array in the embryos. The genes on this array can, for example, rescue a genetic defect or to introduce a phenotype [167,168]. One of the most widely used phenotypic markers is green fluorescent protein (GFP). The gene that encodes GFP is from the bioluminescent jellyfish *Aequorea victoria*. Martin Chalfie was

the first to express the GFP coding sequence in another eukaryotic system: *C. elegans* [169], for which he received the Nobel Prize.

Precise and targeted editing of genomes has always been challenging, especially in *C. elegans*, but the discovery of the CRISPR/Cas9 system has taken biomedical research by storm [157,158]. CRISPR systems are adaptable immune mechanisms used by many bacteria. When a bacterium gets infected for the first time it incorporates a sequence from the invader into its own genomic CRISPR locus. Upon a next infection with the same pathogen, the invader sequence is transcribed and processed into a mature CRIPS RNA, which forms a complex with Cas9. This complex then targets the invading DNA and induces DSBs resulting in the destruction of the pathogen [170]. This system has been adapted by researchers and can now be used to induce targeted DSBs at almost any location of choice in a wide variety of organisms, ranging from *C. elegans* to mammals [Reviewed in 171]. The CRISPR/Cas9 technique can be applied to generate targeted gene knockouts or specific mutations, and to introduce foreign sequences at precise genomic locations. I applied this novel technology in the work described in this thesis; in chapters 3 and 4 I have used it to generate new knockout alleles of the REV-1 gene and to introduce a specific amino acid substitution mutation in the gene encoding PCNA (PCN-1 in *C. elegans*). Generating this PCNA mutation would not have been possible without CRISPR/Cas9.

The isolation and subsequent expression of GFP in different species and the discovery, development, and application of the CRISP/Cas9 system are both iconic victories for fundamental biological research. They show that studies in simple model organisms can have profound impact on applied research and medicine. Nowadays, gene therapy is already used to combat genetic disease and it can be expected that further development of sophisticated genome editing techniques will improve such treatments greatly. In the future we may come to rely more and more on genome editing techniques for our survival; further genetic adaptation of food crops may be necessary to sustain the ever-growing human population, for example. In any case, the field of genetic research will have a sensational and challenging future.

Aim and outline of this thesis

In this thesis I will argue that TLS is a mechanism that not only maintains replication potential and thereby promotes survival in the presence of DNA damage, but also preserves genome stability. When I started the research described in this thesis it became apparent that TMEJ is particularly important for the repair of replication associated breaks, and this thesis provides strong support of this notion.

Chapter 2 was initially titled *Nightcrawlers – When daylight kills*. In this chapter we show that a worm, when stripped of three DNA damage response mechanisms, completely loses the ability to live in the presence of regular daylight. It directly

illustrates that our natural environment on earth is inducing a harsh burden for DNA and it shows how essential the combined efforts of different DNA damage response mechanisms are, thus allowing organisms to live long and prosper in the face of DNA damaging environments.

In **chapter 3** we show that the TLS polymerase REV-1 protects genomic stability under challenged and unchallenged conditions and we confirm that Polθ mediated end-joining repairs replication associated DSBs in REV-1 deficient animals.

Chapter 4 provides evidence how TLS factors Polζ and PCNA also provide genome stability. In addition, we show that mutations accumulate in animals that lack all Y-family polymerases as well as Polζ, yet they are able to grow and reproduce.

In **chapter 5** I present a novel assay that I developed together with Robin van Schendel to specifically look at mutagenesis at single ICLs. The approach is based on the assays that have been used to study ICL repair in Xenopus egg extracts and provides a new tool to study the complex ICL repair mechanisms.

In the general discussion in **chapter 6** I will reflect on the conclusions of the experimental chapters and discuss how these impact our current understanding of evolution, and diseases like cancer and ageing. I will also consider the future perspectives of this research.

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