CHARACTERIZATION AND FUNCTIONAL STUDY
OF DNA POLYMERASE BETA DURING ZEBRAFISH DEVELOPMENT

A dissertation presented

by

Xiaojie Yang

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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology in the Graduate School of Arts and Sciences of Northeastern University, August, 2009
ABSTRACT

Base excision repair (BER) is the most common DNA repair mode in nature. It is very important for repairing alkylation damage, oxidative damage and AP sites produced by either environmental or endogenous agents. The BER pathway and many of its key components have been very well characterized by in vitro reconstituted biochemical assays using mammalian recombinant proteins. DNA polymerase beta (Pol β) is the major DNA polymerase in this pathway. The bifunctional enzyme, which has both 5’-dRP lyase and DNA synthesis activities, is indispensable for mammalian development, but not cell survival. However, the early lethality in PolB−/− mice prevents a further investigation of the mechanism underlying death shortly after parturition.

In this study, I investigated the role of Pol β in early development using a vertebrate model, zebrafish. I first cloned the zebrafish polb gene and characterized its expression pattern at both mRNA and protein levels throughout zebrafish embryogenesis. Functional studies using both knockdown and overexpression were performed thereafter.

Zebrafish polb encodes a very conserved protein, sharing ~80% sequence identity with its human counterpart. Only one gene copy for polb is found in zebrafish genome, and one transcript is present in embryos and adults. Despite the fact that Pol β mRNA is expressed at early developmental stages I examined, Pol β protein is not detectable until after mid-gastrulation stage. The protein level then increases as development progresses.
Although morphogenesis in zebrafish embryos was not affected by either reduction or overexpression of Pol β in very early embryos, Pol β knockdown embryos showed a hypersensitivity phenotype to the alkylation agent, methyl methanesulfonate (MMS). Ectopic expression of eGFP-tagged Pol β protein in the early knockdown embryos can partially restore MMS-resistance. These results imply that the Pol β-dependent BER pathway is crucial for combating alkylation DNA damage, maintaining genome integrity, and promoting organismic survival.

The protein that precedes Pol β in the BER pathway is AP endonuclease (AP endo), which cleaves an abasic site to generate the 3’-hydroxyl group required by the polymerase. A reduction of Pol β protein levels was found in AP endo hypomorphic zebrafish embryos generated by MO knockdown. In contrast, there was no change in AP endo protein levels after downregulating Pol β. This finding suggests a potential regulatory function of AP endo on its downstream partner Pol β.

In short, this is the first study on the zebrafish polb gene. Expression studies and functional characterization during early development confirm its definitive role in DNA repair. However, knockdown zebrafish embryos develop normally during embryogenesis, implying that zebrafish Pol β does not play an essential role in early development.
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consequently obtain a good zebrafish antibody.

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<td>Abbreviation</td>
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<tr>
<td>AAAF:</td>
<td>N-acetoxy-2-acetylaminoacridine</td>
</tr>
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<td>AD:</td>
<td>Alzheimer's disease</td>
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<td>amyotrophic lateral sclerosis</td>
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<td>AP sites:</td>
<td>abasic sites</td>
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<td>AP endo:</td>
<td>AP endonuclease</td>
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<td>BER:</td>
<td>base excision repair</td>
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<td>BRCT:</td>
<td>BRCA1 C Terminus</td>
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<td>CPDs:</td>
<td>cyclobutane pyrimidine dimers</td>
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<td>dpf:</td>
<td>days post fertilization</td>
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<tr>
<td>dRP:</td>
<td>deoxyribose phosphate</td>
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<tr>
<td>DSB:</td>
<td>double strand DNA breaks</td>
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<td>DSBR:</td>
<td>double strand break repair</td>
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<tr>
<td>FEN 1:</td>
<td>flap-endonuclease 1</td>
</tr>
<tr>
<td>hpf:</td>
<td>hour post fertilization</td>
</tr>
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<td>HR:</td>
<td>homologous recombination</td>
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<td>lpBER:</td>
<td>long-patch BER</td>
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<td>mouse embryonic fibroblasts</td>
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<td>EMS:</td>
<td>ethyl methanesulfonate</td>
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<td>MMR:</td>
<td>mismatch repair</td>
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<td>MMS:</td>
<td>methyl methanesulfonate</td>
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<td>MNNG:</td>
<td>N-Methyl-N'-Nitro-N-Nitrosoguanidine</td>
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MNU: methylnitrosourea
MO: morpholino
NER: nucleotide excision repair
NHEJ: nonhomologous end joining
PARP 1: Poly ADP ribose polymerase 1
PD: Parkinson’s disease
Pol β: DNA polymerase beta
PRMT6: arginine methyltransferase 6
RACE-PCR: rapid amplification of cDNA ends- polymerase chain reaction
ROS: reactive oxygen species
RT-PCR: reverse transcription-polymerase chain reaction
SMUG1: single-strand-selective monofunctional uracil-DNA glycosylase 1
spBER: short-patch BER
ssb: single strand breaks
TdT: terminal deoxynucleotidyl transferase
TLS: translesion synthesis
UDG: uracil DNA glycosylase
UV: ultraviolet
ZAP1: zebrafish AP endonuclease 1
INTRODUCTION

1. DNA damage and mammalian DNA repair systems

Living cells are constantly exposed to endogenous and exogenous agents that could cause DNA damage, and therefore affect DNA replication, repair, and recombination. To combat DNA damage, mammalian cells have acquired several DNA repair pathways, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and double strand break repair (DSBR) [1]. Despite some functional overlaps, each of these repair systems is responsible for recognizing and repairing specific DNA lesions. BER is the most important repair pathway for repairing small, non-distorted DNA and abasic (AP) sites arising from spontaneous depurination or removal of damaged base. Most of the lesions introduced by cellular reactive oxygen species (ROS) and alkylating agents can be repaired by BER [2]. In contrast, NER is an error-free repair pathway for fixing bulky or helix-altering lesions created by exposure to radiation or chemical agents. NER is a multisubunit enzyme system that makes dual incisions bracketing the lesion in the damaged strand [3]. The MMR pathway repairs mismatches and loops generated by insertions or deletions. In some cases, mismatch repair of damaged bases can also be mediated by the BER pathway [4]. Last, repair of double strand DNA breaks (DSB), arising from ROS, ionizing radiation, or V(D)J recombination, is mediated by the homologous recombination (HR) or nonhomologous end joining (NHEJ) pathway. HR is a complex process that involves using an intact homologous DNA strand as a template to repair a DSB, whereas NHEJ is a major pathway for DSBR,
which involves religation of the broken ends without any regard to homology, and is relatively error-prone [5]. Failure of DNA repair could result in mutagenesis, and ultimately genome instability and cancer initiation, unless cell death destroys the cell first.

2. Base excision repair (BER)

Base excision repair (BER) is the primary DNA repair pathway that corrects small base lesions arising from oxidative, alkylation, deamination, and depurination/depyrimidination damage. Intensive studies have revealed the essential components in this pathway and how they are regulated [6] [7].

2.1 BER pathway

Complete repair by BER is a five-step process: lesion recognition/base removal, strand incision, end processing, DNA synthesis and DNA ligation. Biochemical studies have identified two BER subpathways, short-patch BER (spBER) and long-patch BER (lpBER). spBER fills in only one nucleotide, while lpBER involves 2-10 nucleotides inserted at the damaged site with resulting strand displacement [8].

Usually, BER is initiated by DNA glycosylases that specifically recognize and remove the damaged base, forming an AP site in one DNA stand. The AP site is incised at the 5’ side by AP endonuclease (AP endo), creating a 3’ hydroxyl group and a 5’ deoxyribose phosphate (dRP). In the next step, the pathway can be driven to either spBER or lpBER. In spBER, Pol ß inserts the correct base at the damaged site and removes the dRP residue by means of its intrinsic 5’-dRP lyase activity. In contrast, in lpBER, 2-10 nucleotides are inserted by Pol ß or the replicative
polymerases Pol δ/ε, followed by cleavage of the displaced strand by flap-endonuclease 1 (FEN I). Finally, Ligase III/XRCC1 complex (for spBER) or Ligase I (for lpBER) ligates the nick to complete the repair. The major BER pathway is illustrated below.

The Major BER Pathway

2.2 Choice of the two subpathways

Two main factors determine the choice of subpathway. Firstly, the choice can depend on the nature of the lesion. If the dRP intermediate after AP endo incision can be efficiently removed by the lyase activity inherent in Pol β to yield a 5'-phosphorylated DNA strand capable of serving as a substrate for DNA ligase, the pathway favors the short patch route [9] [10] [11]. AP sites, 8-oxoGua, thymine
glycol, and ring-alkylated purines are repaired mainly via spBER. In contrast, lpBER occurs when repairing a reduced AP site or DNA single strand breaks [12]. Secondly, the properties of the lesion-specific DNA glycosylases, i.e. monofunctional (glycosylase activity only) or bifunctional glycosylase (possessing additional AP lyase activity for cleaving the phosphodiester backbone 3’ to the AP site generated by the glycosylase activity), can determine the choice of the BER subpathways [13]. For instance, if the AP site is incised by a bifunctional DNA glycosylase, which results in a one-nucleotide gap with a sugar-phosphate group at the 3’-margin of the gap, spBER takes place after AP endo cleavage 5’ to the dRP. Thirdly, recent studies found that BER was under an energy-dependent regulation, and the switch between spBER and lpBER can be regulated by ATP levels after the dRP excision step. Under conditions of ATP shortage, XRCC1 stimulates strand displacement DNA synthesis by Pol β so that lpBER is favored when ATP levels are low. On the other hand, if ATP is sufficient, ligation by Ligase III prevents strand displacement, leading to spBER [14]. Finally, the ratio of different BER proteins seems to be critical to this switch as well. In an in vitro reconstituted BER system, excess AP endo relative to Pol β stimulates strand-displacement DNA synthesis by Pol β, and thus enables pol β to mediate long patch subpathway [15].

Although the two BER subpathways have been well characterized, there are limited descriptions about quantitative distribution and contribution of the two sub-pathways within living cells thus far. The availability of this information would be very helpful for further understanding this cellular process in response to genotoxic stress.
2.3 Regulation of the BER process

2.3.1 Repair complex formation

BER is a highly coordinated, step-wise process. A complicated interaction network occurs during the repair process, forming protein-protein and protein-DNA complexes. Scaffold proteins act as critical mediators for the formation of the repair complexes. XRCC1 acts as a BER scaffold protein, physically interacting with many BER proteins, including Ligase IIIa, Pol β, Poly ADP ribose polymerase 1 (PARP1), AP endo, and a few DNA glycosylases (MPG, OGG1 and NEIL2) [16] [17] [18] [19] [20] [21] [22]. The major BER endonuclease, AP endo, also forms complexes with other DNA repair proteins, including Ligase I, Fen1, PCNA, Pol β and XRCC1 [23] [19] [24] [25] [26]. Pol β has been found interacting with AP endo, XRCC1, PCNA, Fen1 and Ligase I [26] [27] [28] [17] [29] [30] [31] [32] [33] [34] [35] [36]. Thus, in the BER pathway, the repair intermediates are passed between different repair complexes by a ‘hand-off’ mechanism [37]. This highly coordinated process ensures the transfer of potentially toxic repair intermediates to the next protein (complex), and therefore makes BER an efficient repair machinery with minimal opportunity for loss of an intermediate, which might be quite genotoxic.

2.3.2 Post-translation modification

BER is also regulated by the posttranslational modification of the repair proteins. By altering enzyme activity, subcellular localization, or complex formation, these modifications can thus change BER activity and fidelity [7]. The most common modification found in BER proteins is phosphorylation. Most critical proteins, such as OGG1, AP endo, FEN1, are post-translationally phosphorylated.
also modified by acetylation, sumoylation, ubiquitylation and methylation [7]. These modifications change the protein activities to varying degrees. For example, acetylation at Lys$^{72}$ of Pol β by p300 abrogates the 5’ dRP lyase activity [38]. Phosphorylated rat Pol β protein exhibits higher BER activity than the un-phosphorylated form [39] [40]. Methylation of Pol β at Arg$^{83}$ and Arg$^{152}$, promoted by its interaction with arginine methyltransferase 6 (PRMT6), results in an increase of the processivity of its polymerase activity [41] [42].

2.4 Importance of BER pathway

Impaired BER capacity results in accumulation of DNA lesions, which can lead to mutagenesis and various malignancies or to cell death. Previous studies have led to the proposal that BER is involved in protection from carcinogenesis and aging. (For review, see [43]). Recent research connects BER to several other cellular processes, for instance, neuronal development and neurodegenerative disease [44], immunoglobulin gene diversity [45], and antibody class switching [46]. In particular, the functional roles of BER in the nervous system have been well characterized. Studies have shown that defective BER processing can promote post-mitotic neuronal cell death, associated with neurodegenerative disorders, such as Alzheimer’s disease (AD), Parkinson’s disease (PD) and amyotrophic lateral sclerosis (ALS). For example, high levels of oxidative lesions and increased mutations in OGG1 are found in the brain tissue from AD patients, indicating defective BER may play an important role in the progression of AD [47] [48]. However, how exactly these physical conditions interplay with the BER pathway or specific BER proteins is still largely unknown.
2.5 Unanswered questions on BER pathway

Several questions remain to be elucidated for BER. The major question is how DNA repair proteins gain access to the nucleosome-wrapped DNA strand to perform repair. Since most biochemical studies of the BER pathway are carried out on naked DNA, more information on BER-nucleosome interaction could be valuable to understand the repair system in vivo. Several groups have started to explore the capacity of some BER proteins to act on lesions in model nucleosomes in vitro. They demonstrate that human uracil DNA glycosylase (UDG), uracil DNA glycosylase 2 (UDG2) and single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) display reduced efficiency in removing uracil residues from nucleosomes [49] [50]. Beside the finding with DNA glycosylases, it was also found that Pol β activity is largely inhibited by the presence of nucleosomes [49] [50]. By comparison, human NTH1 DNA glycosylase and long patch enzymes Ligase 1 and FEN1 maintained efficient work in the presence of nucleosome-containing substrates [33] [51] [52]. It remains to be demonstrated whether the entire BER pathway can be completed efficiently on lesions bound to nucleosomes, or whether BER enzymes either have additional activities to loosen chromatin or partner with chromatin modification enzymes to gain access to DNA lesions and perform repair.

3. DNA polymerases and X-family DNA polymerases

3.1 DNA polymerases

DNA polymerases are enzymes that incorporate deoxynucleotides into DNA during either replication or repair. DNA polymerases play an indispensable role in
keeping the genome intact and allowing cells or organisms to develop in an organized manner. Five DNA pols, Pol I, II, III, IV and V, have been characterized in prokaryotes. Pol I and Pol II are involved in DNA repair. Pol III is the major polymerase responsible for DNA replication. Pol IV and Pol V are Y family DNA polymerases functioning in translesion synthesis.

Compared to the small number of DNA polymerases existing in bacteria, at least 19 DNA polymerases have been discovered in eukaryotes, including terminal transferase and telomerase. In fact, the polymerase group is still growing with even more putative members being characterized. All eukaryotic DNA polymerases are categorized into four families, A (Pol γ, ν, θ, π), B (Pol α, δ, ε, ζ), X (Pol β, λ, μ, terminal deoxynucleotidyl transferase (TdT)) and Y (Pol η, i, κ, Rev1) family, based on the primary sequence (For review, see [53] [54]). In general, families A and B are high fidelity enzymes involved in DNA replication. Family X includes enzymes involved in DNA repair. Members in Family Y are called translesion synthesis (TLS) polymerases because of their low fidelities on undamaged templates and their ability to replicate through damaged DNA. Depending on the lesion, Family Y polymerases can bypass the damage in an error-free or error-prone fashion, the latter resulting in elevated mutagenesis.

3.2 X-family DNA polymerases

There are four members of the X-family DNA polymerases, Pol β, Pol λ, TdT and Pol μ. The structures of all four Pol X enzymes have been solved by either NMR or X-ray crystallography (For a review, see [55]). Overall conformation and subdomain organization are very similar in the four Pol X enzymes. They all contain a BRCA1 C
terminus (BRCT) domain and a Pol X conserved domain, with the exception that Pol β does not have the N-terminal BRCT domain. The BRCT domain exists in many DNA repair proteins, and potentially functions as a protein-protein interacting moiety.

The expression patterns of these enzymes have been characterized in mammals. In mouse, brain and testis are the two tissues expressing high levels of Pol β mRNA [56], while Pol λ transcript is highly expressed in testis and ovary [57]. The other two Pol X enzymes, TdT and Pol μ, are found in lymphoid cells, indicating a role in the mammalian immune system [58] [59].

In the matter of function, Pol X enzymes show very diverse activities in DNA synthesis and repair. Pol β is the major repair polymerase participating in the BER pathway. TdT is involved in creation of antibody diversity. It adds non-template nucleotides (N-nucleotides) randomly to 3’ DNA ends, thereby generating stretches of random sequence (N-regions) at immunoglobulin gene junctions during V(D)J recombination [60]. Pol λ and Pol μ are novel DNA polymerases in this family discovered in 2000 [61] [62] [57] [59]. Pol λ is implicated in NHEJ [63] [64] and possibly acts as a backup enzyme for Pol β in BER [65] [66]. Pol μ is demonstrated to be involved in NHEJ and V(D)J recombination [67] [68].

To study the role of X-family DNA polymerases in a physiological background, mutant mice have been generated. Null mutant mice for Pol λ or Pol μ are viable, fertile and display a normal hypermutation pattern [69]. Mice with a mutant TdT gene are also viable, but demonstrate the nearly complete absence of N-nucleotides in their B and T cells, suggesting an essential role of TdT in maturation of the immune system [70] [71]. Different from other Pol X enzyme-deficient mice, PolB−/− mice show
a severe phenotype, i.e., perinatal lethality. Extensive apoptosis in the developing nervous system and defective in lung function probably contribute to the early death in these knockout mice [72]. Over all, these studies indicate that the X-family polymerases mainly contribute to double strand break repair and base excision repair.

4. DNA polymerase beta – the primary polymerase in BER

4.1 Enzymatic activity, structure and cellular expression

Mammalian Pol β is a 39 kDa bifunctional enzyme with both polymerase and 5'-dRP lyase activities [9] [73] [74] [75]. This enzyme lacks 3’ or 5’-exonuclease activity, contributing to its low fidelity. It has been demonstrated that high Pol β protein level can induce mutagenesis in cultured cells [76]. A study on the 3’-5’ exonuclease activity of AP endo, using 3’ mispaired recessed DNA or 3’ mispaired nicked DNA as substrates, shows that AP endo preferably and efficiently removes the mismatched nucleotide from nicked DNA, which suggests that AP endo exonuclease activity could perform the proofreading for the error-prone Pol β to minimize the misincorporation during BER [77]. The two Pol β activities are associated with two functional domains. The smaller 8 kDa amino terminal domain carries a 5’-deoxyribose phosphate lyase activity, responsible for removing the sugar phosphate group of the cleaved AP site. The larger 31 kDa carboxyl terminal domain possesses the polymerase activity, incorporating new deoxynucleotides into a gapped DNA strand [78] [74] [79].

Fully active recombinant mammalian Pol β protein can be expressed in
Escherichia coli at high levels [80] [81] [79] [82], so that fine details of the Pol β protein structure could be analyzed by X-ray crystallography. The X-ray crystal structure for rat Pol β, the first crystal structure for a Pol X enzyme, was solved in 1994, in a ternary complex with the polymerase, DNA substrate and an incoming nucleotide bound in the active site [83]. The N-terminal lyase domain is easily identified, separated from the large polymerase domain by a flexible hinge. The crystallographic structure of the polymerase domain is similar to other DNA polymerases with three subdomains resembling a hand that can grasp DNA. The three subdomains are C (catalytic), D (duplex DNA binding) and N (nucleotidyl transferase), corresponding to the palm, thumb and fingers, respectively. Structure-function analysis has identified key residues located in the two separate domains contributing to the catalytic activity of each domain. Site-directed mutagenesis studies reveal that Lys\textsuperscript{72} serves as a catalytic nucleophile in the lyase domain [84]. In the 31 kDa polymerase domain, three conserved aspartates (Asp\textsuperscript{190}, Asp\textsuperscript{192}, and Asp\textsuperscript{256}) position two Mg\textsuperscript{2+} ions necessary for catalysis [85] [86]. Upon dNTP binding, subdomains and side chains undergo significant movement in order to make contact with the DNA minor groove. The minor groove interactions are necessary to maintain the fidelity of the polymerization. Key residues important for this interaction are Tyr\textsuperscript{271}, Phe\textsuperscript{272}, Asn\textsuperscript{279} and Arg\textsuperscript{283} [55]. Overall, structural features determine the biological function of Pol β.

Until the present study, Pol β was presumed to be a housekeeping enzyme found in all vertebrate cells. It is expressed at a constant low level, and is independent of the cell cycle and DNA replication [87]. However, Pol β expression
can be induced after treatment with genotoxic reagents. For example, Pol β mRNA levels in exponentially growing CHO cells are elevated three to five fold after treatment with methyl methanesulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or N-acetoxy-2-acetylaminofluorene (AAAF) via quantitative RNA dot blot analyses [88]. In mice, Pol β mRNA is universally expressed in all tissues with relatively high level in the testis and brain [56]. The expression pattern directs this research to discover additional functions of Pol β protein, besides its definitive role in BER.

4.2 Biological studies on Pol β knockout mice and Pol β null MEFs cell lines

Knockout mice carrying a targeted disruption in both copies of the Polβ gene show growth retardation and die shortly after birth due to respiratory failure. Histological examination of PolB−/− developing embryos reveals significant neuronal cell death, as well as the lack of inflation of air-way passages in the neonates [72]. As mentioned above, Pol β is very abundant in the mammalian brain [56]. Thus, Pol β appears to be essential for the repair of DNA damage in neurons and the postnatal survival of embryos. Moreover, neuronal apoptosis observed in Pol β-deficient mice can be rescued by tumor suppressor gene p53 deficiency. Nevertheless, p53 knockout does not reverse the developmental defects in the CNS, and PolB+/− p53−/− double knockout embryos do not survive after birth [89].

Although Pol β null mice are not viable, Pol β is not essential for the viability of mammalian cells. Pol β null mouse embryonic fibroblast (MEF) cell lines were established from murine embryos homozygous for the Polβ deletion mutation [90]. Mutagen sensitivity analysis reveals that these cells are hypersensitive to alkylating
agents, such as MMS, MNNG, methylnitrosourea (MNU), and ethyl methanesulfonate (EMS). However, \( PolB^- \) cells are not sensitive to UV radiation or gamma rays, indicating that Pol \( \beta \) does not play an indispensable role in NER. They also exhibit normal mismatch repair capacity [90]. In a separate study, Pol \( \beta \) knockdown mammalian cells, generated using RNA interference (RNAi) technology, which reduces the Pol \( \beta \) protein and mRNA level to undetectable levels, recapitulates the same hypersensitivity to DNA damaging agents in \( PolB^- \) MEFs [91]. Surprisingly, the dRP lyase domain alone is able to reverse the observed hypersensitivity to monofunctional alkylating agents, suggesting that the removal of the dRP group is a critical step during BER [92].

Further study shows that spBER is severely compromised in the \( PolB^- \) MEFs, but Pol \( \beta \)-deficient mouse fibroblasts extracts retain the ability to perform efficient long patch BER [90] [93]. In addition, a higher level of single strand breaks (ssb) are also present in MMS treated \( PolB^- \) cells as compared with wild-type cells. These MMS-induced ssb are rejoined at a slower rate than in control cells, suggesting an essential role of Pol \( \beta \) in repairing MMS-induced ssb. Taken together, these data support an essential role of Pol \( \beta \), especially its dRP lyase activity, for repairing methylation damage to DNA.

### 4.3 Pol \( \beta \) overexpression studies

Elevated expression level of Pol \( \beta \) is frequently found in human tumor cells, especially in prostate, breast, ovarian and colon cancer [94] [95] [96]. Furthermore, overexpression of BER proteins affects the overall efficiency of the BER pathway and ultimately gives rise to severe consequences, like cancer. In the case of Pol \( \beta \), ...
ectopic expression of Pol β causes a mutator phenotype in mammalian cells. It induces apoptosis, enhances mutagenesis, and makes cells more sensitive to oxidative damage and ionizing radiation [97] [98]. Deregulated Pol β can compete with error-free DNA polymerases during DNA replication, repair or recombination pathways, thus affecting genome stability [99] [100] [101]. Also, excess Pol β can interfere with normal BER by competing with Pol δ and Pol ε, thereby enhancing the probability for misincorporation [102]. Consequently, the presence of excess error-prone Pol β protein could promote tumorigenesis.

Given that previous reports imply that an imbalance in Pol β protein level may cause a functional deficiency in the BER pathway, and a particular sensitivity to oxidative DNA damage [99] [97] [98] [100], Sobol et al. developed a bicistronic tetracycline-responsive transgenic system to overexpress Pol β in mice. These mice develop severe cortical cataracts beginning within 4 days after birth. The authors proposed that either a deficiency in the BER pathway or the involvement of Pol β in lens epithelial cell differentiation might contribute to the phenotype [103]. However, no further data are available to elucidate the underlying mechanism for the cataract phenotype at this time.

4.4 Roles of Pol β in other cellular processes

4.4.1 Cancer Predisposition

As mentioned above, Pol β overexpression has been observed in human cancers. Meanwhile, human Pol β polymorphism is another common phenomenon in many cancers. Polymorphism in DNA repair genes can alter the biochemical properties of the protein, result in deficiency in DNA repair, and contribute to cancer
initiation [104]. Pol β variants have been found in over 30% of human tumors [105]. For example, the R137Q variant found in familial colorectal cancer is associated with decreased BER efficiency, possibly because of decreased polymerase activity and impaired interaction with PCNA [106]. The implication of Pol β variants in human cancer makes Pol β a potential therapeutic target. Studies of developing Pol β inhibitors with high specificity and affinity are in progress [107] [108]. Several inhibitors have been identified by NMR chemical shift mapping [109]. Among these inhibitors, pamoic acid is the most active and specific one. Pamoic acid inhibits lyase and polymerase activities of purified Pol β on a uracil-containing DNA substrate, and increases sensitivity to MMS [109]. Hazan et. al. combined computational approaches and NMR techniques and generated a detailed 3D model of the complex of pamoic acid with the 8 kDa domain of the DNA polymerase. Pamoic acid occupies the site where single-stranded DNA binds to Pol β [108]. Thus far, pamoic acid is the most promising Pol β inhibitor.

4.4.2 Nervous system

Reduced Pol β expression level and function are observed in rat brain during postnatal development and in aged mouse brain, while other BER proteins, e.g. AP endo, XRCC1, Ligase 1 and 3, do not change relative to age [110] [111]. A role for Pol β in the nervous system is also supported by the data generated from PolB−/− mice, which show abnormal neuronal development and neonatal lethality [72]. Therefore, a role for Pol β in development and maintenance of the neurological system is a distinct possibility.

4.4.3 Meiosis
The finding of high expression levels of Pol ß in mammalian testis triggers the investigation of a potential role of Pol ß in meiosis. Antibody staining reveals that Pol ß protein is present on homologous chromosomes during prophase I of meiosis in mouse spermatocytes [112]. Further study shows that Pol ß colocalizes with the glutamate receptor interacting protein 1 (GRIP1) on meiotic bivalents of both spermatocyte and oocyte nuclei [113]. These data support the participation of Pol ß in meiosis.

4.5 Other BER mouse knockouts

Although the BER pathway has been very well characterized through in vitro reconstitution assays with purified proteins from mammalian sources, the biological role of BER is still not fully understood. Murine knockouts for most of the key BER proteins have been generated in order to dissect the physiological role of BER and its individual components. Disruption of any of Apex1, PolB, Fen1, Xrcc1, Lig1 and Lig3 genes leads to lethality during embryogenesis [114] [115] [116] [117] [118] [119] [120] [121]. In contrast, DNA glycosylase-deficient embryos, including Nth1, Ogg, Nth1Ogg double mutant, Udg, Aag, Myh, MyhOgg double mutant, are viable and show normal development without obvious phenotypes [122] [123] [124] [125] [126] [127] [128] [129] [130]. The lack of severe phenotype observed for DNA glycosylase-deficient mice suggests there are probably backup mechanisms for dealing with the removal of damaged bases. Other repair systems in the cell, such as mismatch repair and translesion synthesis, can also complement the DNA glycosylase-initiated BER activity [131]. Another interesting finding is that in some cells, deficiency in certain DNA glycosylases leads to resistance to alkylating
agents. For instance, myeloid progenitor bone marrow cells derived from $Aag^{-/-}$ mice, display an alkylation-resistant phenotype [132]. MMS or MNU induced retinal degeneration in mice can be suppressed by a mutation in $Aag$ genes [133]. Taken together, these data suggests that unrepaired alkylation lesions can be well tolerated and that the initiation of BER and accumulation of repair intermediates due to deficiency in downstream enzymes is more lethal to the cell than leaving the damaged bases unrepaired.

5. BER studies in zebrafish

5.1 Advantages of using zebrafish system

Zebrafish has been a popular vertebrate model for biological studies for the last few years. The main advantages of using zebrafish embryos are as follows:

- The zebrafish genome has been sequenced and is available at University of California, Santa Cruz (UCSC) ([ftp://hgdownload.cse.ucsc.edu/goldenPath/danRer4/](ftp://hgdownload.cse.ucsc.edu/goldenPath/danRer4/)).
- Zebrafish and mammals exhibit a high degree of similarity with respect to molecular mechanisms of development and cellular physiology.
- Fertilization and development occurs externally, allowing for direct observation of the whole process of embryogenesis by light microscopy.
- Embryonic development in zebrafish is rapid. Embryogenesis is complete five days after fertilization.
- Gene expression can be suppressed by the use of morpholino (MO) antisense oligonucleotides, which offers a reverse genetic approach to study
gene functions. Conversely, microinjection of capped mRNA or plasmids can restore the expression of specific proteins throughout the embryo or in a tissue-specific manner.

- A large number of embryos can be obtained at minimal cost.

Due to the above advantages and others not listed here, zebrafish has become an ideal model to perform genetic screens and facilitate gene function studies. Recently, it has been widely used in human disease study, drug discovery, and chemical toxicity studies [134] [135].

**5.2 BER activity during early development**

The BER pathway is very well characterized in mammalian cell lines. However, there is very little literature describing the BER pathway and its repair activity during early embryogenesis. Recently, the Strauss lab published the first studies on BER activity during zebrafish embryogenesis [136]. Clearly, BER is active throughout embryogenesis, even in unfertilized eggs. BER is less active in prehatching embryos than in later embryos and adults. Its activity reaches adult level after 3 dpf, as embryos hatch from their chorions. More importantly, instead of Pol β, aphidicolin-sensitive replicative polymerases play a major role in the gap-filling step for both short and long patch repair in embryos before 3 dpf. After 3 dpf, however, embryos rely on Pol β almost entirely for the DNA synthesis of BER. In addition, BER proceeds normally in AP endo knockdown cells, suggesting that another standby enzyme possessing AP endo activity can serve in the BER pathway, and that the failure of BER is not the cause of the observed embryonic lethality in zebrafish AP endo knockdown embryos. Over all, BER is active during zebrafish early
development and in adults, strengthening the impression that BER is an important physiological process during vertebrate development.

**5.3 AP endo in zebrafish development**

Two separate studies in mice document the lethality phenotype after *Apex1* knockout. Xanthoudakis *et. al.* demonstrate that knockout embryos die shortly after implantation at E5.5 [114]. Although zygotic transcription turns on at the 2-cell stage in mouse embryos, E5.5 is otherwise roughly equivalent to the midblastula transition (MBT) in zebrafish. Another study by Ludwig *et. al.* shows knockout embryos develop normally through E7.5, but decline and die by E9.5 [116]. No homozygous null cell lines have been developed to date.

Zebrafish AP endonuclease 1 (ZAP1) is also essential for development. Decreasing ZAP1 levels by antisense morpholino (MO) to 10-30% of controls causes embryonic death shortly after the MBT, which is consistent with the data generated in the mouse system [137]. The early death can be rescued by introducing the wild-type human AP endo protein, but not the endonuclease activity-deficient form of human protein, which indicates a requirement for the endonuclease activity of this protein in vertebrate development. ZAP1 mutant embryos, in which the level of ZAP1 has been reduced to ~ 50%, are viable, but develop with abnormal morphology. Further histological examination reveals that zebrafish AP endo is involved in heart, blood and brain development ([137] and unpublished data).

**6. Pol β in zebrafish development – studies in this thesis**
The importance of BER proteins in cell physiology and the fundamental roles of AP endo and Pol β in mouse development lead to my hypothesis that Pol β may also play an important role in zebrafish development. Moreover, investigating the role of Pol β in zebrafish development would expand the understanding of the importance of this protein in very early embryogenesis where it has not yet been studied.
MATERIALS AND METHODS

1. Zebrafish husbandry and breeding

Wild-type zebrafish were purchased from Aquatic Tropicals (Plant City, FL). Fish were raised and kept at standard laboratory conditions of 28°C on a 14 hr dark, 10 hr light cycle. To collect eggs, adult male and female zebrafish (1:2 ratio) were placed in a small tank with a divider in the center to separate males from females on the day before breeding. After the divider was removed the next morning, the fish spawned. Fertilized eggs were collected, cleaned, staged [138] and then used for experiments.

2. Cloning of zebrafish *polb* gene by RT-PCR (Reverse transcription-polymerase chain reaction) and RACE (rapid amplification of cDNA ends)-PCR.

Primers (Table 1) were designed to amplify the zebrafish *polb* coding sequence according to the sequence in the Sanger Database (http://www.ensembl.org, Ensembl Gene ID: ENSDARG00000003749). Primers were synthesized by Genelink, Inc. (Hawthorne, NY). The cDNA template was prepared from total RNA using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Total RNA, isolated from adult fish by TRIzol® reagent (Invitrogen, Carlsbad, CA), was reverse transcribed to first-strand cDNA. PCR reaction mix contained 1 µl cDNA template, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer, 1 µl dNTP mix (10 mM each), 0.5 µl ‘Phusion’ hot start high fidelity DNA polymerase (New England Biolabs, Ipswich, MA) and 10 µl 5X HF (high fidelity) buffer provided with the DNA
polymerase in a total volume of 50 µl PCR reaction. The PCR program included denaturation at 98°C for 1 min, followed by 30 cycles of 98°C for 15 s, 65°C for 30 s, 72°C for 40 s, and completed with additional elongation at 72°C for 5 min.

To obtain the full length cDNA sequence including 5' and 3' cDNA ends, the GeneRacer™ Kit (Invitrogen, Carlsbad, CA) was used according to the instructions in the manufacturer’s manual. 3' RACE template cDNA was prepared by reverse transcription of adult fish total mRNA using GeneRacer™ Oligo dT primer. 5' RACE template cDNA was prepared by reverse transcription of decapped adult fish mRNA with a gene specific primer (GSP), which is the reverse primer used to amplify the coding region. 3' RACE PCR reaction mixes contained 2 µl cDNA template, 1 µl 10 µM GSP1, 1 µl GeneRacer™ 3' Primer, 1 µl dNTP mix (10 mM each), 0.5 µl Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO), 1.5 µl MgCl₂ and 5 µl 10 X PCR buffer provided with the DNA polymerase in a total volume of 50 µl PCR reaction. For the 5' RACE PCR reaction, the same mix was prepared as for 3' RACE PCR, but with 1 µl 10 µM GSP3, 3 µl GeneRacer™ 5' Primer. The PCR program included denaturation at 94°C for 3 min, followed by 5 cycles of 94°C for 30 s, 72°C for 3 min, 5 cycles of 94°C for 30 s, 70°C for 30 sec, 72°C for 3 min, 25 cycles of 94°C for 30 s, 68°C for 30 sec, 72°C for 3 min, and completed with additional elongation at 72°C for 5 min. After the RACE-PCR, the PCR products were subjected to a nested RACE-PCR using nested primers in order to determine the specificity of the amplified products. The PCR program for nested RACE PCR included denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 65°C for 30 sec, 72°C for 2 min, and completed with additional elongation at 72°C for 10 min. Finally, PCR
products were cloned into pGEM-T vector (Promega, Madison, WI), and sequenced (Tufts university core facility, Boston, MA).

Table 1: Primers for amplification of polb full-length cDNA. Sequences are list from 5' to 3'.

<table>
<thead>
<tr>
<th>PCR</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>coding region</strong></td>
<td>ATGAGTAAACGAAAAAGCGCCA CAAGAATCCC</td>
<td>TCACTCGCTGGGTCTTTCCGGCTCT CTGTA</td>
</tr>
<tr>
<td>5' RACE</td>
<td>GeneRacer 5’ primer CGACTGAGCACGAGGACACT GA</td>
<td>GSP1 GACCGATCCAGTCACTCTG</td>
</tr>
<tr>
<td>5' nested RACE</td>
<td>GSP2 GGACACTGACATGGACTGAAG GAGTA</td>
<td>GSP1 GACCGATCCAGTCACTCTG</td>
</tr>
<tr>
<td>3' RACE</td>
<td>GSP3 TACAGAGACGGAAGGACG CACCGAGTGA</td>
<td>GSP2 ATCTTTCACCCGACTCCATC</td>
</tr>
<tr>
<td>3' nested RACE</td>
<td>GSP4 TACAGAGACGGAAGGACG CACCGAGTGA</td>
<td>GSP4 TACAGAGACGGAAGGACG CACCGAGTGA</td>
</tr>
</tbody>
</table>

3. Genomic DNA extraction

Genomic DNA was isolated from adult zebrafish following the protocol in *The Zebrafish Book* [139]. Briefly, one adult fish was ground in liquid nitrogen. Fish homogenate was then transferred to extraction buffer that consisted of 10 mM tris-HCl, pH8.0, 100 mM EDTA, 0.5 % SDS and 200 µg/ml proteinase K. After 5 h (or overnight) incubation at 50°C, the suspension was centrifuged at 13,000 rpm for 10 min to remove particulate matter, and supernate was collected and subjected to one phenol and two phenol-chloroform extractions, followed by addition of 1/10 volume of 3 M NaAc and ethanol precipitation. Genomic DNA was dissolved in TE buffer and then stored at -20°C. Genomic DNA concentration was determined on a Perkin-Elmer Lambda 3B UV/VIS spectrophotography.
4. DNA electrophoresis, neutral transfer and Southern blotting

About 30 µg genomic DNA was digested with restriction enzyme *EcoR I* or *EcoR V* in the presence of the appropriate buffer (New England Biolabs, Ipswich, MA) in a 50 µl volume for 2 h (or overnight) at 37°C. The digested DNA was resolved in a 0.7% agarose gel without ethidium bromide at 25 V for 20 h. Before transfer, the lane containing DNA ladders was cut off, submerged in 0.5 mg/ml ethidium bromide in TBE for 15 min, viewed on a UV illuminator and photographed for later molecular weigh estimation.

Neutral transfer to an Immobilon-NY+ nylon membrane (Millipore, Bedford, MA) was performed by means of Whatman® Schleicher&Schuell Turboblotter™ rapid downward transfer system (GE, Piscataway, NJ). The detailed transfer procedure is described in the manufacturer's instructions. After 5h transfer, DNA was immobilized with a Stratalinker® UV crosslinker (Stratagene, La Jolla, CA) on the autocrosslink mode, which exposed the membrane to 120,000 microjoules 254-nm light. The membrane was then stored at 4°C for probing.

To prepare radioactive DNA probe, Prime-It® II random primer labeling kit (Stratagene, La Jolla, CA) was used to incorporate α[^32P] dCTP into the polb coding region derived from PCR amplification. Labeled probe was purified with Nick™ columns (GE Healthcare, Piscataway, NJ).

Southern blot procedure was performed following the manual for ExpressHyb™ hybridization solution (BD Bioscience, San Jose, CA). Briefly, the membrane was prehybridized in 10 ml hybridization solution at 60°C for 1 h, and then hybridized in 10 ml hybridization solution with radioactive DNA probe for 2 h at 60°C. After
hybridization, the blot was washed in wash solution 1 (20X SSC, 0.05% SDS) at room temperature for a total 40 min wash with 2-3 times replacement of the wash solution. The blot was further washed in washing solution 2 (0.1X SSC, 0.1% SDS) at 50 °C for 40 min with one change of fresh solution. All steps of hybridization and washing were performed with continuous shaking. Finally, the membrane was exposed to a storage phosphor screen overnight, which was developed with a Molecular Dynamics Storm 840 PhosphorImager (GE Healthcare, Piscataway, NJ).

5. Total RNA isolation, electrophoresis, transfer and Northern blotting

Total RNA was extracted from various stages of embryos and adult fish using TRIsol® reagent (Invitrogen, Carlsbad, CA). Adult fish were ground in liquid nitrogen, and then 50 mg homogenate was processed in 1 ml TRIsol reagent. As for embryos, about 50 embryos were homogenized in 1 ml TRIsol reagent by passing the embryos through a 22½ gauge needle attached to a syringe. After homogenization, total RNA was extracted as described in the manufacture’s instructions, and then subjected to phenol/chloroform purification to improve the quality.

About 30 µg total RNA for each sample was resolved in a 1.2 % denaturing agarose gel containing 18% deionized formaldehyde at 70 V for 3 h. After electrophoresis, RNA was transferred using the same transfer system used for Southern blotting. After immobilizing the RNA to the membrane, the membrane was stored at 4°C until probing was performed.

The same DNA probe used in Southern blot analysis was prepared for Northern blots to probe polb mRNA. The membrane was first prehybridized at 68°C by
immersing the membrane in ExpressHyb™ solution for 1.5 h. Hybridization was performed at 68°C for 3 h, followed by two washes, one at room temperature and the other at 55°C for one hour each. Finally, the blot was exposed overnight, and was developed as described previously for Southern blots.

6. Real-time RT-PCR

Total RNA, extracted from different stage embryos, was subjected to digestion with RNase-free DNase to avoid any genomic DNA contamination, and then reverse transcribed using High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Primers were designed using the Primer 3 software (developed by Steve Rozen, Helen J. Skaletsky, 1996, 1997), available on-line at http://frodo.wi.mit.edu. Real-time PCR was performed in an ABI PRISM 7000 Sequence Detection System thermal cycler (Applied Biosystems, Foster City, CA) in a total volume of 20 µl. The reaction mix contained 1x SYBR® Green PCR Master Mix, 250 nM each of forward primer and reverse primer. The thermal cycle parameters used were: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. All the samples were analyzed in triplicate and the mean value of these measurements was normalized to β-actin that served as an internal control.

Table 2: Primers used in real-time RT-PCR:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>polb</td>
<td>TCCCTGAACGAAGGAATCAC</td>
<td>ATCTTTGCACCGACTCCATC</td>
</tr>
<tr>
<td>bactin</td>
<td>CAACAGAGAGAAGATGACAGAGATCA</td>
<td>GTCACACCATCACCAGAGTCCATCAC</td>
</tr>
</tbody>
</table>

7. Preparation of zebrafish Pol β rabbit polyclonal antibody
The immunogens and the detailed immunization protocol were designed and performed by 21 Century Biochemicals, Marlboro, MA. Sequences of the two immunogens were as follows: Immunogen 1: acetyl-CLDGAGAKIAEKIDEFL-amide; Immunogen 2: acetyl-CFYIQWKYREPDRSCOOH. These two separately conjugated peptides were co-injected into two rabbits. Six antisera were collected from each rabbit at day 52, 56, 73, 77, 84, 90 after initial injection. Antiserum 5 was pooled from two rabbits and subjected to affinity purification using the starting conjugated peptides, yielding two antibodies specific to each peptide.

8. Protein extraction, gel electrophoresis, transfer and Western blotting

Adult fish were ground in liquid nitrogen, and then transferred to 1X lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF) supplemented with one tablet of Complete, Mini, EDTA free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) per 10 ml lysis buffer that also included 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 1 µg/ml pepstatin. After incubation for 30 min on ice, the homogenate was centrifuged at 13,000 rpm for 20 min at 4°C. Protein concentrations were determined using the Bradford dye binding procedure (Bio-Rad, Hercules, CA). Fish embryos were homogenized directly in lysis buffer with a Kontes Pellet Pestle® micro grinder, followed by the same procedure as for adult fish.

For Pol β immunoblotting, 100 µg fish protein extract was resolved on a NuPAGE® Novex 4-12% Bis-Tris precast protein gel at 200 V for 1 h, and then transferred onto a PVDF membrane through the XCell II™ Blot Module. Transfer was performed at 30 V for 1 h. Instead of 100 µg, 80 µg protein extract was used in
AP endo Western blot analysis. All reagents for protein electrophoresis and blotting were purchased from Invitrogen, Carlsbad, CA. After transfer, the PVDF membrane was blocked in 5% dry milk dissolved in TBST (1X TBS with 0.1% Tween 20) for 1 h at room temperature or overnight at 4 °C. After blocking, the membrane was washed twice in TBST for 5 min each. Initially, an anti-rat Pol β mouse monoclonal antibody (Thermo scientific, Fremont, CA) was used for detecting zebrafish Pol β protein expression during zebrafish early development. All subsequent Pol β Western blots used the custom rabbit anti-zebrafish Pol β polyclonal antibody made by 21st Century Biochemicals as described in Material and Methods Section 7, unless indicated otherwise. Rabbit anti zebrafish AP endo antiserum generated by Sigma-Genosys (The Woodlands, TX) (Wang, Y. 2006) was used for AP endo Western blotting. Anti-β-Actin antibody was purchased from GeneTex, Inc. (Irvine, CA.). The membrane was incubated with the primary antibody in TBST with 1% dry milk for 1 h at room temperature, followed by 4X 15 min washes in TBST. The membrane was then incubated with the corresponding secondary antibody (Sigma-Aldrich, St. Louis, MO) in TBST with 1% dry milk. After 4X15min washes, the membrane was incubated with the chemiluminescence reagent (Perkin Elmer, Waltham, MA) for 1 min, and then exposed to CL-XPosure™ X-ray films (Thermo Scientific, Rockford, IL). Western blot images were scanned into the computer and quantified using ImageJ software (free online download from NIH, http://rsbweb.nih.gov/ij/).

9. Morpholinos (MOs) and microinjection

Zebrafish Pol β specific MOs were designed and synthesized by GeneTools, LLC (Philomath, OR). Two morpholinos were prepared: MO1-
CTTGTTGCGCTTTTCGTTCATCAT, targeting the ATG site, and MO2-GGCCAGCTCTGAAACAAACAACACA, against the intron1/exon2 junction. Two nl MO was injected into 1-2 cell stage embryos, using phenol red as an injection indicator. Injection volume was determined by calibration performed on a 1X 0.01 mm stage micrometer (Thermo scientific, Fremont, CA). Injected embryos were raised at 28.6°C to the desired developmental stages. Phenotypes were examined daily using a Leica stereomicroscope (Bannockburn, IL) and photographed.

10. pCS2+ -eGFP-zfpolb plasmid construction, *in vitro* transcription and overexpression experiment

The enhanced GFP gene (eGFP) was amplified from p3E-eGFPpA vector with the primer set: eGFP-BamH I-For- CGGGATCCATGGTGAGCAAGGGCGAGGAG, and eGFP-EcoR I-Rev- CGGAATTCCTTGTACAGCTCGCTGCCATGCC. The resulting sequence was then cloned into the pCS2+ zebrafish expression vector between the BamH I and EcoR I cloning site. Zebrafish polb gene was amplified from first-strand cDNA as described in Materials and Methods, section 1, using the primer set: polb-EcoRI-For-AAAAAAAAGAATTCATGAGTAAACGAAAAGC, and polb-Xho I-Rev-AAAAAAAAGAATTCATGAGTAAACGAAAAGC. The zebrafish polb coding region was then cloned into pCS2+ -eGFP plasmid, at the C terminus of the eGFP gene.

To obtain eGFP tagged polb message, *in vitro* transcription was performed using Ambion® mMESSAGE mMACHINE® SP6 Kit, followed by purification with the MEGAclear™ Kit (Applied Biosystems, Austin, TX). Five hundred and forty pg RNA was microinjected into 1 cell stage wild-type embryos. eGFP expression and embryonic development were examined daily by fluorescence microscopy. To
confirm the overexpression of Pol β protein, Western blot analysis was performed to quantify the protein level using both zebrafish specific Pol β antibody (prepared by 21st Century Biochemicals, Malboro, MA) and anti-GFP antibody (Cell Signaling Technology, Inc. Danvers, MA).

11. Methyl methanesulfonate (MMS) treatment

Wild-type, MO knockdown and zfpolb mRNA rescued embryos were subjected to MMS treatment at 4 hpf. Rescue experiments were performed by co-injection of MO2 and eGFP-zfpolb mRNA into the embryos. Acute treatment was performed by treating embryos in MMS solution for 1 h, and then washing out MMS with fish water for 3 times. Thereafter, embryos were allowed to continue developing in fish water. Alternatively, chronic treatment was performed by leaving embryos in MMS diluted in fish water for 5 days. Embryos were examined daily for developmental progress, and fish water or MMS solution was changed daily. Death was evaluated as cessation of heartbeat and circulation. Percent survival was recorded as surviving embryos at 5 dpf divided by total number of embryos subjected to the treatment.
RESULTS

1. Zebrafish *polb* gene encodes a protein highly homologous to the human counterpart.

Zebrafish *polb* coding region was cloned from first strand cDNA reverse transcribed from adult fish total RNA. A single band about 1000 bp appearing on an agarose gel represented the expected product (Fig. 1A). In order to obtain the cDNA ends, 5' and 3' RACE-PCR were performed using a random primer amplified with first strand cDNA as template. One fragment was obtained from each reaction (Fig. 1B). These PCR products were subcloned individually into a cloning vector, and subsequent sequencing results indicated that the combined fragments encoded the full-length cDNA for the *polb* gene, as they match the *polb* cDNA sequence available in Ensembl database (Ensembl transcript ID: ENSDART00000002764). Therefore, the full-length zebrafish *polb* cDNA was successfully cloned. The cloned coding region was translated into amino acids, and then aligned with the human Pol β protein using CLUSTALW, a multisequence alignment tool provided by the European Bioinformatics Institute. There is about 80% sequence identity between the zebrafish and human Pol β proteins (Fig. 2). Moreover, all the key residues, highlighted in the protein sequence, were completely conserved in zebrafish. The high homology probably implied a potential functional similarity of zebrafish Pol β and mammalian Pol β proteins.
2. Southern blot assay revealed a single copy of polb gene in the zebrafish genome.

Zebrafish has a tetraploid ancestry, so that some of its genes are duplicated, for example, transcription factor hoxb1 and runx2 [140] [141]. Our lab also reported that the BER gene apex1 is duplicated in zebrafish [137].

Southern blot assay was carried out to obtain the number of copies of the polb gene in the zebrafish genome (Fig. 3). Restriction analysis was first performed on the genomic sequence in the Ensembl database by the NEBcutter (http://tools.neb.com/NEBcutter2/) to obtain the expected theoretical fragment sizes. Locations of the EcoRI and EcoRV sites are illustrated in Figure 3A. EcoRI digestion revealed two major bands. The upper band corresponded to the fragment produced from cutting at position c and d, while the lower band matched the fragment between positions b and c. EcoRV digestion produced four major bands. These bands from bottom to top corresponded to genomic fragments e-f, f-g, c/d-e, a-g (due to partial digestion), respectively. Given that the sizes of all fragments generated from EcoRI and EcoRV digestion can be assigned to the polb genomic sequence in the Ensembl database, zebrafish polb gene has only one copy.

3. Pol β mRNA shows a dynamic temporal expression pattern during embryogenesis.

To characterize the mRNA expression pattern, I examined the existence of any other mRNA variants and the expression level during zebrafish embryogenesis by means of Northern blotting analysis and qRT-PCR. Northern blotting showed one major transcript for polb gene in 1.5 hpf, 24 hpf, 48 hpf embryos and adult fish, with
the size of transcript matching the 1594 bp cDNA reported in the Ensembl database (Fig. 4A). Thus, there was a single polb transcript produced from the polb gene.

Pol β mRNA was present throughout zebrafish embryogenesis (Fig. 4), as revealed by both Northern blot analysis and qRT-PCR. In 1 hpf and 1.5 hpf early cleavage embryos, a large amount of Pol β message was detected, compared with embryos at other stages during the first 24 hours of development. Expression peaked at the beginning of the MBT stage (~3 hpf). Thus, the observed burst of polb expression could be due to the activation of its zygotic transcription at the MBT. After MBT, the transcript level decreased gradually, with the lowest expression present in 24 hpf embryos. These embryos only had 1/8 of the total amount of Pol β message found in the 3 hpf embryos (Fig. 4B). Therefore, Pol β mRNA was expressed in a dynamic pattern during embryogenesis.

4. Pol β protein expression is not correlated with its mRNA expression, and is deficient in blastula and early gastrula embryos.

Protein expression levels provide valuable information on a protein’s activity and function. To characterize the protein levels during early development, Western blotting was performed on protein extracts from various stage embryos. Since zebrafish Pol β protein shares very high homology with human counterpart (Fig. 2), I used either a mouse monoclonal antibody (clone 18S) targeting rat Pol β but cross-reacting with most mammalian Pol βs, or a rabbit polyclonal antibody prepared against a zebrafish epitope (see section 5). As seen in Figure 5, no Pol β protein was detected in unfertilized eggs, cleavage, blastula, and early gastrula stages, suggesting a dispensable role of Pol β in very early embryogenesis. The results are
in contrast to qRT-PCR described above, namely that Pol β mRNA was expressed throughout these early developmental stages (Fig. 4B). After initial detection of the protein level at 13 hpf, Pol β protein levels increased until 3 dpf.

5. Two antisera and one affinity purified antibody obtained from custom antibody production showed high reactivity to the zebrafish Pol β protein.

In initial studies, I used a commercial mouse monoclonal anti-rat Pol β antibody to detect the protein from zebrafish. As the antibody cross-reacted with numerous proteins in addition to the one predicted on the basis of the cloned sequence, a specific antibody was generated through custom antibody production (See details in Materials and Methods). To select peptides with potential antigenicity, zebrafish Pol β was mapped to the human Pol β 3-D structure. Two peptides shown in Figure 6A were selected. Peptide 1 was located at the N-terminus of the protein, while peptide 2 was the last few amino acids at the C-terminus. All bleeds collected from two rabbits and two affinity-purified antibodies were screened for reactivity to Pol β via western blotting. The results are summarized in Figure 6B. High reactivity was found in Bleed 6 produced from each rabbit and affinity-purified antibody 2, which was defined as As-6 and Ab-2, respectively. Western blotting results for the two affinity-purified antibodies are illustrated in Figure 6C. Ab-1 did not recognize zebrafish Pol β in embryonic extracts, while Ab-2 demonstrated specificity and avidity. At 1:1000 dilution, Ab-2 detected a band at about 40 kDa, close to the 39 kDa Pol β protein, in both 3 dpf and 1 dpf wild-type embryos, but not in 1 dpf Pol β knockdown embryos, confirming that the detected band was Pol β. Meanwhile, unlike the commercial antibody used initially, this antibody produced low
background. Thus, Ab-2 antibody was chosen to examine Pol β protein expression levels during early zebrafish development to confirm the detected pattern by the commercial anti-mammalian Pol β antibody. Western blotting results confirmed the same expression pattern detected by this zebrafish-specific antibody and the anti-mammalian Pol β antibody. Therefore, Ab-2 was used in the subsequent Western blot experiments. In addition, Ab-2 can recognize human Pol β protein (Fig. 9B).

6. Characterization of the efficacy of two Pol β specific MOs.

Use of antisense MO is a standard method to study gene function in zebrafish. Two MOs were designed to knock down Pol β protein expression, MO1, the translation blocker, and MO2, the splicing inhibitor. The sequences are described in Materials and Methods, section 9. In initial experiments, cytotoxicity was determined by microinjecting a range of MO concentrations. Western blotting was then used to measure the degree of Pol β knockdown in injected embryos (Fig. 7A). Injection of ~8.4 ng MO reduced Pol β protein to a point where it was barely detectable, compared with the Pol β level detected in wild-type embryos. As a splicing inhibitor, MO2 efficiently blocked the splicing of the polb pre-mRNA at the junction of intron 1 and exon 2, resulting in retention of intron 1, as shown by RT-PCR (Fig. 7B). Furthermore, MO1 suppressed Pol β translation up to 4 days (Fig. 7C). Thus, both MOs were effective in reducing Pol β protein level in zebrafish embryos.

7. Knocking down Pol β protein does not affect the general morphology of early developing embryos.

After validation of the efficacy of the MOs, the role of Pol β protein in development was studied by examining morphological changes in early zebrafish
embryos for up to 5 days. Following microinjection with 8.4 ng MO 1 or 2, embryos were raised at 28.6°C and photographed at various developmental stages (Fig. 8). There were no obvious abnormalities in knockdown embryos, except that 84% and 61% larvae microinjected with MO1 and MO2, respectively, did not inflate their swim bladders after 6 days. In addition, antibody staining with MF 20, which recognizes sarcomeric myosin in both skeletal and cardiac muscle, alcian blue cartilage staining, and o-dianisidine red blood cell staining all showed no difference between Pol β knockdown embryos and wild-type embryos (Data not shown). Hence, Pol β did not play an indispensable role during morphogenesis.

8. Overexpression of Pol β protein does not affect early zebrafish development.

Pol β overexpression or polymorphism has been detected in many human tumors, and it has been shown that overexpression of Pol β can lead to altered BER efficiency and a mutator phenotype [105]. Here, I examined whether overexpression Pol β at very early developmental stage resulted in any morphologically detectable effect on development. To elevate the Pol β protein level in early embryos, ~500 pg capped and polyadenylated zebrafish Pol β mRNA tagged with eGFP message was microinjected into zebrafish embryos immediately after fertilization. eGFP expression was detected as early as 4 hpf (Fig. 9A-a, e), strongly expressed at 1 dpf (Fig. 9A-b a, f), and then diminished after 3 dpf (Fig. 9A-c,d,g,h), probably due to the half life of the injected mRNA and protein turnover. During the first 5 days of development, embryos did not show any morphological abnormalities. Western blotting analysis confirmed the overexpression of the fusion protein at 1 dpf (Fig. 9B). Introduction of
a same amount of human Pol β mRNA into each embryo also did not affect the early
development (Data not shown), although expression of the human protein was
detected easily (Fig. 9B). Therefore, overexpression of Pol β did not affect early
embryogenesis.

9. Methylation agent MMS arrested zebrafish embryonic development at early
cleavage stage in a dose dependant fashion.

The alkylation agent MMS damages DNA in a way that requires BER for repair
[142]. Since Pol β is normally an important component of BER, I examined whether
knockdown of this protein would result in enhanced sensitivity to MMS. In sea
urchins that exposure to high concentrations of MMS arrests embryonic
development at the first cell division [143]. Here in zebrafish, embryos exposed to 50
mM MMS immediately after fertilization failed to progress beyond the 8-cell stage.
Embryos exposed to 10 mM MMS progressed as far as the 32-cell stage (Data not
shown). Thus, exposure to MMS arrested cell division in zebrafish embryos, just as it
did in sea urchin embryos.

10. Zebrafish embryos lacking Pol β protein are more sensitive to MMS than
control treated embryos.

Since mammalian cells lacking Pol β show hypersensitivity to mutagens [144]
[91]), the consequences of knockdown of Pol β on sensitivity to MMS were
examined for zebrafish. Sensitivity was assessed by cumulative death following
acute or chronic exposure to MMS within the first 5 days of development. In acute
treatment, 4 hpf embryos were exposed to MMS solution for 1 hour, while in chronic
treatment, same stage embryos were exposed to MMS continuously for 5 days. Pol
β knockdown embryos were more sensitive to MMS, demonstrated by lower survival rates than the untreated embryos under both acute and chronic conditions (Fig. 10A, B). Especially at concentrations of 1.5 mM (acute) and 0.10 mM (chronic) MMS, survival rate decreased dramatically in knockdown embryos, while the untreated embryos maintained good viability. The median lethal dosage, LC$_{50}$ was calculated and shown in Fig. 10C. The knockdown embryos have the lower LC$_{50}$ values of 0.079 mM and 1.05 mM MMS for chronic and acute conditions, respectively, than those in untreated control embryos.

To confirm that the hypersensitive phenotype in knockdown embryos was related to the lack of Pol β protein, eGFP tagged wild-type zebrafish polb mRNA was co-injected with MO2 into 1-cell stage embryos. The use of this MO did not interfere with the expression of introduced eGFP-zfpolb mRNA, because MO2 blocks a splicing site in the pre-mRNA without any effect on the mature mRNA. Ectopic expression of the Pol β protein partially restored the resistance to MMS in knockdown embryos (Fig. 10A, B). LC$_{50}$ values of rescued embryos were 0.098 mM and 1.43 mM for chronic and acute treatments, respectively, which were intermediate between untreated control and knockdown embryos (Fig. 10C). However, it was not surprising that full rescue could not be accomplished since ectopic expression started to decrease after it reaches highest expression at 1 dpf (Fig. 9).

11. **Knockdown of Pol β does not affect the AP endo protein level. However, ZAP1 mutant embryos lack Pol β protein.**
As noted earlier, Pol β is a key polymerase functioning in BER. Thus, knocking down Pol β might cause some change in the BER pathway in developing embryos. This possibility is under study in our lab. However, I chose to examine whether there were any changes in the expression level of the major endonuclease in BER, ZAP1, in Pol β deficient embryos. Western blot analysis showed no indication of any effect of knocking down Pol β on the levels of this enzyme (Fig. 12A). In contrast, knocking down ZAP1 resulted in a substantial reduction of Pol β protein level (Fig. 12B). These results suggested that AP endo was an important regulator of the BER pathway by regulating the enzyme immediately downstream from it.
Figure 1: Molecular cloning of zebrafish polb full length cDNA.  
**A.** Amplification of zfpolb coding region from first strand cDNA by RT-PCR.  
**B.** Cloning of zfpolb 5' and 3' cDNA ends by RACE-PCR. Primer sequences are listed in table 1 in the Materials and Methods.
**Figure 2:** Zebrafish and human Pol β protein sequences were aligned using ClustalW2. Sequence identity is 80%. Functional residues, shaded in grey, are all conserved. Identical amino acids in the two proteins are marked with an asterisk (*), conservative substitutions with a colon (:), and semi-conservative substitutions with a period (.)
Figure 3: One single copy of zebrafish polb gene is present in zebrafish genome demonstrated by Southern blot analysis.

A. Illustration of the theoretical locations of EcoRI and EcoRV sites in the polb genomic sequence. ‘0’ indicates the first nucleotide in the gene. Negative numbers indicate upstream sequence.

B. Southern blot of zebrafish genomic DNA digested with EcoRI (Lane 1) and EcoRV (Lane 2) and probed with α[^32]P dCTP labeled full length polb cDNA.
**Figure 4:** Dynamic expression of polb message during early development.

**A.** Northern blot of total RNA extracted from different stage embryos and adults. Each lane was loaded with 30 µg total RNA and probed with α[\(^{32}\)P] dCTP labeled polb cDNA.

**B.** qRT-PCR quantitation of polb mRNA expression within the first 24 hour of development. Data were generated from 2 independent experiments. Asteric (*) indicates significant difference (p<0.05) from value at 1 hpf.
Figure 5: Pol β protein expression during zebrafish early development. Developmental stages are indicated as hpf (hour post fertilization). ‘0 hpf’ stands for unfertilized eggs. ‘A’ stands for adult fish. Lower panel provides the quantitation of the Western blot results after normalizing the Pol β to β-actin.
**Figure 6:** Preparation and validation of zebrafish specific Pol β antibody.

A. Illustration of sequences and locations of the two peptides used in antibody production. N-amino terminus; C-carboxyl terminus, aa-amino acids. Courtesy by Alex Abyzov.

B. Summary of antibody screening results of 5 bleeds (1, 2, 3, 4, 6) from each rabbit and 2 affinity-purified antibodies produced from Bleed 5. Reactivity represents the detection of Pol β in a Western blot test.

C. Western blotting showed success for one of the two affinity-purified antibodies, Ab-2, in detection of zebrafish Pol β in total protein extract. dpf – days post fertilization; WT-wild type; KD-Pol β knockdown with 0.75 mM (~12.6 ng) MO2.

---

**A.**

![Image of antibody structure with sequences highlighted]

**Peptide 1 (61–77 aa):** KLDGVGAK1AEKIDEFL  
**Peptide 2 (324–339 aa):** FEYIQWKYREPKDRSE

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**B.**

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<tr>
<th>Antibody</th>
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<tr>
<td>Bleed 1, 2, 3, 4 from each rabbit</td>
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<tr>
<td>Bleed 6 from each rabbit</td>
<td>YES 1:1000 dilution</td>
</tr>
<tr>
<td>Ab-1, Affinity-purified (Purified with peptide 1 from Bleed 5)</td>
<td>NO</td>
</tr>
<tr>
<td>Ab-2, Affinity-purified (purified with peptide 2 from Bleed 5)</td>
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C.

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- Pol β
Figure 7: Both Pol β mropholinos MO1 and MO2 can effectively block Pol β translation in early embryos.

A. Western blotting showed that the two morpholinos knock down Pol β expression in a dose-dependent fashion. Protein extracts were prepared from 1 dpf embryos.

B. RT-PCR showed that MO2, a splicing blocker targeting intron1 and exon 2 junction, produced a polb splicing variant that retained intron 1 (upper band), besides the normal transcript (lower band). Beta-actin shown in lower panel served as control. RT-PCR was performed on first strand cDNA prepared from 1 μg total RNA extracted from 1 dpf wild-type (WT) or knockdown (KD) embryos. KD embryos were microinjected with ~8.4 ng MO2. Inverted Image.

C. Western blotting demonstrated that MO1 effectively blocks Pol β translation up to 4 dpf. At 5 dpf, the expression was resumed. WT- extracts from wild type embryos; KD – extracts from Pol β knockdown embryos microinjected with ~8.4 ng MO1.
Figure 8: Pol β does not affect general morphogenesis during zebrafish early development. Note that at 6 dpf, some Pol β knockdown larvae fail to inflate their swim bladders. WT-wild-type. KD-knockdown; embryos were microinjected with 2 nl 0.5 mM (8.4 ng) MO2 at 1-2 cell stage.
Figure 9: Zebrafish embryos overexpressing Pol β proteins show no obvious abnormalities during morphogenesis.

A. Bright field pictures (a-d) and corresponding fluorescent images (e-h) show normal development in embryos overexpressing eGFP-Pol β fusion protein.

B. Western blotting confirmed the translation of microinjected mRNAs. Total protein was extracted from 1 dpf embryos. Lane 1: wild-type embryos; Lane 2: embryos microinjected with 540 pg human POLB mRNA; Lane 3: embryos microinjected with 540 pg eGFP tagged zfpolb mRNA. Since anti-zebrafish Pol β antibody (Ab-2) recognizes both human and zebrafish Pol β, this antiserum was used for Western blot analysis. β-Actin detected by anti-β-Actin was used as loading control.
**Figure 10**: Pol β is required for MMS resistance in zebrafish embryos. Pol β knockdown embryos were more sensitive to MMS-induced DNA damage than control embryos. Ectopic expression of eGFP tagged Pol β protein partially restored the resistance in knockdown embryos. **A.** Acute treatment was performed by exposing 4 hpf embryos to MMS solution for 1 hour. **B.** Chronic treatment was performed by leaving the 4 hpf embryos in MMS solution for five days. Data are the averages of two to three independent experiments. **C.** The median lethal concentration, LC$_{50}$, for both acute and chronic treatment, is derived from data presented in Figure 10 A and B.
Figure 11: Western blot analysis of AP endo level in Pol β knockdown embryos, and Pol β level in AP endo knockdown embryos.

A. AP endo protein level did not change in Pol β knockdown embryos. Protein extracts were prepared from 1 dpf wild-type (WT) or knockdown (KD) embryos. Pol β knockdown embryos were microinjected with MO1 at various dosages.

B. AP endo hypomorphic embryos had reduced Pol β protein level, compared with wild-type embryos. Protein extracts were prepared from 3 dpf embryos. AP endo hypomorphic embryos were created by microinjecting 0.15 mM AP endo TB (translation blocker) MO at 1-2 cell stage.
DISCUSSION

Overview of this study

DNA polymerase ß is a highly conserved enzyme found in many species, from yeast (Saccharomyces cerevisiae, namely Pol IV) to lower Cnidarians such as jellyfish, and to higher mammals. Pol ß plays an essential role in the base excision repair pathway, which removes simple base lesions and abasic sites, and thus protects the genome from a variety of exogenous and endogenous DNA damage agents. Mammalian Pol ß has been studied intensively in terms of structure, activity, catalytic mechanism and biological functions [145] [146] [147] [148] [149] [8]. In the present study, I present the first work on the zebrafish polb gene. First, I cloned and characterized this gene and examined its expression in different developmental stages. Second, I investigated its role in zebrafish development by using the morpholino knockdown technique. A list of the important findings in my study includes:

1. The zfpolb gene sequence indicates that it encodes a member of the very conserved DNA polymerase ß family.

2. Despite the fact that zebrafish has a tetraploid ancestry, Southern blot analysis indicates that there is only one gene copy in the zebrafish genome.

3. Temporal expression of the zfpolb mRNA during embryogenesis begins in embryos immediately after fertilization, peaks at the MBT, and then steadily decreases with time during the first day of development. However, gene expression continues through adulthood.
4. The Pol β protein level is not correlated with its mRNA level. No Pol β protein is detectable prior to mid-gastrulation. After that, Pol β translation rate increases as development progresses. However, a reduced level of Pol β protein is seen in adult fish, compared with that in embryos.

5. Pol β deficiency results in a hypersensitivity phenotype to MMS in developing embryos. High dosages of MMS halt embryonic development at the 8-32 cell stage. Re-introducing Pol β protein into the knockdown embryos partially rescues the hypersensitivity phenotype.

6. Antisense MO knockdown experiments show that lack of Pol β protein does not affect early patterning and morphogenesis in zebrafish. Alternately, ectopic expression of an eGFP-tagged Pol β protein in wild-type embryos does not lead to any obvious morphological change.

7. Pol β protein levels are decreased in AP endo knockdown embryos, suggesting a possible regulation of Pol β expression by AP endo.

**Zebrafish polb gene encodes a conserved protein.**

According to the complete cDNA sequence for zebrafish polb in the Ensembl database, I designed polb gene-specific primers and successfully cloned the zfpolb cDNA from the first strand cDNA made from adult fish (Fig. 1). Translated Pol β protein has 339 amino acids with a molecular weight about 39 kDa. It shares about 80% sequence identity with the human orthologue. Most importantly, all the key residues involved in catalytic activity characterized in the mammalian protein are conserved in zebrafish (Fig. 2). Pol β is found in all vertebrate species as a
conserved 39 kDa protein lacking 3’ and 5’-exonuclease activities, but containing 5’-dRP lyase activity and nucleotidyl transferase activity [75]. The Ensembl database lists gene information on 42 Pol β orthologues and presents evidence that the sequence identity among these proteins is between 70% (in squirrel) to 100% (in gorilla and chimpanzee), with an exception for yeast POL IV, which only has 13% homology with the human protein (http://www.ensembl.org/Homo_sapiens/Gene/Compara_Ortholog?g=ENSG000000070501). The high conservation of Pol β among various species indicates that Pol β probably performs conserved functions in these organisms. Mammalian Pol β has been characterized comprehensively as a major BER polymerase responsible for not only single nucleotide incorporation but also removal of 5’-deoxyribose phosphate during the repair process [150]. It is reasonable to assume that zebrafish Pol β possesses similar activities and functions to its mammalian counterpart. The Strauss laboratory has already demonstrated a Pol β-like activity in BER found in zebrafish extract [136]. Nevertheless, enzymatic studies on zebrafish Pol β would provide direct evidence for its polymerase and lyase activities.

The zebrafish genome only has one copy of polb gene.

During vertebrate evolution, three whole genome duplication (WGD) events occurred. It is thought that the third WGD gave rise to a number of duplicate genes that exist exclusively in teleosts today. In fact, after the third WGD, the majority of duplicated genes lost the second copy. However, zebrafish still have about 2900 pairs of duplicate genes [151]. The two duplicated genes either share the function of
the original gene, or mutations arise, providing new functions to one of the gene copies or leading to a pseudogene [152]. Determination of the number of gene copies is important for downstream functional studies by a reverse genetics approach. I performed Southern blotting by probing the restriction digested genomic DNA with the full-length polb cDNA. The results show clearly that there is no second copy for the polb gene in zebrafish genome.

Among other BER proteins characterized in zebrafish, ZAP1, the enzyme immediate upstream of Pol β, has two copies, though they encode exactly the same protein [137]. With two or more copies, one has to consider designing one or more MOs in order to knock down all copies. Having only one copy of polb gene makes it straightforward for morpholino design.

**Zebrafish Pol β mRNA and protein levels are not coordinated during zebrafish embryogenesis**

*Pol β mRNA is expressed in a dynamic pattern during very early development.*

Expression patterns provide information for target protein activity at the examined stages. Temporal changes in zfpolb mRNA expression during the first day of zebrafish development were quantified by real-time RT-PCR (Fig. 4). First of all, ample polb mRNA is present in embryos shortly after fertilization, i.e., in 1 hpf and 1.5 hpf stage embryos. The mRNA level continues to increase until it reaches a peak at MBT (∼ 3 hpf), and decreases steadily thereafter. The amount of mRNA in 24 hpf embryos is only about 1/8 of the amount in 3 hpf embryos, suggesting a slower transcription rate relative to degradation in these embryos. A similar reduction was
also confirmed by Northern blot assay. After 24 hpf, a slight increase was seen in 48 hpf embryo and adult fish. Thus, polb mRNA is expressed in a dynamic pattern in early embryos. This dynamic expression pattern is very consistent with the data generated from a study on transcriptome analysis of zebrafish embryogenesis by means of microarrays [153]. The microarray data show that a high level of polb mRNA is detected in 3 hpf and 9 hpf embryos, compared with the other stages within the first 24 hours of development. The lowest polb mRNA level is present in 24 hpf embryos. 

*Unlike many other zebrafish genes, zygotic transcription of polb gene is turned on before MBT.*

Zebrafish MBT begins at ∼3 h after fertilization, and is characterized by cell cycle lengthening, loss of cell synchrony, appearance of cell motility, and activation of zygotic transcription [154]. Nevertheless, recent evidence in both *Xenopus* and zebrafish supports a new notion that onset of the zygotic transcription can occur prior to the MBT [155] [153]. In the case of Pol ß, a relatively high amount of mRNA is present in pre-MBT stage embryos (1 hpf and 1.5 hpf), probably representing the maternal message, since zygotic transcription of Pol ß is not activated by 1.5 hpf (See below). It was known decades ago that the accumulation of many substances in the egg for later use by the embryo was a general feature of oogenesis [156] [157] [158]. Right at the beginning of the MBT (∼3 hpf), an expression peak of Pol ß mRNA was observed which could be due to the activation of transcription from the zygotic genome. However, to account for the large accumulation of message, the rate of accumulation would have to be rather fast if zygotic transcription initiates at 3
hpf. This observation raises a question whether polb is a special gene with an earlier onset of zygotic transcription. In fact, such a speculation is supported by the microarray study mentioned previously [153], which shows that Pol β is one of the 125 genes, among a total of 16,416 genes analyzed, with an onset of expression from 2 hpf (64 cells) onwards and having a pre-MBT transcript accumulation. Thus, the results of my qRT-PCR assays are consistent with the microarray data, namely that polb mRNA is found at all stages during early embryogenesis, and zygotic transcription is activated prior to the MBT which leads to a transcript accumulation at 3 hpf.

*Pol β protein level does not correlate with mRNA level during embryogenesis.*

In the next step, I examined the Pol β protein expression pattern during early zebrafish development. Pol β protein is not detected until after 6 hpf (shield stage). The level increases thereafter as development progresses (Fig. 5). This pattern is distinct from that of the polb mRNA, which has been discussed above (Fig. 4). Lack of correspondence between mRNA and protein is not uncommon. In *Drosophila melanogaster*, several maternal mRNAs, synthesized during oogenesis and found in fertilized eggs, are translated in the embryos in an extremely complex fashion [159]. Similarly, in *Xenopus*, most of the oocyte's mRNAs are not translated in the oocyte and will be used only during embryogenesis, or later in development [160].

From my Western blotting data, it seems that translation of Pol β initiates between 6 hpf (shield stage) and 13 hpf (8-somite stage). It would be interesting to find out whether the precise start time occurs at 9 hpf (90% epiboly), the stage that the mRNA expression peaks in the previous microarray study [153].
Pol β protein expression pattern correlates nicely with its activity in embryonic BER.

It is interesting to find that Pol β is missing in embryos before 6.5 hpf (mid-gastrulation), in spite of the presence of the mRNA encoding the protein. After that, the protein level increases with the relative highest level detected at 3 dpf. These observations raise a question about whether Pol β levels correlate with BER activity in embryos. The Strauss lab has characterized the BER activity during early development, and found that the replicative polymerases predominate in BER in embryos before 3 dpf [136]. Thus, lack of Pol β protein in embryos before 6.5 hpf coincides with a dispensable role of Pol β in BER. Moreover, the increased level of Pol β protein correlates with a polymerase switch from replicative polymerases to Pol β in the BER assay. In general, the Pol β protein level coordinates with its participation in BER during early zebrafish development.

An early study on repair polymerases during *Misgurnus fossilis* (loach) embryogenesis reported that Pol β activity was extremely low in eggs and embryos up to the gastrula stage (5.25-10 hpf) [161]. Whereas neither the mRNA nor protein expression patterns for loach Pol β during embryogenesis were provided in that study, I speculate that, just as zebrafish embryos, very early loach embryos have little or no Pol β protein. The similarity between loach and zebrafish embryos suggests that lack of early translation might be a general feature for Pol β during vertebrate embryogenesis, so that it plays a nonessential role in early embryonic BER. This notion is important for understanding the role of BER in development, and could be evaluated by investigating whether or not mammalian embryos lack Pol β prior to gastrulation.
Pol β is not essential for early patterning and morphogenesis during early zebrafish development

Pol β knockdown does not interrupt normal morphogenesis.

The studies described above demonstrate that Pol β protein is not detectable in unfertilized eggs and very early stage embryos, and thereafter its level increases and reaches a high level at 3 dpf. This expression pattern led me to examine whether this protein is involved in zebrafish patterning and morphogenesis. Using a standard reverse-genetics approach, known as anti-sense MO, I investigated the effect of Pol β knockdown on zebrafish embryogenesis. Pol β knockdown embryos appear normal throughout the first 5 days of development, compared with stage-matched wild-type embryos (Fig.8). Preservation of normal morphology in Pol β knockdown embryos indicates that Pol β does not play an essential role in zebrafish early patterning and morphogenesis.

Early brain development is not affected by Pol β knockdown.

In the absence of Pol β, murine embryos display extensive apoptosis in post-mitotic neuronal cells at the cortex region during cortex neurogenesis, i.e., from E12.5 to E14.5. p53 knockout rescues the neuronal apoptosis but does not completely restore the normal brain cyto-architecture. These data suggest that mammalian Pol β plays an essential role in neurogenesis [72] [89]. To test whether Pol β knockdown zebrafish elicit the same phenotype, I examined gross brain morphology microscopically throughout zebrafish embryogenesis and stained 1 dpf Pol β knockdown fish embryos by acridine orange for evidence of apoptotic neuronal cells (Data not shown). The results show no difference between control and
knockdown embryos. Apoptosis in embryos older than 1 dpf was not examined in my study. It is possible that the observed neurological defect in mammals could arise at later developmental stages in zebrafish. In fact, neurologically, mouse E12.5, when apoptosis is detected in developing mouse brain, is comparable to zebrafish 2-3 dpf, as highly comparable early forebrain gene expression and GABA cell patterns are observed at these stages in mouse and zebrafish, respectively [162] [163]. Zebrafish neurogenesis occurs in two successive (primary and secondary) waves [164]. Primary neurogenesis occurs during late gastrulation, and builds the first functional embryonic and larval neuronal scaffold [165]. In 2 dpf zebrafish embryos, secondary neurogenesis begins to take over the primary system through a refined and increasingly complex network [166]. At 2 dpf, neuroblasts are dedicated for proliferation, while at 3 dpf, there is an obvious overall increase in the degree of differentiation in the entire zebrafish brain [163]. Therefore, the impact of Pol ß knockdown on zebrafish embryonic brain development should be evaluated at 2 and 3 dpf, or even later stage embryos. Combined with histological examination of the brain architecture, the question whether Pol ß is involved in neural development in zebrafish could be elucidated. If loss of Pol ß does not affect neurogenesis in zebrafish, it might indicate another protein(s) can compensate for the function of Pol ß in zebrafish, or that there is a basic difference between mammalian and fish brain development.

*Uninflated swim bladder might be a result of Pol ß knockdown.*

Despite a lack of obvious morphological changes in knockdown embryos by 3 dpf, a swim bladder defect arises at 5 dpf in knockdown larvae. About 84% MO1-
injected and 61% MO2-injected embryos develop this abnormality (Data not shown). Subsequent observations until 7 dpf confirm that the knockdown fish do not inflate their swim bladder later in development, indicating that developmental delay is not a cause for an uninflated swim bladder at 5 dpf. However, failure to inflate the swim bladder is actually a common feature in mutant zebrafish generated in large-scale mutagenesis screens. Normal embryos without an inflated swim bladder usually die from failure to swim and feed [167] [168]. Also, some environmental factors can affect swim bladder inflation, such as water quality and light intensity [169]. Together these observations indicate that the swim bladder is a very delicate organ that is readily affected by either exogenous or endogenous conditions. Hence, it is difficult to determine whether this observation is due to a loss of Pol β. Nevertheless, there is a strong indication that zebrafish could develop this defect after Pol β knockdown, according to the finding in Pol β-deficient mice. PolB−/− neonates die from respiratory defects. Their lungs fail to inflate with air and their alveolar air spaces are smaller than those of their wild-type littermate [72]. Since the swim bladder is considered homologous to the mammalian lung in an evolutionary sense [170], an uninflated swim bladder in fish could be comparable to an uninflated lung in mammals.

**Pol β is an important component in the BER pathway.**

*Zebrafish Pol β knockdown embryos display increased sensitivity to MMS, comparable to that observed in Pol β null mouse embryonic fibroblasts (MEFs).*

As BER is a major pathway for repair of small base modifications, such as deamination, alkylation and oxidation, this protection might be disrupted by down-
regulation of Pol ß, the major polymerase in this pathway. I, therefore, examined how Pol ß knockdown zebrafish embryos react to the DNA damage agent, MMS, an environmental alkylation agent. The knockdown embryos subjected to either a one-hour period of acute exposure (0-2.5 mM) or 5-day period chronic exposure (0-0.15 mM) display a reduced survival rate at 5 dpf, compared with wild-type control embryos. The observed MMS hypersensitivity phenotype in Pol ß knockdown embryos indicates that Pol ß plays an important role in combating alkylation DNA damage, maintaining a fine-tuned developmental process and promoting organismic survival during vertebrate development. This finding in zebrafish coincides with the findings in mammalian cells. Research data in Pol ß null murine embryonic fibroblasts (MEFs) support a fundamental and specific role of Pol ß in repairing alkylation damage (reviewed in the INTRODUCTION of this thesis). Although complementary or back-up repair mechanisms contribute to the repair of other types of DNA damage in mammalian cells in a Pol ß null background [93] [171], they cannot completely compensate for the loss of function of Pol ß in protecting cells against alkylation DNA damage. The consistency of the hypersensitivity phenotype observed in MEFs and zebrafish embryos implies that MEFs probably share many characteristics with zebrafish embryos, especially since MEFs were initially derived from embryos. MMS treatment in PolB<sup>-/-</sup> MEFs also induces mutagenesis, accumulation of single-strand breaks (ssb), apoptosis and chromosomal breakage [90] [149] [172] [93]. These deleterious cellular consequences might also happen in zebrafish embryos, and thereby disrupt development and lead to embryonic death.
Overall, my study extends the definitive role of Pol β in repair of methylation DNA damage on a whole-organism level in a vertebrate other than mammals.

Hypersensitivity phenotype is partially rescued by restoring the expression of wild-type zebrafish protein in MO knockdown embryos

To confirm that the hypersensitivity phenotype is specific to Pol β, I performed rescue experiments by introducing eGFP-zfpolb mRNA into knockdown embryos, under conditions where the mRNA is translated into protein. Then I exposed the ‘rescued’ embryos to MMS in either an acute or chronic fashion as described previously. Zebrafish embryos can be partially rescued, indicating that at least part of the sensitivity is due to Pol β loss. Such an extent of rescue is lower than that achieved in Pol β null MEFs, where the survival rate can be reversed to close to that of wild-type MEFs [92]. A few reasons might be considered to account for incomplete rescue in zebrafish embryos. First, wild-type Pol β protein was introduced as a fused form of eGFP-zfpolb mRNA, which has a short half-life. Combined with turnover of the newly-made protein, eGFP expression gradually diminished after 24 hpf. Thus, the embryos might not be supplied with sufficient Pol β protein throughout the experimental period. Also, the eGFP tag might interfere with the activity of the protein. Second, the translation of the introduced mRNA was not under developmental control, i.e., tissue-specific and time dependent expression were not achieved. Finally, embryos are likely to have more complicated regulatory patterns than cultured cells, which might cause the differential rescue results.

In addition, in Pol β null MEFs, the hypersensitivity in Pol β null MEFs can be reversed by the Pol β lyase domain without the polymerase domain. Thus, the 5’-
dRP lyase activity of Pol β seems to be crucial for removing cytotoxic repair intermediates, and thus attenuating the sensitivity to MMS-induced damage [92]. My rescue experiment in zebrafish could also be performed with the dRP domain of Pol β protein alone, which should confirm the importance of the dRP activity for Pol β in zebrafish BER.

**Overexpression of Pol β protein in wild-type embryos does not affect morphogenesis in early zebrafish embryos.**

Pol β overexpression or polymorphism is widely detected in many human cancers, suggesting a role of Pol β in preventing genomic instability and tumorigenesis (See review [105]). Overexpression of Pol β in cells leads to altered BER efficiency and a mutator phenotype. However, it is not clear whether upregulating Pol β in an organism during early development would have any consequences. In this study, I examined the effect of elevated Pol β protein on early zebrafish development by introducing an eGFP-tagged zfpolb mRNA or human POLB mRNA into wild-type embryos at the 1-cell stage. Visualization of green fluorescence and detection of the fusion protein in Western blotting indicate successful translation of the injected message. However, I did not observe any obvious phenotypes during the first 5 days of development.

Sobol et. al. developed a bicistronic tetracycline-responsive transgenic system for overexpression Pol β in mice [173]. These mice develop severe cortical cataracts beginning within 4 days after birth. The authors proposed that either a reduction of BER activity from imbalanced Pol β levels or an unidentified role of Pol
ß in lens epithelial cell differentiation induced cataractogenesis. However, Pol ß overexpression in zebrafish does not cause any obvious morphological alterations in eye development. Compared with the transgenic mouse system, Pol ß overexpression in zebrafish is transient. After ectopic mRNA is translated or degraded 1 or 2 days later after initial microinjection, the overexpression level goes down. Whether overexpression of Pol ß results in functional abnormalities in the zebrafish eye remains to be explored.

**Upstream BER protein might regulate downstream protein expression, exemplified by a reduced level of Pol ß after knocking down AP endo.**

The whole BER pathway is considered as a highly coordinated process with upstream protein complexes recruiting downstream enzymes [7] [37]. Any change in the upstream components probably could easily alter the DNA repair function of downstream components. In addition, among BER proteins, AP endo is the one with multiple other functions. Although these functions are not as well characterized as its role in BER, AP endo stimulates the DNA binding activity of a series of transcription factors, such as AP-1 (Fos/Jun), NFκB, HIF-1α, p53, CREB, ATF, Egr-1 [174], [175], and may even act as a 'redox chaperone' [176]. Recent research data show AP endo itself can act as a transcription factor by binding to the negative Ca$^{2+}$ response elements (nCaRE) in the promoters of target genes [177]. The multiple functions discovered for AP endo led me to test whether downregulation of AP endo protein might result in a change in expression of a downstream enzyme, such as Pol ß. My results show that partial loss of AP endo in zebrafish early development prevents
appearance of Pol β. At the same time, the AP endo protein level in the Pol β knockdown embryos does not change (Fig.11). Hence, it is clear that a change in at least one upstream component in the pathway can and does affect the downstream components. Also, the data imply a possible regulation of Pol β by AP endo either directly or indirectly, a result that has not been demonstrated in previous studies.

Pol β gene expression is known to be regulated by CREB-1, Sp1, ATF2d and NF-κB among others [178] [179] [180] [181] [182] [183]. Very sparse information is available on post-transcriptional regulation of Pol β. CREB-1 message level is reduced 2.43 fold in AP endo hypomorphs, compared with control embryos (Wang and Strauss, unpublished data), while there is no direct information for Sp1, ATF2d and NF-κB at this time. Therefore, it is reasonable to speculate that downregulation of CREB-1 by AP endo results in repression of Pol β transcription, and eventually leads to a reduced Pol β protein level. Since this regulation occurs at the transcriptional level, a demonstration of the change in Pol β mRNA level would provide further evidence for this possibility, though it would not verify that the reduced expression of Pol β is entirely specific to CREB-1.
CONCLUSIONS AND SIGNIFICANCE

In this study, the zebrafish polb was characterized at gene, mRNA and protein levels. The single copy zebrafish polb gene expresses in a very dynamic pattern during early development. While Pol β transcript is present in all stage embryos, it is likely that zygotic transcription for polb gene is initiated prior to MBT, which is the typical developmental stage for activation of zygotic transcription. Although transcripts accumulate to a relatively high level at 3 hpf, the transcription level fluctuates with development with a lower level at 24 hpf than at previous stages. In spite of the presence of mRNA throughout embryogenesis, Pol β protein expression is not coordinated with its mRNA level. A deficiency in Pol β protein is found in embryos before 6.5 hpf. Thereafter, protein level increases, with a high level detected at 3 dpf. Pol β is not required for BER before 3 dpf regardless of its presence. Nevertheless, a switch from aphidicolin-sensitive replicative polymerases to Pol β occurs at 3 dpf when I detected high level of Pol β protein in those embryos [136].

Functional studies in zebrafish by either knockdown or overexpression in early embryos do not reveal a critical role of this protein in early patterning and morphogenesis. Moreover, Pol β knockdown zebrafish embryos survive and appear normal, with the exception of a swim bladder defect, whereas mouse knockouts die shortly after birth due to lung dysfunction. The discrepancies between zebrafish and mouse studies imply that Pol β is critically involved in a later stage of development.
Alternately, the difference might reflect a real difference in embryonic development of fish and man.

Although Pol β is not important for early development, it is critical for cells to respond to alkylation DNA damage, which is known to produce lethal and mutagenic lesions. As demonstrated in this study, MMS, an environmental mutagen, disrupts zebrafish development and leads to early death, when Pol β is absent in early embryos. Thus, Pol β guards living cells and organisms from DNA damage arising from alkylation agents, maintains genome integrity and normal development, prevents deleterious consequences, like embryonic death or possible cancer later during the organism’s life span.
Future Directions

Based on the work in this study, I propose two future directions here for further dissecting the biological functions of Pol β during zebrafish early development.

1. Anatomic and histological investigation can be performed for detecting ‘invisible phenotypes’ in zebrafish knockdown or overexpressed embryos.

   Although Pol β does not affect general morphology observed in my study, two organs needs to be further studied, brain and eye, since these two organs display abnormal development in mice when Pol β is knocked out or overexpressed. Anatomic and histological examination could provide direct evidence for any developmental abnormalities.

2. Further studies can be carried out to establish the role of Pol β in embryonic BER.

   In this study, I have shown that the MMS hypersensitivity phenotype in Pol β knockdown embryos can be rescued by wild-type proteins. It is important to find out whether the dRP lyase domain alone can achieve the same degree of rescue, because lyase activity of Pol β can reverse the MMS sensitivity in Pol β-deficient MEFs to an extent close to wild-type cells [92]. This experiment would provide information whether the removal of the toxic dRP residue created by upstream AP endo incision is critical for embryonic survival.

   Moreover, our lab has shown that early embryonic BER does not require Pol β though Pol β takes over the function of DNA synthesis in BER after 3 dpf [136]. Further study needs to be carried out to reveal which polymerase(s) substitute(s) and compete(s) with Pol β to perform DNA synthesis before 3 dpf. Since the dRP
lyase activity for Pol β is critical for the BER function, Pol λ and Pol ι are two candidate polymerases which possess the dRP lyase activity [66] [184], and have been implicated in BER in previous studies [65] [185] [186] [187] [188].

Finally, it would be very interesting to find out what would happen to overall BER activity in zebrafish embryos lacking Pol β. Whether there would be a deficiency in BER activity? Or can other polymerases functionally compensate for Pol β? In vitro BER assays using extracts from morpholino knockdown embryos could be used to answer these questions.

In all, these experiments would provide new insights to understand the role of Pol β in early development.
REFERENCES


