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**TRANSLATION SYNTHESIS:
CELLULAR AND ORGANISMAL FUNCTIONS**

Piya Temviriyankul

Cover: Ancient Buddhist temple mural depicting a Thai daily life scene at Wat Phumin, Nan province, Thailand.

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**TRANSLESION SYNTHESIS:
CELLULAR AND ORGANISMAL FUNCTIONS**

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“And in that moment, I swear we were infinite”

The Perks of Being a Wallflower, Stephen Chbosky

ABBREVIATIONS

9-1-1	Rad9-Rad1-Hus1
(6-4)PPs	(6-4) pyrimidine-pyrimidone photoproducts
ATM	ataxia-telangiectasia mutated protein
ATR	ataxia- telangiectasia and Rad3-related protein
ATRIP	ATR-interacting protein
BER	base excision repair
BPDE	benzo[<i>a</i>]pyrene diolepoxide
Chk1	checkpoint kinase 1
CPDs	cyclobutane pyrimidine dimers
DA	DNA damage avoidance
DDR	DNA damage response
DDT	DNA damage tolerance
DSB	DNA double-strand break
γ -H2AX	phosphorylation of histone H2AX at serine 139
4-HNE	4-hydroxynonenal
GG-NER	global genome nucleotide excision repair
HR	homologous recombination
KAP1	KRAB-ZFP-associated protein 1
MEFs	mouse embryonic fibroblasts
MN	micronuclei/micronucleus
NER	nucleotide excision repair
NHEJ	non-homologous end joining
PCNA-Ub	ubiquitinated proliferating cell nuclear antigen
Pol η	DNA polymerase Eta
Pol ι	DNA polymerase Iota
Pol κ	DNA polymerase Kappa
Pol ζ	DNA polymerase Zeta
RPA	replication protein A
ssDNA	single-stranded DNA
TLR	translesion replication
TLS	translesion synthesis
UBM	ubiquitin-binding motif
UBZ	ubiquitin-binding zinc finger motif
UV	ultraviolet light

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CHAPTER

1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS



A stable genome is of utmost importance for proper functioning of living cells. Genome instability is strongly correlated with formation of cancer cells and cellular premature aging. Endogenous factors as well as exogenous factors, such as exposure to DNA-reactive agents may contribute to the induction of genome instability. In some cases, exposure to genotoxic agents, such as those present in diet, sunlight and also endogenous sources, is inevitable. Studies on the cellular response to components in diet and various wavelengths in sunlight are important for taking measures in relation to food safety, cancer prevention and healthy aging. In this introduction, I will focus on the cellular response to food-derived genotoxins and ultraviolet light and the relationship between diet, irradiation, cancer, and aging.

FOOD, SUNLIGHT AND CANCER

In 2008, the World Health Organization (WHO) reported that cancer, cardiovascular diseases and infectious diseases are the most prominent causes of death worldwide (WHO, 2008). Particularly, cancer is a leading cause of death in economically developed countries, including the Netherlands. The burden of cancer is increasing because of increasing life-span and the cancer-associated lifestyle of people (Jemal *et al.*, 2011). It has been estimated that environmental factors, such as diet, smoking and exposure to sunlight, contribute to 90-95% in cancer development, whereas the contribution of genetic factors is about 5-10% (Anand *et al.*, 2008). Six hallmarks of cancer have been proposed, including [i] sustaining proliferative signaling, [ii] resisting cell death, [iii] evading growth suppressors, [iv] enabling replicative immortality, [v] inducing angiogenesis and [vi] activating invasion and metastasis (Hanahan *et al.*, 2011).

Cancer is a disease of failure of the regulation of cell growth and death (Croce, 2008; Hanahan *et al.*, 2011). The underlying causes of this failure are the generation of genetic changes by endogenous or exogenous sources in oncogenes and tumor suppressor genes that, among others, control cell cycle, cell growth and cell transformation. Genetic changes can range from base pair substitutions to chromosomal rearrangements. Functional imbalance between oncogenes and tumor suppressor genes, driven by genetic changes, enhances cell proliferation and impairs programmed cell death, leading to uncontrolled immortal malignant cells that are capable of invading and metastasis.

Diet and dietary components are thought to be factors causative for the increase in cancer incidence worldwide. The American Institute of Cancer Research and World Cancer Research Fund (AICR-WCRF) recommends to limit consumption red meat, processed meat, alcohol and moldy cereals in order to reduce cancer (American Institute for Cancer Research. *et al.*, 2007; Ferguson *et al.*, 2008). Evidence is accumulating that avoiding exposure to them possibly reduces diet-mediated mutagenesis (Ferguson *et al.*, 2008). It has been estimated that diet contributes to 30-40% of all cancers in males, and probably up to 60% in females (Wynder *et al.*, 1977). Certainly, high intake of alcohol, grilled red meat and polyunsaturated fat is linked with an increased risk

for breast, esophagus, liver and colon cancer (Khan *et al.*, 2010), whereas intake of fungal contamination of staple foods is a major cause of liver cancer, one of the most diagnosed cancer in males and females worldwide (Ferlay *et al.*, 2010; Jemal *et al.*, 2011). Thus, diet is not only a good source for important nutritional compounds, like proteins or vitamins, which are essential for our body, but also can be a source for substances or their precursors that are carcinogenic (Ferguson *et al.*, 2008). Some food compounds can be identified as carcinogens because they are capable of covalently interacting with genomic DNA, resulting in DNA adducts that distort the geometry of DNA, and subsequently induce DNA mutations, genomic instability and probably cancer (Goldman *et al.*, 2003). Food-derived genotoxins are found in a wide range of human daily foods (Ferguson *et al.*, 2008), for instance [i] constituent of natural foods, such as alcohol, ethyl carbamate, methyleugenol, pyrrolizidine alkaloids (PAs), [ii] contaminants during planting or harvesting, such as aflatoxin B₁, fusarium toxin and 1-methylcyclopropene (MCP), [iii] contaminants generated during food processing, e.g. polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HCAs), nitrosamines and acrylamide, [iv] contaminants during food preservation or storage, such as nitrosamines, 1,3-dichloro-2-propanol (DCP) and lipid peroxidation, and [v] components illegally utilized for food coloring, such as Sudan I and Red2G (Benford *et al.*, 2010; Dybing *et al.*, 2008; Ferguson *et al.*, 2008).

Two examples which illustrate the correlation between components in diet and cancer are consumption of alcoholic beverages and of nuts contaminated with fungi. Consumption of alcohol increases the cancer risk in different ways. First, upon entering the body, ethanol typically undergoes metabolic activation, resulting in the DNA-reactive compound acetaldehyde. Indeed, DNA lesions induced by acetaldehyde, like acetaldehyde-derived deoxyguanosine adducts, have been detected *in vivo*. This type of adduct stimulates the DNA damage response via the Fanconi anemia pathway (Abraham *et al.*, 2011; Langevin *et al.*, 2011) and activates phosphorylation of histone H2AX on serine 139 (γ -H2AX), a general DNA damage marker (Marietta *et al.*, 2009). Moreover, rats exposed to alcohol for four weeks display significant induction of micronuclei, a genomic instability marker (Kotova *et al.*, 2012). Therefore, the International Agency for Research on Cancer (IARC) classifies acetaldehyde, generated endogenously or from alcoholic beverages, as a carcinogen. Second, alcoholic beverages are contaminated with ethyl carbamate that is formed during fermentation by yeast. Ethyl carbamate is metabolically converted into vinyl carbamate epoxide which is able to interact with DNA resulting in the formation of DNA lesions, 1,N⁶-ethenodeoxyadenosine and 1,N⁴-ethenodeoxycytidine. There is a clear evidence that ethyl carbamate is a mutagenic and carcinogenic agent, causing lung and liver cancer in rats and mice (Forkert, 2010).

Whole grain, groundnuts, tree nuts and dried fruits are often contaminated with aflatoxin B₁ (AFB₁), which is a mycotoxin and has been classified as a highly carcinogenic compound in man. After metabolic activation, AFB₁ is converted into unstable AFB₁-8,9-exo-epoxide, which reacts mostly with the N7 position of guanine (AFB₁-N7-Guanine)

(Martin *et al.*, 1977). In budding yeast, this DNA lesion delays S-phase progression, indicating an adverse effect on genome replication (Fasullo *et al.*, 2010). Feeding rats with AFB₁ induces liver, lung, kidney and colon cancer. Molecular analysis reveals that AFB₁ induces DNA mutations, especially G>T transversion, for example at codon 249 in the p53 tumor suppressor gene. This mutation is commonly found in liver cancer diagnosed in people from the areas of high exposure to AFB₁, supporting its carcinogenic role (IARC, 2002). A region of p53, about 570 bp, is very sensitive for mutations and numerous mutational hot spots have been discovered in this region (Friend, 1994).

Besides diet, ultraviolet (UV) radiation is a significant factor contributing to cancer. The UV component in sunlight is considered to be the most prominent and ubiquitous physical carcinogen on earth (de Gruijl, 1999). The first reports of skin cancer are from the 19th century describing that sailors and vineyard workers, who habitually were exposed to sunshine during their work, exhibited skin carcinomas [in review (de Gruijl, 1999)]. Skin cancer is the most common tumor diagnosed in the United States and Europe (Gilchrest *et al.*, 1999). Many epidemiological studies indicate clearly that prolonged exposure to solar UV irradiation is associated with skin malignancies and skin cancer [in reviews (Diepgen *et al.*, 2012; Kanavy *et al.*, 2011; Osmola-Mankowska *et al.*, 2012)]. There are three different types of UV light, depending on their wavelength: UVA, UVB and UVC. Although UVC is highly mutagenic, UVC is not present in our environment as the ozone layer of the earth efficiently absorbs it. However, UVA and UVB are mutagenic and carcinogenic in mice (de Laat *et al.*, 1997; Dumaz *et al.*, 1997). UV light is able to interact with DNA, to provoke cellular inflammation and to induce DNA mutations. UVC light covalently links two adjacent pyrimidine bases, resulting in two types of DNA damages, the mildly helix-distorting cyclobutane pyrimidine dimer (CPDs) and the strongly helix-distorting (6-4) pyrimidine pyrimidone photoproduct, [(6-4) photoproduct; (6-4)PPs] [in review (Pfeifer *et al.*, 2005)]. CPD lesions are more mutagenic than (6-4)PPs, possibly because they are slower repaired, less toxic and they can deaminate to uracil or thymidine which is ultimately highly mutagenic. This is confirmed by expression of either CPD photolyase or (6-4) photolyase in cells to remove CPDs or (6-4)PPs-induced by UVB, respectively. The result showed that CPDs contribute to about 80% of UVB-induced DNA mutations (You *et al.*, 2001). Additionally, C>T and CC>TT transitions at dipyrimidine sites have been found in the p53 tumor suppressor gene in skin tumors, supporting a relevant role of UV light in tumorigenesis (Kress *et al.*, 1992).

DNA REPAIR PATHWAYS

Several DNA repair pathways will be activated in order to eliminate DNA damages before they are encountered by replisome complexes, which can induce mutations and/or genome instability. It is unclear how cells select a particular DNA repair pathway, but it seems to be largely dependent on the architecture of damaged DNA.

Nitrosamines in diet and model compounds, such as *N*-alkyl-*N*-nitrosoureas (MNU), methyl methanesulfonate (MMS) and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) (Mitra *et al.*, 1993), are capable of transferring their methyl group to nucleotides, especially guanine and adenine, yielding non-helix-distorting lesions. Alkylation damage such as 7-methylguanine, 3-methyladenine and 7-methyladenine (Beranek, 1990) are efficiently recognized and removed by the Base Excision Repair (BER). In addition, most endogenous-induced DNA lesions, such as 8-oxoG, depurinated or deaminated bases, are also efficiently eliminated by BER.

Bulky DNA lesions, induced by components in diet, such as acetaldehyde, AFB₁, PAHs, products of lipid peroxidation or UV irradiation (CPDs and (6-4)PPs), severely distort the geometry of the DNA helix compared to DNA lesions induced by simple alkylating agents. The bulky DNA adducts are mainly eliminated by the Nucleotide Excision Repair (NER). In NER, DNA adducts are excised, yielding DNA gaps of about 30 nucleotides in length. These ssDNA gaps are eventually filled and ligated. The importance of NER is demonstrated by human diseases that result from genetic defects in NER. Cockayne syndrome, Trichothiodystrophy and Xeroderma pigmentosum (XP) are three examples of NER-associated diseases. All these syndromes display high sensitivity to UV light and the XP patients show increased susceptibility to skin cancer.

Generally, food-derived genotoxins are prone to generate DNA mutations by modifying or binding to DNA. However, other food compounds may induce DNA breaks in an indirect way, like Genistein found in plants, which inhibits topoisomerase II (Bandelet *et al.*, 2007; Michael *et al.*, 2006). Topoisomerase II is an enzyme that incises the DNA double strand helix to relax supercoils during DNA replication and transcription, hence prolonged inhibition of topoisomerase II causes DNA breaks. Etoposide, an anti-cancer drug, also inhibits Topoisomerase II. Homologous Recombination (HR) and Non-Homologous End-Joining (NHEJ) are involved in repairing DNA breaks by joining the broken sugar-phosphate backbone, but not for repairing damaged bases (Hoeijmakers, 2001). The HR pathway is an error-free process that principally functions in S-phase cells, while NHEJ is mutagenic process as it can create insertions, deletions and translocations when wrong DNA ends are joined. NHEJ is basically uncoupled from genome replication.

Hydroxynonenal (HNE), a product of lipid peroxidation, and some cancer drugs, such as mitomycin C and cisplatin, are able to covalently link the two strands of a double stranded DNA molecule, yielding DNA interstrand crosslinks (ICLs) (Huang *et al.*, 2010). These types of DNA lesions are very cytotoxic as they disrupt strand separation of DNA during replication and transcription, respectively. The mechanism underlying ICLs repair is only partly revealed, which may be due to the complexity of ICL adducts and of the many pathways involved. In mammalian cells, a specific set of genes is shown to be uniquely involved in the ICLs repair. Mutations in these genes results in a rare autosomal recessive disease, Fanconi anemia. The characteristics of this disease are hematological abnormalities, growth retardation, microcephaly and cancer.

DNA DAMAGE TOLERANCE PATHWAYS

Although cells possess a comprehensive set of DNA repair pathways, DNA lesions can escape repair and block both DNA replication performed by DNA polymerase δ or ϵ and transcriptional RNA elongation by RNA polymerase II. Owing to the high template stringency of DNA polymerases and their narrow active site that only accommodates normal nucleotides, Polymerases δ and ϵ are unable to replicate across most DNA damages, such as certain alkylated bases or bulky DNA lesions. Arrested replication forks at DNA lesions may result into replication fork collapses and formation of DNA breaks, which are very cytotoxic if not repaired. To complete DNA replication, enabling cellular survival, cells trigger a set of so-called DNA damage tolerance (DDT) pathways (Friedberg, 2005). Of note, DDT does not completely restore DNA to its original context and architecture, thus the DNA lesion remains on the DNA. DDT tolerates DNA damage until a suitable DNA repair pathway can remove the damage. Two DDT pathways have been identified which differ in their ability to cause DNA mutations: translesion DNA synthesis (TLS) which may create DNA mutations (discussed below) and template switching-dependent DNA synthesis (or damage avoidance [DA]) (Fig. 1, adapted from Baynton *et al.*, 2000). During template switching, cells utilize the undamaged DNA strand as a template to avoid DNA lesions. Consequently, this process is an error-free process (Chang *et al.*, 2009). There are several data indicating the existence of DA in bacteria and yeast; DA in mammalian cells, however, is enigmatic. Furthermore, it remains unclear how cells decide to make a choice between TLS or DA to cope with DNA lesions in template strand during replication (Fig. 1).

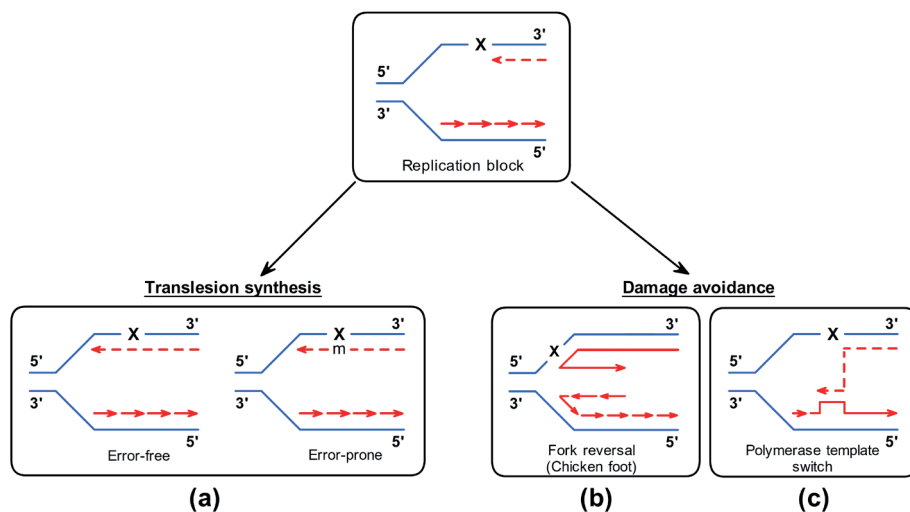


Figure 1 | Overview of DNA damage tolerance pathways. (a) Translesion synthesis (TLS); specialized DNA polymerases replicate across damaged template DNA, resulting in either error-free or error-prone bypass. (b) and (c) DNA damage avoidance (DA) by either template switching or recombination.

ACTIVATION OF DNA DAMAGE TOLERANCE PATHWAYS

In yeast, an arrested replication fork at a DNA adduct leads to the formation of a single-stranded DNA (ssDNA) patch coated with RPA, which can be subsequently recognized by the Rad6-Rad18 complex that monoubiquitinates PCNA on lysine 164 (K164) (Bienko *et al.*, 2005; Hoege *et al.*, 2002). Mono-ubiquitination of PCNA by Rad6-Rad18 stimulates the mutagenic branch of DDT, which is TLS, whereas poly-ubiquitination of this sliding clamp by Mms2-Ubc13-Rad5 complex elicits the error-free damage avoidance (DA) pathway in yeast (Andersen *et al.*, 2008; Bienko *et al.*, 2005; Stelter *et al.*, 2003). However, it is unclear how the Mms2-Ubc13-Rad5 complex promotes the DA pathway. Possibly, Rad5 may employ its helicase activity to unwind stalled replication forks and then promote fork regression (Blastyak *et al.*, 2007). Yeast Rad6 and Rad18 mutants are very sensitive to a variety of DNA-damaging agents, like UV and alkylating agents. In addition, these mutants display a hypomutable phenotype for UV, indicating that Rad6 and Rad18 are involved in regulation of TLS-mediated lesion bypass (Cejka *et al.*, 2001). This mechanism is evolutionary conserved and is present in mammalian cells, since Rad18 and two isoforms of Rad6, Rad6A and Rad6B, have been identified. Similar to yeast, human cells defective for Rad18 exhibit hypersensitivity to UV, MMS and mitomycin C (Tateishi *et al.*, 2000). In human and mouse cells, PCNA-Ub can easily be detected after blocking of DNA replication by DNA damages. Also, PCNA poly-ubiquitination has been detected in higher eukaryotes (Hofmann *et al.*, 1999; Koken *et al.*, 1991; Motegi *et al.*, 2008; Tateishi *et al.*, 2000; Unk *et al.*, 2008). Two mammalian proteins, HLTF and SHPRH, have been identified as putative Rad5 homologues. These two proteins, like yeast, facilitate PCNA polyubiquitination at K164 in a RAD18-dependent manner by interacting with both Rad6/Rad18 and Mms2/Ubc13 complexes. Loss of HLTF or SHPRH in human cells results in a hypersensitivity phenotype to MMS, indicating their role in DDT. Mouse embryonic fibroblasts (MEFs) with targeted disruptions of either HLTF or SHPRH and mouse primary B cells with deletions in both HLTF/SHPRH still show some residual PCNA poly-ubiquitination at K164 (Krijger *et al.*, 2011a; Motegi *et al.*, 2008). These findings indicate the existence of alternative proteins promoting PCNA poly-ubiquitination in higher eukaryotic cells.

TRANSLESION SYNTHESIS: A MUTAGENIC DNA DAMAGE BYPASS MECHANISM

During TLS, specialized TLS polymerases are capable of incorporating nucleotides opposite, and beyond, the covalent DNA template damage caused by an endogenous or exogenous genotoxic agent. After TLS, normal replication by DNA polymerase δ or ϵ can resume. TLS, therefore, results in perpetuation of replication in the presence of damaged templates. Nevertheless, these specialized TLS polymerases replicate past the lesion in either an error-prone or error-free manner due to their specific properties

(Prakash *et al.*, 2005). TLS polymerases share the same protein folding pattern as replicative polymerases (Prakash *et al.*, 2005; Waters *et al.*, 2009), although they have some different features compared to replicative polymerases. TLS polymerases have no 3'-to-5' exonuclease activity (also called proofreading activity). Furthermore, TLS polymerases have a more open and larger active site than normal replicative polymerases. This feature allows TLS polymerases to accommodate various kinds of DNA lesions, like bulky DNA adducts and even two covalently linked bases, such as thymine-thymine dimers induced by UV light (Ling *et al.*, 2003). Taken together, these properties result in a mutation rate that can be 1,000 fold higher than that of replicative polymerases (Waters *et al.*, 2009).

In mammalian cells, TLS polymerases comprise of conserved Y-family DNA polymerases η (Pol η), Pol ι , Pol κ and Rev1, and a B-family DNA polymerase Pol ζ . Interestingly, Pol ζ belongs to the same family as replicative polymerases, Pol δ and ϵ . Nevertheless, in contrast to Pol δ and ϵ , Pol ζ is able to perform both TLS and to extend from mismatched or distorted primer-template pairs generated by Y-family DNA polymerases as it lacks proofreading activity (Waters *et al.*, 2009). The amino acid alignment data show that all TLS polymerases share an evolutionary conserved polymerase domain, which is crucial for their biological activity (Fig. 2).

Closer inspection of the Y-family TLS polymerases reveals that they all contain ubiquitin binding motifs, either an UBM (ubiquitin-binding motif) or an UBZ

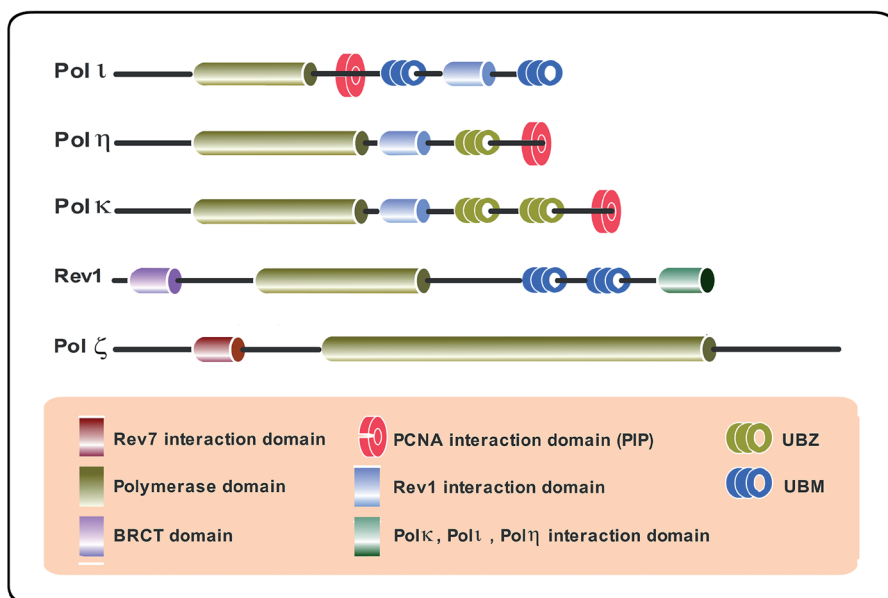


Figure 2 | An overview illustrating protein domains of mammalian TLS polymerases (Prakash *et al.*, 2005; Waters *et al.*, 2009).

(ubiquitin-binding zinc finger motif), indicating their susceptibility to ubiquitin-mediated post-translational modification. Furthermore, in Pol η , Pol ι and Pol κ , a Rev1-binding motif is capable of interacting with the C-terminal domain of Rev1, whereas the interaction of Pol ζ , via its accessory subunit, Rev7, to both the C-terminal and the N-terminal (BRCT) part of Rev1 has been reported (Akagi *et al.*, 2009; D'Souza *et al.*, 2006; Kikuchi *et al.*, 2012; Ohashi *et al.*, 2004). The N-terminal region of Rev1, which contains a BRCT domain, distinguishes Rev1 from other TLS polymerases (Gerlach *et al.*, 1999). This domain has been found in various proteins involved in the DNA damage response, and has been shown to bind to DNA and to phosphorylated proteins (Yu *et al.*, 2003). However, a recent study challenges these findings. In yeast, a mutated phosphate-binding pocket of the Rev1 BRCT domain does not alter the sensitivity and mutation frequencies in response to UV light, implying that the phosphate-binding pocket is not essential for Rev1 function *in vivo*. The authors propose that instead of binding to phosphorylated proteins, the Rev1 BRCT domain may interact with another protein, independent from its phosphorylation state (Pryor *et al.*, 2013). All Y-family DNA polymerases contain PCNA-binding ability. Pol η , Pol ι and Pol κ undoubtedly utilize their PCNA-interacting protein (PIP) domain for binding to PCNA, whereas it remains elusive whether Rev1 directly interacts with PCNA, since both the N-terminal BRCT domain and the C-terminal domain have been reported for their capability to interact with PCNA (Guo *et al.*, 2006; Ross *et al.*, 2005; Wood *et al.*, 2007). However, a recent *in vitro* study indicates that the suggested interaction of the N-terminal BRCT domain with PCNA is caused by DNA impurities (de Groote *et al.*, 2011). Moreover, the UBM motif of Rev1 binds to PCNA in response to DNA damages as a mutation in this domain abolishes the interaction between Rev1 and PCNA, resulting in strongly attenuated damage-induced mutagenesis (Wood *et al.*, 2007).

In contrast to other TLS polymerases that incorporate all four nucleotides, Rev1 possesses only DNA-dependent deoxycytidyl transferase activity, primarily incorporating deoxycytidines across guanine, abasic sites and guanine adducts (Masuda *et al.*, 2002; Nelson *et al.*, 1996a; Zhang *et al.*, 2002). The biological function of this evolutionary conserved activity of Rev1 is still enigmatic, although it is not required for UV-associated mutagenesis (Lawrence, 2004). Since Rev1 binds to many different TLS polymerases and Rev1's catalytic activity is dispensable for TLS at some DNA adducts, it is hypothesized that Rev1 may function as a scaffold protein for other polymerases and may play a central role in TLS (Lehmann, 2005; Prakash *et al.*, 2005).

Because of the properties of TLS polymerases, TLS is an inherently mutagenic process. For example, loss of either Rev1 or Rev3 in mammalian cells results in a hypomutable phenotype following UV exposure (Gibbs *et al.*, 2000; Li *et al.*, 2002). Therefore, this process must be tightly controlled. Several mechanisms have been proposed to restrain the activity of TLS polymerases, including their cellular concentration, their low processivity and their recruitment mechanisms [in review (Sale *et al.*, 2012)].

SUBSTRATE SPECIFICITIES OF DNA TRANSLESION SYNTHESIS POLYMERASES

Owing to the large active site of TLS polymerases, it is not a wonder that each TLS polymerase is able to replicate across various types of damaged DNA templates with an overlap in substrate specificities with other TLS polymerases. Table 1 shows the involvement of each TLS polymerase in translesion synthesis across different kinds of well-defined DNA lesions *in vitro* and *in vivo*. Unfortunately, most of these data are fragmentary studies, and different cell types, doses of DNA damaging agents and experimental methodologies were used. Thus far, the relative *in vivo* contribution of each TLS polymerase in TLS and DNA damage responses to specific genomic lesions is problematic to evaluate. In Chapter 2, using a single cell type, the *in vivo* roles of all key mammalian TLS polymerases in bypassing two food-derived genotoxins, benzo[*a*]pyrene diolepoxide (BPDE) and hydroxynonenal (HNE), are described. The advantage of using BPDE and HNE is that both of them do not require metabolic activation, so they are able to directly interact with genomic DNA. Using a similar approach, the *in vivo* roles and DDR of TLS-related proteins in response to UV light are reported in Chapter 3 of this thesis.

Table 1 | The involvement of each TLS polymerase in translesion synthesis across different kinds of DNA lesions *in vitro* and *in vivo* (Choi *et al.*, 2006; Choi *et al.*, 2010; Frank *et al.*, 2002; Fukuda *et al.*, 2009; Gibbs *et al.*, 2005; Haracska *et al.*, 2000; Haracska *et al.*, 2002a; Haracska *et al.*, 2003; Haracska *et al.*, 2001; Hashimoto *et al.*, 2012; Hendel *et al.*, 2008; Jansen *et al.*, 2009a; Jansen *et al.*, 2009b; Johnson *et al.*, 2000; Klarer *et al.*, 2012; Lupari *et al.*, 2012; Maddukuri *et al.*, 2010; Masutani *et al.*, 1999a; Masutani *et al.*, 2000; Nelson *et al.*, 1996b; Pence *et al.*, 2010; Rechkoblit *et al.*, 2002; Shachar *et al.*, 2009; Sherrer *et al.*, 2011; Stafford *et al.*, 2009; Szuts *et al.*, 2008; Tissier *et al.*, 2000; Vaisman *et al.*, 2003; Washington *et al.*, 2002; Wolffe *et al.*, 2006; Yoon *et al.*, 2009; Yoon *et al.*, 2010; Zhang *et al.*, 2002; Zhang *et al.*, 2001; Zhang *et al.*, 2000)

DNA lesions	Pol α		Pol κ		Pol η		Rev1		Pol ζ	
	<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>
CPDs	±	-	±	+	+	+	-	-	+	±
(6-4)PPs	+	+	-	-	±	±	-	+	+	+
O ⁶ -MG*	+	n.d.	±	+	+	n.d.	+	n.d.	+	n.d.
PhIP**	+	n.d.	+	n.d.	+	n.d.	+	n.d.	n.d.	n.d.
M ₁ dG***	+	n.d.	+	n.d.	+	n.d.	+	n.d.	n.d.	n.d.
Abasic sites	+	n.d.	-	n.d.	+	±	+	+	+	+
BPDE-dG	±	n.d.	+	+	±	+	n.d.	+	-	+
HNE-dG	+	n.d.	+	n.d.	±	n.d.	n.d.	n.d.	n.d.	n.d.

* = O⁶-Methylguanine

** = 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyrimidine

*** = 3-(2-Deoxy-β-D-erythro-pentofuranosyl)pyrimido-[1,2-*a*]purin-10(3H)-one

n.d. = not determined

MECHANISMS OF DNA TRANSLESION SYNTHESIS: POLYMERASE SWITCH MODEL

The polymerase switch (direct bypass) model proposes that multiple TLS polymerases can interact with mono-ubiquitinated PCNA (PCNA-Ub) concurrently, enabling instant switching from polymerase Pol δ or ϵ to a TLS polymerase to enable bypass of DNA lesions on a trial-and-error basis (McCulloch *et al.*, 2008; Pages *et al.*, 2002) (Fig. 3). Following the initial insertion of a nucleotide opposite DNA damage by a

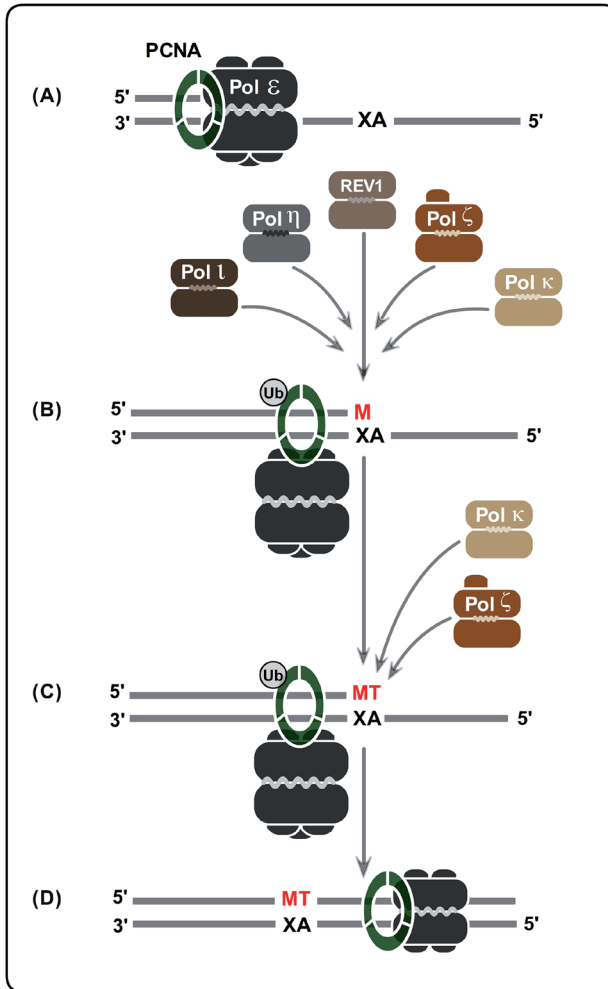


Figure 3 | Overview of the polymerase switch model. (A) Replicative DNA polymerase cannot replicate across damaged template DNA. (B) PCNA is mono-ubiquitinated, resulting in a recruitment of TLS polymerases to bypass DNA damages. (C) After bypass by a suitable TLS polymerase, Pol κ or Pol ζ extend primer terminus. (D) PCNA is deubiquitinated and subsequently replicative DNA polymerase re-start DNA replication.

suitable TLS pol, Pol ζ , or possibly Polk performs extension from the primer terminus which afterwards may activate deubiquitinating enzymes, such as USP1 and Ubp10, to remove ubiquitin from PCNA (Gallego-Sanchez *et al.*, 2012; Huang *et al.*, 2006). After removing mono-ubiquitin from PCNA-Ub, Pol δ is eventually replaced into its normal position, thereby re-launching the replication machinery.

TLS polymerases intrinsically associate with unmodified PCNA via the PIP domains of Pol η , Pol ι and Polk *in vitro*, but not *in vivo* (Haracska *et al.*, 2002b; Vidal *et al.*, 2004), indicating a weak interaction. Upon ubiquitination of PCNA, the interaction of TLS polymerases with PCNA-Ub is enhanced by their UBM/UBZ domains (Bienko *et al.*, 2005). In particular, Pol η has been shown to specifically interact with PCNA-Ub in chromatin in response to UV in living cells (Kannouche *et al.*, 2004; Watanabe *et al.*, 2004). The crystal structure of PCNA-Ub revealed that PCNA-Ub has a distinctive orientation, ubiquitin molecules on PCNA are radially extended away from the PCNA molecules, providing a platform for TLS through ubiquitin-interacting domains (Zhang *et al.*, 2012). As discussed above, mammalian Rev1 is able to interact with PCNA via its UBM domain, while its C-terminal domain binds to Pol η , Pol ι , Polk and Rev7 (the accessory subunit of Pol ζ). An *in vitro* competition study using a fixed amount of purified Rev1 and Polk showed that by increasing the amount of Rev7, Rev1:Polk complexes decreased, whereas Rev1:Rev7 complexes increased, supporting polymerase switching (Guo *et al.*, 2003). Possibly, Rev1 facilitates polymerase switching in response to DNA damages. In this model, PCNA-Ub seems to be a central player contributing to TLS. Nonetheless, mutated PCNA at K164 in chicken and mouse cells does not totally abolish TLS activity, implying the existence of PCNA-Ub-independent TLS in vertebrate cells (Edmunds *et al.*, 2008; Hendel *et al.*, 2011; Krijger *et al.*, 2011b; Temviriyankul *et al.*, 2012). Despite the fact that PCNA-Ub is required for Rev1 activity in yeast (Garg *et al.*, 2005; Wood *et al.*, 2007), chicken Rev1 and PCNA-Ub are not epistatic following exposure to UV or MMS implying different roles in response to DNA damage (Edmunds *et al.*, 2008). These observations raise the question whether TLS polymerases function exclusively at stalled replication forks.

MECHANISMS OF DNA TRANSLESION SYNTHESIS: GAP-FILLING MODEL

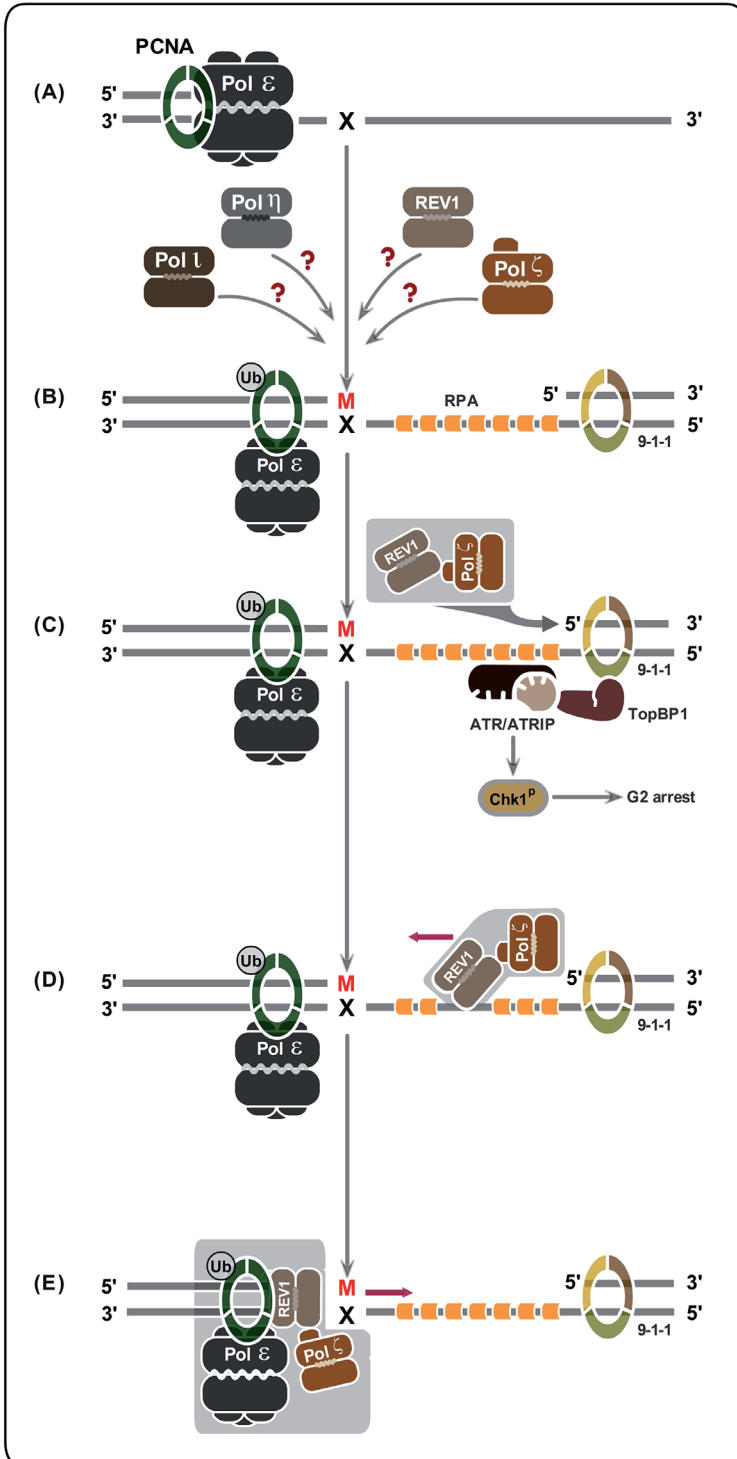
The gap-filling model proposes that damaged DNA is replicated discontinuously, resulting in DNA molecules containing ssDNA gaps that will be filled later by TLS polymerases (Fig. 4) (Jansen *et al.*, 2007).

The first indication for this model was published in 1968 (Rupp *et al.*, 1968). NER deficient strains of *E.coli* exposed to UV showed small fragmented DNA patches compared to control cells. These small patches were eventually converted into high molecular weight DNA. The interpretation was that the newly synthesized daughter

strands consisted initially of DNA strand discontinuities (DNA gaps), presumably opposite the UV lesions, which were subsequently sealed. In 2006 (Lopes *et al.*, 2006), electron microscope analysis to visualize replicating DNA in a NER-deficient strain of *Saccharomyces cerevisiae* (*S. cerevisiae*) exposed to UV revealed the existence of DNA gaps, generated along the replication duplexes in both leading and lagging strands, up to 400bp in size. Furthermore, these gaps were not instantly filled-in after passage of the replication fork. To gain more evidence about the factors involved in response to UV lesions, genes encoding TLS Pols Rev1, Rev3 and Pol η , checkpoint proteins Mec1 and Rad53 and the recombination protein Rad52 were mutated. Interestingly, all mutant yeast strains showed a significant increase of ssDNA gaps compared to the control strain, indicating the roles of TLS Pols, checkpoint signaling and recombination in a gap filling process. Other recent and clear support for this model comes from budding yeast in which TLS activity was studied along the cell cycle. The data showed that Rev1 and Pol ζ are involved in TLS of UV lesions in the G2/M phase of cell cycle, uncoupled from genome replication (Daigaku *et al.*, 2010). These data are supported by findings showing that yeast Rev1, and human Pol ζ , are largely expressed in the G2/M phase (Bhat *et al.*, 2013; Waters *et al.*, 2006), indicating that TLS is not entirely S-phase dependent and may carry out gap-filling during late S till the M phase of cell cycle (Diamant *et al.*, 2012).

The finding of the post-replicative internal gaps after UV irradiation suggests the ability of replication forks to re-prime downstream of DNA damages, leaving ssDNA gaps behind. Indeed, replication forks can restart and re-initiate downstream of UV lesions both in *E.coli* and mammalian cells (Bridges *et al.*, 1974; Elvers *et al.*, 2011; Heller *et al.*, 2006; Lehmann, 1972). The 5' primer terminus downstream of the ssDNA-containing DNA lesion is not only an indication for DNA replication downstream of stalled replication forks, but also is thought to be a docking platform for the alternative processivity clamp, the so called 9-1-1 clamp (Rad9-Rad1-Hus1 in human or Ddc1/Mec3/Rad17 in *S.cerevisiae*) [in review (Parrilla-Castellar *et al.*, 2004)]. The 9-1-1 complex is loaded onto the 5' primer terminus downstream of the ssDNA-containing DNA lesion and promotes phosphorylation of the ATR-ATRIP complex (ATR = ataxia telangiectasia and Rad3-related protein, ATRIP = ATR-interacting protein) involved in DDR. This process occurs independent of PCNA ubiquitination (Chang *et al.*, 2006; Parrilla-Castellar *et al.*, 2004). The 9-1-1 clamp is required for the DNA damage response (DDR) which subsequently delays cell cycle progression, providing more time for cells to cope with replication stress. Furthermore, the 9-1-1 complex may act as a landing platform for TLS polymerases.

Figure 4 | Overview of a hypothetical gap-filling model. (A) Replicative DNA polymerase cannot replicate across damaged template DNA. (B) PCNA is mono-ubiquitinated, resulting in a recruitment of TLS polymerases to bypass DNA damages. However, since DNA lesions are difficult to bypass, replicative DNA polymerase re-start DNA replication downstream of damaged DNA, leaving a ssDNA gap behind. This ssDNA will be coated by RPA, while the 9-1-1 complex loads at the new 5' primer terminus. (C) Rev1:Pol ζ complex binds to the 9-1-1 clamp and ATR activation leads to cell cycle arrest. (D) Rev1:Pol ζ complex may translocate to PCNA. (E) Rev1:Pol ζ complex performs TLS and quench DDR.



For example, Pol ζ (via Rev7) and Polk have been shown to interact with this alternative clamp (Kai *et al.*, 2003; Sabbioneda *et al.*, 2005). Loss of Rad9, Rad1, or Hus1 results in a decrease in Pol ζ -dependent spontaneous mutagenesis. These results suggest that the 9-1-1 complex, in addition to its role in DDR, may directly regulate the entrance of TLS polymerases to post-replicative ssDNA gaps. To provide more evidences, the UV response of yeast cells mutated in Rev1, Rev3, 9-1-1 clamp or ATR was determined. A defect either in a component of the 9-1-1 complex or ATR in NER-deficient cells decreases mutagenic TLS efficiency, as these mutants become hypomutable for UV light. This was accompanied with a reduced level of Rev1 phosphorylation and Pol ζ efficiency (Pages *et al.*, 2009). Combined, to bypass UV lesions and to fill in ssDNA gaps, coordination between DDR proteins, especially the 9-1-1 complex, and TLS polymerases is essential.

The BRCT region of Rev1 is demonstrated to bind at the 5' primer-template junction flanking a single-strand DNA gap *in vitro* (de Groote *et al.*, 2011), although there is some dispute about the relevance of Rev1 in binding to 5' primer-template junctions (de Groote *et al.*, 2011; Pryor *et al.*, 2013). Thus, Rev1, via its BRCT region and in complex with Pol ζ and Polk, may use the 5' end of a primer-template junction at a post-replicative gap as a docking site, then Rev1 may translocate along the ssDNA gap back to the 3' end of the stalled replication fork to catalyze gap-filling (Jansen *et al.*, 2007). In support of a possible role in post-replicative gap-filling, UV-irradiated Rev1- and Rev3-deficient MEFs display ssDNA gaps in G2 cells (Jansen *et al.*, 2009a; Jansen *et al.*, 2009b).

Although there are several lines of research supporting one or the other model, cells might employ both the polymerase switching (direct bypass) and the gap-filling mode of TLS to bypass DNA adducts, depending on the architecture of DNA lesions (Jansen *et al.*, 2007). In the polymerase switching model, replicative polymerases are in complex with TLS Pols and PCNA, suggesting that this process is replication dependent, whilst the gap-filling model can operate in the S and G2 phases as shown by an assay that measured TLS activity on a gapped plasmid in the different phases of the cell cycle (Diamant *et al.*, 2012). In support, TLS was also found to be high in the G2 phase compared to the S-phase cells (Bhat *et al.*, 2013; Waters *et al.*, 2006). UV-induced mildly helix-distorting CPDs are efficiently bypassed in a relatively error-free manner by Pol η via direct bypass (discussed above). Moreover, it was found that although CPDs cannot be eliminated in rodent cells, the S-phase progression is normal (Meyn *et al.*, 1977). This indicates that the rapid and effective bypass of CPDs by Pol η may preclude re-priming and DDR activation, resulting in normal S-phase progression. Cells might also employ polymerase switching to bypass UV-induced (6-4)PPs. However, owing to the poor ability of TLS polymerases to accommodate (6-4)PPs, a strongly helix-distorting DNA lesion, bypassing of (6-4)PPs is time-consuming process and might not be finished during S-phase, unlike CPDs. Also, S-phase cells are vulnerable to agents or events that interfere with cell division. Possibly, to prevent replisome dissociation, replicative DNA polymerase re-primers downstream of strongly helix-distorted DNA damage, leaving ssDNA behind. Consequently, these patches of ssDNA activate DDR to delay cell cycle progression, thereby allowing TLS at post-replicative gaps (Jansen *et al.*, 2007).

DNA DAMAGE RESPONSES (DDR) OR CHECKPOINT ACTIVATION

In addition to recruiting TLS Pols, the employment of the 9-1-1 complex to post-replicative gaps triggers cell cycle checkpoint activation. Briefly, the RPA-coated patch of ssDNA will recruit a complex of protein kinases, the so-called ATR-ATRIP complex. The ATRIP protein binds to the basic part of RPA via its acidic part (Ball *et al.*, 2007). However, this interaction is just enough for a co-localization of ATR-ATRIP complex to the gapped DNA structure, but is not capable of stimulating ATR. To fully stimulate ATR, the C-terminal domain of Rad9, a component of the 9-1-1 complex, is hyperphosphorylated, leading to a conformational change suitable for recruitment of an ATR activator, TOPBP1 (DNA topoisomerase 2-binding protein 1). TOPBP1 consists of an ATR activation domain which physically interacts with and activates ATR [in review (Cimprich *et al.*, 2008)], resulting in the phosphorylation of a downstream kinase protein, Chk1 (Checkpoint kinase1). Phosphorylated Chk1 inhibits origin firing, pauses the formation of new replication forks, slows down replication fork progression, stabilizes stalled replication forks and finally delays cell cycle progression [in reviews (Cimprich *et al.*, 2008; Parrilla-Castellar *et al.*, 2004)]. This DDR pathway is evolutionary conserved, from yeast to man, indicating it is indispensable. Indeed, a hypomorphic mutation in the ATR gene leads to Seckel syndrome, characterized by growth retardation and microcephaly [in review (Cimprich *et al.*, 2008)]. In conclusion, DDR activation provides more time for cells to deal with DNA damages, thereby preventing replication fork collapse and formation of DNA double strand breaks (DSBs) that are very cytotoxic.

FATE OF STALLED REPLICATION FORKS

Even though stalled replication forks are protected by DDR, prolonged stalled replication forks and ssDNA gaps are able to evade the protection afforded by the DDR process, resulting in the dissociation of replisome complexes from stalled replication forks (so called replication fork collapse). The collapse usually leads to the formation of DSBs and cell death. Cells employ several mechanisms to process stalled replication forks in order to re-start DNA replication and to prevent genomic instability. Bacteria use recombination to restart stalled replication forks, leading to the re-loading of replisome complexes, but in eukaryotic cells restart of arrested forks seems to be more complicated and less clear. After replisome dissociation, replication forks can be reversed, giving rise to four-branched DNA molecules, although exonuclease I (ExoI) that degrades single-stranded DNA in a 3' to 5' direction was shown to prevent replication fork reversal (Cotta-Ramusino *et al.*, 2005). A reversed fork can be recognized and cut by the nucleases Mus81-Eme1/Mms4 [in review (Osman *et al.*, 2007)], leading to the formation of DSBs that may provoke DSB repair (Branzei *et al.*, 2009). The yeast Mus81 mutant displays hypersensitivity to UV and MMS, but not to ionizing radiation, indicating that this protein is needed in response to stalled

replication forks, while Mus81 is dispensable for DSB repair (Interthal *et al.*, 2000). Intriguingly, even though cells are equipped with several mechanisms to bypass DNA damages, it seems as if those mechanisms might not be effective enough to bypass all DNA damages before damaged cells enter the mitotic phase. This may lead to either a mitotic arrest and cell killing or transfer of DNA strand discontinuities through mitosis into the subsequent cell cycle, as suggested by recent studies (Ichijima *et al.*, 2010; Liu *et al.*, 2012). In Chapter 5, the fate of ssDNA gaps that persist after low dose UV exposure is described. The data suggest that these persistent ssDNA gaps generated during the first S-phase may lead to generation of DSBs in the second S-phase. In *E. coli*, it was observed that DSBs can directly be generated from nicked DNA in a replication-dependent manner (Kuzminov, 1995; Kuzminov, 2001).

TRANSLESION SYNTHESIS AND DNA REPAIR PATHWAYS

Although the central role of TLS polymerases is to bypass damaged DNA templates that halt replication forks, non-canonical roles of TLS polymerases have also been discovered.

In vitro and *in vivo* data show that Polt has a weak ability to bypass UV-induced DNA damages, but may act as a backup TLS polymerase in XPV cells that lack Polη. Interestingly, knockdown of Polt in human cells resulted in hypersensitivity to oxidative stress, like H₂O₂ and menadione (Petta *et al.*, 2008). Most DNA lesions-induced by oxidative stress are removed by BER and NER. DNA polymerase β (Polβ) plays an important role in gap-filling in BER. Proper functioning of BER in Polβ-depleted cell extracts can be restored by Polt complementation (Bebenek *et al.*, 2001). Polt is capable of interacting with XRCC1, a BER protein, after treatment with oxidative stress. Knockdown of Polt, therefore, leads to reduced BER activity. Another direct evidence for a role of Polt in BER is that Polt has 5'-deoxyribose phosphate (dRP) lyase activity *in vitro* (Bebenek *et al.*, 2001), which is required for BER. Taken together, these findings indicate the role of Polt in BER, possibly acting as dRP lyase.

Polk is found in all domains of life, suggesting its vital roles for many organisms. However, except for proficient bypass of BPDE lesions, Polk exhibits only a weak capability to bypass several types of DNA lesions, similar to Polt. In addition, Polk has been shown to function in gap-filling during NER in non-dividing cells (Ogi *et al.*, 2006). Furthermore, irradiation of MEFs with laser light that generates both single-strand breaks (SSBs) and DSBs leads to recruitment of GFP-tagged Polk to the site of damaged DNA. Polk^{-/-} cells also exhibit a persistence of γ-H2AX, a DNA damage marker for replication stress and DSBs, after treatment with H₂O₂. Therefore, Polk may play a role in repair of DNA breaks induced by oxidative stress (Zhang *et al.*, 2013).

It was thought that Polη might be restricted to perform only TLS. Nonetheless, Polη may also function in DSB repair by promoting DNA strand invasion during HR

(McIlwraith *et al.*, 2005), although Pol η ^{-/-} cells are not sensitive to DSBs induced by IR. This suggests a minor role of Pol η in HR.

Loss of Pol ζ , however, results in a cellular phenotype similar to HR-deficient cells (Sharma *et al.*, 2012), shedding some light on a role of Pol ζ in DSB repair. In yeast, HA-tagged Rev1 and Rev3 are found at sites of DSBs. The recruitment of Rev1 to DSBs does not depend on PCNA ubiquitination, but rather is ATR dependent (Hirano *et al.*, 2006). Even if Rev1 and Pol ζ are recruited to a DSB, a role of these two TLS polymerases in DSB repair remains elusive. In yeast, Rev3 helps to repair DSBs, but also generates point mutations near a DSB (Holbeck *et al.*, 1997). A recent study using siRNAs against human Rev1 or Rev3 measured the HR activity in response to DSBs. The results reveal that Rev1 and Rev3 are essential for HR to repair DSBs (Sharma *et al.*, 2012). In conclusion, these studies suggested that Rev1 and Rev3 are recruited to DSBs with the intention of promoting HR. Defects either in Rev1 or Rev3 result in IR-induced chromosomal aberrations (Sharma *et al.*, 2012). However, at present, it remains unclear how Rev1 and Rev3 function in DSB repair.

TRANSLESION SYNTHESIS AND CHRONIC DEGENERATIVE DISEASES

As described above, cells utilize TLS polymerases to restore DNA replication in the presence of DNA lesions, resulting in mutagenesis. The consequences of genomic instability range from chronic inflammation, atherosclerosis and premature aging to cancer (so-called chronic degenerative diseases) (Andreassi *et al.*, 2000; Hasty *et al.*, 2003). However, until now, there is only one example of cancer associated with a TLS defect, xeroderma pigmentosum variant (XPV).

Patients suffering from XPV, caused by mutations in Pol η , are very sensitive to sunlight and display a high predisposition to skin cancer (Johnson *et al.*, 1999; Masutani *et al.*, 1999b). XPV patients develop skin cancer at the age of around 20-30 without neurological defects. Indeed, isolated cells from XPV patients display slight UV sensitivity and a hypermutable phenotype (Maher *et al.*, 1976). These phenotypes could be eliminated by complementation of the XPV cells with a plasmid expressing human Pol η , confirming the protective roles of Pol η in genome maintenance (Elvers *et al.*, 2011; Masutani *et al.*, 1999b; Yamada *et al.*, 2000). Several data reveal that Pol η bypasses TT CPDs, the most abundant DNA lesion induced by UV in an error-free manner by adding adenine nucleotides (Hendel *et al.*, 2008; Shachar *et al.*, 2009; Sary *et al.*, 2003; Yagi *et al.*, 2005; Yoon *et al.*, 2009). At deaminated CC-CPDs, Pol η incorporates adenine, resulting in C to T transitions. UV-induced (6-4)PPs are also bypassed by Pol η , albeit at a minor extent (Hendel *et al.*, 2008; Yoon *et al.*, 2010). It has been reported that Pol ι may bypass UV-induced lesions in the XPV cells in an error-prone manner (Wang *et al.*, 2007). After exposure to UVC, by comparing XPV cells and derivatives that express reduced levels of Pol ι by 50%, the normal XPV cells display a higher mutation frequency compared to the derivatives, whilst the mutation

patterns are identical. Therefore, Pol ι is suspected to be a player that might initiate UV-induced oncogenesis in the XPV patients. This study suggests that more error-prone TLS leads to enhanced cancer susceptibility. Remarkably, mice defective for both Pol η and Pol ι (Pol $\eta^{-/-}$ Pol $\iota^{-/-}$) seem to develop skin cancer faster than single knockout mice, either Pol $\eta^{-/-}$ or Pol $\iota^{-/-}$ (Dumstorf *et al.*, 2006). In this study, the UV-induced mutation frequencies in Pol $\eta^{-/-}$, Pol $\iota^{-/-}$ and Pol $\eta^{-/-}$ Pol $\iota^{-/-}$ primary MEFs and the onset UV-induced skin tumor were determined. The highest mutation frequencies were found in Pol $\eta^{-/-}$, while the lowest was in Pol $\iota^{-/-}$. In Pol $\eta^{-/-}$ Pol $\iota^{-/-}$ primary MEFs, the mutation frequencies are just five-fold lower than Pol $\eta^{-/-}$, consistent with the above finding that Pol ι acts as an error-prone DNA polymerase in the absence of Pol η . Nevertheless, Pol $\eta^{-/-}$ Pol $\iota^{-/-}$ mice develop UV-induced skin cancer significantly faster than Pol $\eta^{-/-}$ mice. This interesting finding will be discussed below as a recent publication shows that DNA mutations are not the sole factor contributing to skin tumorigenesis (Tsaalbi-Shtylik *et al.*, 2009). Similarly, another mouse study has discovered that Pol ι might restrain the onset of UV-induced skin cancer (Ohkumo *et al.*, 2006). They showed that Pol $\eta^{-/-}$ mice specifically develop epithelial (fibroblast) skin tumors, whereas Pol $\iota^{-/-}$ mice solely develop mesenchymal (keratinocyte) tumors in response to UV exposure. The authors claim that CPDs are most likely the responsible lesions in skin cancer for both cell types, but the specific roles of Pol η and Pol ι in epithelial cells and keratinocytes are still elusive. Nevertheless, this study indicates the involvement of Pol ι in prevention of UV-induced mesenchymal tumors.

Tumorigenesis consists of two main steps; tumor initiation by DNA mutations and tumor promotion by proliferation. Thus, loss of TLS polymerases may delay the onset of cancer as mutagenesis is reduced. This hypothesis was investigated by using mice with a disruption in NER (Xpc) or in the BRCT domain of Rev1 together with Xpc (Rev1BRCT/Xpc) exposed to relatively low UV doses. The BRCT region of Rev1 is required for error-prone TLS of UV-induced DNA damages (Jansen *et al.*, 2009a). Since Rev1BRCT-deficient cells are hypomutable for UV, it was tempting to speculate that Rev1BRCT/Xpc mice would develop less skin cancer than the Xpc mice. Unexpectedly, the double mutant mice (Rev1BRCT/Xpc) developed squamous cell carcinomas faster than the single knockout Xpc mice (Tsaalbi-Shtylik *et al.*, 2009). Also, high induction of DDR and inflammation were observed in Rev1BRCT/Xpc, compared to the Xpc control. This important finding indicates that a defect in error-prone TLS, despite possibly reducing mutation-initiated skin cancer, supports tumor promotion by induction of the DDR and inflammation, which eventually results in the acceleration of skin tumorigenesis. Collectively, this result suggests that TLS polymerases initiate cancer, but simultaneously inhibit cancer promotion by quenching DDR and inflammation.

Besides cancer, premature aging is associated with genomic instability found in species varying from nematodes to mice and man [in review (Hasty *et al.*, 2003)]. High levels of endogenous DNA damage can be found in aged tissues and this accumulation of DNA damage is correlated with aging (Kirkwood *et al.*, 2000). The DNA lesions induced by endogenous metabolic end-products, like reactive oxygen species (ROS),

are hypothesized to act as aging promoters. Binding of ROS to DNA creates more than 75-100 different types of DNA damages, such as nucleotide modifications, DNA strand cross-links and DSBs (Hasty *et al.*, 2003). DDR pathways would decelerate the aging process. Indeed, several diseases caused by germline defects in DDR genes display significant aging phenotypes (Table 2). TLS may also play a role in bypassing naturally occurring endogenous DNA damages. For instance, Rev1 and Pol η can replicate across a DNA lesion mimicking naturally occurring lipid peroxidation-induced DNA adducts *in vitro* (Wolfe *et al.*, 2006; Yang *et al.*, 2009). Moreover, Rev3 mutant mice are embryonic lethal, supporting the involvement of Rev3 in translesion synthesis of endogenously induced DNA lesions *in vivo* (Van Sloun *et al.*, 2002). However, the correlation between TLS and aging is not yet evidenced. Fortunately, Rev1 mutant mice are viable, which allows to analyze the role of Rev1 in preventing premature aging and aging-associated cancer in response to endogenous DNA damaging agents (Chapter 6).

Table 2 | Most commonly described human progeroid syndromes, adapted from (Hasty *et al.*, 2003)

Syndrome	Incidence	Life-span (years)	Aging phenotypes	DNA repair genes defect
Werner	<1/100,000	~50	alopecia, osteoporosis, diabetes, graying of hair, sclerosis, malignancies	RecQ-like exonuclease
Rothmund-Thomson	<1/100,000	Unclear	alopecia, cataracts, osteoporosis, graying of hair, poikiloderma	RecQ-like exonuclease
Cockayne	~1/100,000	~20	cataracts, retinal degeneration, cachexia, hearing loss	CSA or CSB
Trichothiodystrophy	<1/100,000	~10	osteoporosis, cataracts, cerebella ataxia	XPB, XPD and TTDA
Ataxia telangiectasia	<1/60,000	~20	sclerosis, poikiloderma, immunodeficiency, graying of hair, cerebella ataxia	ATM

AIM AND OUTLINE OF THE THESIS

The overall purpose of this thesis is to determine the *in vivo* roles of DNA damage tolerance (DDT) in response to DNA damages induced by food-derived genotoxins, ultraviolet light and endogenous lesions. Translesion synthesis (TLS) is a DDT pathway, which bypasses unrepaired DNA lesions and rescues genome duplication. Until now, five DNA polymerases have been implicated in TLS. Although some *in vitro* and *in vivo* studies have shown roles of prokaryote and eukaryote TLS polymerases in bypassing food-derived genotoxins and UV light, a study using a complete set of TLS polymerases is definitely lacking. In addition, the relative *in vivo* contribution of each TLS polymerase in TLS and DNA damage responses to specific genomic lesions is problematic to assess due to differences in cell lines, agent doses and experimental

methodologies. In this thesis, I employed mouse embryonic fibroblasts (MEFs) with defined mutations in TLS polymerases and in a DNA sliding clamp, PCNA, coupled with a comprehensive set of methodologies to unveil the relative *in vivo* contribution of TLS-associated proteins. Moreover, the fate of unreplicated DNA adducts during two cell cycles and the function of TLS in preventing organismal aging were studied.

Chapter 1 provides a general introduction for this thesis.

Chapter 2, the *in vivo* roles of TLS polymerases in response to food-derived genotoxins, BPDE and HNE, were determined. Therefore, this chapter is the first report describing the *in vivo* functions of a complete set of key mammalian TLS polymerases in response to food-derived genotoxins.

UVC induces CPDs and (6-4)PPs that can be considered as models for mildly and severely helix-distorting DNA adducts, respectively. **Chapter 3** describes the *in vivo* roles of TLS-related genes in response to the same UV dose under comparable conditions.

Chapter 4 deals with the observation from Chapter 3 that Pol η -deficient MEFs display a backup system to relieve replication fork stalling at photolesions and quench the DDR. In this chapter, a backup role of Pol ι and Pol κ in Pol η -deficient MEFs after exposure to UV light is reported.

TLS-deficient cells may accumulate ssDNA gaps. **Chapter 5** describes the fate of these ssDNA gaps and provides a mechanistic understanding how these gaps are converted into DSBs. TLS deficiencies lead to DDR and genomic instability. Since both DDR and genomic instability are correlated with premature aging, a mouse model was generated to study the role of TLS in premature aging. The results of this study are described in **Chapter 6**. A general discussion is presented in **Chapter 7**.

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CHAPTER

2

DIFFERENT SETS OF TRANSLESION SYNTHESIS DNA POLYMERASES PROTECT FROM GENOME INSTABILITY INDUCED BY DISTINCT FOOD-DERIVED GENOTOXINS

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ABSTRACT

DNA lesions, induced by genotoxic compounds, block the processive replication fork but can be bypassed by specialized translesion synthesis (TLS) DNA Polymerases (Pols). TLS safeguards the completion of replication, albeit at the expense of nucleotide substitution mutations. We studied the *in vivo* role of individual TLS Pols in cellular responses to benzopyrene diolepoxide (BPDE), a polycyclic aromatic hydrocarbon, and 4-hydroxynonenal (4-HNE), a product of lipid peroxidation. To this aim, we used mouse embryonic fibroblasts with targeted disruptions in the TLS-associated Pols η , ι , κ and Rev1, as well as in Rev3, the catalytic subunit of TLS Pol ζ . After exposure, cellular survival, replication fork progression, DNA damage responses (DDR), and the induction of micronuclei were investigated. The results demonstrate that Rev1, Rev3 and Pol η are essential for TLS and the prevention of DDR and DNA breaks reflecting genome instability in response to both agents. Conversely, Pol κ and the N-terminal BRCT domain of Rev1 are specifically involved in TLS of BPDE-induced DNA damage. We also describe a novel role of Pol ι in TLS of 4-HNE-induced DNA damage *in vivo*. We hypothesize that different sets of TLS polymerases act on structurally different genotoxic DNA lesions *in vivo*, thereby suppressing genomic instability associated with cancer. Our experimental approach may provide a significant contribution in delineating the molecular bases of the genotoxicity *in vivo* of different classes of DNA damaging agents.

INTRODUCTION

Exposure to environmental genotoxic agents is associated with the induction of replicational stress, genomic alterations such as nucleotide substitutions, amplifications, deletions, and rearrangements, and cancer. Nucleotide substitution mutations are generated by DNA translesion synthesis (TLS), an evolutionarily conserved DNA damage tolerance pathway that is responsible for most DNA damage-induced mutations (Waters *et al.*, 2009). TLS is catalyzed by specialized DNA polymerases (Pols) that insert a nucleotide opposite replication-blocking DNA lesions. Mammalian cells express multiple TLS Pols, most notably the Y family DNA Pols Rev1, η , ι and κ and the B-family Pol ζ that consists of a catalytic subunit Rev3 and an accessory unit Rev7 (Waters *et al.*, 2009). TLS Pols display a low selectivity of nucleotide incorporation and lack proofreading activity, and therefore, TLS is an inherently mutagenic process. By allowing the completion of replication of damaged DNA templates, TLS prevents persistent replication fork arrest and quenches S phase checkpoint activation and apoptosis, mediated by the Atr/Chk1 pathway (Jansen *et al.*, 2009a; Jansen *et al.*, 2009b).

Epidemiological studies have indicated that diet is an important modifying factor in cancer risk in man. Accordingly, consumption of polyunsaturated fat and alcohol is linked with an increased risk for cancer of breast, esophagus, and liver, whereas high intake of red meat is correlated with an increased risk of gastrointestinal cancers (Khan *et al.*, 2010). Grilled meat, barbecued food, and burnt toast contain significant levels of benzo[a]pyrene (B[a]P), a polycyclic hydrocarbon that is a well-established carcinogen in animal models and is considered to be a causative agent for various cancers in humans (Rybicki *et al.*, 2004). In eukaryotic cells, B[a]P is converted metabolically into benzo[a]pyrene dilepoxide (BPDE) that mainly interacts with the N2 position of guanosine, although reaction products with adenosine are also found (Cheng *et al.*, 1989). BPDE-induced DNA lesions induce predominantly nucleotide substitutions at G.C base pairs in mammalian cells. Such mutations are indeed found in tumors from mice exposed to B[a]P (Ruggeri *et al.*, 1993), and in the genome of lung cancer cells from an individual who was exposed to B[a]P via tobacco smoke (Plesance *et al.*, 2010). The latter tumor additionally contained a considerable number of genomic rearrangements. Moreover, exposure to BPDE contributes to the deletion of chromosome 9p21, which is frequently found in bladder cancer (Gu *et al.*, 2008). Together, these findings strongly suggest that B[a]P-induced carcinogenesis involves both nucleotide substitutions and double-stranded DNA breaks (DSBs) that underlie large-scale genome instability.

Another important diet-related mutagen is 4-hydroxynonenal (4-HNE) that is formed during processing and storage of food (Kanner, 2007). In addition, cellular levels of this α,β -unsaturated aldehyde increase after consumption of red meat, fish, shellfish, and vegetable oils (Surh *et al.*, 2005). Most of the cellular 4-HNE, however, is generated by peroxidation of polyunsaturated fatty acids following endogenous oxidative stress.

In DNA, 4-HNE mainly induces poorly repaired N¹,N²-propanodeoxyguanosines. In addition, low yields of exocyclic etheno adducts at guanine, adenine, and cytosine are produced following oxidation of 4-HNE to its epoxide (Poli *et al.*, 2008; Schaur, 2003). Similar to BPDE, 4-HNE may induce nucleotide substitutions and genome instability in mammalian cells (Eckl, 2003; Poli *et al.*, 2008). Suggestive evidence of a link between 4-HNE-induced nucleotide substitutions and cancer is provided by the finding that in the human p53 gene 4-HNE preferentially reacts with the bases of codon 249, which is a mutational hotspot in hepatocellular carcinoma (Hu *et al.*, 2002).

Although multiple *in vitro* studies with purified proteins have shown that BPDE- and 4-HNE-induced DNA adducts are bypassed in a mutagenic fashion by various TLS Pols with different efficiencies (Ogi *et al.*, 2002; Wolffe *et al.*, 2006; Yang *et al.*, 2009; Zhang *et al.*, 2002), little is known about the contribution of TLS Pols in protection from BPDE- and 4-HNE-induced genome instability *in vivo*. Here, we set out to study the role of individual TLS Pols in cell survival and replication fork progression and in the suppression of DNA damage responses (DDR) and DSB formation *in vivo*, following exposure to BPDE- and 4-HNE. To this end, we used mouse embryonic fibroblasts (MEFs) lines with defined deletions in the Y family DNA Pols η , ι , κ , and Rev1 as well as in Rev3. In addition, we included a MEF line containing a deletion of the N-terminal BRCT region of Rev1, which is important for recruitment of Rev1 to stalled replication forks during an early mutagenic TLS pathway (de Groote *et al.*, 2011; Jansen *et al.*, 2009a; Jansen *et al.*, 2005). Our data fit a model describing specialized roles for Pol κ and Pol ι and a general role for Rev1, Rev3, and Pol η in suppressing DDR and genome instability induced by B[a]P and 4-HNE.

RESULTS

Toxicity of BPDE and 4-HNE to TLS Pol-Mutant Cell Lines

To determine the role of different TLS Pols in the cellular responses to structurally divergent environmental and food- derived genotoxins, we assayed cell proliferation in MEF lines with deficiencies for individual TLS Pols following exposure to BPDE and 4-HNE (Figs. 1A and B). After exposure to BPDE, proliferation of MEFs deficient for Rev1 or Rev3 was strongly inhibited, compared with wild type (WT) MEFs (Fig. 1A), suggesting an important function of both Rev1 and Rev3 in protecting cells from BPDE-induced cytotoxicity. A comparable sensitivity was found for Rev1 BRCT MEFs that contain a homozygous deletion of the N-terminal BRCT region that was previously found to be largely dispensable for cytotoxicity of UV light (Jansen *et al.*, 2009a). MEFs deficient for Pol η or Pol ι displayed moderate sensitivity to BPDE. A similar result was found for MEFs deficient for Pol κ , supporting the proposed role of Pol κ in TLS of BPDE-induced DNA damage (Ogi *et al.*, 2002). Completely different contributions of most TLS Pols in cell survival were found for exposure to 4-HNE (Fig. 1B). Thus, 4-HNE inhibited cell proliferation very strongly in MEFs defective for Pol η , Pol ι , or

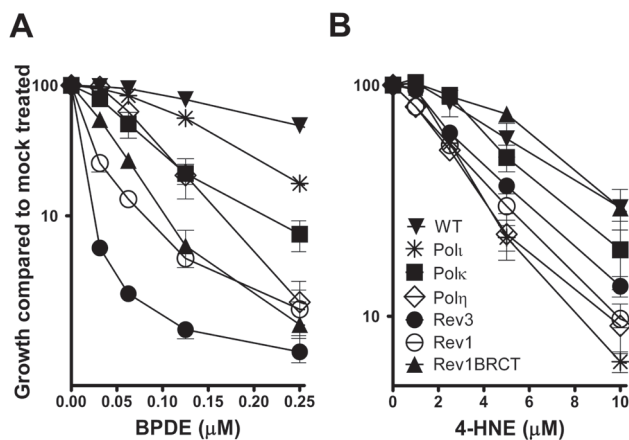


Figure 1 | Effect of BPDE and 4-HNE on the proliferation of MEFs with disruptions in TLS Pols. Cells were treated with different concentrations of BPDE (A) and 4-HNE (B) for 20 min and 2h, respectively. After 3 days, the number of cells was determined. The number of unexposed cells was set at 100%. Error bar, SD ($n=3$).

Rev1, whereas proliferation of cells deficient for Polk upon 4-HNE treatment was only mildly affected. Interestingly, Rev1 BRCT MEFs showed the same sensitivity as WT cells suggesting that, in contrast to BPDE-induced toxicity, the BRCT region of Rev1 is not involved in protection against 4-HNE-induced cytotoxicity (Fig. 1B). Similar to BPDE, 4-HNE was toxic to Rev3-deficient MEFs, suggesting a general function of Rev3 in the cellular response to 4-HNE and BPDE. When combined, these data demonstrate differential involvement of individual TLS proteins in protection against cytotoxicity induced by BPDE and 4-HNE.

Differential Roles of TLS Pols in Replication Fork Progression

To study the underlying cause for the differential BPDE- and 4-HNE-induced toxicity, we asked whether the MEF lines with different TLS deficiencies vary in replication fork progression after BPDE or HNE treatment. To test this, we used the ADU assay that measures the progression of replication forks. In this assay, replication forks are pulse labeled with [^3H]Thymidine immediately before or after genotoxin treatment. The persistence of radioactivity at isolated ssDNA ends, which are derived from replication forks, indicates their prolonged stalling.

Compared with mock-treated cell lines (Fig. 2A), replication fork progression at 2h after BPDE treatment was delayed to some extent in all cell lines (Fig. 2B). Fork progression was particularly impeded in MEF lines defective for Rev1 or Rev3. At 6h after BPDE exposure, persistent fork stalling was still observed in Rev1- and Rev3-deficient MEFs as well as in Rev1 BRCT MEFs (Fig. 2C). These data indicate that both Rev1, via its BRCT region, and Rev3 are involved in replicative bypass of BPDE-induced DNA

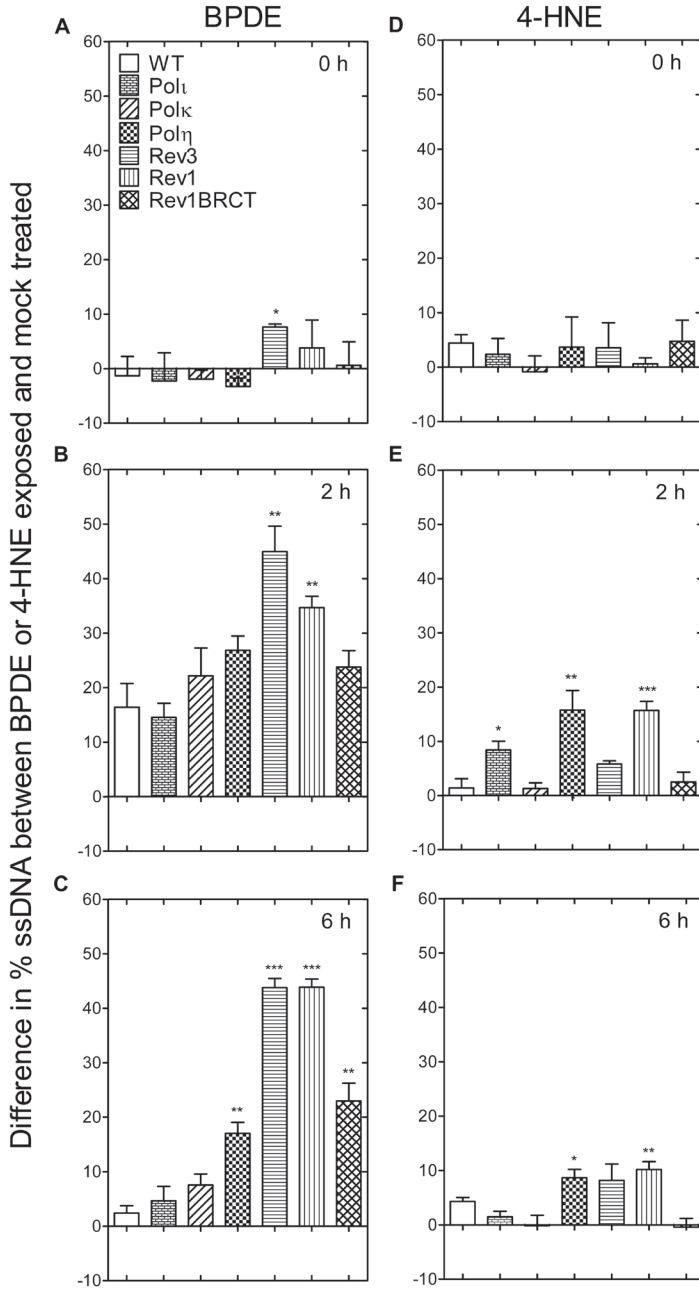


Figure 2 | Inhibition of replication fork progression by BPDE and 4-HNE treatment. Inhibition of replication fork progression in different MEF lines at 0h (A and D), 2h (B and E) and 6h (C and F), following 15 min of BPDE treatment or 1h of 4-HNE treatment. Error bar, SEM ($n=3$). Statistical significance was analyzed against WT at specific time point by Student's *t*-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

lesions. Increased fork stalling was also found for MEFs deficient for Pol η , albeit to a lesser degree than MEFs defective for Rev1 or Rev3 (Fig. 2C). In contrast, compared with WT MEFs, Polk- and Polt-deficient MEFs displayed no significant reduction in fork progression at 6h following BPDE exposure (Fig. 2C), indicating that Polk and Polt do not play major roles in the bypass of BPDE-induced DNA lesions.

We also performed ADU assays with the cell lines after treatment with 4-HNE. Compared with immediately after treatment (Fig. 2D), at 2h after treatment significantly reduced replication fork progression was observed in MEF lines defective for Pol η , Polt, and Rev1 (Fig. 2E). Only in Pol η - and Rev1-deficient MEFs, the replicational delay was maintained for an additional 4h (Fig. 2F). These data indicate that TLS of 4-HNE-induced DNA damage requires TLS Pols Pol η , Rev1, and, possibly, Polt. The other TLS Pols play a minor role in fork progression after 4-HNE treatment. In contrast to BPDE-treated Rev1 BRCT MEFs and consistent with the cytotoxicity data, 4-HNE-treated Rev1 BRCT MEFs displayed similar replication fork progression as mock-treated cells (Figs. 2D-F). This result provides further evidence that the BRCT region of Rev1 is dispensable for bypass of 4-HNE-induced DNA damages. Consequently, the BRCT region of Rev1 is only involved for lesion bypass at specific types of DNA damages, such as those induced by BPDE or, to a lesser extent, UVC light (Jansen *et al.*, 2005). Importantly, replication fork progression in cells that were treated with the solvents THF or ethanol was not affected in any of the cell lines (data not shown).

Activation of Chk1 in TLS-Deficient Cells Exposed to BPDE and 4-HNE

Arrested replication forks may trigger the activation of the DDR kinase Atr that, among others, phosphorylates the effector protein kinase Chk1 at Serines (S) 317 and 345 (Ward *et al.*, 2004; Zhao *et al.*, 2001). To test whether the BPDE- and 4-HNE-induced replication fork arrests correlate with Chk1 activation in the various TLS-deficient MEF lines, we quantified Chk1 phosphorylation at S345 (Chk1^{S345-P}) following exposure to 0.5 μ M BPDE or 15 μ M 4-HNE (Figs. 3A and C). Within 8h after exposure to BPDE, strong induction of Chk1^{S345-P} was found in MEF lines deficient for Rev1, Rev1 BRCT, or Rev3, which very well correlates with the sensitivity and the robust replication fork arrests observed in these cell lines (Figs. 2A-C). Activation of Chk1 was attenuated in Pol η - or Polk-deficient MEF lines, corresponding with the reduced sensitivity and replication fork blockage. BPDE-induced Chk1^{S345-P} in Polk-deficient cells has also been reported elsewhere (Bi *et al.*, 2005). WT MEFs and Polt-defective MEFs, cell lines that showed no strong sensitivity and no significant replication fork delay, presented only low levels of Chk1^{S345-P} following BPDE treatment (Figs. 3A and C). In contrast to BPDE, exposure to 4-HNE did not result in the induction of Chk1^{S345-P} in any of the MEF lines tested (data not shown). This finding may be explained by the relatively low frequencies of DNA lesions induced by this food compound under the conditions used (Figs. 2D-F).

Formation of γ -H2AX in TLS-Mutant Cells Exposed to BPDE and 4-HNE

Arrested replication forks may eventually collapse, resulting in the formation of DSBs that underlie translocations and genome instability. Phosphorylation of histone H2AX at S139 (γ -H2AX) may reflect its activation by ssDNA (de Feraudy *et al.*, 2010) but is generally used as a marker for the formation of DSBs (Rogakou *et al.*, 1998). To investigate DSB formation in response to BPDE or 4-HNE treatment in the MEF lines, defective for individual TLS Pols, we therefore investigated the formation of γ -H2AX in the different mutant MEF lines, upon treatment with either BPDE or 4-HNE. The highest induction of γ -H2AX was found in BPDE-exposed MEF lines defective in Rev1, Rev1 BRCT, or Rev3. Also, Pol η -defective MEFs exhibited a significant induction of γ -H2AX (Figs. 3B and C). In these cell lines, levels γ -H2AX increased rapidly within 8h after BPDE treatment and continued to increase more slowly at later time points. Thus, the induction of γ -H2AX was correlated with the level of sensitivity and inhibition of

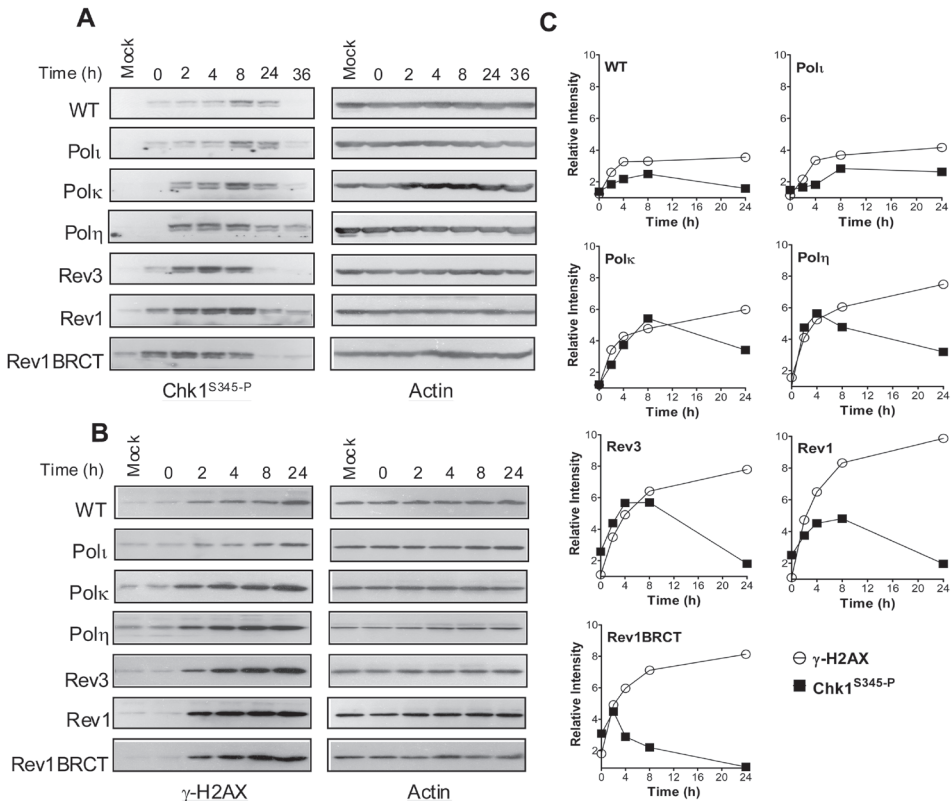


Figure 3 | Effect of BPDE on DNA damage responses of TLS-deficient MEFs. Western blot analysis of whole cell extracts prepared at different times after exposure of cells to 0.5 μ M BPDE for 15 min. 0h: cell extract prepared immediately after treatment. Blots representing expression of Chk1^{S345-P} (A, left panel), γ -H2AX (B, left panel) and β -actin (A and B, right panels). Quantification of Chk1^{S345-P} ($n=2$) and γ -H2AX ($n=2$) relative to the loading control β -actin (C).

replication fork progression in the different TLS Pol-mutant cell lines. Compared with the other MEF lines, WT- and Pol ι -defective cells induced much less γ -H2AX upon BPDE exposure (Figs. 3B and C), which parallels the relative insensitivity and absence of fork arrests in these cells. Interestingly, the magnitude of γ -H2AX formation also correlates with that of Chk1^{S345-P}, although the latter peaks earlier than the formation of γ -H2AX in most cell lines (Fig. 3C). Together, these data suggest that persistent replication fork arrests at BPDE-induced DNA lesions trigger the formation of other types of DNA damages, possibly DSBs, which induce the phosphorylation of H2AX.

Exposure of MEFs to 4-HNE resulted in a strong and time-dependent induction of γ -H2AX in cells defective in Rev1, Rev3, Pol η , or Pol ι (Figs. 4A and B). Interestingly, this induction occurred a few hours later than in MEFs exposed to BPDE. WT MEFs, Rev1 BRCT MEFs, as well as MEFs deficient for Polk hardly produced γ -H2AX in response to 4-HNE exposure (Figs. 4A and B). Although these results correlated well with the observed 4-HNE sensitivities, the correlation with the inhibition of replication forks was less clear (Figs. 2D-F), possibly because only a small number of arrested replication forks might be sufficient to induce the formation of γ -H2AX that can be visualized by Western blotting.

Induction of Genome Instability after BPDE or 4-HNE Treatment

To provide direct evidence for the formation of BPDE- or 4-HNE-induced DSBs, we monitored the generation of MN in the various TLS-defective MEF lines, using the cytokinesis-blocked micronucleus assay. MN are a well-established readout for both chromosome breaks and chromosome loss and therefore for genome destabilization (Fenech, 1993). In initial experiments, all MEF lines were exposed to the same dose of

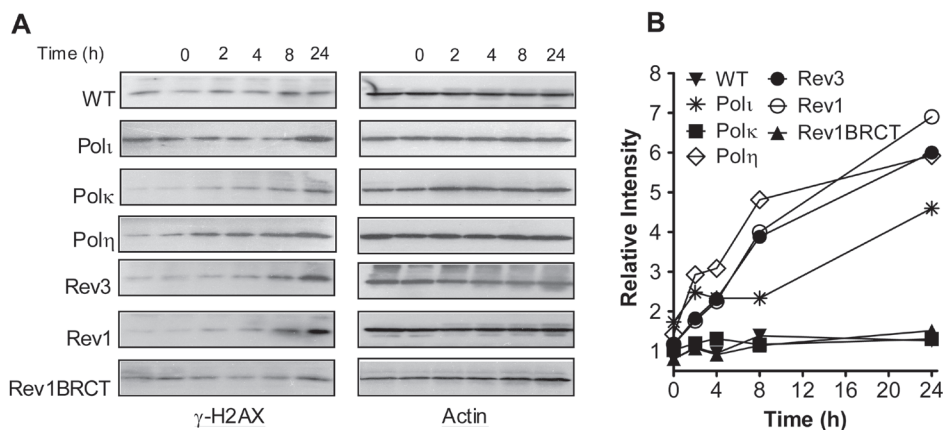


Figure 4 | Effect of 4-HNE on DNA damage responses of TLS-deficient MEFs. Western blot analysis of whole cell extracts prepared at different times after exposure of cells to 15 μ M 4-HNE for 2h. 0h, cell extract prepared immediately after treatment. Blots representing expression of γ -H2AX (left panel) and β -actin (right panel) (A). Quantification of γ -H2AX ($n=2$) relative to the loading control β -actin (B).

BPDE (0.05 μ M) or 4-HNE (10 and 20 μ M). These exposure levels, however, strongly inhibited cell cycle progression in some of the TLS-mutant MEF lines, resulting in low numbers of binucleated cells and, consequently, an underestimation of MN formation (data not shown). For this reason, MN formation was measured at equitoxic doses, resulting in 70% cell killing for each MEF line (Figs. 1A and B). In Figure 5, the MN frequencies for each MEF line were normalized for the dose used. Cells were cultured for 24h following BPDE exposure, after which cytochalasin B was added to block cytokinesis, resulting in binucleated cells that were scored for the presence of MN. Under this condition, a moderate BPDE-induced increase in MN formation was found for WT and for Polt⁻, η -, and κ -deficient MEFs cells as compared with solvent (THF)-exposed cells (Fig. 5A), indicating that BPDE-induced DNA lesions cause DSBs in these cells. However, the induction of MN was much more pronounced for Rev3⁻ and Rev1-defective MEF lines, exposed to BPDE. Similar experiments, using equitoxic

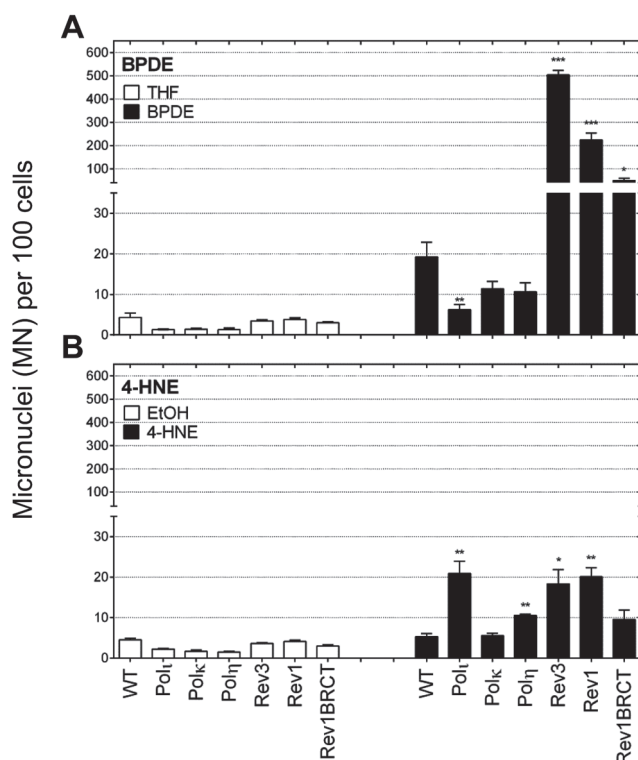


Figure 5 | Effect of BPDE and 4-HNE on induction of micronuclei (MN) in TLS-deficient MEFs. Cells were treated with equitoxic doses of BPDE or 4-HNE resulting in 70% cell killing and assayed for MN in binucleated cells fixed 24h after addition of cyt-B. The frequencies of MN were normalized for the doses used. Bars represent the number of MN per 100 cells after exposure to solvent THF and BPDE (A), and to solvent EtOH and 4-HNE (B). Error bar, SEM ($n=3$). Statistical significance was analyzed by Student's *t*-test against WT. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

doses of 4-HNE, showed moderate but significant increases of MN formation only in Polt-, η -, Rev1-, and Rev3-deficient MEFs (Fig. 5B). In conclusion, these data suggest that the formation of DSBs occurs after cellular exposure to food genotoxins that can block elongating replication forks. Furthermore, Rev1 and Rev3 are major players in protecting mammalian cells for DSB formation following BPDE or 4-HNE exposure.

DISCUSSION

Exposure to DNA-damaging compounds induces cytotoxicity, nucleotide substitution mutations, and genomic instability and, ultimately, the development of cancer. In the present study, we found specialized and general roles of TLS Pols in replicating genomic DNA, averting DDR, and curtailing genome instability, following exposure of mammalian cells to the food and environmental genotoxins BPDE and 4-HNE. We used direct acting BPDE instead of B[a]P to exclude the effect of possible differences in expression of appropriate cytochrome P450 isomers between the various MEF lines.

The importance of Rev1 and Rev3 in replicative bypass of BPDE- and 4-HNE-induced genomic DNA damage is supported by (i) the colocalization of Rev1 with the replication processivity clamp proliferating cell nuclear antigen in human cells upon BPDE exposure (Mukhopadhyay *et al.*, 2004), (ii) the involvement of Rev1 in BPDE-induced mutagenesis (Mukhopadhyay *et al.*, 2004), (iii) the essential role of Pol ζ in the replicative bypass of a site-specific BPDE-N2 guanine adduct in a gapped plasmid in mammalian cells (Shachar *et al.*, 2009), and (iv) the participation of both Rev1 and Rev3 in mutagenic TLS across a 4-HNE-induced heptanone-etheno cytosine adduct *in vivo* (Yang *et al.*, 2009). The importance of Rev3 in bypassing BPDE- and 4-HNE-induced DNA lesions most likely resides in its function of extending mismatched nucleotides, induced by one of the Y family Pols opposite a wide variety of DNA lesions (Waters *et al.*, 2009). Probably, Rev1 plays a noncatalytic role in the bypass of BPDE- and 4-HNE-induced lesions because the spectra of nucleotide substitution mutations induced by these lesions do not reflect the incorporation of deoxycytosines, characteristic of the catalytic activity of Rev1. More likely, Rev1 may act as a scaffold to control the activity of other TLS Pols that interact with the C-terminus of Rev1 (Guo *et al.*, 2003). The phenotype of Rev1 BRCT MEFs that display a defect in TLS of BPDE, but not of 4-HNE reveals a separation of function. Possibly, the majority of 4-HNE-induced DNA lesions require the Rev1 catalytic domain, which is still active in Rev1 proteins with a defective BRCT region (Nelson *et al.*, 2000). We found that Pol η is involved in TLS of both 4-HNE- and BPDE-induced DNA damage. Possibly, Pol η mediates TLS opposite 4-HNE-induced DNA adducts at adenine (Schaar, 2003) because 4-HNE-induced DNA adducts at cytosine and guanine are no substrates for nucleotide incorporation by Pol η *in vitro* and *in vivo* (Wolfe *et al.*, 2006; Yang *et al.*, 2003). Exposure of mammalian cells to BPDE results in the relocation of Pol η to nuclear foci and BPDE-induced adducts at guanines form substrates for Pol η -mediated

error-prone bypass *in vivo* (Langie *et al.*, 2007). Our findings suggest that Pol ζ is more important for TLS at BPDE lesions than Pol η . The mild phenotypes of Pol η -deficient MEFs in response to BPDE are probably caused by redundancy between Pol η and Polk in the insertion of nucleotides opposite BPDE-induced DNA lesions during TLS (Shachar *et al.*, 2009).

In our studies, Polk-deficient MEFs displayed only moderate phenotypes in response to BPDE. However, Polk is believed to play an important role in TLS of BPDE adducts *in vitro* and *in vivo* (Shachar *et al.*, 2009). Possibly, redundancy between Pol η and Polk prevents the persistent accumulation of stalled replication forks in the Polk-deficient cells, whereas Chk1^{S345-P}, γ -H2AX, and MN may result from a transient defect in TLS at BPDE adducts. Alternatively, phosphorylation of Chk1 and formation of γ -H2AX may reflect persistence of ssDNA gaps that are not filled by Polk during nucleotide excision repair of helix distorting DNA lesions in G1 phase cells (Ogi *et al.*, 2010). Although Polk-defective MEFs displayed a mild sensitivity to 4-HNE (Fig. 1B), 4-HNE-induced DNA lesions did not provoke detectable DDR, indicating that Polk is not important in TLS of 4-HNE-induced DNA adducts in mammalian cells.

The function of Polt in TLS and DDR is not well understood. It was reported that Polt may play a role in the repair of oxidative DNA damage (Petta *et al.*, 2008) and in error-prone TLS of UV damages in cells lacking Pol η (Wang *et al.*, 2007). We now find that Polt-deficient MEFs are extremely sensitive to 4-HNE, strongly suggesting that Polt is important for efficient bypass of 4-HNE-induced DNA damage. In support, 4-HNE-dG adducts are excellent substrates for Polt-mediated TLS *in vitro* (Wolffe *et al.*, 2006). The induction of MN in Polt-deficient cells suggests that the inability to bypass 4-HNE lesions *in vivo* may lead to stalled replication forks that ultimately result in the formation of cytotoxic DSBs (Fig. 5B).

The differences in DDR between the various MEF lines were more pronounced following exposure to BPDE than to 4-HNE. Most likely, the intracellular concentration of 4-HNE is relatively low due to its strong lipophilic properties that will result in 4-HNE being concentrated preferentially in cell membranes (Poli *et al.*, 2008). Furthermore, 4-HNE reacts preferentially with proteins, whereas BPDE reacts mostly with nucleic acids (LoPachin *et al.*, 2009; Singh *et al.*, 2002). Together, this may result in relatively low levels of DNA adducts in MEFs exposed to exogenous 4-HNE as compared with those induced by BPDE. Thus, in HNE-exposed cells, it may take longer when elongating replication forks encounter DNA adducts and fewer replication forks get stalled (Fig. 2), resulting in slower kinetics of γ -H2AX formation as compared with BPDE-exposed cells (Figs. 3B and C and 4A and B).

All data together suggest that structurally different DNA adducts induced by food-derived genotoxins will be bypassed by different TLS Pols, but a core complex consisting of Rev1, Rev3, and, possibly, Pol η is essential for TLS at most stalled replication forks. In the absence of one of these specific or general TLS Pols, stalled replication forks may

collapse, resulting in H2AX phosphorylation and the formation of DSBs that underlie the induction of genome instability (Table 1). Our study tempts us to propose that different TLS Pols, despite being intrinsically mutagenic, restrain carcinogenesis by protecting cells from genomic instability induced by different food-derived and other environmental genotoxins. Finally, we conclude that the set of MEF lines, with targeted deficiencies in individual TLS polymerases, combined with assays that address survival, TLS, DDR, and DSB induction are useful tools in the study of the molecular basis of the genotoxicity of environmental and food-derived agents.

Table 1 | Summary of cellular responses of MEF lines to BPDE or 4-HNE

Cell line	BPDE			4-HNE		
	Sensitivity	Replication fork inhibition	Induction of DNA breaks	Sensitivity	Replication fork inhibition	Induction of DNA breaks
WT	+	+	+	+	+	+
Polt	+	+	+	+++	++	++
Polk	++	++	++	+	+	+
Polη	+++	+++	+++	+++	+++	+++
Rev3	+++	+++	+++	++	+	+++
Rev1	+++	+++	+++	+++	+++	+++
Rev1BRCT	+++	+++	+++	+	+	+

MATERIALS AND METHODS

Cell culture and Chemicals. Immortalized MEFs homozygous for a targeted deletion of the N-terminal BRCT region and MEF lines deficient for Rev1 or Rev3 were described previously (Jansen *et al.*, 2006; Jansen *et al.*, 2005). Day 13.5 embryonic fibroblasts from Polη- or Polt-deficient mice were kept in culture until spontaneous transformation. Polη-deficient mice have been described previously (Delbos *et al.*, 2005). The generation of Polt-deficient mice will be published elsewhere (Aoufouchi, S., Delbos, F., De Smet, A., Reynaud, C.-A., and Weill, J.-C., in preparation). Polk-deficient cells were generated as previously reported (Ogi *et al.*, 2002). All MEF lines were cultured in MEF medium as described (Jansen *et al.*, 2009a). BPDE (Biochemical Institute for Environmental Carcinogens, Grosshansdorf, Germany) was dissolved in anhydrous tetrahydrofuran (THF, Sigma-Aldrich) at a concentration of 150mM and stored at -20°C prior to use. 4-HNE (Merck) was dissolved in ethanol (EtOH) (10 mg/ml) and kept at -80°C until use.

Cell proliferation assay. Into each well of a 6-well plate, 5×10^4 exponentially growing cells were seeded in MEF medium and cultured overnight. The cells were exposed in serum-free medium (SFM) to 0–0.25μM BPDE for 20 min at 37°C or to 0–10μM 4-HNE for 2h at 37°C. After incubation, cells were gently washed twice with PBS and cultured in MEF medium for 3 days. Cells were then trypsinized and counted using a Z2 Coulter particle and size analyzer (Beckman Coulter).

Alkaline DNA unwinding assay. Replication fork progression was measured using a slightly modified alkaline DNA unwinding (ADU) assay (Johansson *et al.*, 2004). Briefly, 5×10^4 cells

per well were seeded in a 24-well plate and cultured overnight in MEF medium. Prior to BPDE treatment, cells were pulse labeled with [^3H]thymidine (2 $\mu\text{Ci/ml}$; 76 Ci/mmol) in MEF medium for 15 min and washed once with PBS. BPDE was added to 1 ml SFM at a final concentration of 1.5 μM and the cells were incubated at 37°C. After 15 min, SFM was aspirated and replaced with complete medium. For 4-HNE, cells were treated with 15 μM 4-HNE in SFM for 1h, after which the cells were washed and immediately pulse labeled for 15 min with [^3H]thymidine (2 $\mu\text{Ci/ml}$; 76 Ci/mmol) in MEF medium. THF and ethanol were included as solvent controls. At different times, up to 6h, cells were washed twice with 0.15M NaCl and incubated on ice in the dark with an ice-cold denaturation solution (0.15M NaCl and 0.03M NaOH) for local denaturation of DNA at replication forks. After 30 min, the reaction was stopped by adding 1 ml of 0.02M Na_2HPO_4 . Following sonication for 30s using a Sonifier 250 apparatus (Branson), the lysates were stored overnight at -20°C after addition of SDS (final concentration of 0.25%). Single-stranded DNA (ssDNA), representing replication forks, and double-stranded DNA (dsDNA), representing replicated DNA, were separated using a hydroxyl apatite column. The ssDNA was eluted with 0.13M K_2HPO_4 pH 6.8, and the dsDNA was eluted with 0.25M K_2HPO_4 pH 6.8. The radioactivity in both eluates was determined by liquid scintillation counting (Perkin-Elmer).

Western blotting. Per 9 cm dish 10^6 cells were seeded and cultured overnight in MEF medium. Subsequently, cells were washed twice with PBS and exposed in SFM to 0.5 μM BDPE for 15 min or 15 μM 4-HNE for 1h. After washing with PBS, cells were cultured in MEF medium. At different times cells were lysed using Laemmli lysis buffer. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C extra, Amersham Biosciences). The membranes were incubated overnight at 4°C with rabbit monoclonal anti-phospho Chk1 (S345) antibodies (Cell signaling), a mouse monoclonal anti-phospho-H2AX (S139) antibody (Millipore) and a mouse monoclonal anti-actin antibody (Oncogene). After incubation with secondary antibodies conjugated to peroxidase (Bio-Rad), proteins were visualized by enhanced chemiluminescence detection. At least two completely independent experiments were performed for each genotoxin.

Cytokinesis-blocked micronucleus assay. To investigate the induction of DSBs and associated genome instability we employed the cytokinesis-blocked micronucleus assay. Per 9 cm dish 1.5×10^6 cells were plated and cultured overnight. Then, 75,000 cells were seeded on an autoclaved glass slide (76x26 mm) and cultured for 24h. Cells were washed twice with 10 ml PBS and exposed to equitoxic doses of BPDE or 4-HNE in SFM at 37°C for 20 min and 2h, respectively. THF and ethanol were included as solvent controls. After exposure, cells were gently washed once with PBS and cultured in MEF medium. Twenty-four h after exposure, 3 $\mu\text{g/ml}$ cytochalasin B (cyt-B) (Sigma-Aldrich) was added to inhibit cytokinesis. After 24h, cells were fixed in 3.7% cold paraformaldehyde: 10% Triton-X100 (20:1) at 4°C for 15 min. Then, slides were washed thrice with ice-cold PBS and subsequently nuclei were stained in the dark with DAPI (17.5 ng/ml) (Sigma-Aldrich) for 15 min. To dehydrate the cells, the slides were soaked in the dark in 70%, 90% and absolute ethanol for 5 min each. Before scoring, slides were mounted using Citifluor AF1 (Citifluor, UK). The percentage of binucleated cells that contained one or more micronuclei was determined using a fluorescence microscope and the Metafer 4 program (Metasystem, Germany).

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CHAPTER

3

TEMPORALLY DISTINCT TRANSLESION SYNTHESIS PATHWAYS FOR ULTRAVIOLET LIGHT-INDUCED PHOTOPRODUCTS IN THE MAMMALIAN GENOME

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ABSTRACT

Replicative polymerases (Pols) arrest at damaged DNA nucleotides, which induces ubiquitination of the DNA sliding clamp PCNA (PCNA-Ub) and DNA damage signaling. PCNA-Ub is associated with the recruitment or activation of translesion synthesis (TLS) DNA polymerases of the Y family that can bypass the lesions, thereby rescuing replication and preventing replication fork collapse and consequent formation of double-strand DNA breaks. Here, we have used gene-targeted mouse embryonic fibroblasts to perform a comprehensive study of the *in vivo* roles of PCNA-Ub and of the Y family TLS Pols η , ι , κ , Rev1 and the B family TLS Pol ζ in TLS and in the suppression of DNA damage signaling and genome instability after exposure to UV light. Our data indicate that TLS Pols ι and κ and the N-terminal BRCT domain of Rev1, that previously was implicated in the regulation of TLS, play minor roles in TLS of DNA photoproducts. PCNA-Ub is critical for an early TLS pathway that replicates both strongly helix-distorting (6-4) pyrimidine-pyrimidone ((6-4)PP) and mildly distorting cyclobutane pyrimidine dimer (CPD) photoproducts. The role of Pol η is mainly restricted to early TLS of CPD photoproducts, whereas Rev1 and, in particular, Pol ζ are essential for the bypass of (6-4)PP photoproducts, both early and late after exposure. Thus, structurally distinct photoproducts at the mammalian genome are bypassed by different TLS Pols in temporally different, PCNA-Ub-dependent and independent fashions.

INTRODUCTION

Translesion synthesis (TLS) DNA polymerases are specialized enzymes that catalyze DNA synthesis across DNA lesions that form strong impediments for processive DNA polymerases δ and ϵ (Guo *et al.*, 2009; Waters *et al.*, 2009). TLS DNA polymerases are found in archaea, bacteria and eukaryotes, indicating that TLS is a conserved DNA damage tolerance pathway that is beneficial for cells and organisms. Indeed, in mammalian cells with DNA damage, TLS allows completion of genome duplication prior to cell division, quenches S phase checkpoint activation mediated by the Atr/Chk1 signaling pathway, prevents gross genome instability and contributes to cell survival (Guo *et al.*, 2009; Waters *et al.*, 2009). The most important group of mammalian TLS polymerases comprises the Y family DNA polymerases Pol η , ι , κ and Rev1 and the B family DNA polymerase Pol ζ , which consists of a catalytic subunit Rev3 and an accessory subunit Rev7 (Guo *et al.*, 2009; Waters *et al.*, 2009). The ability of Y family DNA polymerases to insert a nucleotide opposite the DNA lesion results from various atypical properties, including unusual polymerization mechanisms, the lack of an induced fit upon nucleotide binding and the absence of 3'-to-5' exonuclease activity, required for proofreading (Guo *et al.*, 2009; Waters *et al.*, 2009). Accordingly, a Y family DNA polymerase frequently inserts an incorrect nucleotide opposite the damaged nucleotide. Nucleotides inserted by the Y family Pols are excellent substrates for extension by Pol ζ . Like the Y family DNA polymerases, Pol ζ lacks proofreading activity, implicating that TLS is an inherently error-prone process that contributes significantly to spontaneous and genotoxin-induced mutagenesis (Guo *et al.*, 2009; Waters *et al.*, 2009).

Although the precise mechanism of DNA damage bypass by TLS DNA polymerases is not fully understood, mono-ubiquitination at lysine (K) 164 of the DNA sliding clamp Proliferating Cell Nuclear Antigen (PCNA-Ub) is thought to be an important event to elicit TLS (Arakawa *et al.*, 2006; Hoege *et al.*, 2002; Kannouche *et al.*, 2004; Langerak *et al.*, 2007; Stelter *et al.*, 2003; Watanabe *et al.*, 2004). Indeed, cells expressing mutant PCNA that cannot be ubiquitinated at K164 (PCNA^{K164R}) show impaired recruitment of TLS DNA polymerases *in vivo*, display aberrant DNA synthesis and delayed cell cycle progression, and increased sensitivity, following exposure to different genotoxic agents (Hendel *et al.*, 2011; Hoege *et al.*, 2002; Krijger *et al.*, 2011; Niimi *et al.*, 2008; Sabbioneda *et al.*, 2008). PCNA-Ub improves the physical interaction of this sliding clamp with Y family TLS DNA polymerases via specific domains located at their C-termini (Bienko *et al.*, 2005; Waters *et al.*, 2009).

Many *in vitro* studies on the function of individual TLS DNA polymerases of higher eukaryotes show that these enzymes exhibit overlapping substrate specificities (Waters *et al.*, 2009). Moreover, the *in vivo* function of TLS polymerases has been extensively addressed using chicken and mammalian cell lines with defects in individual TLS polymerases (Auclair *et al.*, 2010; Edmunds *et al.*, 2008; Gueranger *et al.*, 2008; Okada *et al.*, 2005; Petta *et al.*, 2008; Shachar *et al.*, 2009; Simpson *et al.*, 2003; Szuts *et al.*,

2008; Yoon *et al.*, 2010; Ziv *et al.*, 2009). However, the relative *in vivo* contribution of each vertebrate TLS DNA polymerase in TLS and DNA damage responses to specific genomic lesions is difficult to assess, despite the considerable progress that has been made in recent years. This difficulty is mainly due to the use of (i) cell lines of different origins which complicates comparisons of data (Gueranger *et al.*, 2008; Hendel *et al.*, 2011; Jansen *et al.*, 2009a; Jansen *et al.*, 2005; Krijger *et al.*, 2011; Okada *et al.*, 2005; Petta *et al.*, 2008; Yoon *et al.*, 2010; Ziv *et al.*, 2009), (ii) RNA interference techniques that may not completely silence the expression of the TLS polymerase of interest (Yoon *et al.*, 2010; Ziv *et al.*, 2009), (iii) cells that display an atypical phenotype in somatic hypermutation, a TLS-dependent process operating at the variable regions of immunoglobulin genes (Faili *et al.*, 2002; Gueranger *et al.*, 2008; McDonald *et al.*, 2003; Simpson *et al.*, 2003), and (iv) episomal DNA templates with site-specific lesions, which may not be fully representative of DNA damage in chromatinized genomic DNA (Shachar *et al.*, 2009; Szuts *et al.*, 2008; Yoon *et al.*, 2010; Ziv *et al.*, 2009).

UV light is a well characterized skin mutagen and carcinogen, inducing strongly helix-distorting (6-4) pyrimidine-pyrimidone ((6-4)PP) photoproducts as well as mildly distorting cyclobutane pyrimidine dimers (CPDs) (Ikehata *et al.*, 2011). These lesions obstruct processive DNA polymerases and, consequently, are substrates for TLS polymerase-mediated bypass. Here, we report on an integrative approach to study the involvement of key TLS polymerases, and of PCNA-Ub for TLS across CPDs and (6-4) PPs in genomic DNA and for DNA damage signaling, cell cycle progression, genome stability and cell proliferation. We used a defined cell type, *i.e.* mouse embryonic fibroblasts (MEFs), with defined deletions in the Y family DNA Pols η , ι , κ and Rev1 as well as in Rev3, the catalytic subunit of the B family DNA Pol ζ . In addition, we used MEF lines with a mutant PCNA that cannot be ubiquitinated (PCNA^{K164R}) or with a deletion of the N-terminal BRCT region of Rev1. This domain is involved in regulating a mutagenic TLS pathway that operates early after UV treatment (Jansen *et al.*, 2009a; Jansen *et al.*, 2005). To adequately compare the different cellular responses, we performed most experiments with the same UV dose. We report both common and quantitatively and temporally distinct roles for PCNA-Ub, Pol η , Rev1 and Rev3 in different TLS pathways across both photoproducts *in vivo*, thereby extinguishing DNA damage signaling, preventing gross genome instability and protecting against cytotoxicity.

RESULTS

TLS Polymerases Differentially Contribute to UVC Toxicity

To compare the involvement of individual TLS polymerases and of PCNA modification in cellular responses to UVC light, we exposed MEFs that contained a defect either in each TLS polymerase or in PCNA modification (PCNA^{K164R}) to different doses of UVC light and measured cell proliferation. Compared to wild type MEFs, the MEF line defective for Rev3 was extremely sensitive to UVC, indicating an indispensable

function of Rev3 in protecting against UVC-induced cytotoxicity (Fig. 1). PCNA^{K164R} MEFs as well as MEFs deficient for Pol η or Rev1 displayed moderate sensitivity to UVC. PCNA-Ub is important for recruitment of TLS polymerases to sites of DNA damage (Hendel *et al.*, 2011; Krijger *et al.*, 2011). Therefore, the UVC sensitivity of PCNA^{K164R} MEFs may be due to the difficulty in activating TLS polymerases that function in PCNA-Ub-dependent TLS across photoproducts. MEF lines deficient for Polk, Pol ι and the N-terminal BRCT region of Rev1 (Rev1BRCT MEFs) were only slightly more sensitive to UVC compared to wild type MEFs, indicating that Polk, Pol ι and the BRCT domain of Rev1 are mostly dispensable for tolerance to UV light. All together, these data reveal marked differences in the contribution of individual TLS proteins and of PCNA modification in protection toward cytotoxicity induced by UVC light.

Activation of DNA Double Strand Break (DSB) Response Proteins in TLS-deficient and PCNA-Mutant Cells Exposed to UVC

To obtain mechanistic insight in the different sensitivities of the TLS-defective MEF lines to UVC, we performed immunoblotting to investigate phosphorylation of histone H2AX at S139 (γ -H2AX) and of KRAB-ZFP-associated protein 1 at S824 (KAP1^{S824-P}), that may reflect the induction of both ssDNA and DSBs (de Feraudy *et al.*, 2010; Rogakou *et al.*, 1998; White *et al.*, 2006; Ziv *et al.*, 2006). As shown in Figs. 2A and B, following exposure to 5 J/m² UVC, all tested cell lines displayed similar kinetics of γ -H2AX accumulation, *i.e.* γ -H2AX was detectable already 2h after treatment and levels gradually increased to a maximum at 24-36h. Yet, the absolute levels of γ -H2AX

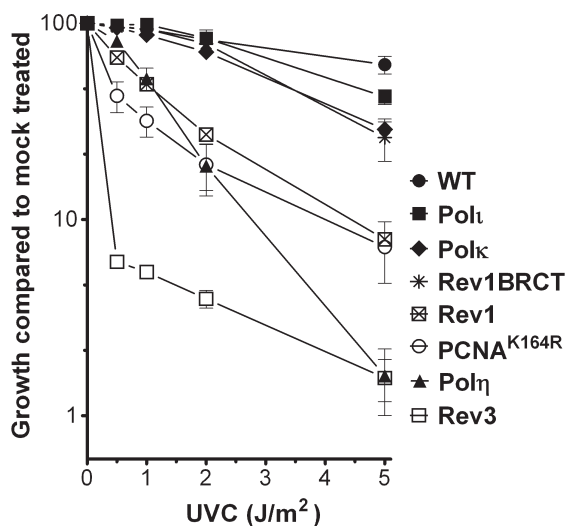


Figure 1 | Effect of UVC on the proliferation of MEFs with defined defects in TLS-related genes. Cells were treated with various doses of UVC. After 3 days, the number of cells was determined. The number of unexposed cells was set at 100% to calculate relative cell growth. Error bar, SD ($n=3$).

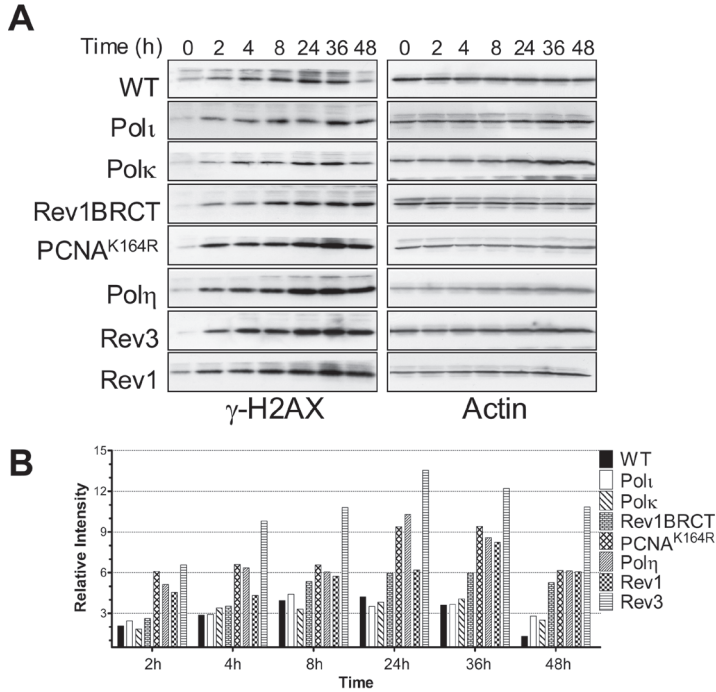


Figure 2 | Effect UVC on formation of γ -H2AX in MEFs with defined defects in TLS-related genes. Whole cell extracts prepared at different times after exposure to UVC (5 J/m²) were used for western blot analysis. 0h: cell extract prepared instantly after exposure. (A) Blots representing expression of γ -H2AX (left panel) and β -actin (right panel). (B) Quantification of γ -H2AX upon UVC exposure relative to the loading control β -actin.

differed considerably among the cell lines. MEFs deficient for Pol ι or Pol κ showed almost no difference in the formation of γ -H2AX as compared to wild type MEFs, in concordance with their moderate sensitivities to UVC. The Rev1BRCT MEFs displayed an intermediate increase in γ -H2AX levels. The highest induction of γ -H2AX was found for MEFs deficient in Rev3. Also PCNA^{K164R} MEFs and MEFs deficient in Rev1 or in Pol η displayed a pronounced induction of γ -H2AX, peaking at 24-36h after UVC treatment. Accumulation of KAP1^{S824-P} coincided with that of γ -H2AX in most cell lines (Figs. 2A, B and S1). Together, these results suggest that the sensitivities of TLS-defective MEF lines correlate with the induction of DNA breaks.

UVC Induces Genome Instability in Rev1, Rev3, Pol η and PCNA-Ub Mutants

The induction of DSBs can be directly measured by analyzing the formation of micronuclei using the cytokinesis-blocked micronuclei assay (Fenech, 1993). In this assay, the use of the cytokinesis inhibitor cytochalasin B results in the accumulation of binucleated, post-mitotic, cells. Here, we have determined the formation of micronuclei in such post-mitotic, TLS-defective or PCNA-mutant, MEFs after UVC

exposure. Since insufficient numbers of binucleated cells were obtained to reliably determine micronuclei formation in some of the MEF lines after exposure to 5 J/m² UVC, we set out to determine micronuclei using equitoxic doses of UVC that lead to 30% cell survival for all MEF lines. After normalization for the different UVC doses, to relate the frequencies of micronuclei to photolesion densities, the highest frequencies of micronuclei were found in PCNA^{K164R}, Rev1- and Rev3-deficient MEFs (Fig. 3). These data indicate that PCNA-Ub, Rev1 and, in particular, Rev3 are very important in preventing genome instability following UVC exposure

Differential Involvement of TLS Polymerases and PCNA Modification in TLS Across UVC-Induced DNA Damages

The formation of DSBs might reflect collapsed replication forks after their stalling at photoproducts. Such stalling results in persistent ssDNA ends, either opposite the lesion, or downstream of the lesion, where processive replication may have reprimed. To measure persistent ssDNA ends, we employed the alkaline DNA unwinding (ADU) assay. Compared to mock treatment, in wild type MEFs, Rev1BRCT MEFs and MEFs deficient for Pol ι or Pol κ , replication fork progression recovered to rates similar to mock-treated cells beyond 4h after treatment (Fig. 4). This result indicates that Pols ι and κ and the N-terminal BRCT region of Rev1 do not play a major role in bypass of genomic photoproducts. Conversely, compared with wild type MEFs, Pol η -deficient MEFs displayed pronounced persistence of ssDNA during the first 2h after UVC exposure, whereas at later times maturation of nascent DNA slowly recovered (Fig. 4). Also PCNA^{K164R} MEFs displayed a slow recovery of DNA maturation following an initial delay that was less pronounced than the delay in

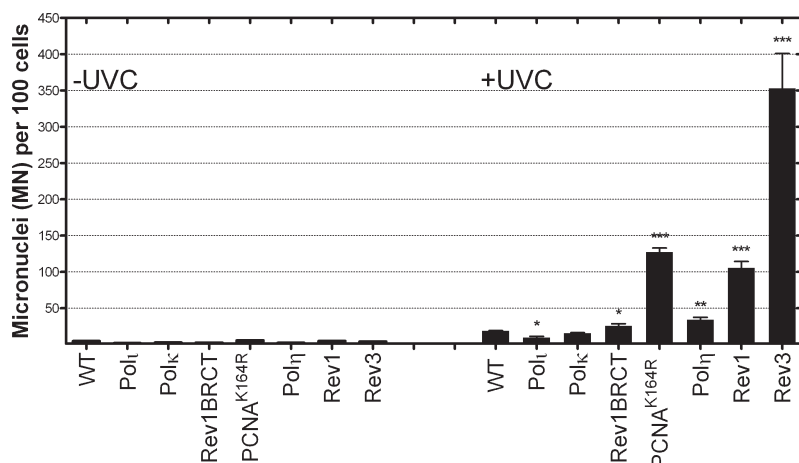


Figure 3 | Frequencies of UVC-induced micronuclei (MN) in MEFs with defined defects in TLS-related genes. MN induction in binucleated cells, fixed 24 h after cyt-B treatment, was determined in MEFs exposed to a dose of UVC resulting in 30% cell survival. The frequencies of MN were corrected for the doses used. Bars represent the number of MN per 100 cells after exposure to UVC or mock treatment ($n=3$). Error bar, SEM. Statistical significance was analyzed by Student's *t*-test against treated WT. **, $p < 0.01$; ***, $p < 0.001$.

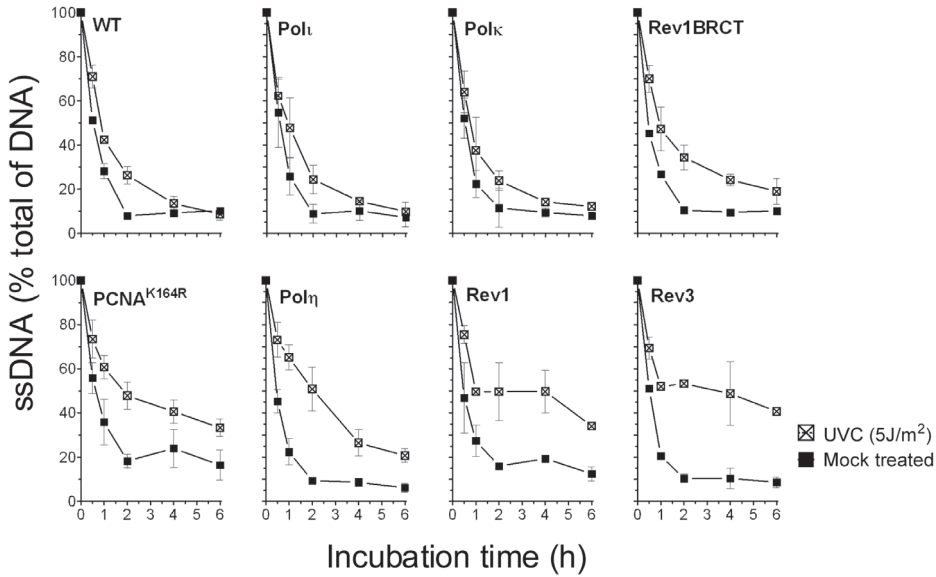


Figure 4 | Relative inhibition of replication fork progression upon UVC exposure in MEFs with defined defects in TLS-related genes. Replication fork progression in different MEF lines was measured up to 6h upon UVC exposure (5 J/m²) or mock treatment (*n*=3). The amount ssDNA (% total DNA) is related to stalled replication forks and was set at 100% at 0h. Error bar, SD.

the Pol η -deficient cells. Together these data suggest that in these cell lines an alternative, PCNA-Ub-independent, pathway completes synthesis of most, albeit not all, nascent DNA. In contrast, maturation of nascent DNA was almost completely abolished in MEFs deficient for Rev1 or Rev3, already after 1h following UVC exposure (Fig. 4). This result signifies that Rev1 and Rev3 are essential for replicative bypass at photoproducts and may act independently from PCNA-Ub and Pol η .

Activation of DNA Damage Signaling is Correlated with Defects in Photoproduct Bypass

Upon the generation of ssDNA, the essential DNA damage signaling kinase Atr phosphorylates the serine/threonine kinase Chk1 (Smits *et al.*, 2010). To test whether the observed differences in replication fork progression among the various UV-exposed MEF lines correlate with the extent of Atr activation, we determined the levels of Chk1, phosphorylated at Serine (S) 345 (Chk1^{S345-P}), in UV-exposed cells, using immunoblotting. Within 8h after 5 J/m² UVC treatment, rapid and robust induction of Chk1^{S345-P} was observed in MEFs deficient for Pol η , Rev1 and Rev3 as well as for PCNA^{K164R} MEFs (Fig. 5), the same cell lines that display significant defects in replication fork progression. Wild type MEFs and MEFs deficient for Polt showed only weak induction of Chk1^{S345-P} in response to UVC, whereas MEFs defective in Polk or Rev1BRCT MEFs displayed moderate Chk1^{S345-P} signals. Of note, phosphorylation

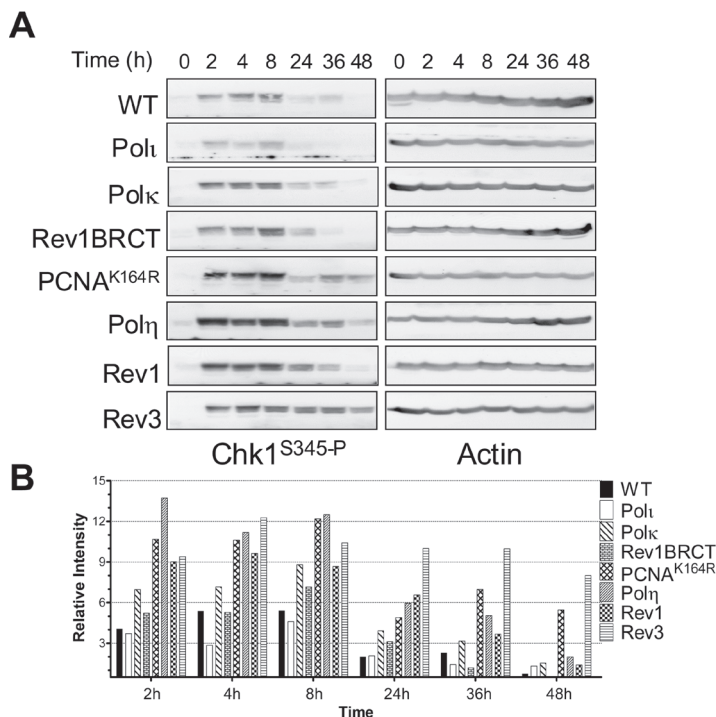


Figure 5 | Effect of UVC on DNA damage checkpoint responses in MEFs with defined defects in TLS-related genes. Whole cell extracts prepared at different times after exposure to UVC (5 J/m²) were used for Western blot analysis. 0h: cell extract prepared instantly after exposure. (A) Blots representing expression of Chk1^{S345-P} (left panel) and β-actin (right panel). (B) Quantification of Chk1^{S345-P} upon UVC exposure relative to the loading control β-actin.

of Chk1 maximizes much earlier than that of KAP1 and H2AX, suggesting that DSBs originate from persisting ssDNA regions (Figs. 2 and 5).

Different TLS Mutants are Differentially Affected in S Phase Progression After UVC Exposure

To relate the extent of replication fork stalling with the induction of cell cycle responses, we determined cell cycle progression of the cells pulse-labeled during S phase with BrdU, immediately after UVC exposure (Figs. 6 and S2A–D). At 4h after labeling, all mock-treated MEF lines displayed the appearance of BrdU-positive cells in the subsequent G1 phase, indicating that cell cycle progression of undamaged MEF lines was not affected by the TLS defects (Fig. S2D). Compared to untreated cell populations, treatment with 5 J/m² UVC induced only mild delays in cell cycle progression in wild type MEFs and in MEFs deficient for Polt, Polk and Rev1BRCT (Figs. 6 and S2D). Polη-deficient MEFs, however, displayed a much stronger accumulation during mid-S phase (Fig. 6). In contrast to Polη-deficient MEFs, UV-exposed PCNA^{K164R} MEFs progressed

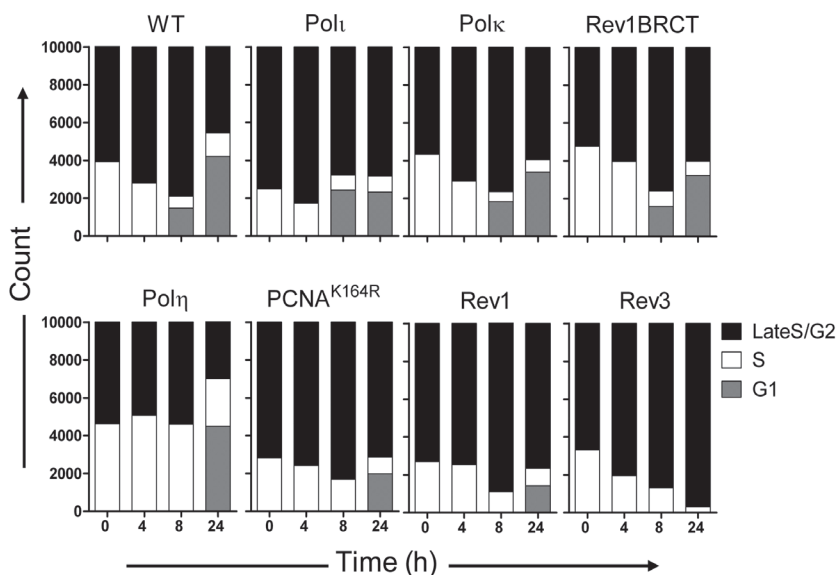


Figure 6 | Cell cycle progression of MEFs with defined defects in TLS-related genes. Cells were pulse-labeled with BrdU, immediately after UVC exposure (5 J/m^2). Then, BrdU-positive cells in G1, S and late S/G2 phases were quantified, at different times up to 24h after treatment.

more rapidly through the S phase, accumulating only at the late S/G₂ phase (Fig. 6). This result may indicate that Polη plays an early role in TLS, independent of PCNA-Ub, a hypothesis that is consistent with the more pronounced defect in replication fork progression of the Polη-deficient MEFs, as measured by the ADU assay (Fig. 4). Similar to PCNA^{K164R} MEFs, MEFs deficient for Rev1 or Rev3 strongly accumulated only at the late S/G₂ phase, following UVC exposure. All UV-exposed mutant MEF lines were capable of entering a new cell cycle, except Rev3-deficient MEFs that arrested indefinitely at the G₂-phase (Fig. 6).

Identification of Genomic Substrates for PCNA-Ub and Individual TLS Polymerases

The prolonged formation of Chk1^{S345-P} in PCNA^{K164R} MEFs and MEFs defective for Polη, Rev1 and Rev3 after UVC exposure (Fig. 5) suggests the persistence of ssDNA containing photoproducts following replication fork stalling. We recently described an innovative approach to identify the specific genomic photoproducts that are substrates for individual TLS polymerases. This approach depends on immunostaining of non-denatured DNA using monoclonal antibodies that recognize either (6-4)PPs or CPDs, embedded in ssDNA (Jansen *et al.*, 2009a; Jansen *et al.*, 2009b). Here, we further developed this method to compare the timing and substrate specificities for Rev1, Polη- and PCNA-Ub-mediated DNA damage bypass in replicating cells. Thus, MEFs were exposed to 5 J/m^2 UVC and pulse-labeled with EdU, enabling to identify cells that were replicating during UVC exposure. These cells were fixed and stained under

non-denatured conditions for EdU and for CPD or (6-4)PP, after UVC treatment. Wild type MEFs were included as controls. Neither CPDs nor (6-4)PPs were detected in wild type MEFs at 2h or 8h after UVC treatment, suggesting efficient replication across both genomic lesions (Fig. 7). In contrast, in nuclei of EdU-positive MEFs deficient for Pol η , unreplacated CPDs were clearly detectable at 2h after UVC exposure whereas at 8h staining for CPDs was strongly reduced (Fig. 7). Since CPDs are hardly repaired in mouse cells (Van Sloun *et al.*, 1999; Yagi *et al.*, 1984), these results suggest that Pol η is mainly involved in an early bypass pathway at CPDs. Moreover, in Pol η -deficient MEFs a backup pathway most likely replicates CPDs at a later stage. Also PCNA^{K164R} MEFs stained positive for unreplacated CPDs although the staining persisted much longer than in the Pol η -deficient MEFs. This indicates that PCNA-Ub directs a Pol η -independent bypass pathway for CPDs, late after exposure. Little staining of CPD in ssDNA was detected in nuclei of replicating Rev1-deficient MEFs, in agreement with previous results (Jansen *et al.*, 2009a). In contrast, strong staining of (6-4)PPs in ssDNA was seen in Rev1-deficient MEFs that were replicating during UV treatment, both early and late after UVC exposure. As we have previously excluded that Rev1 plays a role

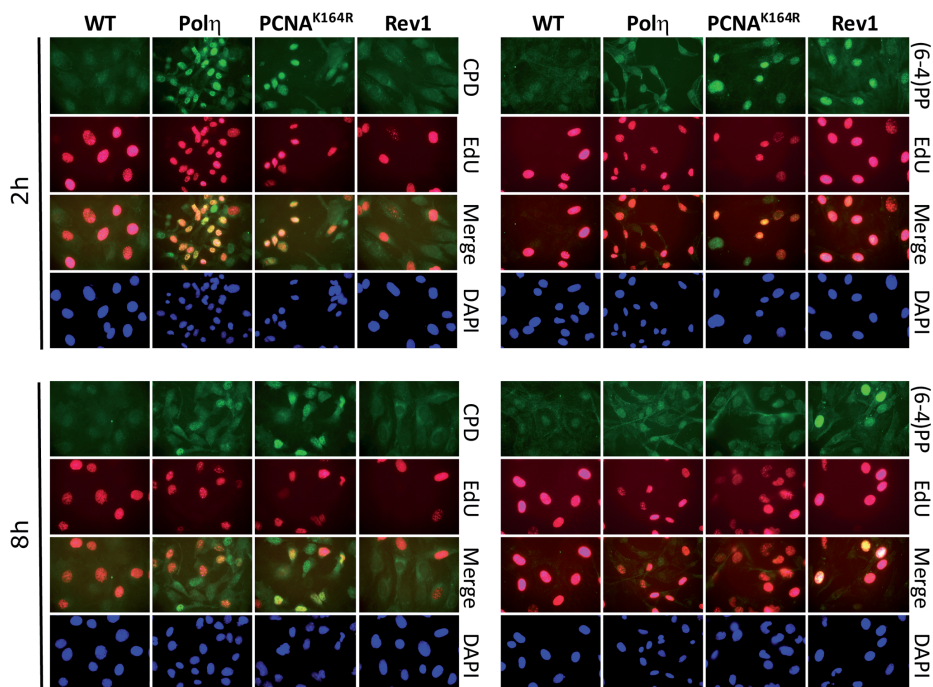


Figure 7 | Different substrate specificities and kinetics of photoproduct bypass for Pol η , PCNA-Ub and Rev1. Cells were exposed to 5 J/m² UVC and immediately pulse-labeled with EdU. Cells were fixed 2h (upper panels) and 8h (lower panels) and immunostained for CPDs (green, left panels) or for (6-4)PPs (green, right panels) in ssDNA of nuclei (DAPI, blue) in replicating (EdU-positive, red) and non-replicating cells at the time of UVC exposure. Merge represents combined stainings for UV lesions and for EdU.

in nucleotide excision repair of (6-4)PPs (our unpublished data), this result confirms previous data suggesting that Rev1 acts both early and late to bypass (6-4)PPs (Jansen *et al.*, 2009a). This result also demonstrates that there is no backup pathway for the bypass of (6-4)PP. Interestingly, PCNA^{K164R} MEFs and, to a minor extent, Polη-deficient MEFs displayed accumulation of unreplicated (6-4)PPs, in the case of PCNA^{K164R} MEFs only early after UV exposure (Fig. 7). This result indicates that both PCNA-Ub and Rev1 are essential for the early bypass of (6-4)PPs, while a Rev1-dependent but PCNA-Ub-independent pathway replicates (6-4)PPs, late after UVC exposure. We noted that some cells that stained positive for UV-damages within ssDNA were EdU-negative. Most likely, these cells had entered S phase after pulse-labeling with EdU. Conversely, EdU-positive MEFs that showed no staining for photolesions in ssDNA, early after UV exposure, were probably labeled with EdU during the end of S phase, precluding the accumulation of stalled replication forks.

DISCUSSION

In this study, we have determined the contribution of all five key mammalian TLS polymerases, and PCNA modification at K164, in replication fork progression, DNA damage signaling, cell cycle progression and genome stability *in vivo*, in response to UVC exposure.

MEF lines defective for Pols ι, κ and the BRCT region of Rev1 displayed very mild phenotypes, indicating that these factors play only minor roles in TLS across genomic photolesions *in vivo*. In support, purified human Polκ is not able to insert nucleotides opposite CPDs and (6-4)PP (Zhang *et al.*, 2000), although there is evidence that Polι and Polκ can act as backups to Polη on a site-specific CPD-containing plasmid (Ziv *et al.*, 2009). Alternatively, the minor UV sensitivity of Polκ-deficient MEFs might be caused by a defect in gap filling during nucleotide excision repair of (6-4)PP (Ogi *et al.*, 2006; Ogi *et al.*, 2010). Polι might be involved in bypassing some (6-4)PPs, as indicated by the analysis of (6-4)PP-induced mutations at episomal vectors in cells treated with Polι siRNA (Yoon *et al.*, 2010). Since Polι plays a significant role in responses to oxidative stress (Petta *et al.*, 2008; Temviriyankul *et al.*, 2012), it might be that Polι protects cells from cytotoxicity induced by long-wave UVA light that, in contrast with UVC, induces oxidative DNA damage. Nevertheless, deficiencies for Pols ι, κ and the Rev1BRCT region may still lead to significant damage responses and biological effects under specific conditions (Dumstorf *et al.*, 2006; Ohkumo *et al.*, 2006; Tsaalbi-Shtylik *et al.*, 2009; Yoon *et al.*, 2010; Ziv *et al.*, 2009).

MEFs deficient for Polη, Rev1 and Polζ, as well as the PCNA^{K164R} mutant, displayed significant defects in responses to DNA photolesions, albeit in quantitatively and qualitatively different fashions. In Polη-deficient cells, we found a defect in TLS at genomic CPDs predominantly early after UVC treatment. Polη-dependent TLS at CPDs quenches Chk1 signaling (Fig. 5) and prevents the accumulation of cells during

mid-S phase (Fig. 6) and genome instability (Figs. 2 and 3). Indeed, Pol η is considered to be the principal TLS polymerase for CPDs and other moderately helix-distorting lesions (Yoon *et al.*, 2010; Ziv *et al.*, 2009). In Pol η -deficient cells the delayed bypass of CPDs (Figs. 4 and 7) may be mediated by Pols ι , κ and ζ , albeit at the expense of higher mutation rates (Ito *et al.*, 2012; Yoon *et al.*, 2010; Ziv *et al.*, 2009). However, since these putative backup pathways are unable to rescue the Pol η defect completely (Fig. 4), some stalled forks will ultimately collapse, resulting in the formation of cytotoxic DSBs in the absence of Pol η (Figs. 1–3).

Similar to Pol η , PCNA-Ub is involved in TLS of CPDs. However, compared with Pol η -deficient MEFs, PCNA^{K164R} mutant MEFs displayed less delay in fork progression early after UVC treatment (Fig. 4) and a less pronounced S phase arrest (Fig. 6). These data suggest that Pol η may partially operate independently of PCNA-Ub during TLS of CPDs, in agreement with previous observations (Hendel *et al.*, 2011; Krijger *et al.*, 2011; Schmutz *et al.*, 2010). Notably, in a previous study we showed that PCNA^{K164R} pre-B cells progressed somewhat slower through S phase compared with Pol η -deficient pre-B cells (Hendel *et al.*, 2011; Krijger *et al.*, 2011). In comparison with the present study, this different result may reflect cell type-specific dissimilarities and stresses the importance of our integrative approach to study DNA damage bypass, DNA damage responses and the induction of genome instability in a defined cell type.

Surprisingly, in addition to a defect in TLS of CPDs, PCNA^{K164R} and, to a minor extent, Pol η -deficient MEFs displayed also a defect in the bypass of genomic (6-4)PP (Fig. 7). The greater dependence of (6-4)PP bypass on PCNA-Ub than on Pol η may explain the higher induction of DSBs in the PCNA-Ub-mutant cells compared with the Pol η -deficient cells (Fig. 3). The nature of this pathway is elusive, although Rev1 and Pol ζ may be involved (Hendel *et al.*, 2011).

Compared with Pol η and PCNA-Ub-defective MEFs, Rev1 or Rev3-deficient MEFs displayed different responses to UVC exposure: (i) Replication fork progression shortly after UVC exposure is less inhibited in Rev1 or Rev3-deficient MEFs (Fig. 4). This difference may reflect the observed dissimilarities in substrate specificities (Fig. 7), since Pol η and PCNA-Ub act primarily on abundant CPDs, whereas *in vivo* Rev1 and Rev3 mainly operate on, less abundant, (6-4)PPs (Jansen *et al.*, 2009a; Jansen *et al.*, 2009b). (ii) Replication forks stalled at (6-4)PP remain obstructed for a long time in MEFs deficient for Rev1 or Pol ζ , indicating that in contrast to CPDs no backup pathway exists for bypass of (6-4)PP (Figs. 4 and 7; (Jansen *et al.*, 2009a; Jansen *et al.*, 2009b; Szuts *et al.*, 2008)). Importantly, this result additionally suggests that (6-4)PP at stalled forks are refractory to repair by NER, contradicting models describing the repair of DNA damage following regression of the stalled replication fork (Atkinson *et al.*, 2009). Moreover, Rev1 and Rev3 strongly suppress the formation of DSBs in mammalian cells (Fig. 3; (Van Sloun *et al.*, 2002; Wittschieben *et al.*, 2006)) by catalyzing TLS and, possibly, by promoting homologous recombination to repair DSBs (Sharma *et al.*, 2012). Furthermore, the much stronger defect in (6-4)PP bypass of MEFs deficient for Rev1 or Pol ζ than of PCNA^{K164R}

MEFs, at 8h after UV exposure, suggests that in mammalian cells the late Rev1 and Pol ζ -dependent bypass of (6-4)PP does not depend on PCNA-Ub, similar to chicken DT40 cells (Edmunds *et al.*, 2008). (iii) Perhaps surprisingly, in contrast to Pol η -deficient MEFs, MEFs deficient for Rev1 or Rev3 progress relatively normally through S phase following UVC exposure, despite accumulation of arrested replication forks, persistence of unreplicated (6-4)PP and concomitant Chk1 activation (Figs. 4–7 and Fig. S2C; (Jansen *et al.*, 2009a; Jansen *et al.*, 2009b)). Collectively, these data corroborate previous results indicating that Rev1 and Rev3 act preferentially at post-replicative gaps, rather than at the stalled replication fork itself, thereby quenching DNA damage signaling (Daigaku *et al.*, 2010; Diamant *et al.*, 2012). In agreement, we have recently described that Rev1 can be recruited to 5' phosphorylated primer-template junctions that can be generated by repriming of postreplicative replication, downstream of the unreplicated lesion (de Groote *et al.*, 2011). It has been proposed that Rev1 functions as a scaffold protein to recruit other TLS polymerases to TLS intermediates (Andersen *et al.*, 2011; Guo *et al.*, 2009; Ohashi *et al.*, 2004; Tissier *et al.*, 2004; Waters *et al.*, 2009). Most likely, Rev3 is recruited to extend 3' DNA ends generated by incorporation opposite (6-4)PP photoproducts (Shachar *et al.*, 2009). Although also Polk may act as an extender TLS polymerase (Yoon *et al.*, 2009; Ziv *et al.*, 2009), our data suggest that in the absence of Rev3 neither Polk nor any other polymerase acts as a backup extender polymerase during genomic DNA damage bypass in mammalian cells. Of note, the induction of high levels of micronuclei in the Rev1, Rev3 and PCNA^{K164R} mutants (Fig. 3) indicates that prolonged stalling of replication forks at (6-4)PP photoproducts is a predominant source of UV-induced DSBs.

In conclusion, we have described an integrative approach to study qualitative and quantitative aspects of TLS, DNA damage responses and the induction of genome instability in a defined cell type. This approach provides novel inroads in the study of DNA damage bypass at the mammalian genome, defining not only the individual actors but also temporally distinct bypass pathways that operate at structurally distinct lesions (Table 1). To delineate these pathways even more accurately, we are investigating MEF lines with double deficiencies in factors involved in TLS.

Table 1 | Contribution of TLS polymerases and ubiquitination of PCNA in temporally distinct bypass pathways operating at genomic CPDs or (6-4)PPs

	CPDs				(6-4)PPs			
	Pol η	PCNA ^{K164}	Rev1	Rev3 ^a	Pol η	PCNA ^{K164}	Rev1	Rev3 ^a
Pathway								
early	+	+	-	ND ^b	±	+	+	ND
late	-	+	-	±	±	-	+	+

^a Taken from (Jansen *et al.*, 2009b)

^b ND, not determined

MATERIALS AND METHODS

Cell culture. Immortalized MEFs homozygous for a targeted disruption of Rev1, the N-terminal BRCT region of Rev1 or Rev3 were described previously (Jansen *et al.*, 2006; Jansen *et al.*, 2009a; Jansen *et al.*, 2009b). MEFs lacking Pol η or Pol ι were isolated from day 13.5 embryos of Pol η or Pol ι deficient mice and cultured until spontaneous immortalization. Pol η -deficient mice have been described previously (Delbos *et al.*, 2005). The generation of Pol ι -deficient mice will be described elsewhere (Aoufouchi *et al.*, in preparation). A MEF line deficient for Pol κ was kindly provided by Dr. Haruo Ohmori (Kyoto University, Japan). A MEF line homozygous for a PCNA^{K164R} mutation was described previously (Krijger *et al.*, 2011). All MEF lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5g/l glucose, Glutamax and pyruvate (Invitrogen), supplemented with 10% fetal calf serum, penicillin (100U/ml), and streptomycin (100 μ g/ml) (DMEM medium) at 37°C in a humidified atmosphere containing 5% CO₂.

Cell proliferation assay. Per well of a 6-well plate, 5×10^4 exponentially growing cells were seeded in DMEM medium and cultured overnight. Cells were washed twice with phosphate buffer saline (PBS) before exposure to various doses of UVC light (Philips TUV lamp, predominantly 254 nm). After exposure, fresh DMEM medium was added and the cells were cultured for 3 days. Then, cells were trypsinized and counted using a Z2 coulter particle and size analyzer (Beckman coulter). At least three completely independent experiments were performed.

Western blot analysis. Per P90 dish, 10^6 cells were seeded and cultured overnight in DMEM medium. Prior to UVC exposure at 5 J/m², or mock treatment, cells were rinsed with PBS. After treatment, cells were cultured in DMEM medium and at the indicated times cells were lysed by adding Laemmli lysis buffer directly onto cells. Proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Hybond-C extra, Amersham Biosciences). The membranes were incubated in blocking buffer (Rockland) and PBS-0.1% Tween 20 in a 1:1 ratio for at least 30 min at room temperature. Thereafter, membranes were incubated overnight with primary antibodies at 4°C and with appropriate peroxidase-conjugated secondary antibodies (Bio-Rad) for 1h at room temperature, subsequently. Proteins were visualized by enhanced chemiluminescence detection (ECL). The following antibodies were used: rabbit monoclonal anti-phospho Chk1 (S345) (Cell signaling), mouse monoclonal anti-phospho-H2AX (S139) (Millipore), mouse monoclonal anti-actin antibody (Oncogene) and rabbit polyclonal anti-phospho Kap1 (S824) (Bethyl Laboratory). At least two completely independent experiments were performed.

Cytokinesis-blocked micronucleus assay. Per P90 dish, 1.5×10^6 cells were plated and cultured overnight in DMEM medium. Then, 7.5×10^4 cells were seeded on a sterile glass slide (76x26 mm) and cultured overnight. Cells attached to the slide were rinsed twice with 10 ml PBS before UVC exposure, followed by further incubation. Mock treated cells were included as experimental controls. To inhibit cytokinesis, 3 μ g/ml of cytochalasin B (cyt-B) (Sigma-Aldrich) was added to the cells, 24h after treatment. Twenty-four hours later, cells were fixed for 15 min using a fixative solution containing 3.5% cold paraformaldehyde and 0.5% Triton-X100. Next, slides were washed thrice with ice-cold PBS and nuclei were stained with DAPI (17.5ng/ml) (Sigma-Aldrich) in the dark for 15 min. To dehydrate the cells, slides were drenched in 70%, 90% and absolute ethanol in the dark for 5 min each, respectively. The number of binucleated cells that contained one or more micronuclei was scored by using a fluorescence microscope (Zeiss, Germany) and the Metafer 4 program (Metasystem, Germany).

Alkaline DNA unwinding (ADU). This assay was slightly adapted from (Johansson *et al.*, 2004). Per well 5×10^4 cells were plated in 24-well plate in DMEM medium and cultured overnight. Then,

the cells were pulse labeled with [^3H]thymidine (2 $\mu\text{Ci/ml}$; 76 Ci/mmol) for 15 min and rinsed once with PBS before exposure to 5 J/m 2 of UVC. At indicated times, up to 6h, cells were rinsed twice with ice-cold 0.15M NaCl and DNA was locally denatured at the DNA replication fork with ice-cold denaturation solution (0.15M NaCl and 0.03M NaOH) for 30 min. To terminate the reaction, 1 ml of ice-cold 0.02M Na $_2$ HPO $_4$ was added. Before storage overnight at -20°C, the samples were sonicated and subsequently SDS was added to a final concentration of 0.25%. Lysates were thawed and loaded onto hydroxyl apatite columns to elute ssDNA (stalled forks) from dsDNA (replicated DNA) using 0.13M K $_2$ HPO $_4$ and 0.3M K $_2$ HPO $_4$ pH 6.8, respectively. The radioactivity in both eluates was counted by liquid scintillation counting (Perkin-Elmer).

Bivariate cell cycle analysis. Exponentially growing MEFs were plated in P90 dishes and cultured overnight. Cells were washed once with PBS and exposed to 5 J/m 2 of UVC or mock treated. After treatment, cells were pulse-labeled with 10 μM Bromodeoxyuridine (BrdU, Millipore) for 30 min at 37°C in the dark. To stop the labeling reaction, cells were rinsed once with PBS and continuously cultured in DMEM medium containing 5 μM Thymidine (Sigma-Aldrich). At desired time points, cells were trypsinized and fixed with 70% ethanol. Before staining, cells were permeabilized by 2M HCl/0.05% Triton-X for 35 min and subsequently neutralized by 1M Tris. Neutralized cells were incubated overnight with a mouse monoclonal antibody against BrdU (Becton Dickinson) at 4°C. Consequently, cells were washed and incubated with a FITC-conjugated rat anti-mouse antibody (BD bioscience Pharmingen) for 1h at room temperature in the dark. Then, cells were centrifuged and resuspended in PBS containing 10 $\mu\text{g/ml}$ of Propidium Iodide and 100 $\mu\text{g/ml}$ of RNase A to stain genomic DNA. Finally, the cells were analyzed by flow cytometry (Guava easyCyte HT, Millipore).

Immunofluorescent detection of unreplicated CPDs and (6-4)PPs in S phase cells. Detection of CPDs or (6-4)PPs in single-stranded DNA templates was performed as previously described with major modifications (Jansen *et al.*, 2009a). MEFs cultured on coverslips were irradiated with 0 or 5 J/m 2 UVC light, followed by 30 min pulse-labeling with 10 μM 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen) in the dark. Two or eight hours later, MEFs were treated with 1% Triton-X100 in PBS for 2 min and subsequently fixed in 2% Formaldehyde/PBS containing 0.5% Triton-X100 for 15 min at room temperature. EdU positive cells were visualized using AlexaFluor 647-conjugated azide following the manufacturer's recommendations (Click-iT $^{\text{TM}}$ Edu imaging kit - Invitrogen). Unreplicated photoproducts were identified in non-denatured DNA using primary mouse monoclonal antibodies against (6-4)PPs (64-M2, CosmoBio) or CPDs (TDM2, CosmoBio). After incubation with secondary Alexafluor488-labeled goat-anti-rat antibodies (Molecular Probes, Inc.), nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). To check the functionality of the primary antibodies, immunofluorescence was performed essentially as described above, except that after EdU detection, cells were fixed with 2% Formaldehyde and denatured with 2N HCl for 10 min. Coverslips were mounted (Vectashield, Vector laboratories) and analyzed by fluorescent microscopy.

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SUPPLEMENTARY FIGURES

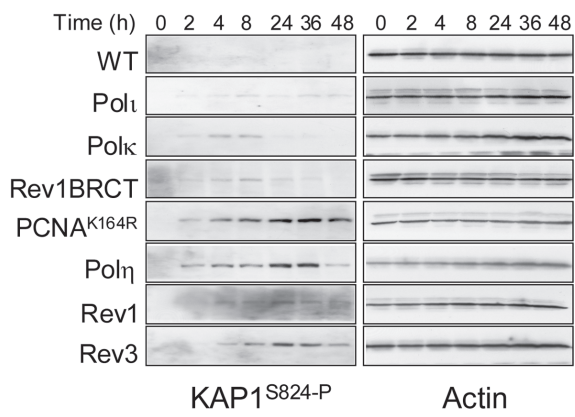


Figure S1 | Effect UVC on KAP^{S824-P} activation of MEFs with defined defects in TLS-related genes. Whole cell extracts were prepared at different times after exposure to UVC (5 J/m²) and used for western blot analysis. 0h: cell extract prepared instantly after exposure. Blots representing expression of KAP^{S824-P} (left panel) and loading control β-actin (right panel).

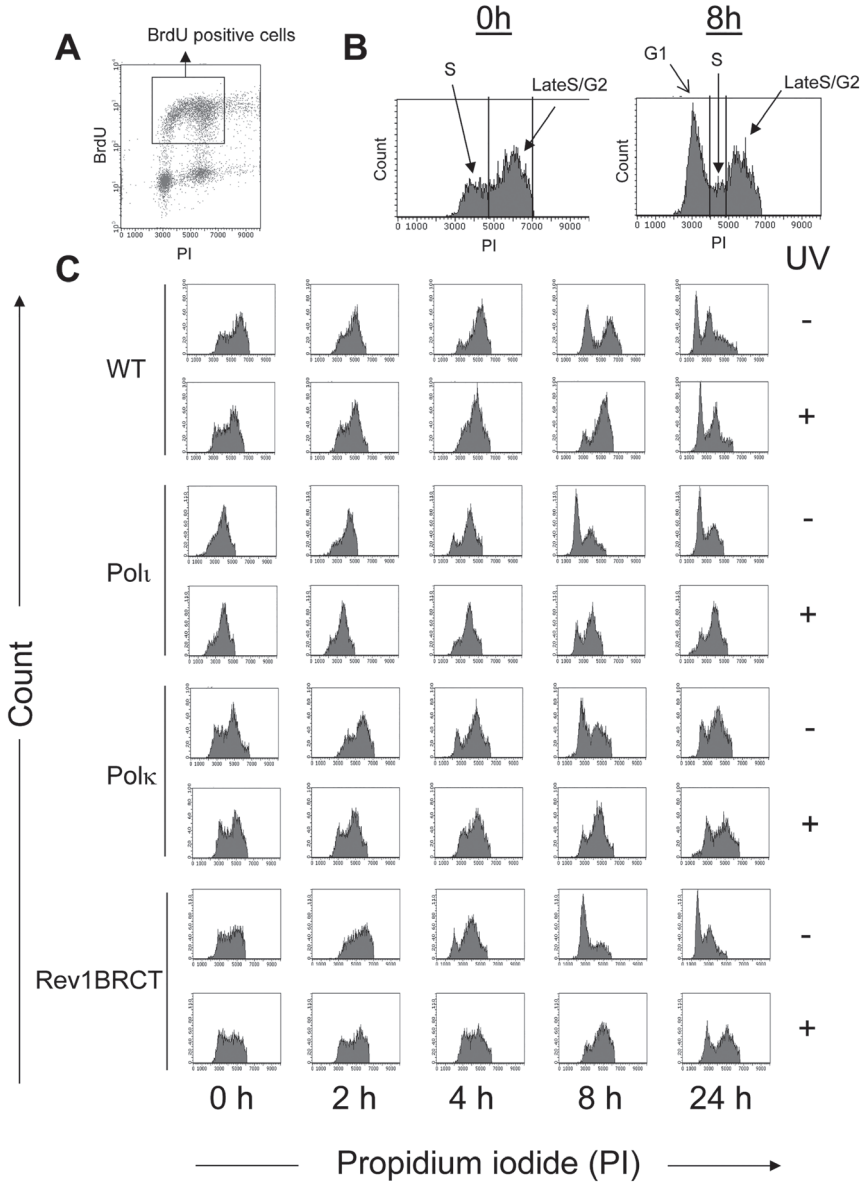


Figure S2 | Bivariate cell cycle analysis for MEFs with defined defects in TLS-related genes. Immediately after mock treatment or UVC exposure (5 J/m^2), cells were pulse-labeled with BrdU. Cells were fixed at different times up to 24h after mock or UVC treatment, stained for BrdU incorporation and DNA content (PI) and analyzed by flow cytometry. (A) Cell cycle profile of MEFs pulse-labeled with BrdU. The population of BrdU-positive cells within the area indicated by a dashed line was used for quantification. (B) Distribution of BrdU-positive cells in G1, S and late S/G₂ stages of the cell cycle. (C) Cell cycle profiles of BrdU-positive wild type MEFs (WT) and various MEF lines containing targeted mutations in TLS-related genes (Polt, κ , η , PCNA^{K164R}, Rev1BRCT, Rev1, Rev3) at 0, 2, 4, 8 and 24h after mock treatment (-UV) or exposure to 5 J/m^2 UVC (+UV). (D) Quantification of BrdU-positive cells in G1, S and late S/G₂ phases at different times up to 24h after mock treatment.

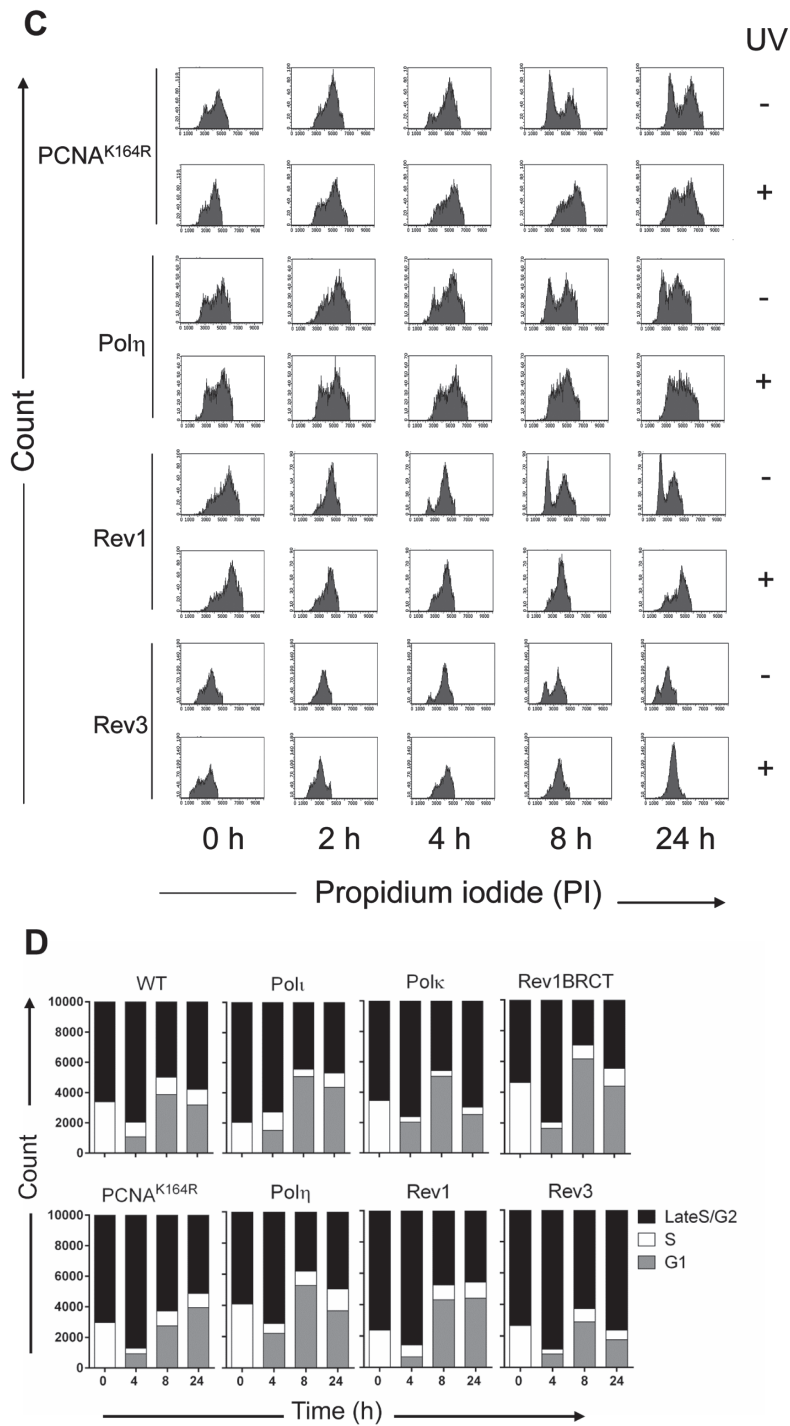


Figure S2 | (Continued)

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CHAPTER

4

REDUNDANCY OF MAMMALIAN Y FAMILY DNA POLYMERASES IN CELLULAR RESPONSES TO GENOMIC DNA LESIONS INDUCED BY ULTRAVIOLET LIGHT

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Manuscript to be submitted

ABSTRACT

Short-wave ultraviolet (UVC) light induces both mildly helix-distorting cyclobutane pyrimidine dimers (CPDs) and severely distorting (6-4) pyrimidine pyrimidone photoproducts ((6-4)PPs). The only DNA polymerase (Pol) that is known to replicate efficiently across photolesions, particularly CPDs, is Pol η , a member of the evolutionary conserved Y family of translesion synthesis (TLS) DNA polymerases. Pol η -deficient mouse embryonic fibroblasts (MEFs) display a defect in TLS at CPDs and (6-4)PPs and consequently an enhanced DNA damage signaling and cell cycle delay upon exposure to UVC light. However, these phenotypes are transient, suggesting redundancy with other DNA damage tolerance pathways. Here we investigated whether Y-family Pols ι and κ may act as backups for Pol η in bypassing genomic CPD and (6-4)PP lesions, by using MEF lines with single and combined disruptions in these Pols. Our data demonstrate that Pol κ plays a dominant role in alleviating stalling of genomic replication forks in Pol η -deficient MEFs, both at CPDs and (6-4)PPs. This dampens DNA damage signaling and cell cycle arrests, and resulted in increased proliferation. Conversely, the contribution of Pol ι is restricted to a subset of the lesions. This study contributes to understanding the mutator phenotype of Xeroderma Pigmentosum-variant, a syndrome caused by Pol η defects.

INTRODUCTION

Exposure to ultraviolet (UV) light induces both mildly helix-distorting *cis-syn* cyclobutane pyrimidine dimers (CPDs) and strongly helix-distorting (6-4) pyrimidine pyrimidone photoproducts ((6-4)PPs) at the genome (Beukers *et al.*, 2008). Both CPDs and (6-4)PPs form blocks for replicative DNA polymerases (Pols), since their active sites are unable to accommodate these photolesions. The only DNA polymerase known to efficiently replicate across CPDs both *in vitro* and *in vivo* is Pol η , a member of the Y family of DNA polymerases, which, in mammalian cells, also includes Pols ι , κ and Rev1 (Guo *et al.*, 2009; Sale *et al.*, 2012; Waters *et al.*, 2009). Pol η is capable of containing a thymine-thymine CPD, the most frequent photolesion, in its enlarged active site (Biertumpfel *et al.*, 2010). In contrast to CPDs, (6-4)TT lesions form a poor substrate for Pol η *in vitro*, as Pol η frequently inserts a G opposite the 3' T but is unable to carry out the subsequent extension step (Johnson *et al.*, 2001). *In vivo*, TLS at (6-4) PP may involve either Pol η or Pol ι , followed by extension by another polymerase, at least at an episomal substrate (Yoon *et al.*, 2010).

The importance of Pol η in DNA damage responses is stressed by patients suffering from the Xeroderma Pigmentosum variant syndrome (XP-V), a rare autosomal recessive human disorder, caused by mutations in the gene that encodes Pol η (Johnson *et al.*, 1999; Masutani *et al.*, 1999). Clinically, XP-V is characterized by photosensitivity of the skin and high susceptibility to develop cancer of sunlight-exposed areas of the skin. After UVC exposure, the conversion of low molecular weight to high molecular weight nascent DNA is much slower in XP-V cells than in normal cells (Lehmann *et al.*, 1975). The TLS defect results in the accumulation of ssDNA regions that activate the Ataxia-telangiectasia-mutated and Rad3-related (Atr) kinase (Despras *et al.*, 2010; Elvers *et al.*, 2011). Activated Atr phosphorylates multiple proteins, including Checkpoint kinase 1 (Chk1) that controls S-phase progression by inhibiting origin firing, slowing down replication fork progression, stabilizing stalled replication forks and delaying cell cycle progression (Feijoo *et al.*, 2001; Zachos *et al.*, 2005). Nevertheless, XP-V cells are only mildly sensitive to UVC light and the defect in the progression of replication at damaged DNA is only transient, suggesting that most lesions are ultimately bypassed in these cells. Since XP-V cells display increased mutagenesis upon exposure to UVC light (Maher *et al.*, 1975), an alternative TLS process presumably operates as a backup to convert ssDNA regions into dsDNA in XP-V cells.

Recently, we have analyzed the *in vivo* roles of individual TLS Pols, including Pol η and other Y family Pols, in the suppression of DNA damage signaling and genome instability in immortalized mouse embryonic fibroblast (MEF) lines upon exposure to UVC light (Temviriyankul *et al.*, 2012). We found that Pol ι and Pol κ -deficient MEFs only displayed minor phenotypes in response to UVC light, whereas Rev1 appears to be mainly involved in the bypass of (6-4)PP (Jansen *et al.*, 2009; Temviriyankul *et al.*, 2012). In addition, we observed that, similar to XP-V cells, Pol η -deficient MEFs display a transient defect in TLS, resulting in the accumulation of cells in mid-S phase and

activation of DNA damage signaling (Temviriyankul *et al.*, 2012). Mainly TLS across genomic CPDs is affected in these cells. Possibly, this transient TLS defect in the absence of Pol η might be due to the Y-family Pols ι and κ that act as backup Pols in bypassing UVC lesions at the genome, and in the suppression of DNA damage responses.

In previous studies the expression of multiple TLS polymerases was reduced using siRNA knock-down strategies while in these cells TLS was investigated only at lesion-containing episomal plasmids (Hendel *et al.*, 2008; Shachar *et al.*, 2009; Yoon *et al.*, 2009; Yoon *et al.*, 2010; Ziv *et al.*, 2009). Here, we have used MEF lines with well-defined single, double and triple deficiencies in Pols η , ι and κ . To provide quantitative data on the UV damage responses in these cell lines, the same UVC dose was applied in most experiments. We report that in Pol η -deficient MEFs exposed to UVC light, Pol κ surprisingly is the predominant TLS polymerase to bypass both genomic CPDs and (6-4)PPs, contributing to (i) alleviating cell cycle arrest, (ii) quenching DNA damage signaling, and (iii) promoting cell survival. Pol ι may play a minor role in TLS of a subset of (6-4)PP.

RESULTS

An Early Role of Pols ι and κ in Photolesion Bypass

Recently, we have shown that replicative bypass of photolesions was delayed rather than abolished in Pol η -deficient MEFs, suggesting the existence of a backup mechanism that almost completely rescues the Pol η defect (Temviriyankul *et al.*, 2012). Here, we tested whether two other Y-family TLS polymerases, *i.e.* Pols ι and κ , are entailed in this backup pathway. To this aim, we compared the responses of Pol η -deficient MEF lines with additional deficiencies in Pol ι , Pol κ or both TLS Pols with wild type and single-mutant MEF lines. We first determined the progression of replicons in the different MEF lines using DNA fiber labeling. This sensitive assay allows the analysis of replicon progression on single DNA molecules, shortly after exposure to UVC. Thus, cells were incubated with Chlorodeoxyuridine (CldU) for 20 min to label replicating DNA, exposed to 0 or to 13 J/m² UVC, and subsequently incubated with Iododeoxyuridine (IdU) for another 20 min. Fibers were generated and stained with specific antibodies for CldU and IdU, visualized by fluorescent microscopy and the lengths of CldU- and IdU-containing tracts were quantified to determine the replication speed and replication fork stalling (Fig. 1A). When undamaged templates are replicated, no obvious differences in the replication speed amongst the cell lines were found (Supplementary Fig. S1). In addition, under mock-treated conditions all cell lines showed the expected ratio of CldU to IdU of 1, indicating that TLS Pols η , ι and κ are dispensable for replication of undamaged DNA templates (Fig. 1B). Following UVC exposure, the ratio of CldU to IdU increased from 1 to approximately 2 in wild type and single mutant Pol ι or Pol κ -deficient MEFs. This result indicates that (i) UVC-induced DNA damage results in reduced replicon progression and (ii) TLS Pols ι and κ are not essential for photolesion

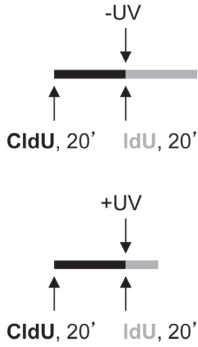
bypass at an early stage after UVC exposure (Fig. 1B). At this stage, compared with wild type and single mutant Pol η or Pol κ -deficient MEFs, a slightly increased spreading of the CldU to IdU ratio was observed in the Pol η -mutant, suggesting reduced fork progression, specifically at damaged forks. Fork progression in the double and triple mutant MEFs was delayed to an even greater extent (Student's t test: $p < 0.01$). These results suggest partial redundancy between these TLS polymerases, early after UVC exposure (Fig. 1B).

A Late Role of Pols ι and κ in Photolesion Bypass

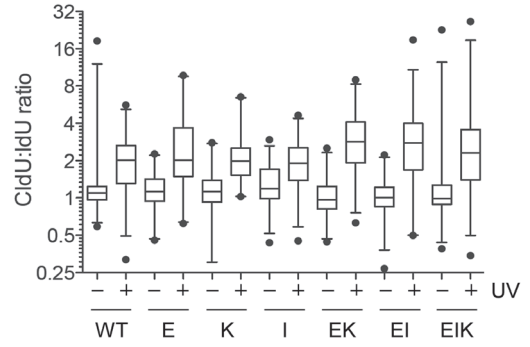
To investigate redundancy between Pols η , ι and κ in TLS at photolesions more sensitively, and also at later times after UVC exposure, we employed an alkaline DNA unwinding assay. In this assay the progression of replicons is determined by measuring the persistence of radioactively labeled ssDNA ends in proliferating cells, pulse-treated with [^3H]thymidine immediately prior to mock-treatment or UVC exposure (Fig. 1C). Previously we have used this assay to show that MEFs with single defects in Pols η , ι and κ are not measurably defective in replication of undamaged DNA templates (Temviriyankul *et al.*, 2012). Interestingly, Pol η -deficient MEF lines with additional defects in Pol ι , Pol κ or both TLS Pols replicate undamaged DNA templates somewhat less efficiently compared to the Pol η single-mutant cells, indicating a defect in TLS endogenous DNA lesions (Fig. 1D, left panel). Upon exposure to 5 J/m 2 UVC, the MEF line deficient for both Pols η and ι displayed a similar replication fork progression as the Pol η single-mutant cells, indicating that fork progression on UV-damaged DNA in Pol η -deficient MEFs does not rely on Pol ι . Conversely, as compared with Pol η single-mutant MEFs, the MEF line deficient for both Pols η and κ displayed strongly reduced fork progression following exposure to UVC (Fig. 1D, right panel). This defective fork progression was not exacerbated by an additional deficiency for Pol ι in these cells. Together, these data strongly suggest that Pol κ , but not Pol ι , can complement the Pol η defect also at later time points after UVC exposure. Nevertheless, also in Pol η , Pol κ doubly-deficient MEFs replicons continue to progress, albeit slowly, revealing that Pol κ is important but not essential for replicative bypass of photolesions in the absence of Pol η .

We wanted to provide an independent approach to study the possible roles of Pols ι and κ as backup polymerases in Pol η -deficient MEFs at later time points after exposure. To this aim, we utilized a sensitive alkaline sucrose gradient-based assay that measures the maturation of newly synthesized daughter strands, specifically beyond the most prevalent genomic CPD lesions, of which the density is represented by the internal ([^{14}C]thymidine-labelled) standard (Fig. 1E). As expected, the generation of nascent DNA molecules was delayed in Pol η -deficient MEFs compared with wild type cells, especially at 2h after exposure (Fig. 1F). At this time point, maturation of nascent DNA in MEFs deficient for both Pols η and ι was indistinguishable from the single Pol η -deficient MEFs, consistent with the alkaline DNA unwinding assay. The defect of the Pol η -deficient MEFs, however, was aggravated when these cells are also deficient

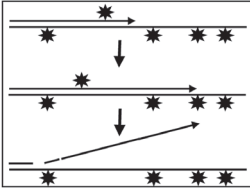
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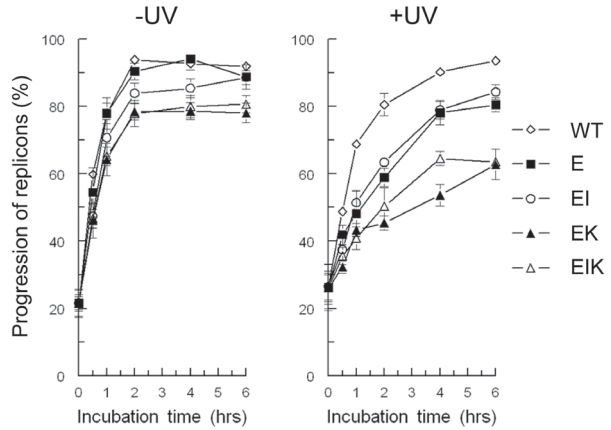
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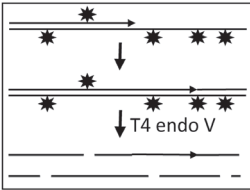
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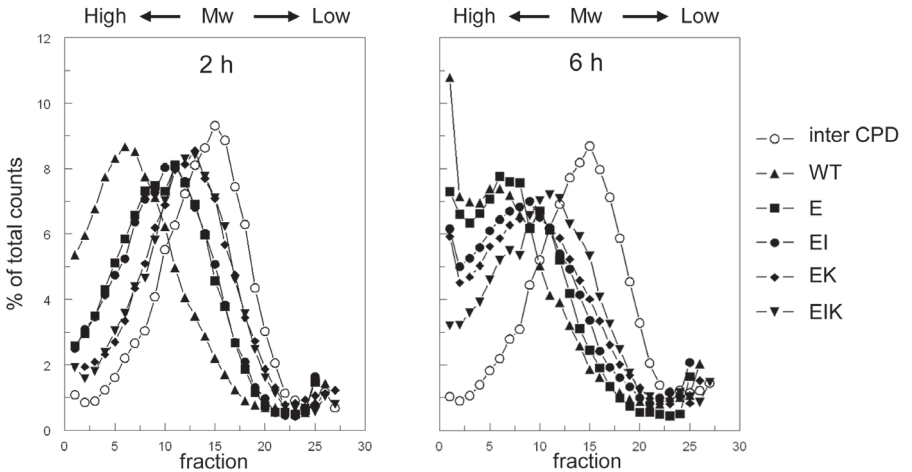
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for Polk (Fig. 1F, left panel). Compared with the MEF line deficient for both Pols η and κ , the triple-mutant MEF line shows a similar deficiency in generating nascent DNA molecules at 2h after UVC exposure (Fig. 1F, left panel). We conclude that, at 2h after UVC exposure, Polk, but not Polt, can complement for the Pol η deficiency. At 6h post-exposure, the percentage of mature DNA molecules was reduced not only in the Pol η + Polk double mutant, but also in the Pol η +Polt double mutant, compared with the Pol η single mutant. In the triple mutant, daughter strand maturation was reduced to an even greater extent (Fig. 1F, right panel). The specific defect in the cell lines with a Polt defect at 6h after treatment suggests that, at least in the absence of Pol η , Polt is required for TLS at a non-abundant lesion type, whereas Polk can complement for the Pol η deficiency in TLS at most photolesions.

Polk Rescues S-phase Progression of Pol η -Deficient MEFs

To investigate redundancy in the roles of Pol η , Polt and Polk in cell cycle progression upon UVC exposure, we determined the incorporation of the nucleotide analog Bromodeoxyuridine (BrdU) in different cell cycle stages of asynchronously growing MEFs. Thus, at different times after mock-treatment or after exposure to 5 J/m² UVC, MEFs were pulse-labeled with BrdU, immediately preceding their fixation. Subsequently, BrdU contents were analyzed by bivariate flow cytometry. Since MEFs deficient for Polt or κ display cell cycle progression similar to wild type MEFs (Temviriyankul *et al.*, 2012), we focused on wild type MEFs and Pol η -deficient MEFs with and without additional deficiencies for Pols ι and/or κ . No major differences were found between the cell lines after mock treatment, indicating that all tested MEF lines proliferate in

◀ **Figure 1 | Both Pols ι and κ are required for replicon progression in Pol η -deficient MEFs, late after UVC exposure.** (A) Schematic representation of DNA fiber labeling with nucleotide analogs CldU and IdU in MEFs that were mock treated (-UV) or exposed to UVC (+UV). (B) Box plot depicting the ratio of lengths of CldU-labeled tracts to IdU-labeled tracts in wild type MEFs (WT) or in MEFs with single, double or triple deficiencies in Pol η (E), Polt (I) and Polk (K), mock treated (-) or exposed to 13J/m² UVC (+). (C) Scheme of the alkaline DNA unwinding assay. Nascent DNA is pulse labelled with [³H]thymidine (dotted line) immediately before the induction of photolesions (triangles) (top). MEFs are then cultured in medium without label (middle). Stalling of a fork at a photolesion results in a DNA end containing [³H]thymidine that is locally denatured using alkaline, followed by sonication and isolation of [³H]thymidine-labelled ssDNA using hydroxyl apatite (bottom). (D) Replication fork progression in mock-treated MEFs (left panel) and in MEFs exposed to 5J/m² UVC (right panel) ($n=4$). Error bar, SEM. (E) Scheme of alkaline sucrose gradient sedimentation using T4 endonuclease V. Template DNA was uniformly labelled with [¹⁴C]thymidine (solid line) followed by exposure to UVC inducing CPD and (6-4)PP photolesions (triangles; top). Elongating daughter strands were pulse labelled with [³H]-thymidine for 30 min (dotted line) and cultured in medium without label (dashed line; middle). At different times, cells were lysed and [¹⁴C]thymidine-containing DNA was cleaved by T4 endonuclease V at a CPD, followed by size fractionation using alkaline sucrose gradients (bottom). The [¹⁴C]thymidine-labelled inter-CPD size distribution serves as an internal standard, since CPDs are not removed in mouse cells. (F) Alkaline sucrose gradient profiles of [³H]thymidine-containing DNA of wild type MEFs (WT, closed triangle), MEFs deficient in Pol η (E; closed square) and of Pol η -deficient MEFs containing an additional defect in Polt (EI; closed circle), Polk (EK; closed diamond) or both TLS polymerases (EIK; closed inverted triangle) at 2 and 6h after exposure to 5J/m² UVC. Also the profile of [¹⁴C]thymidine labelled, CPD-containing fragments is depicted (open circles). Mw, molecular weight.

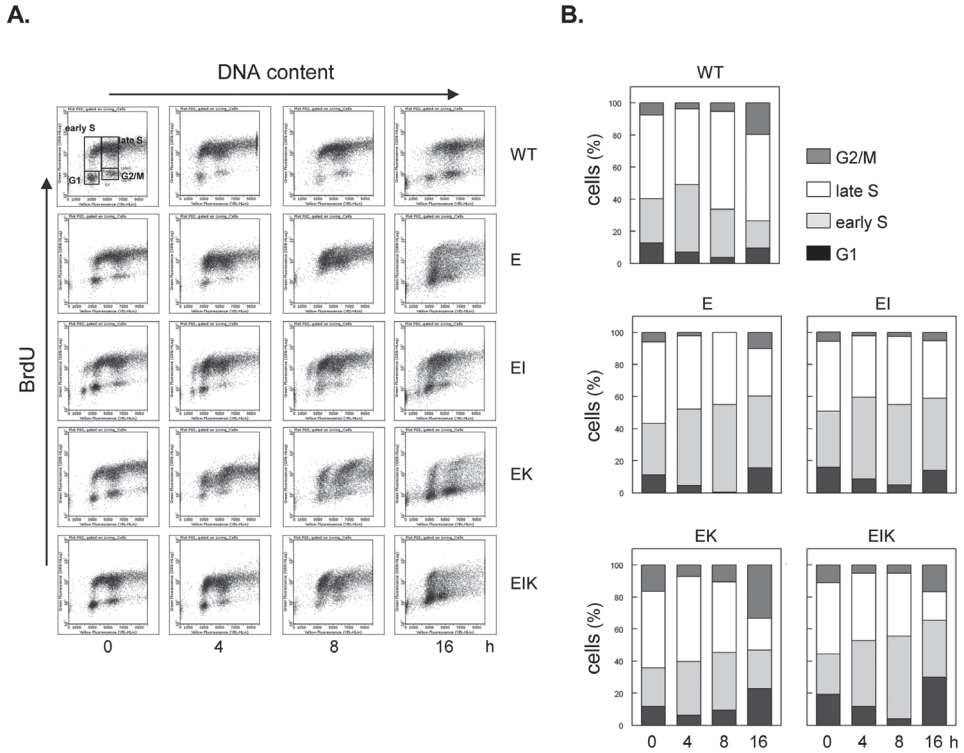


Figure 2 | The replicative activity of Pol η -deficient MEFs exposed to UVC relies mainly on Pol κ . (A) FACS profiles showing BrdU content of wild type MEFs (WT), MEFs deficient in Pol η (E) and Pol η -deficient MEFs containing an additional defect in Pol ι (EI), Pol κ (EK) or both TLS polymerases (EIK) after exposure to 5J/m² UVC. Prior to fixation, MEFs were pulse labelled with BrdU for 30 min, immediately or at 4, 8 and 16h after UVC treatment. BrdU incorporation was determined by immunostaining and DNA content was measured using propidium iodide. (B) Quantification of MEFs in different cell cycle stages, up to 16h after UVC exposure.

a similar fashion in the absence of photolesions (Supplementary Fig. S2). Exposure of cells to UVC, however, revealed marked differences in cell cycle distribution between the MEF lines (Figs. 2A and B). More specifically, compared with UVC-exposed wild type MEFs, Pol η -deficient MEFs displayed increased levels of (early) S phase cells containing low-to-intermediate levels of BrdU (Figs. 2A and B). This phenotype was unaltered when also Pol ι was disrupted indicating that Pol ι does not serve as a quantitatively important backup to Pol η (Figs. 2A and B). In both Pol η -deficient MEFs and MEFs double deficient for Pol η and Pol ι this response was aggravated by the additional deficiency for Pol κ (Figs. 2A and B). Together, these results suggest that S phase progression is perturbed in the absence of Pol η , both early (Fig. 1) and late after UVC exposure (Figs. 2A and B). The residual S phase progression in these cells strongly depends on Pol κ , rather than on Pol ι .

Low Level of Double Strand DNA Breaks in MEFs Undergoing Replication Stress

Persistently stalled replication forks may collapse to double-strand DNA (dsDNA) breaks, underlying genome instability (Lundin *et al.*, 2002; Petermann *et al.*, 2010; Saintigny *et al.*, 2001). To investigate the collapse of replication forks in the mutant cell lines, we assayed for phosphorylation of ataxia-telangiectasia-mutated (ATM) and of heterochromatic KRAB-ZFP-associated protein 1 (Kap1) in MEFs treated with UVC during S phase, as assessed by EdU incorporation, by immunostaining. Phosphorylation of ATM (ATM^{S1981-P}) is an early step in the response to dsDNA breaks (Shiloh *et al.*, 2013). Activation of Atm leads to phosphorylation of Kap1 at S824 (Kap1^{S824-P}), although the formation of Kap1^{S824-P} can also be mediated by other phosphatidylinositol-3 kinase-like kinases, including Atr (White *et al.*, 2006). Except for the Rev1-deficient MEFs, we found only a minor UVC-dependent increase of ATM^{S1981-P} foci in nuclei of all other MEF lines tested, up to 8h after UVC exposure (Figs. 3A and B), suggesting that only few forks collapse at the UVC dose used (5J/m²). Interestingly, strong induction of Kap1^{S824-P} was found in all Polη-defective MEF lines and in MEFs deficient for Rev1, already 2h after UVC exposure (see also below). Of note, with the exception of the Rev1-deficient MEFs, Kap1^{S824-P} levels did not increase beyond 2h after exposure, in agreement with the residual photolesion bypass in these cell lines (Fig. 1).

Quenching of The UV-Induced DNA Damage Response Requires Pols η, ι and κ

We stained the cell lines for phosphorylation of Chk1 (Chk1^{S345-P}) a target for Atr-induced DNA damage signaling at ssDNA tracts (Cimprich *et al.*, 2008). Thus, at different time points prior to staining, cells were exposed to 5 J/m² UVC and immediately pulse-labeled with EdU. We included Rev1-deficient MEFs as a positive control, since these cells exhibit strong and persistent Atr/Chk1 signaling following UVC exposure (Jansen *et al.*, 2009; Temviriyanukul *et al.*, 2012). At 2h after exposure, all MEF lines displayed Chk1^{S345-P}-positive cells among EdU-positive (replicating) cells. The intensity of Chk1^{S345-P} staining in EdU-positive double deficient MEF lines, and to an even greater extent in the triple-deficient line, was higher than in wild type cells and cells deficient for Polι or Polκ (Figs. 4A and B). Furthermore, it should be noted that the extent of Chk1^{S345-P} correlated with that of Kap1^{S824-P} in the different MEF lines, although Kap1^{S824-P} is restricted to a subset of EdU-positive cells (compare Figs. 4A, B with Figs. 3C, D). This result suggests that the formation of Kap1^{S824-P} rather is due to Atr signaling than to the formation of dsDNA breaks. To confirm the presence of ssDNA tracts, we assessed the recruitment to chromatin of the heterotrimeric Replication Protein A (Rpa), which binds to ssDNA and recruits Atr. We observed a similar distinction between the MEF lines with respect to the level of Rpa as shown for Chk1^{S345-P} (see Figs. 4C and D). These results are in agreement with the pronounced replicon stalling in these MEF lines as observed in the replication assays (Fig. 1). At 8h after UVC exposure, the intensity of Rpa staining dropped considerably in all MEF lines, except in the Rev1-mutant and in the triple-mutant MEFs (Figs. 4C and D). In

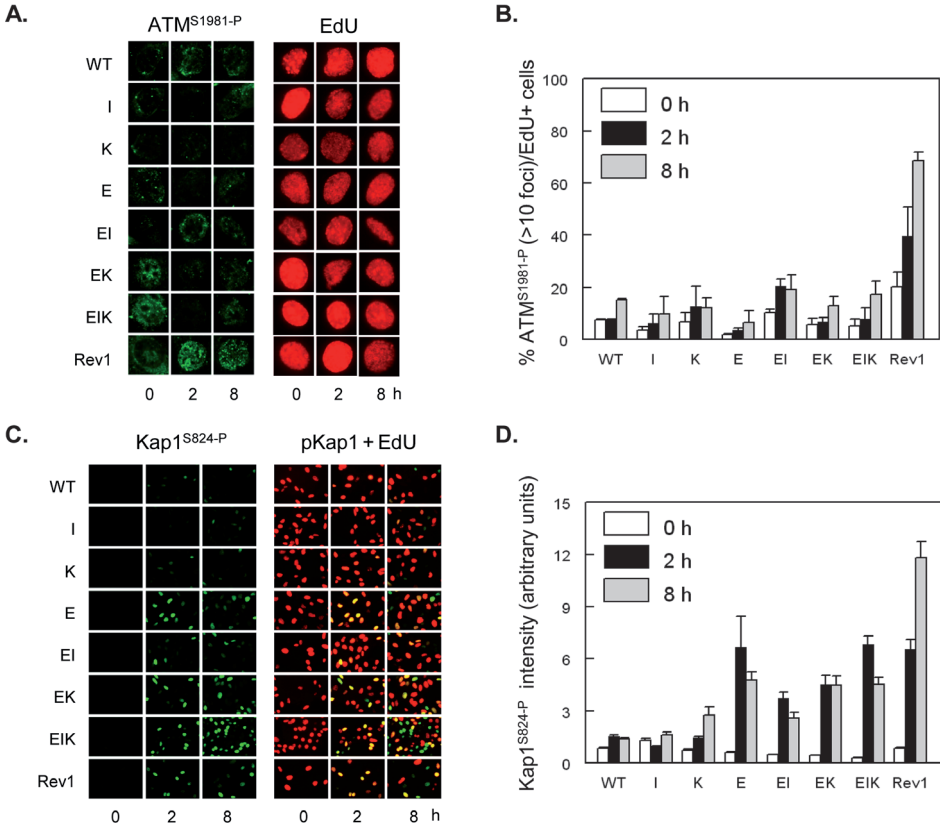


Figure 3 | Few UVC-induced dsDNA breaks in Pol η -deficient MEFs with or without additional deficiencies in Pols ι and κ . (A) Wild type MEFs (WT), MEFs with single, double or triple deficiencies in Pol η (E), Pol ι (I) and Pol κ (K), or MEFs deficient in Rev1 (Rev1) were pulse labelled with EdU for 30 min, prior to exposure to 5J/m² UVC. Then, MEFs were fixed at 0, 2 and 8h after treatment and immunostained for ATM^{S1981-P} (green, left panel) in replicating, EdU-incorporating MEFs (red) at the time of UVC exposure. (B) Quantification of EdU-positive MEFs containing at least 10 ATM^{S1981-P} foci. Error bar, SEM. (C) Similar experiment as in (A), except that MEFs were immunostained for Kap1^{S824-P} (left panels, green) in replicating, EdU-incorporating MEFs (right panels, merge of staining for Kap1^{S824-P} (green) + EdU (red)). (D) Quantification of the intensity of Kap1^{S824-P} signal in EdU-positive MEFs. Error bars, SEM.

conclusion, DNA damage signaling in these mutant cell lines qualitatively reflected their defect in TLS, suggesting that ssDNA at stalled replication forks is the primary determinant of DNA damage responses.

Polk Protects Pol η -Deficient MEFs from UVC Toxicity

To study the biological consequences of prolonged replication fork stalling, enhanced DNA damage signaling and impaired cell cycle progression, caused by defects in multiple Y family Pols, we analyzed cell proliferation at 3 days after exposure to various

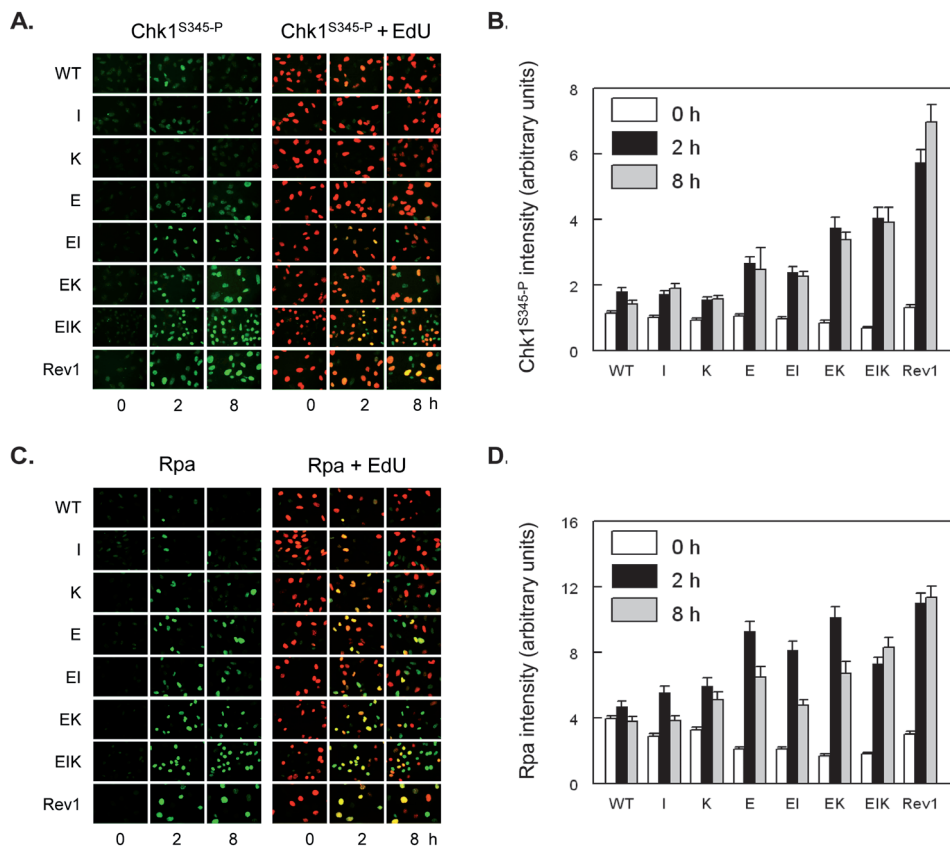


Figure 4 | Quenching of DNA damage responses to photolesions requires Pols η , ι and κ . (A) Wild type MEFs (WT), MEFs with single, double or triple deficiencies in Pol η (E), Pol ι (I) and Pol κ (K), or MEFs deficient in Rev1 (Rev1) were pulse labelled with EdU for 30 min, prior to exposure to 5J/m² UVC. Then, MEFs were fixed at 0, 2 and 8h after treatment and immunostained for Chk1^{S345-P} (left panels, green) in replicating, EdU-incorporating MEFs (right panels, merge of staining for Chk1^{S345-P} (green) and EdU (red)) at the time of UVC exposure. (B) Quantification of the intensity of Chk1^{S345-P} staining in EdU-positive MEFs. Error bar, SEM. (C) Similar experiment as in (A), except that MEFs were immunostained for Rpa (left panels, green). (D) Quantification of the intensity of Rpa staining in EdU-positive MEFs. Error bars, SEM.

doses of UVC. Amongst the MEF lines tested, MEFs deficient for both Pols η and κ as well as the triple mutant MEFs displayed the highest sensitivity to UVC light, whereas Pol η -deficient MEFs and MEFs deficient for both Pols η and ι showed an intermediate UVC sensitivity (Fig. 5). Confirming previous observations (Temviriyankul *et al.*, 2012), the MEF line deficient for Pol κ was slightly more sensitive to UVC light than wild type MEFs, whereas Pol ι -deficient MEFs displayed no increased UVC sensitivity (Fig. 5). These results are again consistent with an important role for Pol κ as a backup TLS polymerase for Pol η .

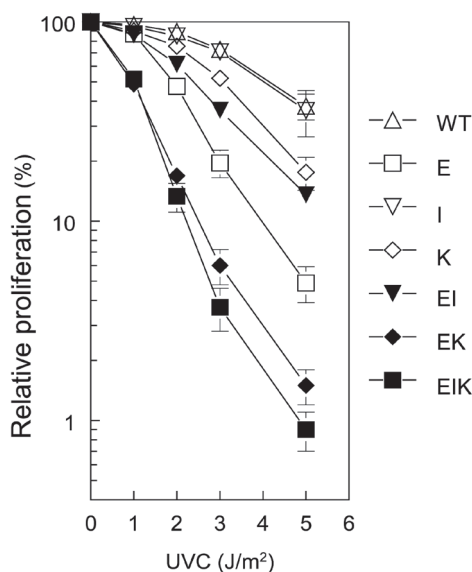


Figure 5 | Effect of UVC on proliferation of MEFs with single, double or triple deficiencies in Pols η , ι and κ . MEFs were exposed to different doses of UVC and 3 days later the number of cells was assessed. The number of unexposed MEFs was set at 100%. Error bars, SEM.

Genomic CPDs are Substrates for Pols ι and κ in Pol η -Deficient MEFs

By employing a novel immunostaining protocol using monoclonal antibodies that recognize CPDs or (6-4)PPs only when embedded in ssDNA we have previously observed that, in Pol η -deficient MEFs, mainly CPDs cause stalling of replication forks (Temviriyankul *et al.*, 2012). We applied this methodology to the current set of MEF lines to study which genomic photolesions are causing the phenotypes associated with MEFs deficient for both Pols η and κ and with the triple mutant MEFs. Thus, cells were pulse-labeled with EdU, to identify the cells that were replicating during UVC exposure, and exposed to 5 J/m² UVC. At 2h or 8h after UVC exposure, cells were fixed and immunostained for CPDs, or for (6-4)PPs, embedded in ssDNA.

Almost no cells positive for CPDs were detected in wild type MEFs and MEFs deficient for Pol ι or κ , indicating efficient bypass across genomic CPDs, independent of Pols ι and κ (Fig. 6A). As expected, EdU-positive MEFs that are deficient for Pol η displayed unreplacated CPDs at 2h and less at 8h after UVC exposure (Fig. 6A and (Temviriyankul *et al.*, 2012)), suggesting transient fork stalling at CPDs. Similar results were observed for Pol η mutant MEFs with an additional deficiency for Pol ι (Fig. 6A). MEFs deficient for both Pols η and κ displayed more EdU+CPD positive cells at 8h upon UVC exposure, indicating that Pol κ does perform TLS at CPDs in the absence of Pol η . Nevertheless, EdU-positive triple-mutant MEFs exhibited the most pronounced staining for unreplacated CPDs at 8h after UVC exposure (Fig. 6A). This

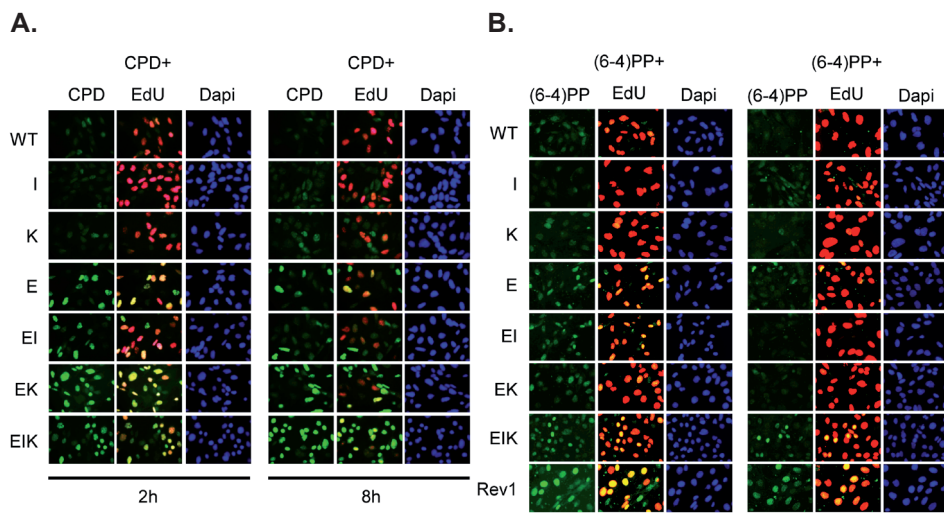


Figure 6 | Pols ι and κ are required for efficient bypass of photoproducts in MEFs deficient in Pol η . (A) Wild type MEFs (WT) or MEFs with single, double or triple deficiencies in Pol η (E), Pol ι (I) and Pol κ (K) were pulse labelled with EdU for 30 min, prior to exposure to 5J/m² UVC. Two and eight hours later, MEFs were fixed and immunostained for CPD (green; upper panels) in ssDNA of nuclei (blue; lower panel) in replicating, EdU-incorporating (red, merged with CPD staining; middle panels) and non-replicating MEFs at the time of UVC exposure. (B) Similar as in (A) except that MEFs were immunostained for (6-4)PP (green; upper panel). Rev1-deficient MEFs (Rev1) were included as a positive control (Jansen *et al.*, 2009; Temviriyankul *et al.*, 2012).

result suggests that, in the absence of Pol η , Pol ι can perform TLS at CPDs, but only when also Pol κ is inactive.

All Y Family Polymerases Contribute to TLS of (6-4)PPs

In contrast to CPD lesions, (6-4)PPs impose a strong helical distortion to the DNA, and the 3' pyrimidine base in the pyrimidine dimer is twisted outwards and unable to engage in base-pairing (Rastogi *et al.*, 2010). Thus far, it has been largely unclear what TLS polymerases are responsible for bypass of these 'severe' lesions at the genome of mammalian cells, although we have previously described a regulatory role for Rev1 (Jansen *et al.*, 2009). Immunostaining of unreplicated (6-4)PPs in EdU-positive cells revealed that TLS of (6-4)PPs appeared perturbed in MEF lines defective in Pol η , as judged by the EdU-positive cells staining for single-stranded (6-4)PPs, at 2h after UVC exposure of these cells (Fig. 6B). Nevertheless, the defect was less pronounced than in MEFs deficient for Rev1 [(Jansen *et al.*, 2009) and Fig. 6B)]. Staining for unreplicated (6-4)PPs was also found for double and, to a greater extent, triple mutant MEFs, at 2 and 8 hours after UVC exposure (Fig. 6B). These results are the first to demonstrate the involvement of these Y-family polymerases in TLS of genomic (6-4)PP and, moreover, suggest that in the absence of Pol η , both Pols ι and κ act as backup TLS polymerases to replicate across (6-4)PPs.

DISCUSSION

In this study, we have comprehensively analyzed the contributions of the three Y family Pols η , ι and κ in TLS, S phase progression, DNA damage signaling, checkpoint activation and survival in response to genomic CPD and (6-4)PP lesions, using single, double, and triple-deficient MEFs. Our results demonstrate that, in the absence of Pol η , Pol κ plays a more important role than Pol ι in responses to genomic photolesions. In support, we and others have shown that Pol κ (but not Pol ι)-deficient mammalian cells are slightly sensitive to UVC light (Ogi *et al.*, 2002; Schenten *et al.*, 2002; Temviriyankul *et al.*, 2012). Although some studies attribute this sensitivity to a defect in NER, at least outside of S phase (Ogi *et al.*, 2006; Ogi *et al.*, 2010), others, using siRNA strategies, provide evidence for a role of Pol κ in TLS of a T.T CPD on episomal substrates *in vivo* (Yoon *et al.*, 2009; Ziv *et al.*, 2009) while no effect was found on TLS across TT (6-4)PP (Yoon *et al.*, 2010). Indeed, Pol κ can extend from a nucleotide inserted across 3'Ts of TT CPDs by another DNA polymerase (Washington *et al.*, 2002). This TLS-related function of Pol κ on abundantly-induced TT CPDs explains the strong defects in replication fork progression, increased staining for CPDs, enhanced DNA damage signaling, slow progression through S phase, and reduced cell proliferation observed in UVC-exposed MEFs deficient for both Pols η and κ . The role of Pol κ as backup for Pol η is not restricted to UV lesions, since also in somatic hypermutation of Immunoglobulin genes Pol κ acts as backup in the absence of Pol η (Faili *et al.*, 2009).

Pol ι plays only a minor role in TLS at genomic photolesions in Pol η -deficient cells, which only is apparent from the delayed maturation of nascent strands upon UVC exposure. However, the triple mutant MEFs displayed the most pronounced phenotypes of all cell lines tested in this study, suggesting that Pol ι is essential for TLS across some UVC-induced DNA lesions in the absence of both Pol η and Pol κ . In support, purified Pol ι can replicate TT (6-4)PPs (Haracska *et al.*, 2001; Vaisman *et al.*, 2003; Zhang *et al.*, 2001) and Pol ι mediates part of the mutagenicity of (6-4)PP *in vivo* (Dumstorf *et al.*, 2006; Yoon *et al.*, 2010). In addition to a subset of (6-4)PPs, also some CPDs might be candidates for Pol ι -mediated TLS in MEFs deficient for both Pols η and κ . Indeed, Pol ι is capable to inserting nucleotides opposite TT CPDs *in vitro* (Haracska *et al.*, 2001; Vaisman *et al.*, 2003; Zhang *et al.*, 2001) although CPDs on an episomal substrate are only poorly bypassed by Pol ι in human (Pol η -deficient) XP-V cells (Yoon *et al.*, 2009; Ziv *et al.*, 2009). Of note, the efficiency by which UV photolesions are induced in the genome strongly depends on the dipyrimidine sequence. Thus, the order of preference for the formation of CPDs is TT > CT = TC > CC, whereas (6-4)PPs are mostly induced at TC and CC dipyrimidines, to a lesser extent at TT dimer sites and not at CT sites (Brash, 1988; Lippke *et al.*, 1981; Mitchell *et al.*, 1992; Rastogi *et al.*, 2010; Tornaletti *et al.*, 1993). Moreover, as these lesion types are structurally highly dissimilar (Rastogi *et al.*, 2010), they may require different sets of TLS polymerases to allow efficient lesion bypass during DNA replication.

In triple-mutant MEFs replication forks are permanently stalled only late after UVC exposure. This indicates that some photolesions can be bypassed independently from

the three Y family polymerases. Thus, the B family TLS Pol ζ or the recently described archaeal-eukaryotic primase called Primase-Polymerase may play a role in an alternate backup TLS pathway (Bianchi *et al.*, 2013; Mouron *et al.*, 2013; Yoon *et al.*, 2009). Nevertheless, as persistent CPDs and (6-4)PPs are observed in the triple-deficient cells, we infer that bypass of some lesions fully depends on the three Y family Pols.

In conclusion, we have unveiled important but redundant roles for the three Y family of TLS polymerases in TLS of genomic CPD and (6-4)PP photolesions. Polk appears the most important backup to Pol η although, to a minor extent, Polt also functions as backup. Nevertheless, also in the triple mutant most photolesions are ultimately bypassed, implicating the existence of yet other redundant pathways.

MATERIALS & METHODS

Cell culture. MEFs lacking Pol η , Polt or Polk were isolated from day 13.5 embryos of Pol η , Polt or Polk-deficient mice (Delbos *et al.*, 2005; Schenten *et al.*, 2002); Aoufouchi *et al.*, in preparation). Crossings between Pol η , Polt and Polk-deficient mice produced 13.5-day embryo that were doubly-deficient for Pol η and Polt, for Pol η and Polk or for all three Pols. From these embryos MEFs were isolated and immortalized following transfection of SV40 large T antigen. Immortalized MEFs homozygous for a targeted disruption of Rev1 were described previously (Jansen *et al.*, 2009). All MEF lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/liter glucose, Glutamax and pyruvate (Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) (MEF medium) at 37°C in a humidified atmosphere containing 5% CO₂.

DNA fiber analysis. Per well of a 6-well plate, 7.5×10^4 MEFs were seeded and cultured overnight in MEF medium. Prior to UVC exposure (13 J/m²), MEFs were incubated in medium containing 25 μ M 5-Chloro-2'-deoxyuridine (CldU) for 20 min at 37°C. After UVC exposure, medium containing 500 μ M 5-Iodo-2'-deoxyuridine (IdU) was added, resulting in a final concentration of 250 μ M IdU and 12.5 μ M CldU. After 20 min at 37°C, cells were trypsinized, 2 μ l of a suspension of 3×10^5 MEFs/ml was spotted onto a microscope slide, incubated for 5 min and lysed with 7 μ l lysis buffer (200mM Tris-HCl pH7.4, 50mM EDTA, 0.5% SDS) for 3 min. Slides were tilted to 15° C to allow the DNA to run down the slide. Next, slides were air dried and subsequently fixed in methanol-acetic acid (3:1). After rehydration, fixed DNA fibers were denatured in 2.5M HCl for 75 min. Incorporation of CldU was detected using rat- α -BrdU antibodies (1:500; BU1/75, AbD Serotec) and Alexafluor-555-labeled goat- α -rat antibodies (1:500; Molecular Probes), whereas incorporated IdU was detected using mouse- α -BrdU antibodies (1:750; Clone B44, BD) and Alexafluor-488-labeled goat- α -mouse antibodies (1:500; Molecular Probes). Finally, slides were mounted in Fluoro-Gel (Electron Microscopy Sciences). Microscopy was performed using a fluorescent microscope (Zeiss).

Alkaline DNA unwinding (ADU). This assay, which measures progression of replicons (Johansson *et al.*, 2004) was performed with minor modifications. The procedure is outlined in Fig. 1C. Per well, 5×10^4 MEFs were plated in a 24-well plate and cultured overnight in MEF medium. After pulse labeling with [³H]thymidine for 15 min, MEFs were washed once with PBS and subsequently exposed to 5 J/m² UVC or mock-treated. Then, at indicated times, DNA at replication forks was locally denatured upon incubation of MEFs with ice-cold denaturation solution (0.15M NaCl and 0.03M NaOH) for 30 min. The denaturation of DNA was terminated by adding ice-cold 0.02M NaH₂PO₄. After sonication, SDS was added to a final concentration

of 0.25% and the samples were stored at -20°C for at least 16h. After thawing, the lysates were loaded onto hydroxyl apatite columns to elute ssDNA using $0.1\text{M K}_2\text{HPO}_4$ (pH6.8) and dsDNA using $0.3\text{M K}_2\text{HPO}_4$ (pH6.8), respectively. Radioactivity in each eluate was determined by liquid scintillation counting (PerkinElmer). Replication progression was calculated by determining the ratio of radioactivity in total DNA: ssDNA.

Alkaline sucrose gradients. The replicative bypass of genomic CPDs and the increase in molecular mass of elongating nascent DNA molecules in MEFs exposed to 5 J/m^2 UVC was determined by a sensitive variant of the alkaline sucrose sedimentation assay as described previously (van Zeeland *et al.*, 1981). The procedure is outlined in Fig. 1E.

Immunostaining. MEFs were cultured overnight on coverslips, incubated with $10\mu\text{M}$ 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen) in MEF medium for 30 min and subsequently exposed to UVC irradiation (5 J/m^2). At indicated times after UVC treatment, cells were fixed and permeabilized as follows: for detection of Rpa, Chk1^{S345-P} and Kap1^{S824-P}, cells were pre-extracted and permeabilized by 0.3% Triton-X in CSK buffer pH7.2-7.5 (10mM HEPES pH7.4, 100mM NaCl, 3mM MgCl₂, 0.3% triton-X100, 300mM sucrose) for 2 min on ice and immediately fixed with 3.7% Paraformaldehyde for 20 min; for detection of ATM^{S1981-P}, cells were fixed and permeabilized with ice-cold methanol:acetone (1:1) for 10 min at -20°C . Cells were blocked with 3% BSA+0.1% tween-20 for at least 30 min to prevent non-specific binding, and subsequently incubated overnight with antibodies against Rpa (Cell Signaling), Chk1^{S345-P} (Cell Signaling), Kap1^{S824-P} (Bethyl Laboratory) or ATM^{S1981-P} (Rockland Immunochemicals) at 4°C . Then, appropriate fluorescent dye-conjugated secondary antibodies were applied and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). To visualize EdU-positive cells, which represent the S-phase cells at a time of UVC treatment, Alexafluor 647-conjugated azide was used according to the manufacturer's recommendation (Click-iT™ Edu imaging kit, Invitrogen). Samples were mounted (Vectashield, Vector laboratories), and images were acquired by wide field fluorescent microscopy (Axioplan M2, Carl Zeiss). Fluorescence intensity and numbers of foci were quantified using ImageJ software (National Institutes of Health). Between 90-135 nuclei per cell line were analyzed for each time point. Detection of CPDs and (6-4)PPs in single-stranded DNA templates was essentially performed as described (Jansen *et al.*, 2009), except that to enable detection of (6-4)PPs the cells were fixed in 3.7% paraformaldehyde for 15 min after extraction with ice-cold 0.3% triton-X100 in CSK buffer for 2 min.

Bivariate cell cycle analysis. Cell cycle progression of MEFs, exposed to 5 J/m^2 UVC or mock-treated and pulse-labeled with BrdU, 30 min prior to fixing the cells, was determined by bivariate cell cycle analysis essentially as described previously (Temviriyankul *et al.*, 2012).

Cell proliferation assay. Proliferation of MEFs was determined 3 days after mock-treatment or exposure to various doses of UVC light (Philips T UVC lamp, predominantly 254 nm) as described previously (Temviriyankul *et al.*, 2012).

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SUPPLEMENTARY FIGURES

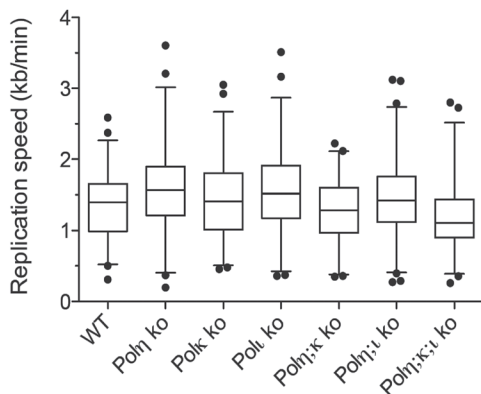


Figure S1 | MEFs with deficiencies in different TLS polymerases display similar replication speed. Lengths of CldU-labeled tracts in DNA fibers of unexposed wild type MEFs (WT) and MEFs with single, double or triple deficiencies in Pol η (η), Pol ι (ι) and Pol κ (κ) were determined. Then, the replication speed was calculated and depicted as box plots.

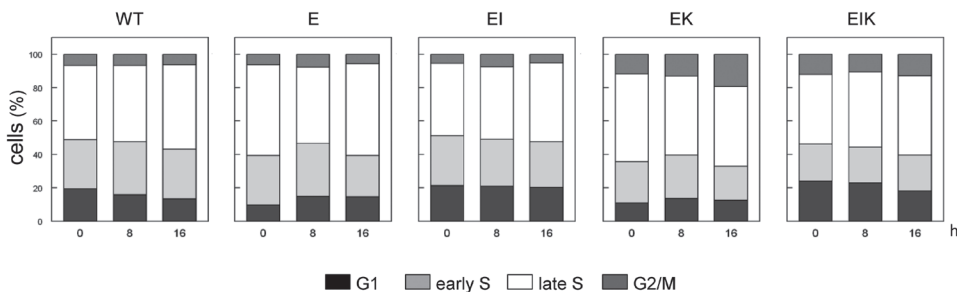


Figure S2 | MEFs with deficiencies in different TLS polymerases proliferate in a similar fashion in the absence of Photolesion. FACS profiles were generated of wild type MEFs (WT), MEFs deficient in Pol η (E) and Pol η -deficient MEFs containing an additional defect in Pol ι (EI), Pol κ (EK) or both TLS polymerases (EIK) that were pulse labelled with BrdU for 30 min, immediately or at 4, 8 and 16h after mock treatment. BrdU incorporation was determined by immunostaining and DNA content was measured using propidium iodide. The percentage of cells in different cell cycle stages was determined, up to 16h after mock treatment.

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CHAPTER

5

GENOME INSTABILITY FOLLOWING MITOTIC TRANSMISSION OF UNREPLICATED DNA LESIONS

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ABSTRACT

Replication stress can lead to the generation of double stranded DNA breaks (DSBs) that are correlated with loss of genetic information, structural chromosomal aberrations and tumorigenesis. Here, we have investigated the mechanistic basis of these structural alterations using mammalian cells defective in nucleotide excision repair and post-replicative bypass of DNA lesions as models. We show that, upon treatment with ultraviolet (UV) light, cells accumulate patches of ssDNA containing (6-4)pyrimidine pyrimidone photoproducts ((6-4)PPs) in genomic DNA. Rather than rapidly collapsing into DSBs, these ssDNA gaps are transmitted through mitosis into the subsequent cell cycle. During the ensuing S phase, the ssDNA gaps are converted into DSBs, leading to genomic instability.

INTRODUCTION

Genomic DNA is continuously damaged by cellular by-products such as oxygen radicals and by exogenous agents such as ultraviolet light (UV), X-rays and genotoxic substances. In replicating cells, DNA lesions that have escaped removal by appropriate DNA repair pathways may form impregnable obstructions for replicative DNA polymerases δ and ϵ , leading to arrested replication forks. Prolonged fork stalling can lead to dissociation of the replisome and fork collapse, resulting in the formation of DNA double-stranded breaks (DSBs) (Chapman *et al.*, 2012). DSBs are believed to arise when stalled replication forks are converted into aberrant DNA structures that are cleaved by structure-specific endonucleases (Hanada *et al.*, 2007; Osman *et al.*, 2007). Persistent DSBs activate DNA damage signaling pathways that induce cell cycle delay, senescence or apoptosis. In addition, the generation of DSBs is strongly correlated with loss of genetic information, as evidenced by the appearance of micronuclei (MN), and with other structural chromosomal aberrations. Such genomic instability is associated with tumorigenesis (Bonassi *et al.*, 2007; Murgia *et al.*, 2008).

To prevent prolonged stalling of replication forks at sites of unrepaired DNA damage, cells are equipped with DNA damage tolerance (DDT) pathways, i.e. DNA damage avoidance (DA) and translesion synthesis (TLS). DDT thereby allows cells to complete replication of damaged DNA templates, precluding genomic instability (Sale *et al.*, 2012; Waters *et al.*, 2009). In bacteria, stalled forks are predominantly and accurately rescued from collapsing by DA, which involves template switching to the undamaged sister chromatid (Berdichevsky *et al.*, 2002; Izhar *et al.*, 2008). In higher eukaryotes TLS appears to be a more dominant DNA damage tolerance pathway (Izhar *et al.*, 2013; Szuts *et al.*, 2008). TLS utilizes specialized DNA polymerases, so-called TLS polymerases, that are capable of inserting nucleotides opposite, and extending beyond, DNA lesions, thereby enabling DNA damage bypass. TLS, though, is an intrinsically error-prone process, due to the extended active sites, the low selectivity of nucleotide incorporation and the lack of proofreading activity of TLS polymerases (Waters *et al.*, 2009). Thus, TLS contributes to cell survival at the cost of genome instability. TLS most likely bypasses mildly helix-distorting DNA lesions directly at the replication fork. Strongly helix-distorting DNA lesions, such as UV-induced (6-4) pyrimidine pyrimidone photoproducts ((6-4)PPs), can be bypassed independently from chromosomal replication, via post-replicative filling of single-stranded DNA (ssDNA) gaps that are caused by repriming of the replisome downstream of the fork-blocking DNA lesion or by convergence of a replication fork from an adjacent origin of replication (Daigaku *et al.*, 2010; Jansen *et al.*, 2009).

The TLS polymerase Rev1 plays a central role in TLS in eukaryotic cells. Rev1 is shown to be essential for the bypass of UV-induced (6-4)PP and of BPDE-induced DNA lesions *in vivo* (Temviriyankul *et al.*, 2012a; Temviriyankul *et al.*, 2012b; Zhao *et al.*, 2006). In addition, cells deficient for Rev1 are hypomutable by different DNA damaging agents, indicating that Rev1 is important for mutagenic TLS across helix-distorting

DNA lesions (Jansen *et al.*, 2009; Washington *et al.*, 2004; Yang *et al.*, 2009; Zhang *et al.*, 2002). It is believed that Rev1 functions mostly as a scaffold protein for other TLS polymerases to perform TLS across fork-blocking DNA lesions (Sale *et al.*, 2012; Waters *et al.*, 2009). Presumably, Rev1 is involved in post-replicative gap-filling in yeast and mammalian cells, since (i) the expression of Rev1 is the highest in the G2 phase in yeast (Waters *et al.*, 2006), (ii) Rev1-dependent TLS can act independent of chromosomal replication (Diamant *et al.*, 2012), and (iii) following UV exposure, Rev1-deficient mouse embryonic fibroblasts (MEFs) accumulate ssDNA gaps containing (6-4)PPs during S phase progression (Jansen *et al.*, 2009).

Recently, we reported that the generation of ssDNA gaps containing (6-4)PPs in proliferating Rev1-deficient MEFs is accompanied with increased DNA damage signaling, a delay in cell cycle progression, enhanced formation of MN and hypersensitivity to UVC light (Temviriyankul *et al.*, 2012b). Thus, Rev1-deficient cells appear good models to study the induction of DNA damage responses and of genome instability, by replication stress. Here, we employed an integrative approach to monitor the fate of ssDNA gaps containing (6-4)PPs and their impact on genomic integrity at well-defined stages during the first and second cell cycles after UVC exposure. To further sensitize these cells to replication stress, MEF lines used here were additionally defective in nucleotide excision repair, by a targeted disruption of the *Xpc* gene (Cheo *et al.*, 1997).

We provide evidence that ssDNA gaps opposite (6-4)PPs can persist in mitotic chromatin. After transfer through mitosis, these lesion-containing gaps are converted into DSBs in the ensuing S phase, which is accompanied with massive DNA damage signaling. These data reveal a novel pathway for the collapse of stalled replication forks. In addition, these results may have profound implications for genotoxicity testing of chemical and physical agents using the MN assay.

RESULTS

Delayed Formation of Micronuclei in *Rev1*^{-/-}*Xpc*^{-/-} MEFs After Exposure to a Low Dose of UVC

To study the fate of stalled forks at UVC-induced (6-4)PPs, we assayed the induction of micronuclei (MN) in *Xpc*^{-/-} and *Rev1*^{-/-}*Xpc*^{-/-} MEFs (Jansen *et al.*, 2009). MN are abnormal extracellular bodies resulting from DSBs-induced chromosome breaks or from mis-segregation of whole chromosomes into daughter cells during cell division. MN thereby are specific and sensitive indicators of chromosome instability, relevant for tumorigenesis (Bonassi *et al.*, 2007; Murgia *et al.*, 2008). MEFs were mock treated or exposed to a low or high dose of UVC (0.4 and 5 J/m²) and immediately cultured in the presence of the cytokinesis-blocking agent cytochalasin-B (cyt-B). Of note, the 0.4 J/m² dose induces similar toxicity in *Rev1*^{-/-}*Xpc*^{-/-} MEFs to the 5 J/m² dose in *Xpc*^{-/-} MEFs (Fig. 1A). After 24h, binucleated (BN) cells, that have undergone mitosis, were

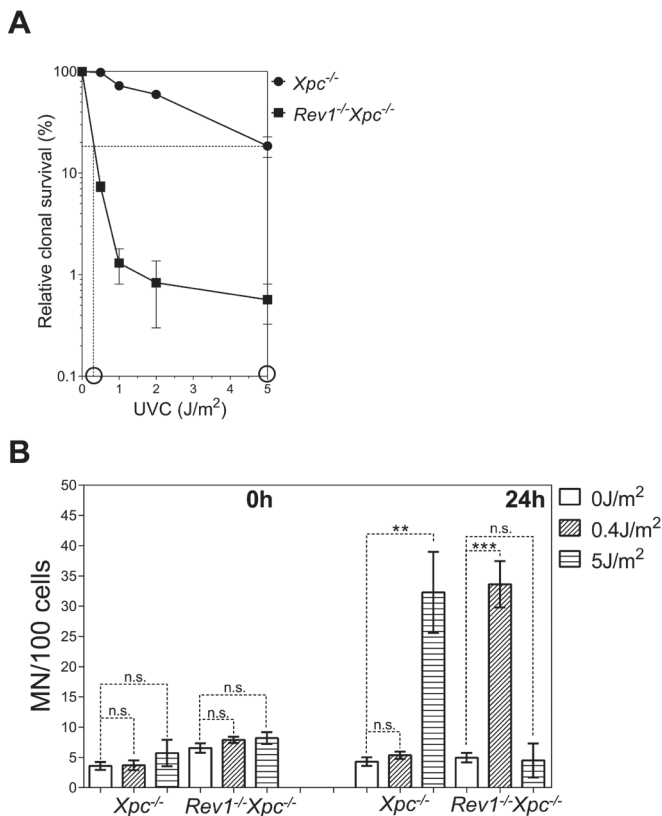


Figure 1 | UVC hypersensitivity and the late formation of MN in *Rev1*^{-/-}*Xpc*^{-/-} MEFs exposed to low doses of UVC. (A) Relative clonogenic survival of cells treated with different doses of UVC. The cloning efficiency of mock-treated cells was set as 100% ($n=3$). Circles indicate an equitoxic UVC dose for *Xpc*^{-/-} and *Rev1*^{-/-}*Xpc*^{-/-} MEFs. Cells were treated with different doses of UVC or mock-treated, and then cyt-B was added, at 0h (left panel) or 24h (right panel) after treatment. Twenty-four hours later, cells were fixed and the induction of MN in binucleated cells was scored. Bars represent the number of MN per 100 cells after exposure to UVC or mock treatment ($n=3$). Error bar, SEM. Statistical significance was analyzed by Student's *t*-test against mock treated. **, $p<0.01$; ***, $p<0.001$, n.s., non significant.

analysed for the induction of MN. Surprisingly, no induction of MN was observed for either cell line following exposure to either dose of UVC (Fig. 1B). We then added cyt-B at only 24h after UVC exposure, followed by an additional incubation of 24h. Under these conditions *Rev1*^{-/-}*Xpc*^{-/-} MEFs, exposed at 0.4J/m², displayed 7-fold more MN than its mock-treated control. *Xpc*^{-/-} MEFs displayed no MN induction at this UVC dose (Fig. 1B). Conversely, at 5J/m² UVC, MN induction in *Xpc*^{-/-} MEFs was approximately 6 times higher than its mock-treated control, whereas *Rev1*^{-/-}*Xpc*^{-/-} MEFs exhibited background levels of MN (Fig. 1B). Together, these results suggest that equitoxic UVC doses induce genome instability, irrespective of the Rev1 status, but in all genetic backgrounds only late after exposure.

Progression of *Rev1*^{-/-}*Xpc*^{-/-} MEFs Into The Second Cell Cycle Upon a Low Dose of UVC

We then investigated cell cycle progression in these MEF lines exposed to 0, 0.4 and 2J/m² UVC. After treatment, replicating cells were pulse-labeled with BrdU, to enable to track cell cycle progression of individual cells. Then, cells were fixed at indicated times. At 8h after mock treatment, all cells progressed to the subsequent G1 phase at a similar rate, suggesting that loss of Rev1 does not affect cell cycle progression of undamaged cells (Fig. 2 and supplementary Fig. S1). Progression of *Xpc*^{-/-} MEFs to the subsequent G1 phase was not affected by exposure to a low dose of 0.4J/m² UVC, whereas exposure to 2J/m² slightly and transiently delayed progression (Fig. 2 and supplementary Fig. S1). *Rev1*^{-/-}*Xpc*^{-/-} MEFs displayed a very transient reduction in the progression towards G1, at 8h after exposure of S phase cells to 0.4J/m² UVC. Very slow recovery and cell cycle progression was seen when *Rev1*^{-/-}*Xpc*^{-/-} MEFs were exposed to 2J/m² UVC, indicating that the DNA damage load irreversibly affects cell cycle progression in *Rev1*^{-/-}*Xpc*^{-/-} MEFs at a higher UVC dose. These results suggest that the delayed induction of MN is correlated with cell cycle progression of damaged cells, upon exposure to equitoxic UVC doses of *Xpc*^{-/-} and *Rev1*^{-/-}*Xpc*^{-/-} MEFs.

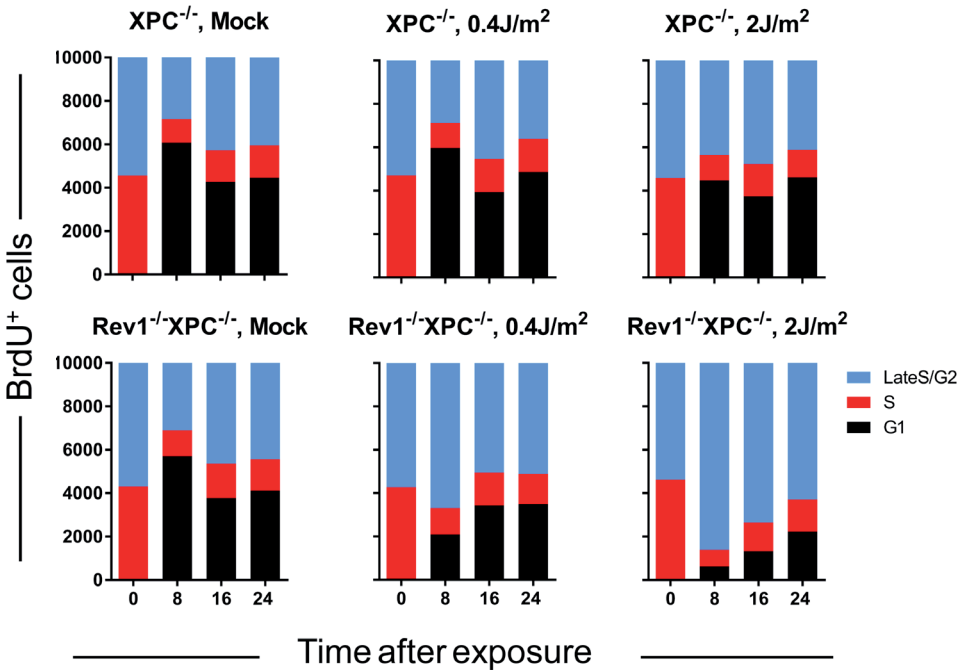


Figure 2 | *Rev1*^{-/-}*Xpc*^{-/-} MEFs can progress to the second cell cycle upon low doses of UVC irradiation. Cells were pulse-labeled with BrdU, immediately after UVC exposure (up to 2J/m²) or mock-treated. Then, cells were analyzed by flow cytometer. BrdU-positive cells in G1, S and late S/G2 phases were quantified, at different times up to 24h after treatment.

UVC Induces Genome Instability Only in The 2nd S phase Upon Exposure

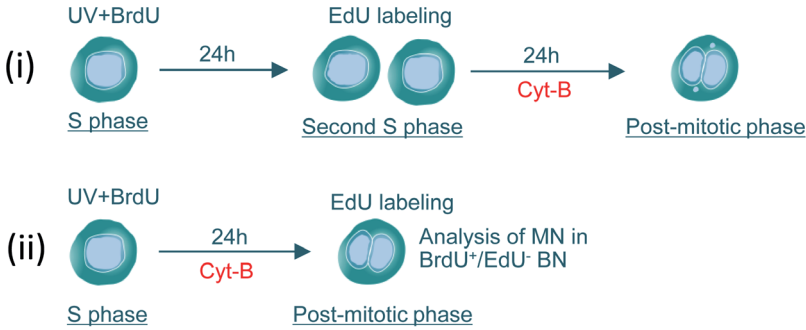
We considered the possibility that UVC-induced genome instability becomes apparent only during the 2nd cell cycle after exposure. This possibility was addressed using a double-labeling MN assay protocol. This protocol relies on the finding that cyt-B inhibits cytokinesis but does not preclude cell cycle progression in daughter nuclei of binucleated cells (Estensen, 1971; Fournier *et al.*, 1975). MEFs were treated with 0 or 0.4J/m² UVC, pulse-labeled with BrdU to mark cells that were replicating during UVC treatment and cultured for 24h in normal medium, allowing the cells to enter the next cell cycle (Fig. 2). Then, the cells were pulse-labeled with the replication marker EdU, to enable to identify cells in the 2nd S phase after exposure, and cultured in the presence of cyt-B for a further period of 24h prior to fixation and analysis of MN induction (Fig. 3A(i) for a scheme). We infer that binucleated cells, double-positive for BrdU and EdU (BrdU⁺/EdU⁺), have finished the 2nd cell cycle upon UVC treatment during S phase.

No induction of MN was seen in BrdU⁺/EdU⁺ *Xpc*^{-/-} control MEFs after either mock or UVC treatment, consistent with the results from the classical MN assay (see above). However, at the same UV dose, binucleated BrdU⁺/EdU⁺ *Rev1*^{-/-}*Xpc*^{-/-} MEFs displayed a significant induction of MN after UVC treatment, *i.e.* about 8 times higher than the mock-treated control and *Xpc*^{-/-} MEFs (Fig. 3B). To exclude the possibility that these MN are derived from DSBs generated in the first, rather than the second, cell cycle, we analyzed MN in MEFs that were exposed to 0 or 0.4J/m² UVC, then pulse-labeled with BrdU, and immediately cultured for only 24h in the presence of cyt-B to enable to visualize MNs in first-cycle cells. Just before fixation, the cells were pulse-labeled with EdU. Binucleated BrdU⁺/EdU⁻ *Rev1*^{-/-}*Xpc*^{-/-} cells have not entered yet the second S phase after UVC exposure (Fig. 3A(ii) for a scheme). In this cell population, the frequency of MN was not increased, excluding the possibility that the MNs arise during the first cycle (Fig. 3C). Together, these results suggest that *Rev1*^{-/-}*Xpc*^{-/-} cells acquire genome instability only during the second cell cycle following exposure during S phase to UVC light.

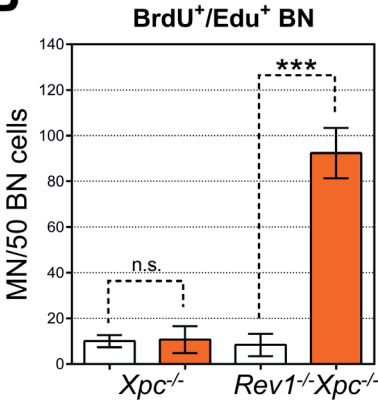
Unreplicated (6-4)PPs Persist Beyond Mitosis

We hypothesized that ssDNA interruptions at (6-4)PP can persist throughout the cell cycle. To investigate this, we first measured DNA strand interruptions in mitotic cells using the alkaline comet assay (McKelvey-Martin *et al.*, 1993). Thus, *Xpc*^{-/-} and *Rev1*^{-/-}*Xpc*^{-/-} MEFs were exposed to 0, 0.4 and 2J/m² UVC followed by pulse labeling with BrdU to mark cells that are replicating during UVC treatment. Cells were then cultured in the presence of nocodazole for 16h to accumulate cells at the first mitosis after exposure. This nocodazole treatment is important to exclude comets of S phase cells from the analysis. Then, cell suspensions were subjected to gel electrophoresis under alkaline conditions and tail moments of BrdU-containing nuclei, representing all DNA strand interruptions, were quantified. For mock-treated cells, both genotypes showed similar tail moments, suggesting the presence of only low amounts of

A



B



C

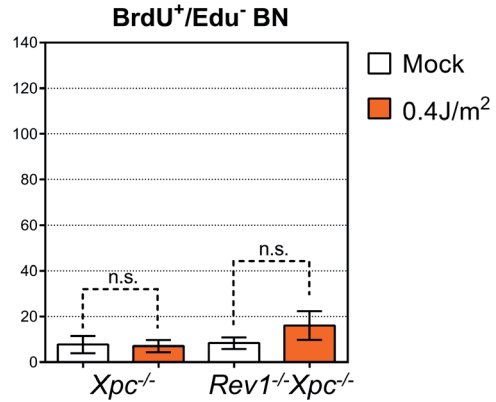


Figure 3 | Induction of DSBs in the 2nd S phase of *Rev1*^{-/-}*Xpc*^{-/-} MEFs exposed to UVC. (A) Experimental schemes to identify BN cells derived from UV-exposed cells that have completed (i) two cell cycles (BrdU⁺/EdU⁺ BN cells) or (ii) one cell cycle (BrdU⁺/EdU⁻ BN cells). (B) Quantification of MN in BrdU⁺/EdU⁺ BN cells. (C) Quantification of MN in BrdU⁺/EdU⁻ BN cells. Bars represent the number of MN per 50 cells after exposure to UVC or mock treatment (*n*=3). Error bar, SEM. Statistical significance was determined by Student's *t*-test. ***, *p*<0.001, n.s., non significant.

DNA strand discontinuities in non-exposed cells (Figs. 4A and B). In *Xpc*^{-/-} MEFs treated with doses of up to 2J/m² of UVC no increase in tail moments was observed. *Rev1*^{-/-}*Xpc*^{-/-} MEFs, however, displayed a UV dose-dependent increase in tail moments in the alkaline comet assay. Neutral comet assays, that detect only DSBs, did not show any increase of tail moments in UVC-exposed *Rev1*^{-/-}*Xpc*^{-/-} MEFs compared with mock-treated cells (Figs. 4A and B), confirming that measurable levels of DSBs are not generated during the first cell cycle after UVC exposure. Based on these results, we infer that the alkaline comets of UVC-exposed *Rev1*^{-/-}*Xpc*^{-/-} MEFs at the cell cycle of exposure represent ssDNA breaks, rather than DSBs resulting from collapsed replication forks.

To investigate whether the alkaline comets in UVC-exposed *Rev1*^{-/-}*Xpc*^{-/-} MEFs reflect ssDNA gaps opposite (6-4)PP, we adapted a recently described and highly sensitive immunostaining technique (Temviriyankul *et al.*, 2012b). As shown in Figure 4C, at 8 hours after exposure, mock-treated *Rev1*^{-/-}*Xpc*^{-/-} cells showed only background staining, similar to mock-treated or UVC-exposed *Xpc*^{-/-} MEFs (Figs. 4A and B). However, after exposure to a dose as low as 0.4J/m² UVC (Fig. 4C), staining for unreplicated (6-4)PPs was observed in *Rev1*^{-/-}*Xpc*^{-/-} MEFs that were positive for EdU (i.e. replicating during UVC exposure) and negative for the mitotic marker H3^{ser10-P}, in agreement with the notion that Rev1 is required for TLS of (6-4)PPs-containing ssDNA gaps (Jansen *et al.*, 2009). In non-mitotic, EdU-positive *Xpc*^{-/-} cells (6-4)PPs embedded in ssDNA were not detected. This indicates that, at these UVC doses, most (6-4)PPs were efficiently bypassed in Rev1-proficient cells. Surprisingly, also EdU positive and mitotic, H3^{ser10-P} positive, *Rev1*^{-/-}*Xpc*^{-/-} cells from this experiment displayed pronounced staining for (6-4)PPs, despite the highly condensed chromatin of mitotic chromosomes (Figs. 4D and E). Thus, we here provide direct proof that chromatin with (6-4)PP-containing ssDNA gaps, which were generated during S phase, can persist into mitosis.

To assess whether (6-4)PP within ssDNA are transferred through mitosis into the G1 phase of daughter nuclei, we immunostained for (6-4)PP in ssDNA in binucleated cells obtained as described above and in Fig. 4F. This revealed a more than 4-fold increase in (6-4)PPs in ssDNA in low-dose-exposed binucleated BrdU⁺/EdU⁻ *Rev1*^{-/-}*Xpc*^{-/-} MEFs, as compared with mock treated MEFs (Fig. 4G). This result strongly suggests that patches of ssDNA containing (6-4)PPs can persist beyond mitosis, to the next cell cycle. Moreover, these ssDNA gaps are likely transferred from G1 into the 2nd S phase, since increased levels of (6-4)PPs in ssDNA were even found in low-dose-exposed binucleated BrdU⁺/EdU⁺ *Rev1*^{-/-}*Xpc*^{-/-} MEFs (Fig. 4G). Alternatively, this result might suggest the generation of additional ssDNA gaps in *Rev1*^{-/-}*Xpc*^{-/-} MEFs following replication fork stalling at persistent (6-4)PPs in the 2nd S phase after UVC treatment.

Unreplicated (6-4)PPs-Containing Cells Evade Checkpoint Signaling

It is commonly believed that cell cycle checkpoints prevent genomic instability (Cimprich *et al.*, 2008). We wondered whether DNA damage signaling was perturbed in *Rev1*^{-/-}*Xpc*^{-/-} MEFs, since these cells progressed to the subsequent cycle, resulting in S-phase-associated accumulation of DSBs and genomic instability, following low-dose UVC exposure. To investigate this, *Rev1*^{-/-}*Xpc*^{-/-} MEFs were pulse-labeled with EdU immediately before exposure to 0 or 0.4J/m² UVC, in order to mark cells that were replicating at the time of exposure. After exposure, the cells were cultured in the presence of nocodazole for 8h. Staining of cells for EdU and histone H3^{ser10-P} revealed a population of *Rev1*^{-/-}*Xpc*^{-/-} MEFs that were EdU positive and H3^{ser10-P} negative. These cells presumably represent pre-mitotic cells that were replicating during UVC exposure. These cells were tested for DNA damage signaling by quantifying the presence of Chk1^{S345-P}, a downstream effector protein of activated Atr checkpoint kinase (Nam *et al.*, 2011) and also for chromatin-bound Rpa, an ssDNA-binding

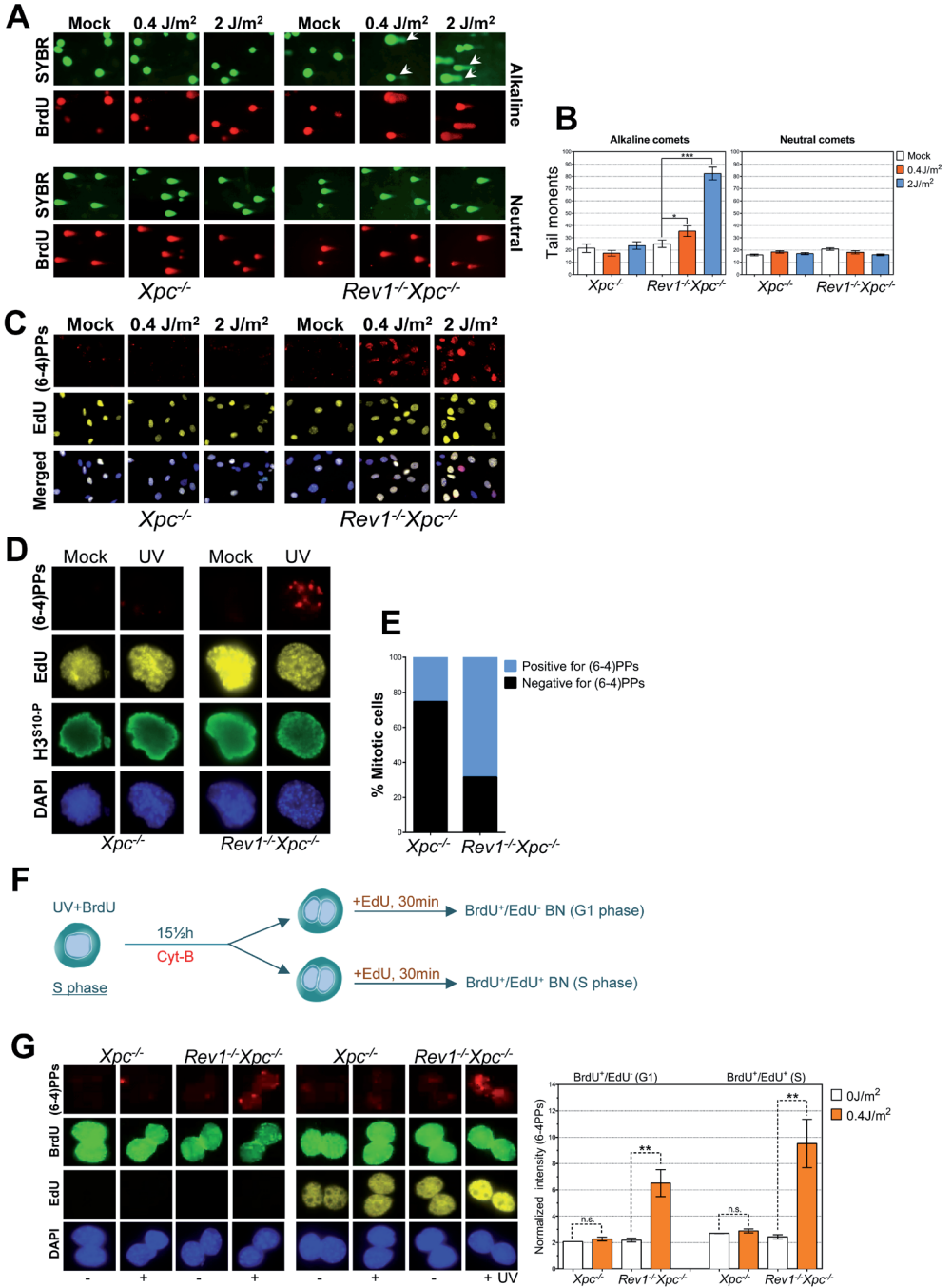


Figure 4 | Patches of ssDNA containing unreplcated (6-4)PPs can persist beyond mitosis. (A) 'Comets' of cells exposed to 0 - 2 J/m² UVC, pulse-labeled with BrdU and arrested with nocodazole for 16h, before electrophoresis under alkaline (upper panels) or neutral (lower panels) condition. Nuclei were identified by

heterotrimeric protein that mediates Atr recruitment (Nam *et al.*, 2011). Enhanced levels of both proteins were only observed in low-dose UVC-exposed *Rev1^{-/-}Xpc^{-/-}* MEFs (Figs. 5A and B), suggesting that persisting ssDNA gaps containing (6-4)PP DNA induce significant damage signaling in the *Rev1^{-/-}Xpc^{-/-}* MEFs. However, this DNA damage signaling apparently does not prevent the cells to progress to the next cell cycle (Fig. 2). Significant levels of Chk1^{S345-P} and chromatin-bound Rpa were also detected in the subsequent G1 phase of *Rev1^{-/-}Xpc^{-/-}* MEFs, whereas these levels further increased in the ensuing S phase (Figs. 5C and D; see Fig. 4F for a scheme).

To test whether the late appearance of MNs are caused by the formation of DSBs during the 2nd cell cycle following UVC exposure, we immunostained BrdU⁺/EdU⁻ and BrdU⁺/EdU⁺ binucleated *Rev1^{-/-}Xpc^{-/-}* MEFs for ATM^{S1980-P}, which is associated with the early response to DSBs (Abraham *et al.*, 2005; Lee *et al.*, 2005). The intensity of the ATM^{S1981-P} signal in BrdU⁺/EdU⁻ binucleated *Rev1^{-/-}Xpc^{-/-}* MEFs was slightly increased after UV treatment (Fig. 5E), suggesting a low amount of UVC-induced DSB formation in G1 phase cells. Thus, although (6-4)PP in ssDNA gaps progress to the 2nd cell cycle after UVC exposure, they hardly collapse to DSBs before or during G1. However, significant staining for ATM^{S1980-P} was detected in BrdU⁺/EdU⁺ binucleated *Rev1^{-/-}Xpc^{-/-}* MEFs (Fig. 5E). Therefore, we conclude that DSBs are generated mainly during the 2nd S phase upon low-dose UVC-exposure, explaining the delayed genome instability upon low-dose UVC exposure. Since the intensity of ATM^{S1980-P} signal in the 2nd S phase coincides with that of Chk1^{S345-P} and chromatin-bound Rpa, we assume that at this stage DNA signaling results from ssDNA induced by enzymatic resection at DSBs (Symington *et al.*, 2011).

In conclusion, we provide evidence that, at low levels of UVC-induced DNA lesions, unreplicated DNA lesions are transmitted through mitosis into the subsequent cell cycle. During the ensuing S phase, these lesions are then converted into DSBs accompanied with massive DNA damage signaling. Finally, these DSBs underlie the formation of MN after the subsequent mitosis (Fig. 6 for a model).

- staining with SYBR and replicating cells at a time of UVC treatment were identified by immunostaining for BrdU. White arrows indicate the DNA discontinuities. (B) Tail moments in BrdU-positive cells for alkaline (left panel) or neutral 'comets' (right panel) ($n=3$). Of note, tail moments of alkaline and neutral 'comets' are different due to the different settings that had to be applied for quantification purposes. (C) Immunostaining of cells under non-denaturing condition for (6-4)PPs, 8h after pulse-labeling with EdU for 30 min and exposure to 0 - 2J/m² UVC. Cells were co-immunostained for H3^{S10-P} to exclude mitotic cells and EdU staining was performed to identify replicating cells. Only non-mitotic, H3^{S10-P} negative, cells are shown. (D) Immunostaining under non-denaturing condition for the presence of (6-4)PPs and in nuclei of cells that were pulse-labeled with EdU, exposed to 0 - 2J/m² UVC and cultured in the presence of nocodazole for 16h. Cells were co-immunostained for H3^{S10-P} to identify mitotic chromatin. (E) Quantification of (6-4)PPs-positive in mitotic cells (H3^{S10-P}-positive cells) ($n=3$). (F) Experimental scheme to discriminate binucleated (BN) cells that are in the second G1 phase (BrdU⁺/EdU⁻ BN) from those that are in the second S phase (BrdU⁺/EdU⁺ BN) after treatment with 0 or 0.4J/m² UVC. (G) Immunostaining under non-denaturing condition for (6-4)PPs in BrdU⁺/EdU⁻ BN cells (G1 phase, left panel) and in BrdU⁺/EdU⁺ BN cells (S phase, middle panel). Right panel depicts the quantification for fluorescence intensity. Error bar, SEM. Statistical significance was determined by Student's *t*-test. **, $p<0.01$, n.s., non significant.

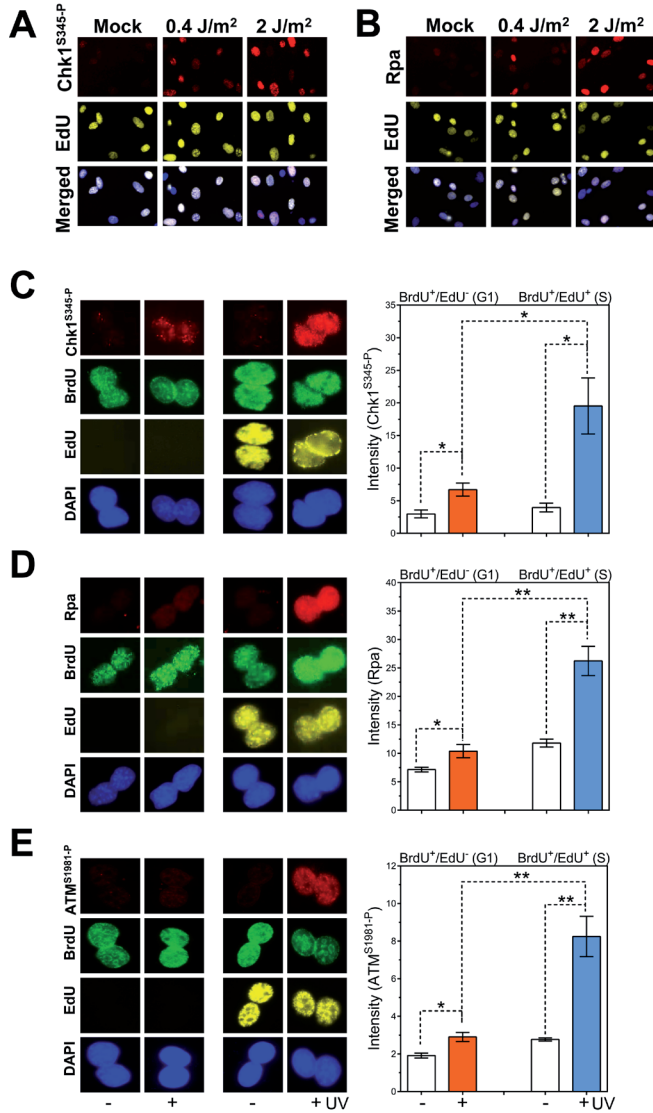


Figure 5 | Checkpoint signaling in cells containing unrepligated (6-4)PPs. (A-B) Immunostaining of *Rev1*^{-/-}*Xpc*^{-/-} cells, 8h after pulse-labeling with EdU for 30 min and exposure to 0 - 2J/m² UVC: (A) Chk1^{S345-P}, and (B) Rpa. Cells were co-immunostained for H3^{S10-P} to exclude mitotic cells and EdU staining was performed to identify replicating cells. Only non-mitotic cells are shown (C) Immunostaining for Chk1^{S345-P} in BrdU⁺/EdU⁻ *Rev1*^{-/-}*Xpc*^{-/-} BN cells (G1 phase, left panel) and in BrdU⁺/EdU⁺ *Rev1*^{-/-}*Xpc*^{-/-} BN cells (S phase, middle panel). Right panel depicts the quantification for fluorescence intensity. (D) Immunostaining for Rpa in BrdU⁺/EdU⁻ *Rev1*^{-/-}*Xpc*^{-/-} BN cells (G1 phase, left panel) and in BrdU⁺/EdU⁺ *Rev1*^{-/-}*Xpc*^{-/-} BN cells (S phase, middle panel). Right panel depicts the quantification for fluorescence intensity. (E) Immunostaining for ATM^{S1981-P} in BrdU⁺/EdU⁻ *Rev1*^{-/-}*Xpc*^{-/-} BN cells (G1 phase, left panel) and in BrdU⁺/EdU⁺ *Rev1*^{-/-}*Xpc*^{-/-} BN cells (S phase, middle panel). Right panel depicts the quantification for fluorescence intensity. Three independent experiments for each marker were performed and approximately 200 BN cells/ experiment were assayed. Error bar, SEM. Statistical significance was determined by Student's *t*-test. *, *p*<0.05 **, *p*<0.01

DISCUSSION

In the present study we have analyzed the origins of genome instability in mammalian cells enduring replication stress following exposure to UV light, an important environmental carcinogen. We have found that patches of ssDNA containing (6-4)PPs in genomic DNA, following exposure to a low dose of UVC, can persist through G2 and mitosis. These ssDNA patches are transmitted to the subsequent cell cycle where they are converted into DSBs only during S phase.

These results contrast previous observations in human cells deficient for TLS polymerase Pol η that display the rapid generation of DSBs after the formation of post-replicative ssDNA gaps following exposure to high-dose UVC light (Elvers *et al.*, 2011), possibly by Mus81/Eme1-dependent cleavage at stalled replication forks (Hanada *et al.*, 2007; Saintigny *et al.*, 2001). We hypothesize that at a high UV dose the massive generation of ssDNA may lead to depletion of Atr, which, in addition to DNA damage signaling, is involved in the stabilization of stalled replication forks (Elvers *et al.*, 2012). This will result in rapid fork collapse and the rapid, rather than delayed, formation of DSBs (Petermann *et al.*, 2010).

How are persistent ssDNA gaps converted into DSBs in the second cell cycle after UVC exposure? Immunostaining for Rpa and phosphorylated Chk1 in non-replicating BN cells (Figs. 5C and D) suggests that ssDNA gaps do activate the Rpa/Atr/Chk1 DNA damage signaling pathway (Fig. 5A and B). However, this does not result in the cessation of cell cycle progression. Moreover, the patches of ssDNA containing (6-4)PPs are not rapidly processed into DSBs, since the level of phosphorylated ATM is not enhanced in these cells, and MN are not observed following the cell cycle of UVC exposure. We therefore propose that, since ssDNA gaps persist through mitosis (Fig. 4D), during the subsequent S phase the replisome encounters gaps in the template, resulting in the generation of a DSB and consequently strong activation of the Rpa/Atr/Chk1 DNA damage signaling pathway (Figs. 5C, D and E). The concomitant phosphorylation of ATM suggests that DSBs arise during the second S phase, at these persisting ssDNA gaps.

So far, the transmission of structures, arising from replicative stress, through mitosis has been shown only after treatment of cells with DNA polymerase inhibitors and only in an indirect manner, *i.e.* by visualizing DNA damage response proteins such as γ -H2AX and 53BP1 (Ichijima *et al.*, 2010; Liu *et al.*, 2012; Lukas *et al.*, 2011). In contrast, our work provides a molecular description of the relevant DNA lesions (*i.e.* ssDNA gaps encompassing a photolesion), as well as a mechanistic basis for the resulting genomic instability, caused by environmentally relevant densities of DNA lesions. To the best of our knowledge, we provide for the first time physical evidence that patches of unreplicated DNA, at mutagenic and clastogenic DNA lesions, can be transferred through mitosis and transmitted to daughter cells, where they exert their clastogenic effects (Fig. 4D and G). We employed *Rev1^{-/-}Xpc^{-/-}* MEFs as a sensitive model, but it

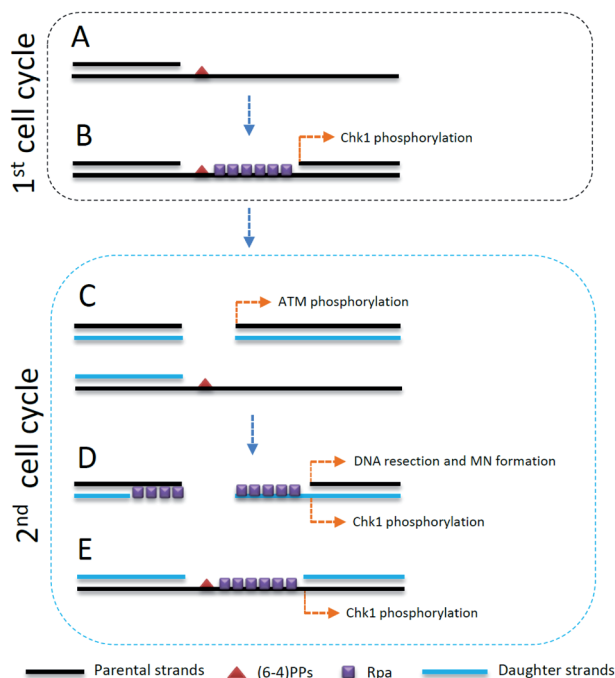


Figure 6 | A schematic diagram representing the formation of DSBs from ssDNA patches in the second cell cycle. (A) (6-4)PPs block DNA replication, resulting in the formation of ssDNA gaps. **(B)** Rpa binds to ssDNA gaps and subsequently Chk1 is activated *via* Atr signaling. Finally, ssDNA gaps are transmitted through mitosis to the next cell cycle. **(C)** Replication across ssDNA gaps leads to the formation of both DSBs and ssDNA containing (6-4)PPs. DSBs are eventually recognized by ATM to provoke DSB repair. **(D)** DSBs are resected, resulting in ssDNA, which is coated by Rpa. Persistent DSBs cause the formation of MN at the end of mitosis. **(E)** ssDNA coated with Rpa activates Atr signaling resulting in Chk1 phosphorylation.

should be emphasized that, at an equitoxic UVC dose, also *Xpc*^{-/-} MEFs displayed delayed genome instability. This result suggests that the delayed mechanism of genome instability described here may represent a physiologically relevant mechanism.

In our study we used a low, physiologically relevant, dose of UVC as a model agent for the study of responses to both mild and severely helix-distorting DNA adducts. Such adducts are induced by many environmental genotoxic agents and cancer medicines, amongst others (Schut *et al.*, 1999; Sinha *et al.*, 2002). Our finding that genotoxic DNA damage can result in the induction of genomic instability only during the second S phase following exposure is highly relevant for clastogenicity testing of such compounds and provides a rationale for the recent chemical testing guidelines released by the Organisation for Economic Co-operation and Development (OECD), including (i) the standard *in vivo* MN test guideline 474 and (ii) the *in vitro* MN test guideline 487, which suggest that the first and the second cell divisions should be analyzed during MN analysis.

MATERIALS AND METHODS

Cell culture. The generation of MEF lines deficient for Xpc (*Xpc*^{-/-} MEFs) or double deficient for Xpc and Rev1 (*Rev1*^{-/-}*Xpc*^{-/-} MEFs) are described previously (Cheo *et al.*, 1997; Jansen *et al.*, 2009). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, Glutamax and pyruvate (Invitrogen), supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) (DMEM medium) at 37 °C in a humidified atmosphere containing 5% CO₂.

Clonogenic survival assay. MEFs were treated with UVC doses up to 5J/m², trypsinized, and counted. A fixed number of cells was plated in p90 dishes and cultured for 10 days at 5% CO₂ and 37°C. Before staining with methylene blue, cells were washed and fixed. To determine the relative clonogenic survival, the cloning efficiency of unexposed cells was set at 100%.

Cytokinesis-blocked micronucleus assay. The assay was performed as previously described (Temviriyankul *et al.*, 2012a; Temviriyankul *et al.*, 2012b), which method was based on previous studies (Bolt *et al.*, 2011; Fenech, 1993). Briefly, 7.5×10^4 cells were plated on a glass slide (76 mm × 26 mm) and cultured overnight. Prior to exposure to 0 - 5J/m² UVC, cells were washed twice with PBS. At 0h or 24h after UVC treatment, Cytochalasin B (cyt-B) (3 µg/ml) (Sigma-Aldrich) was added to the cultures in order to inhibit cytokinesis. Twenty-four hours after addition of cyt-B, cells were fixed using 3.5% paraformaldehyde (4°C) and 0.5% Triton-X100. Then, slides were rinsed with PBS and nuclei were stained with DAPI (17.5 ng/ml). The slides were soaked in 70%, 90% and absolute ethanol for 5 min each, respectively. The binucleated cells (BN) and number of micronuclei (MN) were scored using a fluorescence microscope (Zeiss, Germany) and the Metafer 4 program (Metasystem, Germany). Three independent experiments were performed and mock treated cells were included as a control. To determine whether MN result from DNA breaks in the first or second cell cycle after UVC exposure, the formation of MN was determined in two groups of cells. In the first group, immediately after 0 - 0.4J/m² UVC irradiation, cells were treated with 10 µM Bromodeoxyuridine (BrdU, Millipore) for 30 min after which the cells were cultured in the presence of cyt-B for 23h to generate BN cells. Thus, MN in BN cells with nuclei containing BrdU are derived from S phase cells at the time of UV exposure (first cell cycle MN). To distinguish BN cells with nuclei in G1 phase from those that have entered a new S phase, the cells were incubated in medium containing 10 µM 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen) for 30 min, prior to fixation. Consequently, BN cells with nuclei double positive for BrdU and EdU reflect 2nd S phase cells after UVC exposure. In the second group, immediately after UVC irradiation, cells were pulse labeled with 10 µM Bromodeoxyuridine (BrdU, Millipore) for 30 min, after which the cells were cultured in DMEM medium for 23h without cyt-B, to allow cells to enter the second S phase after UVC exposure. Then, EdU was added and 30 min later, cells were washed with PBS and cultured in the presence of cyt-B for 24h to generate BN cells. MN in BN cells with nuclei containing both BrdU and EdU are derived in cells that have completed two rounds of DNA replication (second cell cycle MN). To visualize BrdU and EdU in DNA of BN cells, the DNA of nuclei was denatured, incorporation of EdU was determined according to the manufacturer's protocol, whereas incorporation of BrdU was detected by immunofluorescence. Then, BN cells were scored for MN.

Bivariate cell cycle analysis. MEFs were seeded in 90 mm dishes at 70% confluence and were cultured overnight. Cells were rinsed with PBS and exposed to UVC or mock treated. Immediately after exposure to 0 - 2J/m² UVC, cells were pulse labeled with BrdU for 30 min in 5% CO₂ at 37°C. Cells were trypsinized and fixed with 70% ethanol at indicated time points. BrdU staining and flow cytometry were performed as described (Temviriyankul *et al.*, 2012b).

Single cell gel electrophoresis assay (Comet assay). DNA strand interruptions and DSBs in replicating cells at a time of UVC exposure were measured by alkaline and neutral comet assay, respectively. In detail, cells were plated to reach about 60-70% confluence. To identify replicating cells at the time of UVC treatment, cells were pulse-labeled for 30 min with BrdU, immediately after exposure to 0 - 2J/m² UVC. Then, the cell population was cultured in the presence of the mitotic spindle poison Nocodazole (300ng/ml) for 16h to accumulate cells in G2/M phase. Single cell suspensions were processed to visualize comets according to the manufacturer's instructions (Trevigen). In order to visualize BrdU-containing chromatin after gel electrophoresis, chromatin was denatured and stained for BrdU using a mouse monoclonal antibody against BrdU (Becton Dickinson) followed by incubation with an Alexafluor555-labeled goat-anti-mouse antibody (Invitrogen). Staining with SYBR green (Invitrogen) was applied to visualize DNA. The comet tail moments of about 180-240 BrdU-containing nuclei from three independent experiments were scored using the Comet software (TriTek).

Immunofluorescence. The antibodies used in immunofluorescence were mouse anti-BrdU (Beckton Dickinson), rabbit anti-BrdU (Rockland immunochemicals), mouse anti-Histone H3^{S10-P} (Millipore), rabbit anti-Histone H3^{S10-P} (Millipore), mouse anti-(6-4)PP (CosmoBio), rabbit anti-Chk1^{S345-P} (Cell signaling), rat anti-Rpa (Cell signaling), mouse anti-ATM^{S1981-P} (Rockland immunochemicals), and appropriate secondary antibodies conjugated with fluorescence dyes (Invitrogen). To detect (6-4)PPs, Rpa and Chk1^{S345-P} in the late S/G2 phase, cells were seeded on coverslips and cultured overnight in 5% CO₂ at 37°C. Prior to exposure to 0 - 2J/m² UVC, cells were pulse-labeled with EdU for 30 min. After UV exposure, cells were cultured in the presence of nocodazole for 8h, to prevent cells from entering a second cell cycle after UV exposure. Then cells were fixed and immunostained for (6-4)PPs, Rpa or Chk1^{S345-P} using appropriate antibodies. To increase the sensitivity of anti-(6-4)PPs staining, Tyramine Signal Amplification (TSA, Perkin-Elmer) was applied, according to the manufacturer's recommendations. The late S/G2 phase cells that were replicating at the time of UV exposure were identified by positive staining for EdU and negative staining for H3^{S10-P}. To detect (6-4)PPs in mitotic cells, cell cultures were treated as described above, with the exception that the cells were cultured in nocodazole-containing medium for 16h to accumulate G2/M phase cells. The mitotic cells, which were replicating at a time of UVC treatment, were identified by positive staining for both EdU and H3^{S10-P}. (6-4)PPs and DNA damage response proteins in BN cells were visualized as follows. Cells were cultured on coverslips in medium with 10 µM BrdU for 30 min, immediately after irradiation with 0-0.4J/m² UVC. Then, cells were cultured in the presence of cyt-B for 15½h. Prior to fixation, cells were pulse-labeled with 10 µM EdU in the presence of cyt-B for 30 min. Since BrdU detection requires a denaturation step, which may destroy protein epitopes, fixed cells were firstly stained for EdU. Then, cells were blocked with 5% BSA+0.1% tween-20 in PBS for 30 min and subsequently incubated overnight with anti-Rpa, Chk1^{S345-P} or ATM^{S1981-P} antibodies diluted in 1% BSA+0.1% tween-20 in PBS at 4°C. Then, appropriate secondary antibodies were applied. After washing, cells were again fixed with 3.7% Paraformaldehyde for 15 min, followed by incubation with 2N HCl for 12 min, in order to denature DNA. Before incubation with anti-BrdU antibodies, coverslips were rinsed intensively to reduced acidity. After incubation with an appropriate secondary antibody against anti-BrdU antibodies, nuclei were stained with DAPI. Images were captured using a wide-field microscope (Axioplan 2, or Axioplan Imager M2, Zeiss). Image intensity was quantified using Fiji (ImageJ) software (National Institutes of Health).

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SUPPLEMENTARY FIGURE

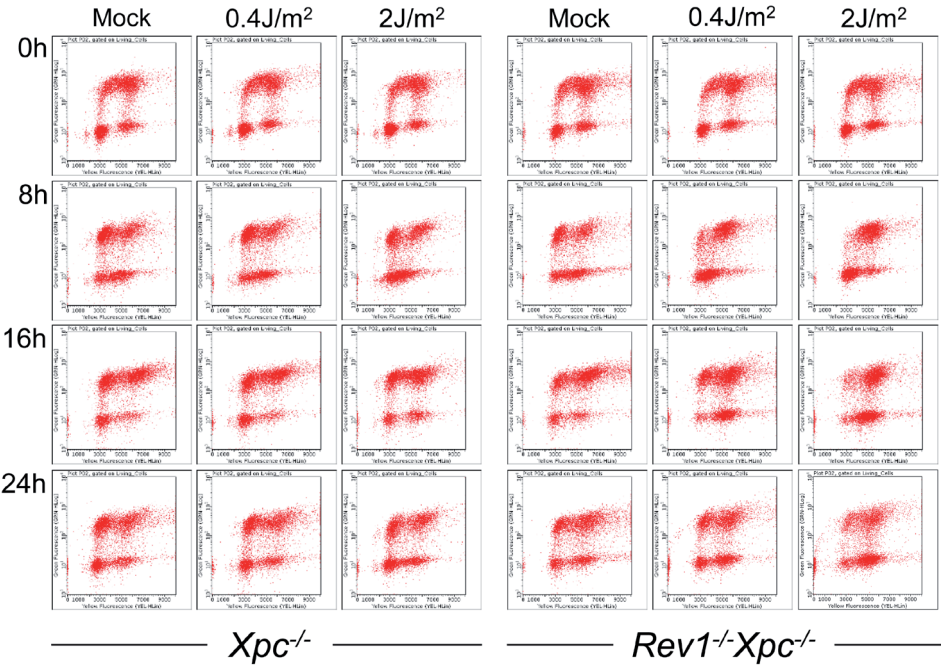


Figure S1 | *Rev1*^{-/-}*Xpc*^{-/-} MEFs can progress to the second cell cycle upon low doses of UVC irradiation. Cells were pulse-labeled with BrdU, immediately after UVC exposure (up to 2J/m²) or mock-treated. At indicated times after exposure, cells were fixed. Then, BrdU incorporation was determined by immunostaining and DNA content was measured using propidium iodide (PI). FACS profiles were generated by flow cytometer and BrdU-positive cells in G1, S and late S/G2 phases were quantified.

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CHAPTER

6

DNA TRANSLESION SYNTHESIS SUPPRESSES AGING AND AGING-ASSOCIATED CANCER

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ABSTRACT

The attrition of stem cells and of proliferating compartments that characterizes aging correlates with the accumulation of endogenous DNA lesions during life. To explore the existence of a causal relation between replicative stress at unrepaired endogenous DNA lesions and aging we used mice deficient for the translesion replication gene *Rev1*. *Rev1*^{-/-} mice displayed stem cell attrition and mild progeroid phenotypes that were exacerbated by simultaneous deficiency of the global-genome nucleotide excision repair (GG-NER) gene *Xpc*. *Rev1*^{-/-}*Xpc*^{-/-} mice displayed chronic replicative stress, cellular senescence and apoptosis in proliferating compartments, and predisposition to aging-associated lymphomas. We demonstrate the involvement of Rev1 in replicative bypass of peroxidated lipid aldehyde-DNA adducts, a known aging marker and GG-NER substrate. Replicative stress did not induce compensatory somatotrophic suppression, unlike transcriptional stress. Combined, these data provide strong support for a causal role in aging of replicative stress at accumulating endogenous bulky DNA adducts.

Aging is associated with the accumulation of endogenous DNA lesions, with the attrition of stem cells and with the loss of proliferative compartments (Chou *et al.*, 2010; Dykstra *et al.*, 2011; Geiger *et al.*, 2009; Hoeijmakers, 2009; Maslov *et al.*, 2009; Rossi *et al.*, 2007; Ruzankina *et al.*, 2008; Wang *et al.*, 2012; Winczura *et al.*, 2012). However, the mechanistic relation between DNA damage and aging largely is elusive. Most damaged genomic nucleotides are repaired by excision repair pathways, including GG-NER that specifically repairs bulky and helix-distorting nucleotide lesions (Hoeijmakers, 2009). During DNA replication, damaged nucleotides that have evaded repair require specialized bypass mechanisms that thereby suppress replicative stress and genome instability. Translesion replication (TLR) comprises a set of specialized DNA polymerases with reduced template stringency and is a major pathway to replicate damaged nucleotides (Sale *et al.*, 2012). Since *Rev1* is a key regulator of TLR (Jansen *et al.*, 2006; Sale *et al.*, 2012), we argued that *Rev1*-deficient (*Rev1*) mice would be useful tools to investigate the involvement of replicative stress at unrepaired endogenous DNA lesions in aging.

Rev1 embryos were present at the expected ratio and displayed no overt defects (Figs. S2A and B). *Rev1* pups were slightly underrepresented and the mice displayed mild growth retardation (Figs. S2A-D), supporting previous data (Jansen *et al.*, 2006). Moreover, the median and maximal lifespans of *Rev1* mice were reduced, and they prematurely developed an aged appearance (Figs. S2E and F). Compared with age-matched wild type littermates, livers of these *Rev1* mice displayed enhanced signs of aging-associated degeneration, including steatosis, karyomegaly, and accumulation of the lipid degradation product lipofuscin (Thoolen *et al.*, 2010) (Figs. S3A and B; Table S1). In all *Rev1* mice gonads were unusually small (Fig. 1A). Ovaries were depleted of follicles and in most testicular tubules spermatogenesis was absent (Figs. S3C and D). Gonads were largely devoid of cells positive for the germ cell marker GCNA1 already in juvenile *Rev1* mice (Wang *et al.*, 1996) (Figs. 1B, Figs. S3E and F), indicating an early germ cell defect. In adult *Rev1* mice, GCNA1 staining appeared normal in the residual follicles and active seminiferous tubules, and these displayed normal spermatogenesis (Figs. S3D and G). Indeed, *Rev1* males occasionally begot progeny.

In most aged *Rev1* mice the cellularity of the bone marrow was reduced, compared with the controls (Fig. 1C and Table S1). To further analyse the hematopoietic compartment of the *Rev1* mice, we isolated quiescent hematopoietic stem cells (HSCs; LSK CD48⁻ CD34⁻ EPCR⁺ CD150⁺) and derived hematopoietic progenitors (LSK and LSK CD34⁺) (Dykstra *et al.*, 2011) from bone marrow of 18-months old mice, prior to the onset of aging pathology. The absolute and relative frequencies of these cell types did not significantly differ between the genotypes (Fig. 1D). *Rev1* HSCs displayed wild type viability upon mitogenic stimulation *in vitro*, as assessed with a cobblestone area forming colony (CAFC) assay (Fig. 1E). However, when grown in cytokine-supplemented cultures, the size of the *Rev1* colonies was reduced, compared with controls, indicating a proliferative defect ($p < 0.001$, Fig. S4). To investigate the *in*

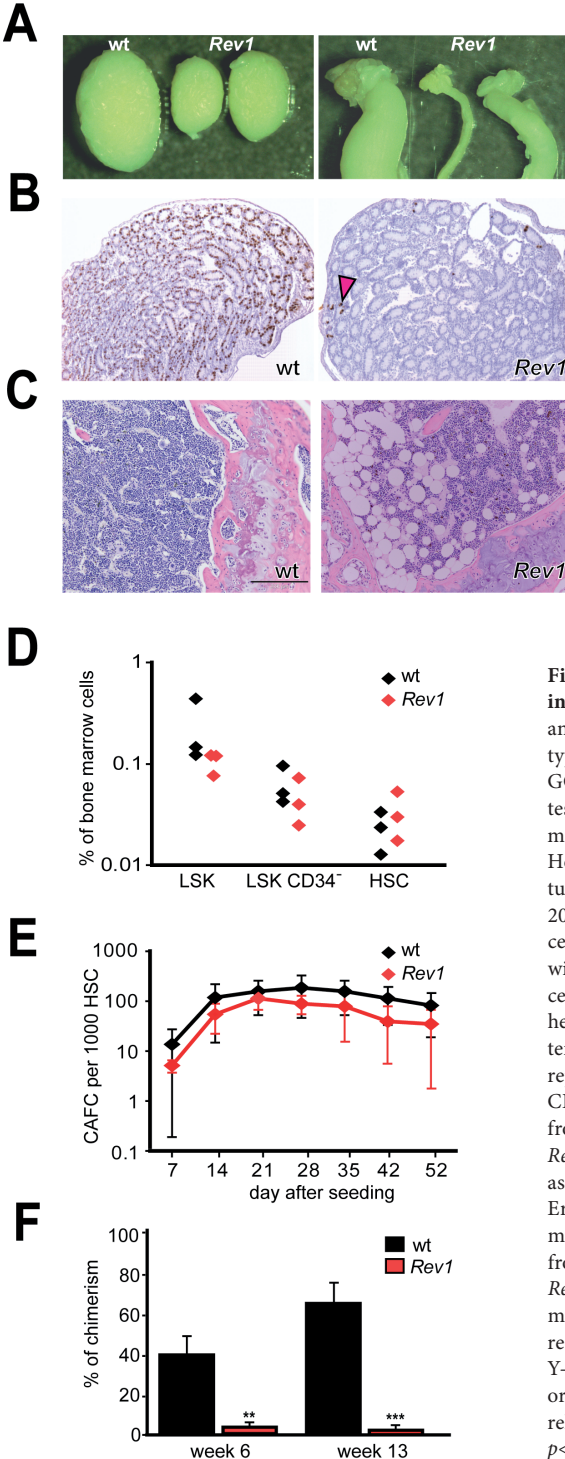


Figure 1 | Germ and stem cell attrition in *Rev1* mice. (A) Bouin-fixed testes (left) and ovaries (right) of 2-month-old wild type and *Rev1* littermates. (B) Staining for GCNA1-positive (brown) germ cells in testes from 6-day-old wild type and *Rev1* males. Sections were counterstained with Hematoxylin. Arrowhead: GCNA1-positive tubules. (C) Section of the femur of a 20-month-old *Rev1* mouse revealing loss of cellularity compared with an age-matched wild type control. Dark blue: hematopoietic cells. Size bar: 200 μ M. (D) Frequencies of heterogeneous Lin⁺Sca-1⁺c-kit⁺ (LSK), short-term repopulating LSK CD34⁺ and long-term repopulating HSC (LSK CD48⁻CD34⁺EPCR⁺CD150⁺) cells, isolated from bone marrow from healthy 18-month-old wild type and *Rev1* mice. (E) *In vitro* proliferation (CAFC) assay of HSCs isolated from bone marrow. Error bars: SD. (F) Competitive *in vivo* bone marrow repopulation assay of HSCs isolated from bone marrow from 3 wild type and *Rev1* donors. For each donor, 200 HSCs were mixed with 1.2×10^6 W41.SJ1 competitors per recipient (3-5 recipients per experiment). The Y-axis depicts the contribution of wild type or *Rev1* cells to the repopulated bone marrow, relative to the competitors. **, $p < 0.01$, ***, $p < 0.001$, two-tailed *t*-test. Error bars: SD.

in vivo functionality of the HSCs, we measured their capacity to reconstitute the bone marrow of lethally irradiated recipients using a competitive repopulation assay. This experiment revealed a highly significant competitive defect of the *Rev1* HSCs (Fig. 1F). A decline in the competitive repopulation capacity of HSCs is characteristic also of physiological aging (Dykstra *et al.*, 2011; Geiger *et al.*, 2009).

The failure of *Rev1* germ cells and HSCs to (re)populate their respective compartments, and the progeroid phenotypes of *Rev1* mice, suggested a causal relation between replicative stress at endogenous DNA lesions, the attrition of proliferating cells, and aging. *Rev1*-mediated TLR is required for the replicative bypass of nucleotide lesions that have escaped repair by GG-NER, as was illustrated by the synergistic hypersensitivity to ultraviolet light of *Rev1Xpc* mice and mouse embryonic fibroblasts (MEFs; Fig. S5). We reasoned that exacerbation of the *Rev1* aging phenotypes by simultaneous GG-NER deficiency would provide evidence of a causal role for bulky nucleotide adducts. To test this, we analyzed cohorts of *Rev1Xpc* double and *Xpc* single-deficient mice. *Xpc* mice develop lung tumors at advanced age, but otherwise do not display spontaneous phenotypes (Melis *et al.*, 2008). *Rev1Xpc* embryos were present at the expected frequency but displayed stochastic abnormalities (Figs. 2A and B). *Rev1Xpc* pups were strongly underrepresented (Fig. 2A). Juvenile *Rev1Xpc* mice displayed no overt abnormalities, besides variably reduced sizes (Fig. 2C and Fig. S5C). However, at different age and with variable severity, degenerative phenotypes became apparent, including cachexia, anemia, and kyphosis (Fig. 2D), and the average and maximal lifespans of the *Rev1Xpc* mice were significantly reduced (Fig. 2E). Eight of these progeroid *Rev1Xpc* mice were sacrificed when moribund (6-13 months old; Fig. 2E and Supplementary Table 1), and analysed for features of aging. Livers of most *Rev1Xpc* mice displayed high levels of steatosis, karyomegaly and accumulated lipofuscin (Figs. 2F-H). Also in kidneys karyomegaly was observed (Figs. S6A and B). The gonads of *Rev1Xpc* mice mimicked those of the *Rev1* mice. The epidermis of all tested *Rev1Xpc* mice was atrophic and keratotic, displaying a reduced number of cell layers (Fig. 2I) suggesting decay of the proliferating basal layer, whereas subcutaneous fat was lacking (Fig. S6C). Most significantly, the bone marrow of *Rev1Xpc* mice was virtually devoid of hematopoietic cells, suggestive of aplastic anemia (Fig. 2J).

The attenuation of lymphoid lineages is a characteristic of aging mice (Dykstra *et al.*, 2011; Geiger *et al.*, 2009). To investigate the lymphoid compartment in spleens from *Rev1Xpc* mice, we quantified B and T lymphocytes using bivariate fluorescent cytometry. In young *Rev1Xpc* mice, T and B cell compartments were normal (Figs. S7G and S8G). In contrast, in spleens of aging *Rev1Xpc* mice the naïve, IgM⁺IgD⁺, B-cell population was strongly reduced, compared with age-matched *Xpc* controls (Figs. 3A and B; Fig. S7). Whereas the CD4⁺ (helper) T cell population appeared normal, the CD8⁺ (cytotoxic) T cell population was attenuated in some of the *Rev1Xpc* spleens (Fig. 3C and Fig. S8). Strikingly, in a significant fraction of the *Rev1Xpc* mice (χ^2 , $p=0.03$), spleens and/or lymph nodes were enlarged (Fig. S8M). Analysis

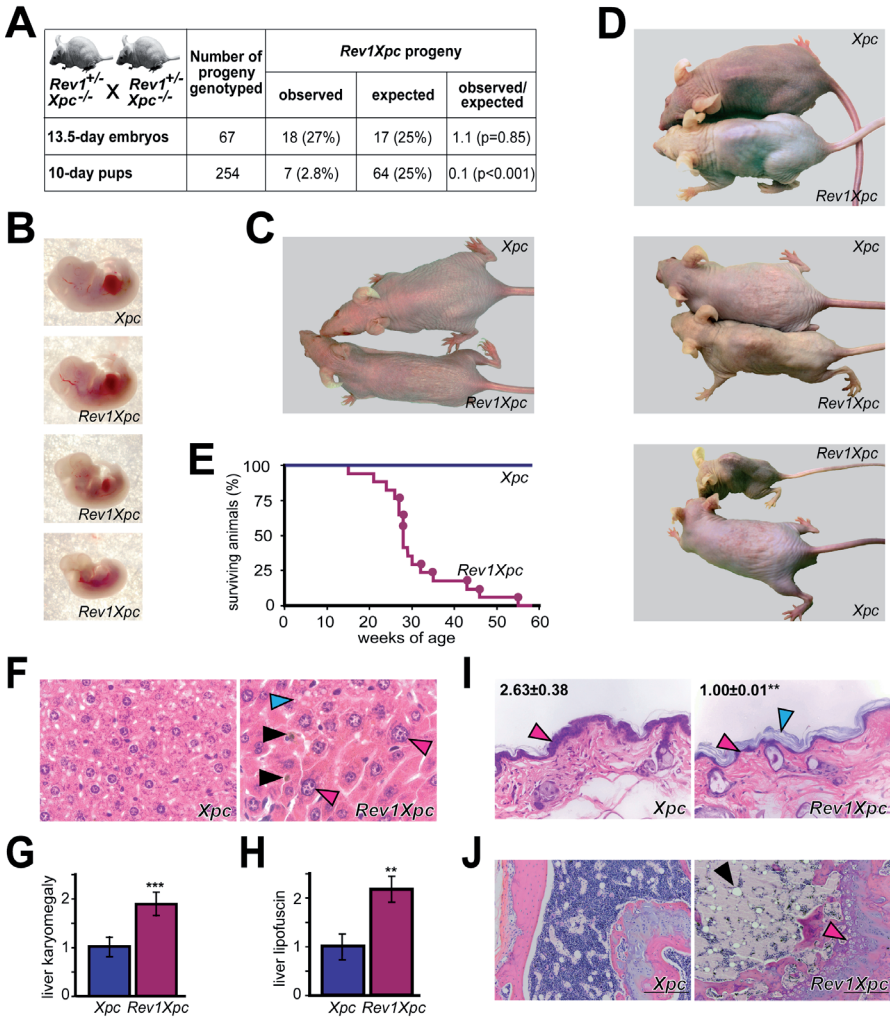


Figure 2 | Progressively degenerative phenotypes of *Rev1Xpc* embryos and mice. (A) Frequencies of live 13.5-day *Rev1Xpc* embryos and pups from *Rev1^{+/-}Xpc^{-/-}* backcrosses. (B) Stochastic aberrations of 13.5-day *Rev1Xpc* embryos. Note developmental defects in forebrain and hindbrain, and defective vascularization, of the two bottom *Rev1Xpc* embryos, whereas the embryonic liver is absent in the bottom embryo. (C) A 2 months old *Rev1Xpc* mouse displaying a reduced body size, but no other overt phenotypes. (D) Overt stochastic features of progeroid *Rev1Xpc* mice include kyphosis, cachexia and anemia. Top and bottom pairs are littermates of 5 months old. The middle pair is the same as shown in Fig. 1C, but here at the age of 12 months. (E) Kaplan-Meier survival curve of the cohorts of *Rev1Xpc* mice and *Xpc* littermates ($n=17$ per cohort). Significantly reduced lifespan ($t_{50}=28$ weeks; Mann-Whitney test, $p<0.001$). Spheres refer to mice subjected to extensive autopsy (Table S1). (F) Hematoxylin-Eosin (HE) staining of liver sections of 8-month old mice. Blue arrowhead: steatosis. Purple arrowheads: karyomegaly (indicative of polyploidization), black arrowheads: lipofuscin inclusions. (G) Relative levels of hepatic karyomegaly. ***: $p<0.001$. Error bars: SD. (H) Relative levels of hepatic lipofuscin. **: $p<0.01$. Error bars: SD. (I) HE staining of skin sections. The numbers refer to the number of epidermal cell layers ($\pm$ SD, **: $p<0.01$; purple arrowheads). Blue arrowhead: keratosis of the *Rev1Xpc* epidermis. Also see Fig. S6C. (J) HE staining of a section from femurs. Black arrowhead: loss of bone marrow (aplastic anaemia) in the *Rev1Xpc* mouse. Purple arrowhead: bone resorption, indicating osteoporosis. Size bars: 200 μ M.

of these spleens revealed the presence of disseminated CD4⁺CD8⁺ (immature T cell) lymphomas (Fig. 3D; Figs. S8J and K), a malignancy common to physiologically aging mice (Frith *et al.*, 1981). In contrast to these proliferating tissues, in (non-proliferating) brains of *Rev1Xpc* mice, no macroscopic or microscopic alterations were found, which differs from mice deficient for the transcription-coupled NER subpathway, in which transcriptional stress causes segmental progeria (Hoeijmakers, 2009; Niedernhofer *et al.*, 2006).

Based on the synergy between the *Rev1* and *Xpc* defects in embryonic, aging and cancer phenotypes we predicted that replicative stress at unrepaired endogenous DNA lesions is increased in the *Rev1Xpc* mice. Indeed, splenocytes from progeroid *Rev1Xpc* mice displayed increased, but variable, levels of phosphorylated H2AX (γ H2AX), a marker of replicative stress and associated double-strand DNA breaks that characterizes senescent cells in aging mice (Wang *et al.*, 2009) (Fig. 3E). Consistently, expression of the senescence and aging-related genes *p16^{INK4A}* (Ruzankina *et al.*, 2008; Geiger *et al.*, 2009) and *Gadd45A* (Passos *et al.*, 2010) was increased (Fig. 3F) and abundant apoptosis was observed in the *Rev1Xpc* spleens (Figs. 3G and H). We conclude that replicative stress at endogenous DNA lesions induces senescence and apoptosis of proliferating cells in *Rev1Xpc* mice.

We then investigated the nature of the endogenous bulky DNA lesions responsible for the progeroid phenotypes of *Rev1* and, more prominent, *Rev1Xpc* mice. The aging-related phenotypes of the skin (Chen *et al.*, 2012), liver (Gorla *et al.*, 2001; Thoolen *et al.*, 2010), germ cells (Turner *et al.*, 2008), HSCs (Yahata *et al.*, 2011) and lymphoid cells (Geiger *et al.*, 2009) have all been associated with oxidative stress. We therefore focused on bulky DNA lesions that can be induced by oxidative stress. Two such lesions prevail: cyclopurines and lipid peroxide-induced hydroxyalkenal (aldehyde)-DNA adducts (Wang *et al.*, 2012; Winczura *et al.*, 2012). HSCs express high levels of detoxifying aldehyde dehydrogenases (Storms *et al.*, 2005), and acetaldehyde can induce aplastic anemia, resembling the spontaneous aplasia of the *Rev1* and *Rev1Xpc* bone marrow (Langevin *et al.*, 2011). *Rev1* and *Rev1Xpc* cells are not sensitive to acetaldehyde (Langevin *et al.*, 2011). Therefore we hypothesized that, rather than simple aldehyde-DNA adducts, hydroxyalkenal-DNA adducts may underlie the phenotypes of *Rev1* and *Rev1Xpc* mice. In support, compared with *Xpc* MEFs, *Rev1Xpc* MEFs were hypersensitive to the oxidative agent Paraquat and to the lipid peroxide 4-hydroxynonenal (HNE; Fig. 4A). We directly investigated a role of *Rev1* in TLR of Heptanone etheno-2-deoxycytidine (H- ϵ dC), a very common hydroxyalkenal-nucleotide adduct that is derived from endogenous, HNE-related, 4-oxononenal (Winczura *et al.*, 2012). This was done by transfection of a plasmid vector containing a site-specific H- ϵ dC into *Rev1* and wild type MEFs (Fig. 4B). In *Rev1* MEFs, the TLR efficiency dropped to 27% of wild type, and the frequency of the incorporation of adenine at the lesion was reduced (X^2 test, $p < 0.001$; Fig. 4B).

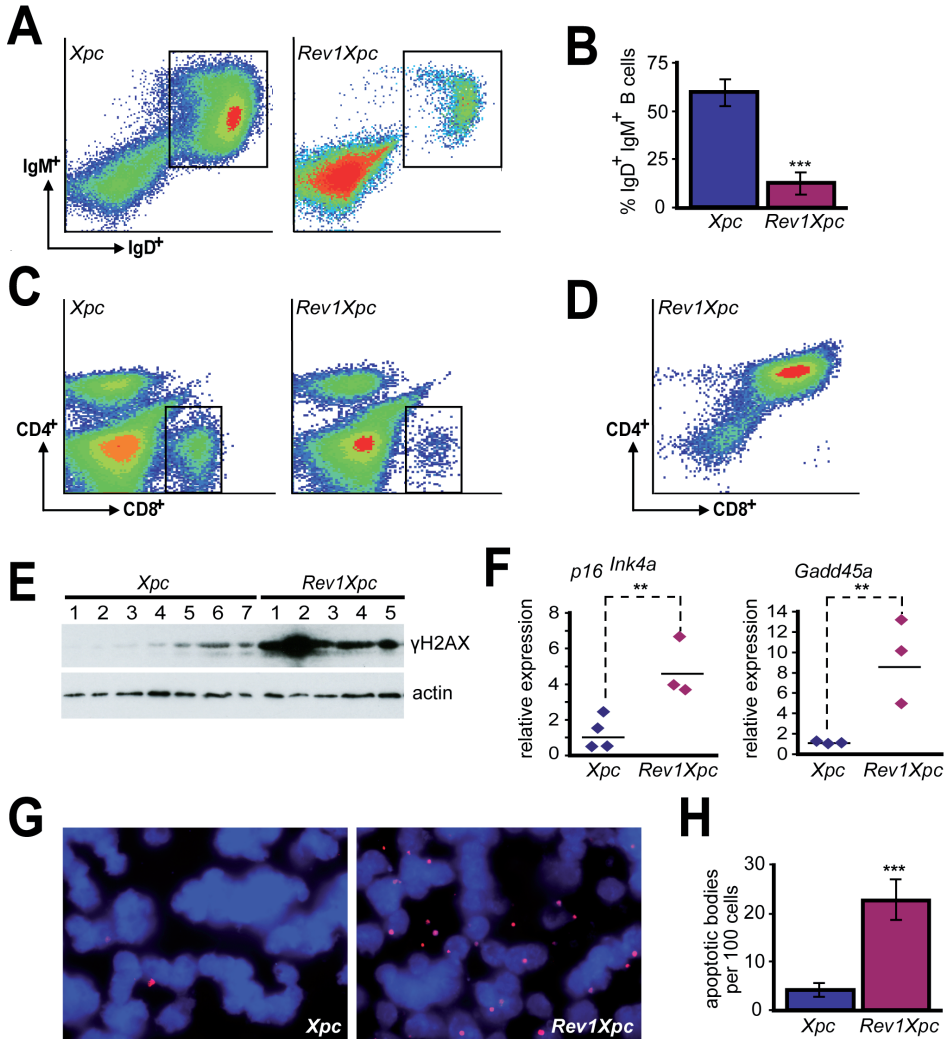


Figure 3 | Decay of lymphocyte populations, increased DNA damage signaling, senescence and apoptosis in spleens from progeroid *Rev1Xpc* mice. (A) Fluorescent cytometry of IgM⁺IgD⁺ B lymphocytes (boxed) from the spleen of a *Rev1Xpc* mouse and an age-matched *Xpc* control. (B) Quantification of IgM⁺IgD⁺ B-cells in spleens from 5 *Rev1Xpc* mice and 6 *Xpc* controls. See Fig. S5. ***: $p < 0.001$. Error bars: SD. (C) Fluorescent cytometry of CD4⁺ and CD8⁺ T lymphocyte populations in spleens of a progeroid *Rev1Xpc* mouse and an age-matched *Xpc* control mouse. See Fig. S6 for examples of stochasticity. (D) CD4⁺CD8⁺ T-cell lymphoma in the spleen of a 6 month-old *Rev1Xpc* mouse. (E) Western blot displaying γH2AX levels in spleens from 5 *Rev1Xpc* mice and 7 age-matched *Xpc* littermates. (F) Expression of cellular senescence and aging-related genes *p16^{Ink4a}* and *Gadd45a* in spleens from *Rev1Xpc* mice compared with spleens from age-matched *Xpc* littermates. **: $p < 0.01$. (G) TUNEL staining for apoptotic bodies (red dots) in a *Rev1Xpc* spleen and an age-matched *Xpc* littermate. (H) Quantification of apoptotic bodies in spleens from *Rev1Xpc* and age-matched *Xpc* mice. ***: $p < 0.001$. Error bars: SD.

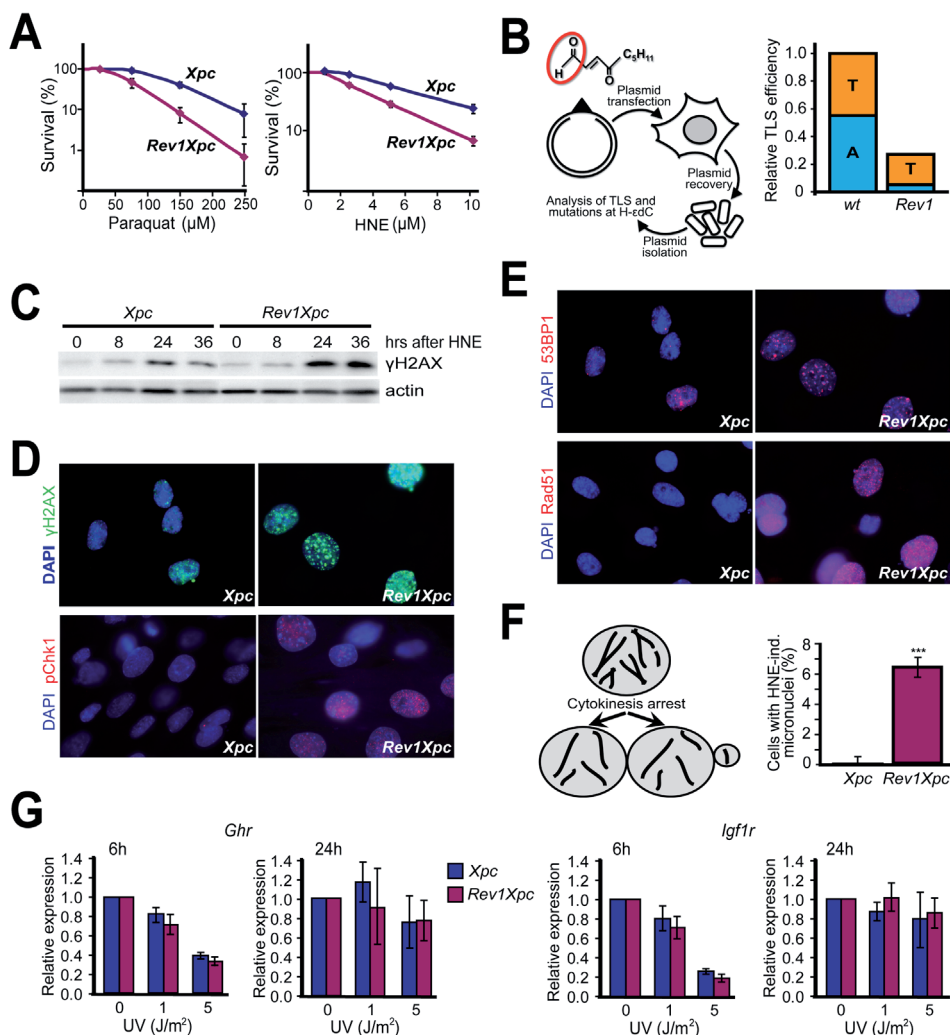


Figure 4 | DNA damage responses and genome instability in *Rev1* and *Rev1Xpc* MEFs. (A) Sensitivity of *Xpc* and *Rev1Xpc* MEFs to the oxidative stress-inducing agent Paraquat and the lipid peroxidation-derived hydroxyalkenal HNE. $n=3$, independent experiments. Error bars: SD. (B) Left panel: assay to measure the efficiency and mutagenicity of TLR at a site-specific H-edC (structure at the top of the panel; the reactive aldehyde group is encircled). The opposing single-stranded DNA gap renders the lesion resistant to repair. Three days after transfection, covalently-closed plasmids are isolated from transfected MEFs and rescued in *E. coli*. Right panel: efficiency and mutagenicity of TLR at the H-edC. T, A: incorporation of thymidine and adenine respectively. (C) Induction of γH2AX in *Xpc* and *Rev1Xpc* MEFs after exposure to 15 μM HNE, analyzed by Western blotting. (D) Immunocytochemical analysis of the replicative stress markers γH2AX and pChk1 in *Xpc* and *Rev1Xpc* MEFs, 24h after exposure to 5 μM HNE. (E) Induction of the double-strand DNA break markers 53BP1 and Rad51 in *Xpc* and *Rev1Xpc* MEFs, 24h after exposure to 5 μM HNE. (F) Left panel: In the cytokinesis-block micronucleus assay cells are arrested during cytokinesis, which allows the quantification of chromatid fragment-containing micronuclei. Right panel: quantification of genome instability at 48h after exposure of *Xpc* and *Rev1Xpc* MEFs to equitoxic HNE doses, corrected for the dose used. $n=3$ independent experiments. ***: $p<0.001$. Error bars: SD. (G) mRNA levels in primary *Xpc* and *Rev1Xpc* MEFs of two key somatotrophic genes at 6 and 24h after exposure to different UV doses, as determined by quantitative PCR. $n=3$ independent experiments. Error bars: SD

In further support of a causal role of *Rev1* in suppressing replicative stress in response to hydroxalkenal-DNA adducts, exposure of *Rev1Xpc* MEFs to HNE induced phosphorylation of the stress markers Chk1 and of H2AX (Figs. 4C and D). Finally, HNE exposure of *Rev1Xpc*, but not *Xpc*, MEFs, induced foci of the double strand DNA breaks markers 53BP1 and Rad51 (Fig. 4E) and micronuclei that contain chromosomal fragments (Fig. 4F). These results indicate that in the absence of *Rev1* replication forks collapse at hydroxalkenal-DNA adducts, resulting in genome instability. We hypothesize that genome instability originating from replicative stress at endogenous bulky DNA adducts sustains the development of lymphomas in *Rev1Xpc* mice.

Transcriptional stress induces a longevity-promoting response by suppressing the somato-, lacto- and thyrotrophic axes (Garinis *et al.*, 2009; Hoeijmakers, 2009; Niedernhofer *et al.*, 2006). We investigated whether also replicative stress induces this response. However, compared with *Xpc* MEFs (Garinis *et al.*, 2009), UV exposure of *Rev1Xpc* MEFs induced no exacerbated or prolonged suppression of the somatotrophic genes *Ghr* and *Igf1r* (Fig. 4G). Furthermore, compared with age-matched *Xpc* mice, the level of the somatotrophic protein IGFBP1 was not altered in livers of progeroid *Rev1Xpc* mice (Fig. S9). Since, unlike replicative stress, transcriptional stress may occur in all aging cells, it may be a more appropriate inducer of the shift to maintenance in aging organisms.

We conclude that replicative stress at endogenous bulky DNA adducts induces the attrition of proliferating stem and differentiated cells that underlies the aging phenotypes of *Rev1* and, more pronounced, of *Rev1Xpc* mice (Fig. S1 for an integrative model). Each progeroid mouse model is believed to emphasize a segment of the pathology of physiological aging (Burtner *et al.*, 2010). The phenotypes of *Rev1* and *Rev1Xpc* mice, therefore, support a causal role for replicative stress also in physiological aging and associated carcinogenesis. Despite the fact that hydroxyalkenal-DNA adducts are substrates for GG-NER, these lesions accumulate to high levels during physiological aging, also in GG-NER-proficient mice and humans (Chou *et al.*, 2010; Wang *et al.*, 2012; Winczura *et al.*, 2012). Given their long lifespan it is not unlikely that replicative stress at bulky DNA adducts is a more prominent determinant of aging and associated cancer in humans than in mice.

MATERIALS AND METHODS

Mice and cell lines. *Rev1* and *Xpc* mice were described previously (Jansen *et al.*, 2006; Melis *et al.*, 2008). *Rev1* ($n=32$) and wild type ($n=55$) cohorts were of mixed 129OLA/SV and C57Bl/6 background. SKH (hairless, albino) *Xpc* and *Rev1Xpc* cohorts ($n=17$ each) were obtained from backcrossing *Rev1^{+/-}Xpc^{-/-}* parents. MEF lines were obtained from 13.5-day embryos by spontaneous immortalization. Survival was measured by clonogenic or proliferation assays.

Histology. Mouse tissues were fixed in Bouin or Harrison's fixative, and paraffin-embedded sections were stained by Haematoxylin and/or Eosin (HE). Pathological examination was performed on coded samples. Karyomegaly and lipofuscin deposits were quantified by grading

4 coded sections per mouse from 0-3, using 8 mice per genotype. Epidermal cell layers were counted on 4 coded sections per mouse, using 8 mice per genotype. Two-tailed T tests were used to calculate significance.

Analysis of the hematopoietic system. Isolation of hematopoietic precursor populations and HSCs and competitive transplantation experiments using HSCs were performed as described (Dykstra *et al.*, 2011). CAFC assays were performed on stromal cells, as described (van Os *et al.*, 2008). Colony sizes were determined using cytokine-supplemented media, as described (Dykstra *et al.*, 2011).

Flow cytometry analysis, immunohistochemistry and Western blotting. Splenocytes were isolated from 6-7 mice per genotype. T cell subsets were stained using anti-CD4 and anti-CD8 antibodies and immature B cells were stained using anti-IgM and anti-IgD antibodies, as described (Jansen *et al.*, 2006). Immunohistochemistry and Western blotting were performed using established protocols. Apoptosis was assessed by TUNEL staining and quantified on 2 coded Cytospin-precipitated splenocyte samples per mouse, using 4 mice per genotype. Significance was assessed by two-tailed T test.

TLR assay. The generation of a site-specific single-stranded H-edC lesion and the determination of the efficiency and mutagenicity of TLR was performed essentially as described for a benzo[*a*]pyrene-dG adduct (Hashimoto *et al.*, 2012) (Fig. 4B).

Cytokinesis-block micronucleus assay. *Xpc* and *Rev1Xpc* MEFs were treated with equitoxic doses of HNE (9 μ M and 5 μ M, respectively). Chromatid breaks were quantified in cells blocked at cytokinesis (Fenech, 1993) (Fig. 4F). Results were analysed using a two-tailed T-test.

Quantitative PCR. Somatotrophic gene expression in UV-treated MEFs and senescence-related gene expression in mice were measured as described (Garinis *et al.*, 2009).

SUPPLEMENTARY FIGURES

6

REV1 CONTRIBUTES TO NORMAL AGING

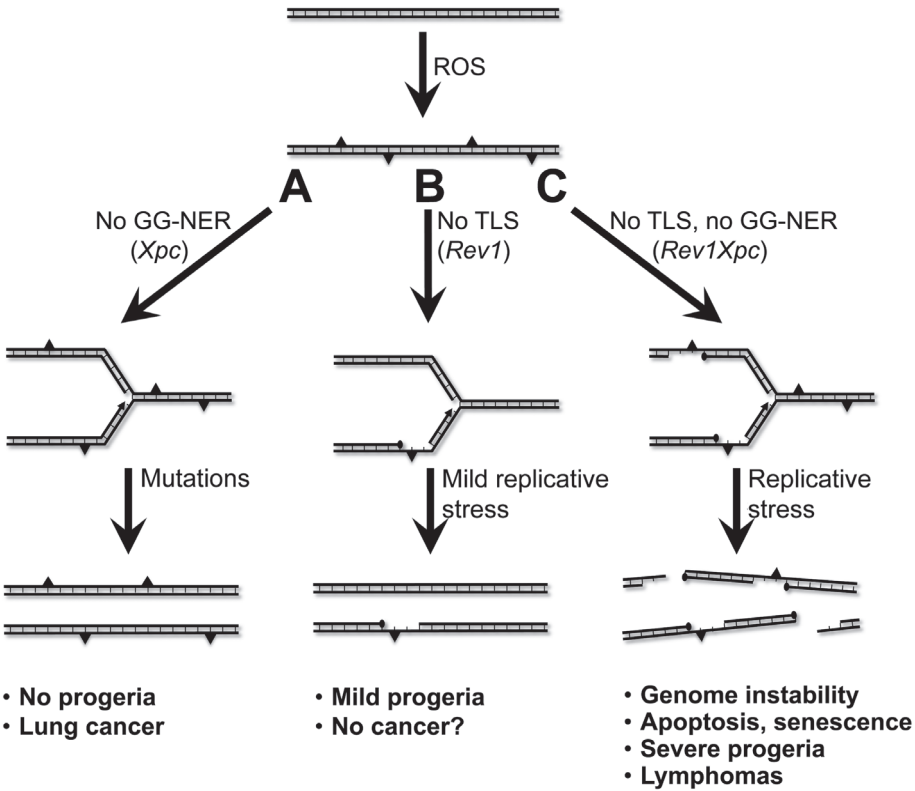


Figure S1 | GG-NER and TLS deficiencies differentially affect cancer predisposition and aging. Top: genomic DNA is damaged continuously by endogenous sources, including ROS that, in addition to base damages, induces bulky nucleotide lesions (triangles). (A) Deficiency of GG-NER of endogenous bulky nucleotide lesions leads to a TLS-dependent accumulation of mutations that may underlie the predisposition to spontaneous lung tumors in old *Xpc* mice (Melis *et al.*, 2008). (B) Deficiency of TLS results in mild replicative stress at endogenous bulky nucleotide lesions that have escaped GG-NER. This is associated with mild progeroid features, but not with obvious cancer predisposition. (C) Combined TLS and GG-NER deficiency leads to severe replicative stress at endogenous bulky nucleotide lesions. This induces genome instability, apoptosis and senescence in proliferating compartments and, consequently, accelerated aging. The genomic instability, combined with escape from apoptosis and senescence, may underlie the development of aging-associated lymphomas.

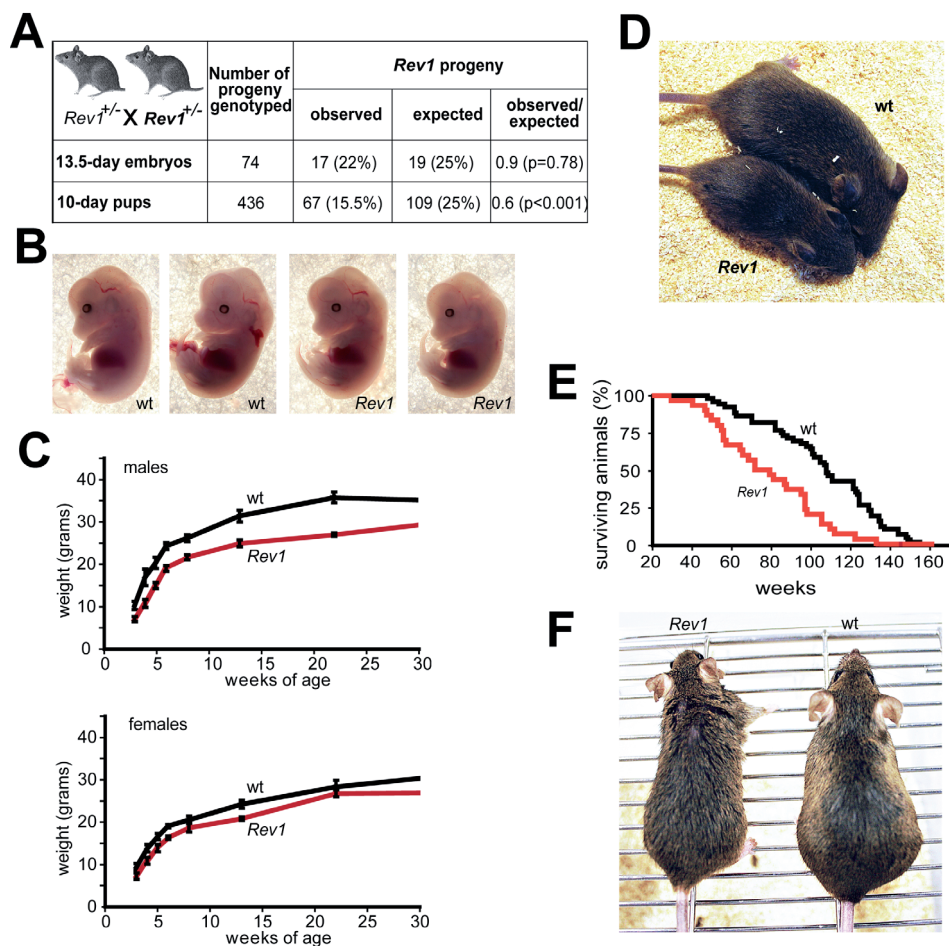


Figure S2 | Phenotypes of *Rev1* embryos and mice. (A) Frequency of 13.5-day *Rev1* embryos and pups from *Rev1*^{+/-} backcrosses. (B) *Rev1* embryos at day 13.5 of gestation are undistinguishable from wild type controls. (C) Body weights of *Rev1* male and female mice and wild type littermates. Data derived from (Jansen *et al.*, 2006). (D) *Rev1* mice of 2 months of age display a variably reduced body size but no other overt phenotypes. Reproduced from (Jansen *et al.*, 2006). (E) Reduced lifespan of *Rev1* mice (*Rev1*: $n=32$, $t_{50}=75$ weeks; wild type: $n=55$, $t_{50}=108$ weeks; $p<0.05$, Mann-Whitney test). (F) An 18-month old *Rev1* mouse displaying ruffling of the fur.

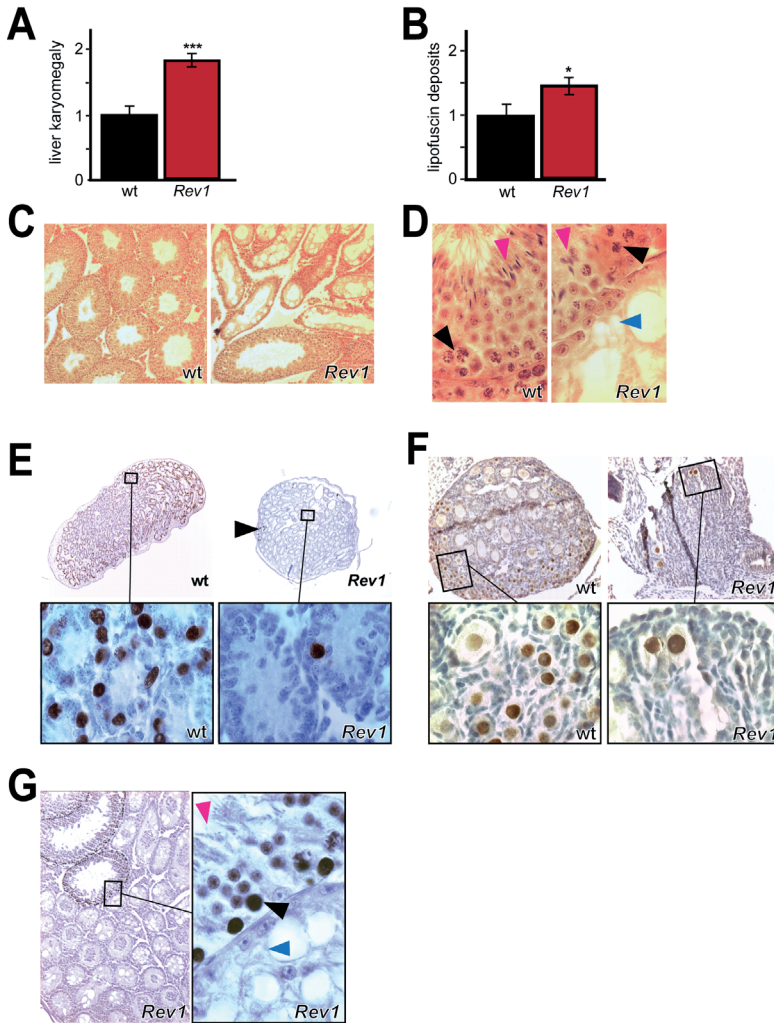


Figure S3 | Phenotypes of *Rev1* liver and gonads. (A) Quantification of hepatic karyomegaly in (18-months old) *Rev1* and age-matched *Xpc* mice. $n = 8$ mice, ***: $p < 0.001$. Error bars: SD. (B) Quantification of hepatic lipofuscin in (18-months old) *Rev1* mice and age-matched *Xpc* controls. $n = 8$ mice, *: $p = 0.011$. Error bars: SD. See the legend to Fig. 2C for experimental details. Bouin-fixed sections reveal a lack of spermatogenic stages in most seminiferous tubules in testis from a 2-month old *Rev1* mouse. (C-D) Residual spermatogenesis (arrowhead) in a testis of a 2-month old wt and *Rev1* mouse. Black arrowhead: meiotic (anaphase) cells, Purple arrowhead: early spermatids. Blue arrowhead: the lower tubule is devoid of spermatogenic stages. (E) Staining for GCNA1 (brown) in testes from a 6 day-old wt and *Rev1*. Loss of GCNA1 staining in *Rev1* male demonstrates the early attrition of germ stem cells. Sections were counterstained with hematoxylin and were the same as depicted in Figure 1B. Arrowhead: GCNA1-positive tubules. Bottom panels: magnified sections displaying rare GCNA1-positive germ cells in the *Rev1* male. (F) Staining for GCNA1 (brown) in ovaries from a 6 day-old wt and *Rev1*. Loss of GCNA1 staining in *Rev1* female demonstrates the early attrition of germ stem cells. Sections were counterstained with hematoxylin. Bottom panels: magnified sections displaying residual GCNA1-positive germ cells in the *Rev1* female. (G) GCNA1 staining (brown) confirms the presence of a low fraction of normal testicular tubules of an adult *Rev1* mouse. Sections were counterstained with hematoxylin. Right panel: magnification displaying GCNA1-positive germ cells (black arrowhead) and early spermatids (purple arrowhead).

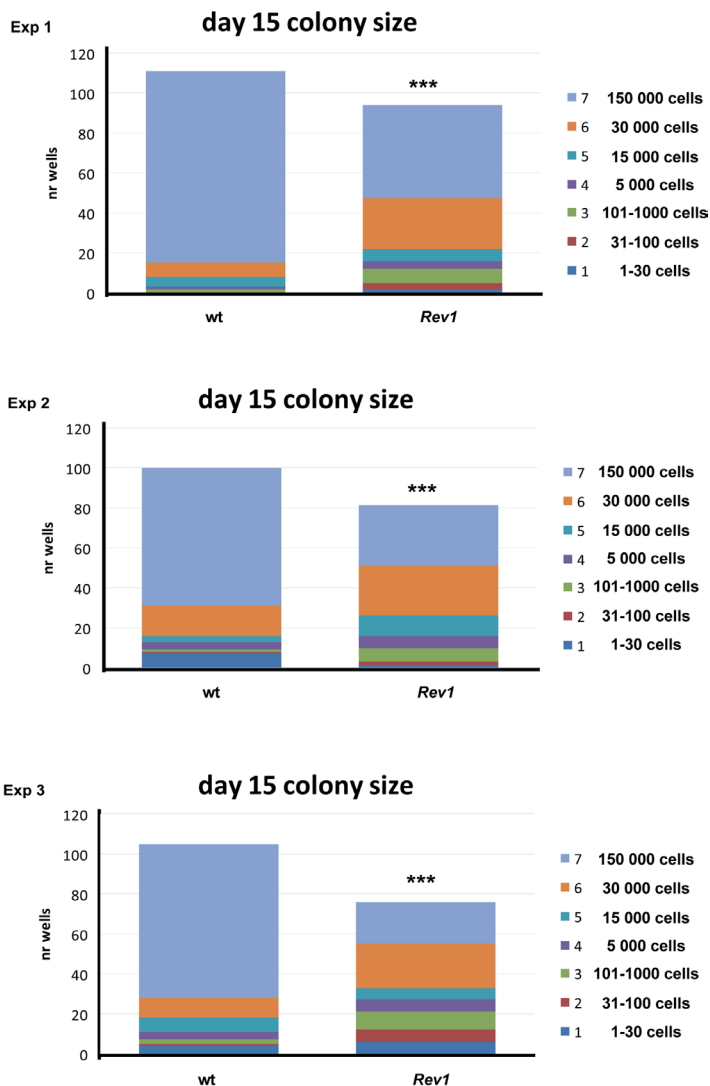


Figure S4 | Reduced sizes of *Rev1* HSC clones. HSCs from three 18-month-old *Rev1* and three wild type mice were seeded in cytokine-supplemented medium and colony sizes were determined at 15 days after seeding. χ^2 test, ***: $p < 0.001$. Error bars: SD.

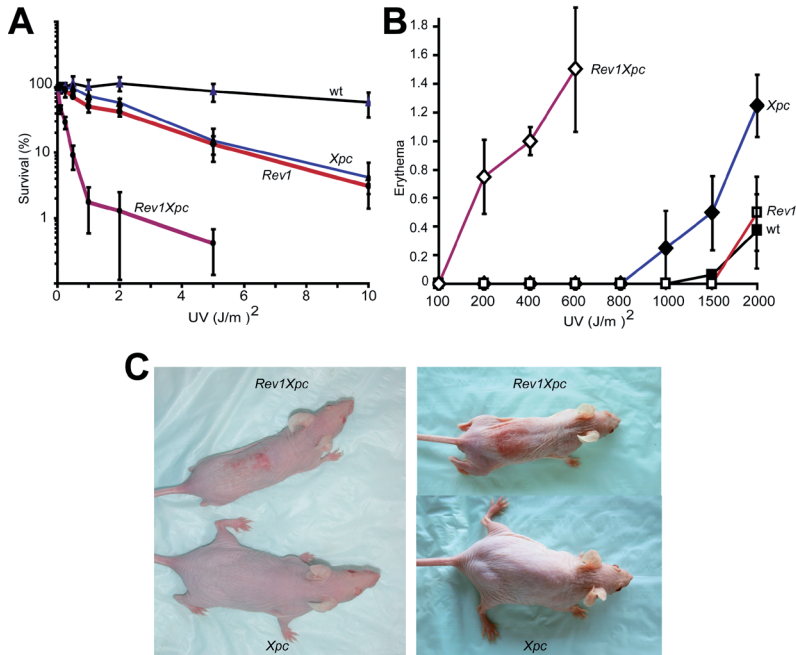


Figure S5 | Sensitivity of *Xpc*, *Rev1* and *Rev1Xpc*-deficient cells and mice to UV-induced photolesions. (A) Clonal survival of UVC-exposed wild type, *Rev1*, *Xpc* and *Rev1Xpc* MEFs. $n=4$ independent experiments. Error bars: SD. (B) Sensitivity of skin of hairless albino wild type, *Rev1*, *Xpc* and *Rev1Xpc* mice, 7 days after local exposure to artificial sunlight. $n=4$ per dose per genotype. Error bars: SD. (C) Induction of erythema in 2-months old *Rev1Xpc* mice, but not in age-matched *Xpc* controls, locally exposed to 40 J/m² of artificial sunlight per day, during 5 subsequent days.

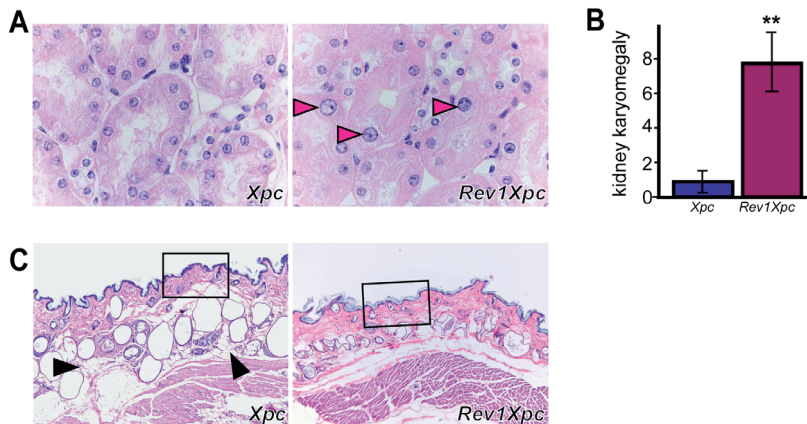


Figure S6 | Degenerative phenotypes in organs of progeroid *Rev1Xpc* mice. (A) HE staining of a kidney section from a *Rev1Xpc* mouse and an age-matched *Xpc* control. Purple arrowheads: karyomegaly. (B) Relative levels of renal karyomegaly in *Rev1Xpc* mice, compared with age-matched *Xpc* controls. $n=8$ mice, **: $p<0.01$. Error bars: SD. (C) HE staining of skin sections. Black arrowheads: subdermal fat in the *Xpc* mouse. Black rectangles: sections magnified in Fig. 2I.

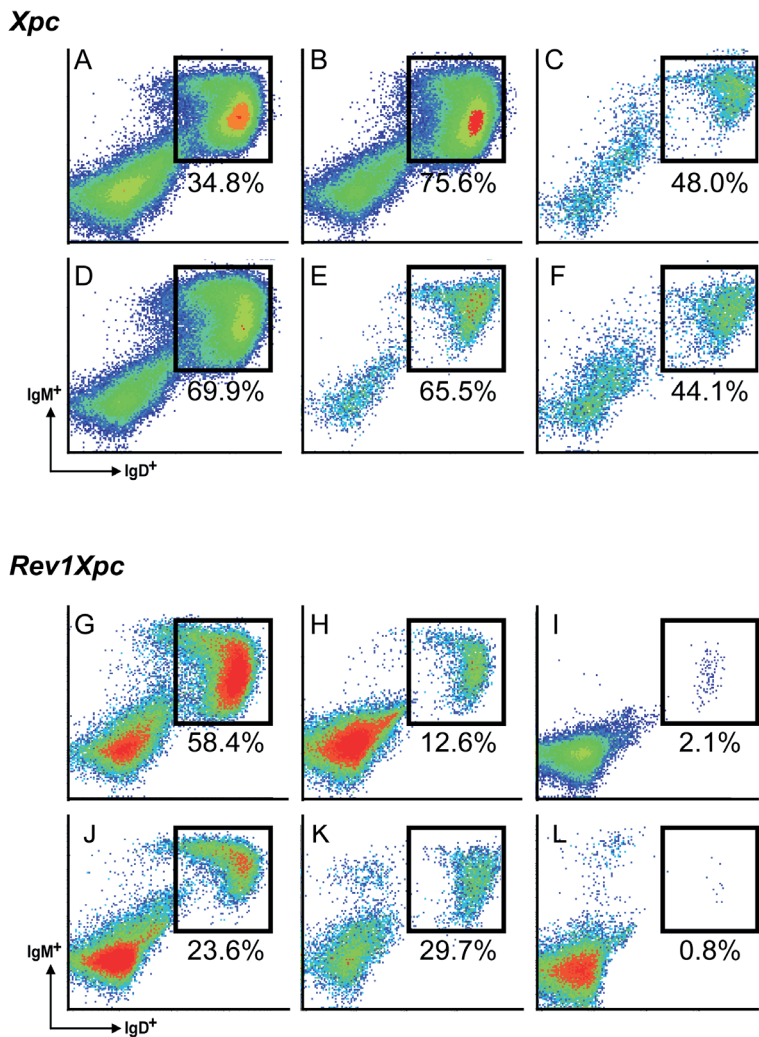


Figure S7 | Analysis of B lymphocytes in spleens of progeroid *Rev1Xpc* mice and age-matched *Xpc* controls. (A-F) Fluorescence cytometry analysis and quantification (% of total cell counts) of IgD⁺IgM⁺ B cells in spleens of 6 adult *Xpc* mice. Panel B represents the same mouse as Fig. 3A (left). (G) Fluorescence cytometry analysis and quantification (% of total cell counts) of IgD⁺IgM⁺ B cells in the spleen of a young *Rev1Xpc* mouse demonstrating that the B cell compartment is normal at young age. (H-L) Fluorescence cytometry analysis and quantification (% of total cell counts) of IgD⁺IgM⁺ B cells in 5 spleens of progeroid *Rev1Xpc* mice (age-matched with the *Xpc* controls analyzed in panels A-F). Panel H represents the same mouse as Fig. 3A (right panel).

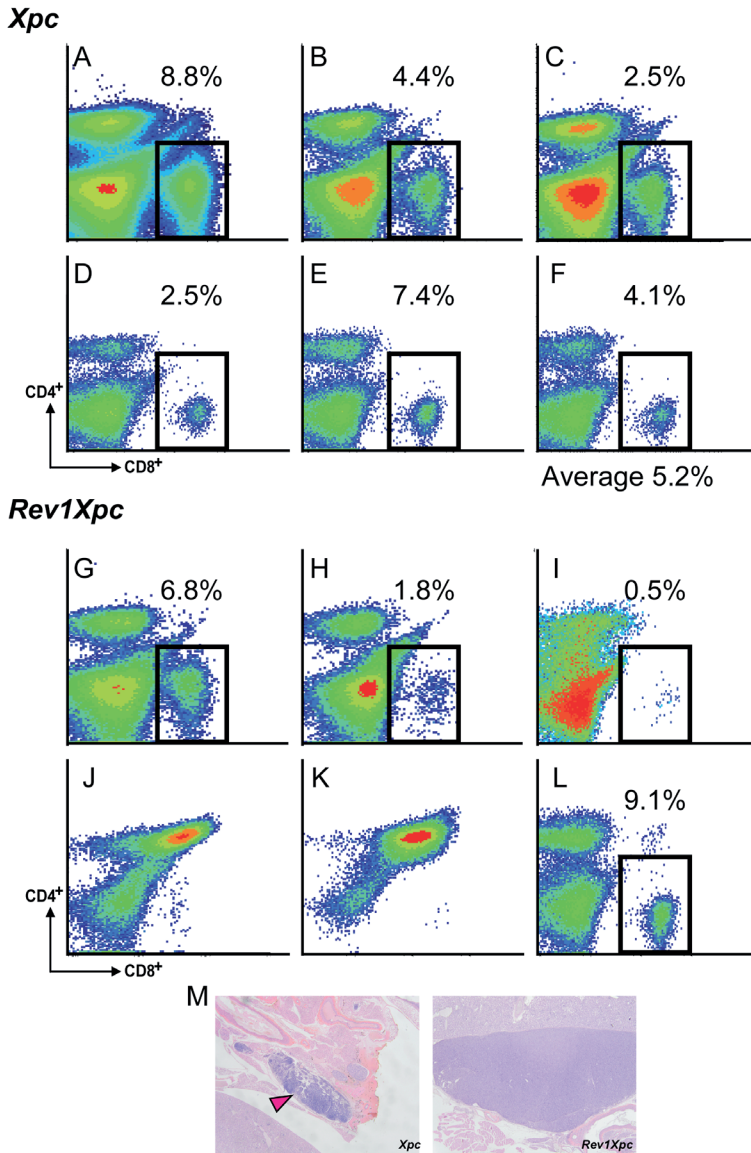


Figure S8 | Analysis of T lymphocytes in spleens of *Rev1Xpc* mice. Mice were the same as in Fig. S7. (A-F) Fluorescence cytometry analysis and quantification (% of total cell counts) of CD4⁺ and CD8⁺ T cells in spleens of 6 adult *Xpc* mice. Panel B represents the same mouse as Fig. 3C. Fluorescence cytometry analysis and quantification (% of total cell counts) of CD4⁺ and CD8⁺ T cells in the spleen of a young *Rev1Xpc* mouse demonstrating that the T cell compartment is normal at young age. (H-I) Fluorescence cytometry analysis and quantification (% of total cell counts) of CD4⁺ and CD8⁺ T cells in spleens of progeroid *Rev1Xpc* mice reveal a reduction in the numbers of CD8⁺ T cells. Panel H represents the same mouse as Fig. 3C (right panel). (J-K) CD4⁺CD8⁺ T cell lymphomas in two progeroid *Rev1Xpc* mice. Panel K represents the same mouse as Fig. 3D. (L) Increased level of CD8 expression (rightward shift) of CD8⁺-positive T cells in a progeroid *Rev1Xpc* mouse. (M) CD4⁺CD8⁺ T cell lymphoma in a mesenteric lymph node in a progeroid *Rev1Xpc* mouse. Purple arrowhead: normal mesenteric lymph node.

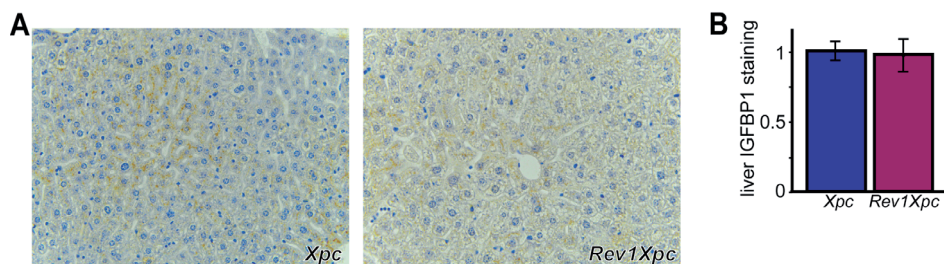


Figure S9 | Expression of the somatotrophic protein IGF1R in liver. A. Expression of IGFBP1 (brown) in livers of a progeroid *Rev1Xpc* and an age-matched *Xpc* mouse. B. Relative levels of IGFBP1 expression in liver of progeroid *Rev1Xpc* mice, compared with age-matched *Xpc* controls. No differential IGFBP1 expression is observed. $n=8$ mice each. Error bars: SD.

Table S1 | Pathological examination of the mouse cohorts

Mouse #, genotype, age (months) ¹ , sex	Liver	Bone marrow	Skin	Other pathologies
007, wt, 28, ♀				Chronic inflammation. Possible B-cell hyperplasia.
008, wt, 28, ♂	Reactive hepatitis. Possible hepatoma.		Mild atrophy of hair follicles and sebaceous glands	Thyroid carcinoma. Possible lymphoma.
010, wt, 25, ♀				Lymphoid hyperplasia.
012, wt, 24, ♂				Early stage B-cell lymphoma.
013, wt, 24, ♂				B-cell hyperplasia.
016, wt, 22, ♀	Hemangioma.			B-cell lymphoma.
018, wt, 19, ♀				Chronic inflammation. Possible B-cell hyperplasia.
185, <i>Rev1</i> , 19, ♀	Fatty vacuolisation. Cytoplasmic glycogenosis. Steatosis. Lipofuscin accumulation. Karyomegaly.	Hypoplasia.		Kidney: chronic inflammation. Hyalin deposits (Hill <i>et al.</i> , 2003) in glomerular membranes. Lymphoid follicular atrophy
186, <i>Rev1</i> , 19, ♀	Fatty vacuolisation. Cytoplasmic glycogenosis. Steatosis. Lipofuscin accumulation. Karyomegaly.	Hypoplasia.	Atrophy of hair follicles and sebaceous glands	B-cell lymphoma. Osteosclerosis. Lymphoid follicular atrophy
187, <i>Rev1</i> , 21, ♂	Fatty vacuolisation. Cytoplasmic glycogenosis. Lipofuscin accumulation. Karyomegaly.	Hypoplasia.		Kidney: Hyalin deposits in glomerular membranes.
005, <i>Rev1</i> , 28, ♀	Steatosis.	Hypoplasia.		B-cell lymphoma. Lymphoid follicular atrophy.
006, <i>Rev1</i> , 28, ♀	Lipofuscin accumulation.		Atrophy of hair follicles and sebaceous glands.	B-cell lymphoma. Hemangioma in ovary.
009, <i>Rev1</i> , 25, ♀		Hypoplasia.	Mild atrophy of hair follicles and sebaceous glands.	Mild tubular degeneration in liver.
011, <i>Rev1</i> , 24, ♂	Steatosis.	Hypoplasia.	Atrophy of hair follicles and sebaceous glands.	Lymphoma
014, <i>Rev1</i> , 24, ♂	Steatosis.		Mild atrophy of hair follicles and sebaceous glands.	Papillary adenoma in uterus.
049, <i>Rev1</i> , 25, ♂	Karyomegaly.	Hypoplasia.	Atrophy of hair follicles and sebaceous glands.	

Table S1 | Pathological examination of the mouse cohorts (*continued*)

Mouse #, genotype, age (months) ¹ , sex	Liver	Bone marrow	Skin	Other pathologies
005, Rev1, 28, ♀	Steatosis.			
006, Rev1, 28, ♀	Lipofuscin accumulation. Karyomegaly.	Hypoplasia.	Atrophy of hair follicles and sebaceous glands.	
002, Xpc, 8, ♀				
003, Xpc, 6, ♀				
004, Xpc, 10, ♀				
043, Xpc, 8, ♀				
046, Xpc, 13, ♂				
047, Xpc, 12, ♀				
048, Xpc, 10, ♀				
052, Xpc, 8, ♀		Aplastic anaemia.	Atrophic epidermis. Lack of subcutaneous fat.	Kidney: karyomegaly.
180, Rev1Xpc, 7, ♀	Mild glycogenosis. Lipofuscin accumulation. Karyomegaly.	Aplastic anaemia.	Atrophic epidermis. Lack of subcutaneous fat	Kidney: karyomegaly, tubular degeneration.
181, Rev1Xpc, 10, ♀	Mild glycogenosis. Lipofuscin accumulation. Karyomegaly.	Aplastic anaemia.	Atrophic epidermis. Lack of subcutaneous fat	Kidney: karyomegaly, tubular degeneration.
182, Rev1Xpc, 13, ♀	Karyomegaly.	Aplastic anaemia.		Kidney: severe karyomegaly.
042, Rev1Xpc, 8, ♂	Steatosis.	Aplastic anaemia.	Atrophic epidermis. Lack of subcutaneous fat	Kidney: severe anisokaryosis, tubular degeneration.
044, Rev1Xpc, 11, ♀	Karyomegaly.			Kidney: tubular degeneration.
045, Rev1Xpc, 13, ♂ (not moribund)	Steatosis.	Aplastic anaemia.	Atrophic epidermis. Lack of subcutaneous fat	Possible lymphoma.
050, Rev1Xpc, 6, ♀	Karyomegaly.	Hypoplasia.	Atrophic epidermis. Lack of subcutaneous fat	Osteoporosis.
051, Rev1Xpc, 7, ♀	Karyomegaly.	Aplastic anaemia.	Atrophic epidermis. Lack of subcutaneous fat	Lymphoma.
053, Rev1Xpc, 6, ♀	Karyomegaly.	Aplastic anaemia.	Atrophic epidermis. Lack of subcutaneous fat.	Osteoporosis.

¹ *Rev1Xpc* and *Rev1* mice were euthanized and autopsied when moribund. The age-matched *Xpc* and wild type littermates were sacrificed before the onset of end-of-life pathology.

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CHAPTER

7

PERSPECTIVES



In this thesis, I have characterized the role of genes involved in TLS at the genome of mammalian cells in response to DNA-damaging agents, using mouse embryonic fibroblast (MEFs) and mice with defined mutations in these genes. To this aim, I have developed a toolbox to study multiple aspects of TLS of the damaged genome, including bypass of different types of lesions, replication fork progression, mutagenesis, DNA damage signaling, genome breakage and cell survival. This work shows comprehensively the relative *in vivo* contributions of a number of mammalian TLS-associated polymerases to replication of damaged DNA and to DNA damage signaling responses when cells are exposed to common food-derived genotoxins and to UV light. Additionally, this thesis provides evidence that ssDNA gaps can be transmitted through mitosis into the subsequent cell cycle and converted into DSBs that underlie genome instability, in a DNA replication-dependent fashion. Lastly, I have shown that Rev1, a key TLS polymerase, is required for preventing premature aging in mice.

THE COMPLEXITIES OF TLS

Many *in vitro* studies of individual TLS polymerases have been conducted and these have yielded important insights into their biochemical properties (Waters *et al.*, 2009), rather than on their functions and contribution to TLS in a living cell. In Chapters 2 and 3, I described the roles of different mammalian TLS polymerases in response to DNA damages at nuclear chromatin. Even if, at the genome, some types of DNA lesions, such as CPDs, may be bypassed by only one TLS polymerase at the replication fork (Yoon *et al.*, 2009), the present idea of TLS is that persistent DNA damage is replicated by the concerted action of at least two TLS polymerases: inserter polymerases and extender polymerases. The inserter TLS polymerase is capable of inserting nucleotides across DNA lesions, while extension beyond the lesions is performed by extender TLS polymerases (Prakash *et al.*, 2002; Shachar *et al.*, 2009; Yoon *et al.*, 2009; Yoon *et al.*, 2010). So far, mammalian Polk and Polζ have been implicated in the latter stage (Haracska *et al.*, 2002; Prakash *et al.*, 2002). Data in this thesis suggest that the extender function of Polk might be limited to specific DNA lesions, since MEFs deficient for Polζ display persistent stalled replication forks at UV-induced (6-4)PPs and at BPDE lesions (Chapters 2 and 3). This suggests that, for these DNA lesions, (i) other TLS polymerases, including Polk, are not capable of acting as an extender polymerase, or that (ii) Polk-mediated extension might be Polζ-dependent.

REGULATION OF TLS

Because TLS is an inherently mutagenic process, its activity should be tightly controlled. This thesis and work by others provide evidence that Rev1 and Polζ are indispensable for bypassing strongly helix-distorting (6-4)PPs (Shachar *et al.*, 2009; Szuts *et al.*, 2008), whereas they have a minor role in the bypass across mildly helix-distorting CPDs. In

addition, CPDs and Benzo[a]pyrene diepoxyde (BPDE)-induced DNA adducts seem to be cognate DNA lesions for Pol η and Pol κ , respectively (Bi *et al.*, 2005; Yoon *et al.*, 2009; Zhang *et al.*, 2002). It is not known how the “optimal” TLS polymerase is recruited to a specific lesion. In yeast and in human cells, arrest of the replication fork by DNA adducts elicits TLS via the mono-ubiquitination of PCNA at K164 (PCNA-Ub), especially for TLS by Pol η across mildly distorting CPDs (Bienko *et al.*, 2010). This may suggest a “tool belt” mechanism whereby all TLS polymerases are bound to PCNA which, upon fork stalling and ubiquitination, selects the best suited TLS polymerase to perform the bypass (Freudenthal *et al.*, 2010). However, data in chapter 3 and from Edmunds *et al* suggest that Rev1 and Rev3-mediated TLS is independent from PCNA-Ub (Edmunds *et al.*, 2008). Therefore, a reasonable possibility could be that the TLS polymerase selection is based on a trial and error basis. In such a model, when a particular TLS polymerase would not be able to appropriately bypass the lesion, another might be invoked, possibly only after repriming of replication downstream of the lesion (see below). Other processes and pathways that may regulate mutagenic TLS include (i) proofreading *in trans* by the replicative polymerases (Bebenek *et al.*, 2001a), (ii) DNA mismatch repair (Tsaalbi-Shtylik *et al.*, in preparation), and (iii) the Fanconi anemia (FA) pathway. The FA pathway has been implicated in the regulation of Rev1 and Rev3 via its FANCC component (Niedzwiedz *et al.*, 2004). In support, it has recently been shown that the FA-associated protein (FAAP20) interacts with Rev1 through its ubiquitin-binding domain (UBZ4) (Kim *et al.*, 2012). Depletion of FAAP20 leads to loss of recruitment of Rev1 to UV damages, indicating that the FA complex may stimulate TLS. Furthermore, the FA complex possesses ubiquitin ligase activity, and it is tempting to speculate that the FA complex may regulate TLS by ubiquitination of TLS-related proteins in response to strongly helix-distorting DNA lesions. In addition, the FA complex may also indirectly influence TLS, since recent data indicate that the FA complex stabilizes stressed replication forks. Accordingly, in the absence of the FA complex, destabilized forks may not be suitable for Rev1-dependent TLS (Lossaint *et al.*, 2013).

Recently, it has been shown that a subunit of the yeast replicative DNA polymerase Pol δ , Pol32, interacts with Pol ζ and that this interaction is required for Pol ζ -mediated TLS (Johnson *et al.*, 2012). Likewise, in human cells, the human ortholog of Pol32, POLD3, interacts with Rev1 and Pol ζ as well as with the ubiquitin-binding protein Spartan. Depletion of Spartan leads to enhanced UV-induced mutagenesis, which is dependent on Rev1 and Pol ζ (Kim *et al.*, 2013). Together, these data suggest a link between the coordination and regulation of replicative and TLS DNA polymerases.

MECHANISTIC BASIS FOR TLS

To bypass DNA lesions, TLS may act in two modes: (i) direct bypass and (ii) post-replicative gap filling. Post-replicative gaps can be generated by re-priming of processive

replication beyond the lesions or by the convergence of an adjacent replicon. Possibly, cells employ both TLS modes to bypass DNA damages, depending on the architecture of DNA lesions. For instance, the mildly-helix distorting CPDs might be competently bypassed by Pol η via direct bypass, while TLS across the strongly helix-distorting (6-4) PPs is thought to involve post-replicative gap filling (Jansen *et al.*, 2007). It was shown by electron microscopy that TLS-deficient yeast cells accumulate ssDNA gaps 30 min after UV exposure, providing evidence for TLS as a gap-filling mechanism (Lopes *et al.*, 2006). It would be intriguing to apply the same approach to human or mouse Xpc mutant cells that are deficient for global-genome nucleotide excision repair (GG-NER) of UV lesions, but proficient for TLS. Consistent with a role in post-replicative gap filling, Rev1 or Rev3 mutant mouse cells show the accumulation of (6-4)PPs-containing ssDNA patches in the G₂ phase (Jansen *et al.*, 2009a; Jansen *et al.*, 2009b). Although also in mammalian cells gap-filling TLS at an episomal substrate can be separated from replication (Diamant *et al.*, 2012), the physiological role of post-replicative gaps at the genome is not established, as it is difficult to exclude the possibility that, in Rev1 or Rev3-proficient cells, CPDs and (6-4)PPs are bypassed directly. ssDNA gaps activate the Atr-mediated DNA damage signaling pathway and the loading of the 9-1-1 complex to 5' primer-dimer termini (Majka *et al.*, 2006; Nam *et al.*, 2011). Furthermore, yeast Rev1, Pol ζ as well as Polk might be recruited to damaged chromatin and interact with the 9-1-1 complex (Jansen *et al.*, 2007; Kai *et al.*, 2003; Sabbioneda *et al.*, 2005; Yu *et al.*, 2003). In yeast, it has been demonstrated that 9-1-1 mutant yeast cells display a hypomutable phenotype to DNA damaging agents (Murakami-Sekimata *et al.*, 2010; Paulovich *et al.*, 1998). This implies that the 9-1-1 complex is at the intersection between TLS and DNA damage signaling in yeast. Recently, it was found that Rev1, via its N-terminal BRCT domain, specifically binds 5' phosphorylated primer-template termini *in vitro*, a result consistent with recruitment of the protein to post-replicative gaps (de Groote *et al.*, 2011). Further study for the interaction between Rev1 or Rev3 and the 9-1-1 complex in mammalian cells might provide more insight into the linkage of TLS and DNA damage signaling.

ISOLATION AND CHARACTERIZATION OF TLS COMPLEXES

Unraveling the composition of TLS polymerase-containing complexes in response to a particular kind of DNA damage in the genome, and dissecting whether such complexes act directly at stalled replication forks or at post-replicative gaps will contribute in understanding TLS at the molecular level. This can be investigated by using different approaches. To study the proximity of photolesions in single-stranded DNA, different DNA polymerases, and the 9-1-1 complex, the proximity ligation assay can be applied. This assay is a highly sensitive, antibody-dependent, technique to investigate the proximity of biomolecules *in situ* (Gullberg *et al.*, 2003). Similarly, chromatin immunoprecipitation (ChIP), using an antibody specific for a protein or DNA adduct in concert with western blotting or mass spectrometry, may be employed

to analyze protein complexes at ssDNA containing CPDs or (6-4)PPs. Additionally, such ChIP analysis also allows the identification of histone modifications, which may provide evidence on the existence of preferred chromatin regions for TLS polymerases. To isolate protein complexes at replication forks, an assay called Isolation of proteins on nascent DNA (iPOND) can be used (Sirbu *et al.*, 2012; Sirbu *et al.*, 2013). This assay relies on the pull-down of DNA replication forks that have incorporated 5-ethynyl-2'-deoxyuridine (EdU) prior to stalling, with associated proteins, which can be followed by western blotting or mass spectrometry. To enhance the specificity, an antibody that specifically binds to single-stranded CPDs or (6-4)PPs may be used to pull down replication complexes at these lesions, prior to iPOND. In addition, epitope-tagged ubiquitin or other post-translational modifications will provide a map showing which proteins are modulated in response to stalled replication forks by specific DNA damaging agents.

GENERATION OF CELL LINES WITH MULTIPLE DEFICIENCIES IN TLS-RELATED GENES: NEXT GENERATION MUTAGENESIS STRATEGIES

After identifying TLS-associated complexes at particular lesions, cells deficient for specific TLS-associated proteins or TLS complexes could be generated in order to study TLS at the genome, using the toolbox developed here. With regard to the generation of cell lines deficient in certain factors, at present two techniques are widely used: siRNA (or shRNA)-mediated knockdown and gene-targeting. The disadvantages of siRNA or shRNA knockdown are efficiency, specificity and stability, while gene-targeting is costly, time-consuming and laborious. The generation of double-mutant cells is even more difficult. Recently, Wang *et al* have demonstrated a novel gene-targeting technology using the CRISPR/Cas system. This technique is highly efficient and can target multiple genes in cells and in mice simultaneously (Wang *et al.*, 2013). Applying this approach to TLS-associated genes will allow the generation of cell lines with various combinations of TLS mutants in a limited time span.

FROM SINGLE STRAND GAPS TO GENOME INSTABILITY

Previous work has shown that *Rev1^{-/-}Xpc^{-/-}* MEFs that are defective in the bypass of (6-4)PPs replicate UV-damaged DNA almost completely, leaving unreplicated (6-4)PPs in ssDNA gaps (Jansen *et al.*, 2009a). In Chapter 5 of this thesis, the fate of these ssDNA gaps was studied. It was found that, at environmentally relevant levels of UV lesions, these gaps, despite activating DNA damage signaling, are transmitted through mitosis into the subsequent cell cycle leading to the formation of DSBs during the ensuing S phase. Yet, high levels of UV damage result in poor cell cycle progression and a strong mitotic arrest ((Jansen *et al.*, 2009a) and data not shown). These data suggest that high levels of ssDNA gaps activate a strong cell cycle checkpoint that precludes

cells from entering a new cell cycle. Apparently, relatively low levels of ssDNA gaps are tolerated in cultured mammalian cells. In the second S phase, ssDNA gaps may stall replisomes resulting in DSBs as reflected by the formation of phosphorylated Atm and the induction of micronuclei (MN). It should be noted, however, that phosphorylated ATM represents only a fraction of DSBs, mainly in heterochromatin (Riballo *et al.*, 2004). In the near future, it would be intriguing to determine whether chromosomal aberrations accompany the induction of MN in the second cell cycle. Furthermore, live cell imaging of *Rev1*^{-/-}*Xpc*^{-/-} MEFs expressing GFP-tagged 53BP1, a DSB marker, allows the analysis of 53BP1 foci formation in the first and second cell cycle after UV exposure. Therefore, such an analysis may provide additional insights in the occurrence of UV-induced DSBs in *Rev1*^{-/-}*Xpc*^{-/-} MEFs. Recently, this approach revealed that in aphidicolin-treated cells 53BP1 foci are formed, when the cells are in the subsequent cell cycle following treatment (Lukas *et al.*, 2011). These data suggest that incompletely replicated DNA in the first cell cycle is transmitted to the daughter cells, comparable with the findings presented in Chapter 5. Surprisingly, *Rev1*-deficient cells are likely defective in the repair of DSBs (Sharma *et al.*, 2012). This might aggravate the genome instability induced by UVC in *Rev1*^{-/-}*Xpc*^{-/-} MEFs. However, also *Xpc*^{-/-} cells accumulate DSBs during the second S phase upon high UVC exposure indicating that the delayed genome instability described in Chapter 5 is not restricted to *Rev1*-deficient cells.

The MN assay is an important standard assay in genotoxicity testing (Kirsch-Volders *et al.*, 2011). The advantages of the assay are its (i) simplicity of scoring, (ii) accuracy, (iii) sensitivity and (iv) rapidity. Nonetheless, this assay requires cycling cells and therefore appropriate doses of test agents are critical to prevent the induction of permanent cell cycle arrests. The Organisation for Economic Co-operation and Development (OECD) guidelines for standard testing for MN recommends the second cell division to be covered in MN analysis (Kirsch-Volders *et al.*, 2011). However, a mechanistic insight related to this advice was lacking. In line with this, the data in Chapter 5 demonstrates that the induction of MN following exposure to agents inducing replication-blocking DNA lesions may require two cell divisions to convert primary DNA damage (a ssDNA gap) into a DSB. Therefore, the testing guidelines released by OECD, including (i) the standard *in vivo* MN test guideline 474 and (ii) the *in vitro* MN test guideline 487, should be obligated.

TLS AND PREMATURE AGING

As already mentioned, stalled replication forks at DNA damage lead to Atr-mediated DNA damage signaling. The bypass of DNA adducts by TLS polymerases results in completion of replication and the quenching of DNA damage signaling. This suggests that there might be an equilibrium between these two mechanisms. It has been demonstrated that TLS, while mutagenic, paradoxically suppresses tumor promotion in mice by mitigating excessive proliferation that likely is caused by DNA damage

signaling associated with chronic replicative stress (Tsaalbi-Shtylik *et al.*, 2009). Perturbation of the balance between mutagenesis, replicative stress and proliferation might lead to pathologies, such as cancer and premature aging. Premature aging correlates with the accumulation of endogenous DNA damages, induced mainly by free radicals (de Boer *et al.*, 2002; Hasty *et al.*, 2003). Lipid peroxidation products, generated endogenously by cells or found in fatty diets, induce free radicals, DNA lesions and large amounts of protein damages. Chapter 2 of this thesis shows that Rev1 and Polt play a role in bypassing DNA lesions induced by hydroxynonenal (HNE), an end product of lipid peroxidation. As described in Chapter 6, *Rev1^{-/-}Xpc^{-/-}* mice display phenotypes of accelerated aging that most likely originates in enhanced replication stress caused by defective TLS at endogenous DNA damages. These endogenous DNA damages might be related, among others, to lipid peroxidation-induced DNA adducts.

Polt has been implicated not only in bypassing HNE adducts (Chapter 2), but it might also act as 5'-deoxyribose phosphate lyase, which is essential for the removal of free radical-induced DNA damages by Base Excision Repair (BER) (Bebenek *et al.*, 2001b). Indeed, depletion of Polt in human cells enhanced their sensitivity to oxidative stress (Petta *et al.*, 2008). At present, it is unclear whether Polt prevents premature aging in mice. It would be interesting to study mice with a defined deletion in the Polt gene.

Although Rev3 performs only a minor role in bypassing HNE-induced lesions (Chapter 2), Rev3 mutant mice display embryonic lethality (Van Sloun *et al.*, 2002; Wittschieben *et al.*, 2000). This suggests that the endogenous DNA damages causing the embryonic lethality of Rev3-deficient mice may not be related to lipid peroxidation products. It has been found that Rev3 mutant chicken DT40 cells are hypersensitive to a nitric oxide inducer, suggesting a role of Rev3 in TLS across nitric oxide-induced DNA damages (Wu *et al.*, 2006). Nitric oxides are generated in cells and interact with oxygen, resulting in the reactive nitrogen species (RNS) that bind to both DNA and proteins. In order to shed light on the role of Rev3 in preventing replication stress induced by nitric oxide and aging, the addition of nitric oxide scavengers to Rev3-defective embryos may prevent embryonic lethality.

Premature aging may be provoked by mutations in DNA of mitochondria, (Lagouge *et al.*, 2013). Mitochondria have no Nucleotide Excision Repair (NER) (LeDoux *et al.*, 1992), thus, there is a possibility that endogenous lipid peroxidation products might induce mutations in mtDNA. In yeast cells, Rev1 and Rev3 are recruited to mitochondria to perform mutagenic TLS (Zhang *et al.*, 2006). At present, a role for mammalian TLS polymerases in mitochondria is not known. Therefore, mutation analysis on mtDNA of TLS-deficient mammalian cells may unveil this process in mammals. Moreover, investigation on the integrity of mitochondria in cells lacking a TLS polymerase might provide a molecular link between TLS, cellular metabolism and aging.

Telomere shortening is also related to premature aging of somatic cells (Lopez-Otin *et al.*, 2013). Telomeres can be classified as a DNA damage trap as they possess limited

DNA repair activity leading to persistent DNA damages, telomere exhaustion and attrition. It is not yet clear whether TLS polymerases play a role in preventing telomere shortening or in preventing DNA damage signaling at persistent ssDNA gaps at telomeres. Further study using mice described in Chapter 6 may provide insights into the role of TLS polymerases in protecting telomeres, and ultimately aging.

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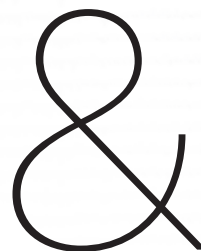
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APPENDIX



SUMMARY
SAMENVATTING
CURRICULUM VITAE
PUBLICATION LIST
ACKNOWLEDGEMENTS

SUMMARY

To cope with DNA damages induced by endogenous and exogenous agents and by spontaneous decay of nucleotides, cells employ both DNA repair and DNA damage tolerance (DDT) mechanisms. Translesion synthesis (TLS) is an important DDT mechanism in mammalian cells. Mammalian TLS is performed by at least five key proteins, *i.e.* TLS DNA polymerases Rev1, η , ι , κ and ζ . These TLS DNA polymerases play roles in bypassing unrepaired DNA adducts during and after S-phase, thereby allowing completion of genome duplication. However, since TLS is a mutagenic process, it must be tightly controlled. Thus far, the *in vivo* role of each TLS polymerase in response to DNA damages in mammalian cells and organisms has largely remained unclear. Furthermore, the relative contribution of each TLS polymerase is also poorly understood. In this thesis, I have used cell lines with single or combined deficiencies in TLS polymerases to explore the absolute and relative *in vivo* contributions of these TLS-polymerases in response to DNA damages induced by food-derived genotoxins and UV light. Furthermore, I have studied the genetic and cellular consequences of unreplicated DNA lesions, resulting from defects in TLS. Using TLS-defective mice, I have addressed the importance of TLS in preventing organismal premature aging.

Chapter 1 provides an introduction to TLS, the main players in this DDT pathway and the different mechanisms by which TLS polymerases may replicate damaged DNA.

Chapter 2 describes the *in vivo* roles of TLS polymerases in response to the food-derived genotoxins Benzo[a]pyrene diepoxyde and Hydroxynonenal (HNE). It was found that Rev1, Pol ζ and Pol η play a central role in TLS when cells are exposed to either one of both agents. Consistent with *in vitro* studies, Pol ι is specifically involved in TLS across HNE-induced DNA damages *in vivo*. Interestingly, in addition to the known role of Pol κ in TLS of BPDE-induced DNA lesions, the BRCT domain of Rev1 is crucial for bypassing BPDE-induced DNA lesions and for quenching DNA damage signaling, although this domain appears to be dispensable for TLS of HNE-induced DNA damages. The study suggests that structurally different DNA lesions are bypassed by various sets of TLS polymerases or require different functional domains of the same polymerase. Failed TLS leads to activation of DNA damage signaling, double-strand DNA breaks (DSBs) and, eventually cell death.

The *in vivo* roles of TLS-related genes in MEFs in response to UVC light are described in **Chapter 3**. UVC light induces CPDs and (6-4)PPs that may be considered as models for mildly and severely helix-distorting DNA lesions, respectively. Using similar doses of UVC light, it was found that Pol ι , Pol κ and the BRCT domain of Rev1 are largely dispensable for the responses to UVC-induced DNA lesions, including cell survival and DNA damage response (DDR). Pol η is mainly required for bypassing CPDs, while Rev1 and Pol ζ are essential for bypassing (6-4)PPs. Deficiencies of these TLS polymerases result in enhanced DNA damage signaling and reduced cell cycle progression. Mono-ubiquitination of PCNA (PCNA-Ub) is predominantly involved in TLS for both CPDs

and (6-4)PPs at early after exposure; however, PCNA-Ub is dispensable for bypassing (6-4)PPs late after exposure, suggesting the existence of PCNA-Ub independent TLS. The results in Chapter 3 suggest the existence of a back-up pathway that restores DNA replication in Pol η -deficient MEFs after UVC irradiation. To gain further mechanistic insights, MEF lines with single, double and triple disruptions of genes encoding Pols η , ι and κ were investigated under the same experimental conditions. These experiments are described in **Chapter 4** and the results show that Pol κ is a major TLS polymerase in TLS across both CPDs and (6-4)PPs in Pol η -deficient MEFs. Interestingly, the triple-mutant cells displayed a more severe defect in TLS at (6-4)PPs compared to Pol η -deficient MEFs with an additional defect in either Pol ι or Pol κ . This finding suggests that multiple TLS polymerases play back-up roles in TLS at severely helix-distorting DNA lesions.

Previously, it was reported that TLS-deficient cells accumulate single-stranded DNA (ssDNA) gaps after exposure to DNA damaging agents such as UVC light. In **Chapter 5** the fate of these ssDNA gaps throughout the cell cycle was analyzed using a comprehensive experimental approach. The results unambiguously show that unreplicated (6-4)PPs within ssDNA gaps generated in both TLS-deficient and TLS-proficient replicating cells, are transmitted through mitosis to the subsequent cell cycle. In the ensuing S phase, these ssDNA gaps are converted into double-strand DNA breaks, the most cytotoxic DNA lesions when not repaired, resulting in the formation of micronuclei (MN). With regard to genotoxicity testing, this study provides a mechanistic basis for the recommendation towards the Organisation for Economic Co-operation and Development to analyze micronuclei formation during two cell cycles after exposure.

Chapter 6 describes a role of DNA replication stress at endogenous DNA lesions in premature aging. Rev1 mutant mice display age-related phenotypes earlier than wild-type mice. These aging-related phenotypes were further accelerated when Rev1-deficient mice were also deficient for Xpc, a protein involved in nucleotide excision repair. These data imply that replicative stress at unrepaired endogenous DNA damages leads to aging. The study furthermore provides experimental evidence that Rev1 acts in TLS across lipid peroxidation-induced bulky DNA lesions that mimic naturally occurring adducts. In this way, Rev1 suppresses replication stress and premature aging.

Finally, a perspective building on, and extending, the results of the studies described in this thesis is provided by **Chapter 7**.

SAMENVATTING

Het DNA van cellen wordt voortdurend beschadigd door blootstelling aan endogene en exogene stoffen die met DNA kunnen reageren, en door spontaan verval. Deze beschadigingen kunnen een blokkade vormen voor de DNA polymerases die het DNA verdubbelen voordat de cel gaat delen. Hierdoor wordt de DNA synthese niet afgemaakt wat tot celdood en genoominstabiliteit kan leiden. Om dit te voorkomen beschikken cellen over zowel DNA-herstel als over DNA-schade tolerantie (DDT) mechanismen. Translesie synthese (TLS) is een belangrijk DDT mechanisme in zoogdiercellen. In deze cellen wordt TLS uitgevoerd door ten minste vijf DNA polymerases: Rev1 en polymerases η , ι , κ en ζ . Deze speciale DNA polymerases zijn in staat om DNA te synthetiseren tegenover (trans) een niet verwijderde DNA beschadiging (lesie) waardoor de verdubbeling van het genoom voltooid kan worden. Echter, omdat TLS een mutageen proces is, is dit mechanisme aan strenge controle onderworpen. Tot dusver is de rol van elk individueel TLS polymerase in respons op genomische DNA schade in zoogdiercellen en organismen grotendeels onduidelijk. In dit proefschrift heb ik in muizencellen de *in vivo* bijdrage onderzocht van TLS polymerases, afzonderlijk of samen, in respons op DNA schade, veroorzaakt door genotoxische stoffen die in voedsel voorkomen, en UV licht. Bovendien heb ik de genetische en cellulaire gevolgen van niet-gerepliceerde DNA beschadigingen bestudeerd. Met behulp van TLS-defecte muizen heb ik het belang van replicatie stress, veroorzaakt door endogene DNA schade, in het voorkómen van veroudering van het organisme bestudeerd.

Hoofdstuk 1 geeft een inleiding over TLS, de belangrijkste spelers in deze DDT route en de verschillende mechanismen waarmee TLS polymerases beschadigd DNA repliceren.

Hoofdstuk 2 beschrijft de rol van TLS polymerases in embryonale muizenfibroblasten (MEFs) na blootstelling aan de uit voedsel afkomstige stoffen Benzo(a)pyreen diepoxiede (BPDE) en hydroxynonenal (HNE). Het blijkt dat Rev1, Pol ζ en Pol η een centrale rol spelen bij de TLS van DNA schade veroorzaakt door beide stoffen. Pol ι blijkt in muizencellen specifiek betrokken te zijn bij TLS van HNE-geïnduceerde DNA schade, wat overeenkomt met resultaten van *in vitro* studies. Naast de bekende rol van Pol κ in TLS van BPDE-geïnduceerde DNA schade blijkt ook het BRCT domein van Rev1 cruciaal voor TLS op BPDE-geïnduceerde DNA beschadigingen en het onderdrukken van DNA schade signalering. Dit domein blijkt overbodig te zijn voor TLS op HNE-geïnduceerde DNA schade. Deze studie suggereert dat structureel verschillende DNA beschadigingen worden gerepliceerd door verschillende combinaties van TLS polymerases, gebruik makend van verschillende functionele domeinen. Verlies van TLS, of mutagene TLS gevolgd door verwijdering van misincorporaties tegenover de DNA lesie door mismatch repair (MMR; Tsaalbi-Shtylik *et al.*, in voorbereiding), leidt tot DNA schade signalering, dubbelstrengs DNA breuken (DSBs) en, bij blootstelling aan hoge doses, tot celdood.

De *in vivo* rol van TLS-gerelateerde polymerases in muis embryonale fibroblasten (MEFs) na blootstelling aan UVC licht worden beschreven in **hoofdstuk 3**. UVC licht induceert twee typen DNA beschadigingen, cyclobutaan pyrimidine dimeren (CPDs) en (6-4)pyrimidine-pyrimidone dimeren [(6-4)PPs], die kunnen worden beschouwd als representatief voor respectievelijk matig en ernstig DNA helix-verstorende beschadigingen. Gebruik makend van gelijke doses UVC licht werd vastgesteld dat Polt, Polk en het BRCT domein van Rev1 grotendeels overbodig zijn voor de cellulaire respons op UVC-geïnduceerde DNA beschadigingen. Polη is vooral nodig voor TLS van CPDs, terwijl Rev1 en Rev3 essentieel zijn voor TLS van (6-4)PPs. Verlies van deze TLS polymerases resulteert in verhoogde DNA schade signalering en verminderde progressie van de celcyclus. Mono-ubiquitineren van PCNA (PCNA-Ub) is voornamelijk betrokken bij TLS op zowel CPDs en (6-4)PPs in een vroege fase na UVC bestraling maar PCNA-Ub is niet noodzakelijk voor het repliceren van (6-4)PP in een laat stadium. Dit suggereert het bestaan van een TLS activiteit in zoogdiercellen die niet afhankelijk is van PCNA-Ub.

De resultaten in **hoofdstuk 3** duiden op het bestaan van een 'back-up' mechanisme dat TLS uitvoert in Polη-deficiënte MEFs na UVC bestraling. Om meer mechanistisch inzicht te verkrijgen werden onder dezelfde experimentele omstandigheden MEFs met enkele, dubbele en driedubbele deficiënties in genen die coderen voor Pols η, ι en κ onderzocht. Deze experimenten worden beschreven in **hoofdstuk 4**, en uit deze resultaten blijkt dat Polk een belangrijke TLS polymerase is in Polη-deficiënte MEFs voor TLS van CPDs alsook van (6-4)PPs. In vergelijking met Polη-deficiënte MEFs met een extra defect in Polι of in Polk vertoonden de driedubvoudige mutante cellen een ernstiger defect in TLS op (6-4)PPs. Deze bevinding suggereert dat TLS van sterk DNA helix-verstorende beschadigingen door meerdere TLS polymerases kan worden uitgevoerd.

Eerdere publicaties hebben laten zien dat blootstelling van TLS-deficiënte cellen aan UV licht leidt tot een accumulatie van enkelstrengs DNA (ssDNA) in het genoom, als gevolg van onvolledige DNA replicatie. In **hoofdstuk 5** is het lot van deze ssDNA gaten in het genoom gedurende de celcyclus uitgebreid geanalyseerd. De resultaten tonen ondubbelzinnig aan dat niet-gerepliceerde (6-4)PPs in ssDNA, gegenereerd in S-fase cellen, via de mitose worden overgedragen naar de volgende celcyclus. In de daaropvolgende S-fase worden deze ssDNA gaten omgezet in cytotoxische dubbelstrengs DNA breuken, wat leidt tot de vorming van micronuclei. Deze studie levert een mechanistische basis voor de analyse van micronuclei formatie gedurende twee celdelingen, zoals aanbevolen door de OECD.

Hoofdstuk 6 beschrijft de correlatie tussen replicatiestress veroorzaakt door endogene DNA schade en veroudering. Sneller dan wild-type muizen vertonen Rev1-mutante muizen fenotypes van veroudering. Deze fenotypes werden verder versneld wanneer Rev1-deficiënte muizen ook deficiënt zijn voor Xpc, een eiwit betrokken bij nucleotide excisie herstel van DNA beschadigingen. Deze gegevens impliceren dat replicatie

stress veroorzaakt door accumulatie van endogene DNA schade leidt tot vroegtijdige veroudering. De studie levert verder experimenteel bewijs dat Rev1 betrokken is bij TLS van endogene DNA beschadigingen mogelijk veroorzaakt door peroxidatie van lipiden. Hierbij onderdrukt Rev1 replicatiestress en voortijdige veroudering.

Ten slotte wordt in **hoofdstuk 7** een perspectief beschreven gebaseerd op de resultaten van in dit proefschrift beschreven studies.

CURRICULUM VITAE

Piya Temviriyankul was born on the 23rd of October 1981 in Chainat and grew up in Suphanburi, Thailand. He completed his secondary school at Kannasootsuksalai School, Suphanburi in March 2000. He obtained Bachelor's Degree in Medical Technology from Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand, in 2005. His scientific project under the supervision of Dr. Usanee Anukool was the screening of *Actinomyces* from diverse soils in northern Thailand and the preliminary search for MRSA-inhibitory agents. In March 2005, he decided to continue his study with a Master Degree in Molecular Genetics and Genetic Engineering at Institute of Molecular Biosciences, Mahidol University (Salaya campus), Nakhon Pathom, Thailand. His project under the supervision of Assoc. Prof. Albert Kettermann was the characterization of recombinant *Drosophila* Delta class Glutathione transferases and their interaction with c-jun N-terminal kinases. In October 2008, he was granted a scholarship by the Office of the Higher Education Commission (SFR-4), Royal Thai Government and he became a Ph.D. student at the Department of Toxicogenetics, Leiden University Medical Center (LUMC), Leiden, the Netherlands. The result of his work is described in this thesis.

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