



Universiteit  
Leiden  
The Netherlands

## **Translesion synthesis : cellular and organismal functions**

Temviriyankul, P.

### **Citation**

Temviriyankul, P. (2014, April 10). *Translesion synthesis : cellular and organismal functions*. Retrieved from <https://hdl.handle.net/1887/25142>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/25142>

**Note:** To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/25142> holds various files of this Leiden University dissertation.

**Author:** Temviriyankul, Piya

**Title:** Translesion synthesis : cellular and organismal functions

**Issue Date:** 2014-04-10

# CHAPTER

# 2

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

Piya Temviriyankul, Matty Meijers, Sandrine van Hees-Stuivenberg, Jan J.W.A. Boei,  
Frédéric Delbos, Haruo Ohmori, Niels de Wind and Jacob G. Jansen

## 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  $\eta$ ,  $\iota$ ,  $\kappa$  and Rev1, as well as in Rev3, the catalytic subunit of TLS Pol $\zeta$ . 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 $\eta$  are essential for TLS and the prevention of DDR and DNA breaks reflecting genome instability in response to both agents. Conversely, Pol $\kappa$  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 $\iota$  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,  $\eta$ ,  $\iota$  and  $\kappa$  and the B-family Pol $\zeta$  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 diolepoxide (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 (Pleasant *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  $\alpha,\beta$ -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.

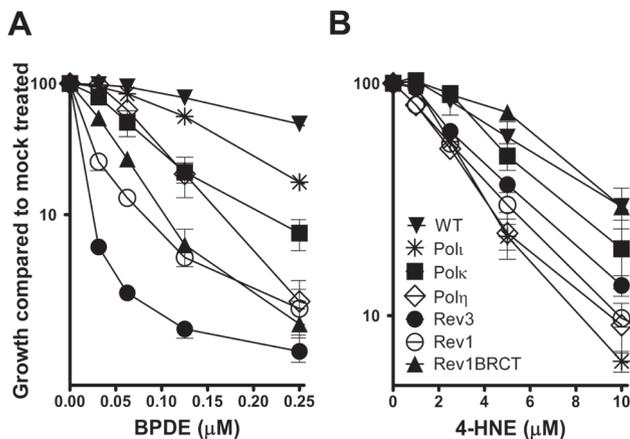
In DNA, 4-HNE mainly induces poorly repaired N<sup>1</sup>,N<sup>2</sup>-propanodeoxyguanosines. In addition, low yields of exocyclic etheno adducts at guanine, adenine, and cytosine are produced following oxidation of 4-HNE to its epoxide (Poli *et al.*, 2008; Schaur, 2003). Similar to BPDE, 4-HNE may 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  $\eta$ ,  $\iota$ ,  $\kappa$ , 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 $\kappa$  and Pol $\iota$  and a general role for Rev1, Rev3, and Pol $\eta$  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 $\eta$  or Pol $\iota$  displayed moderate sensitivity to BPDE. A similar result was found for MEFs deficient for Pol $\kappa$ , supporting the proposed role of Pol $\kappa$  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 $\eta$ , Pol $\iota$ , or



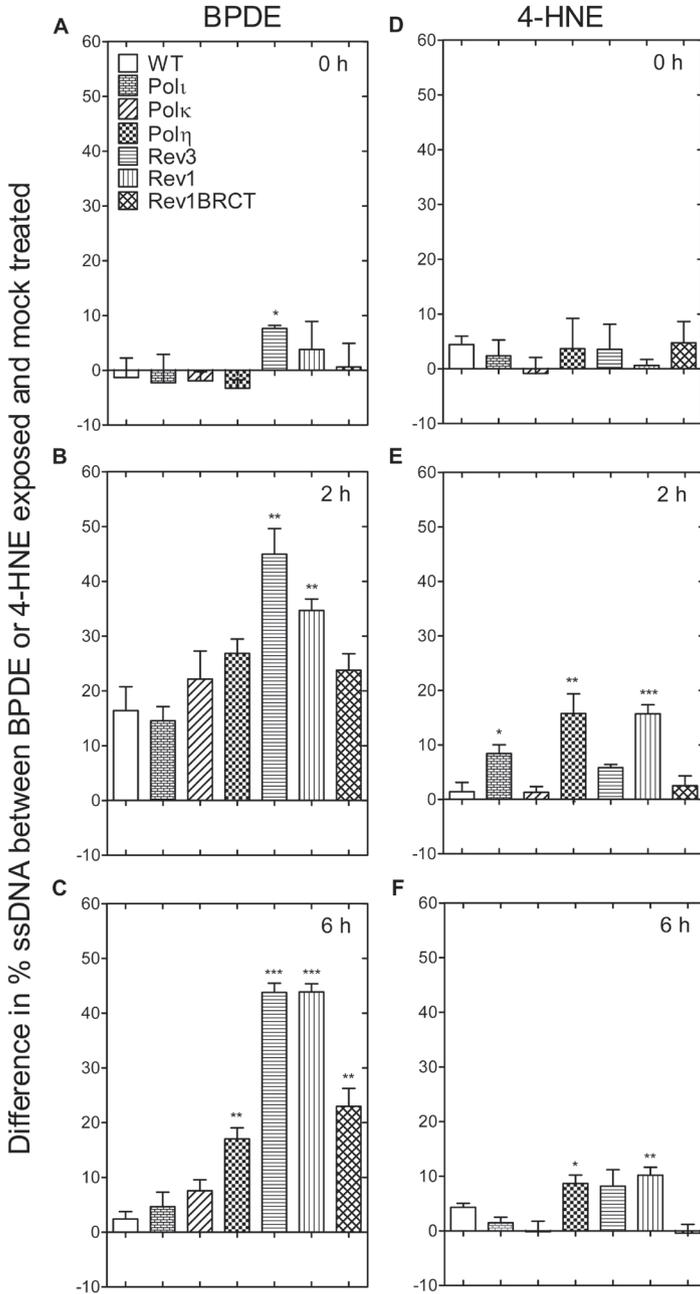
**Figure 1 | Effect of BPDE and 4-HNE on the proliferation of MEFs with disruptions in TLS Pols.** Cells were treated with different concentrations of BPDE (A) and 4-HNE (B) for 20 min and 2h, respectively. After 3 days, the number of cells was determined. The number of unexposed cells was set at 100%. 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 [ $^3\text{H}$ ]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 $\eta$ , 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.

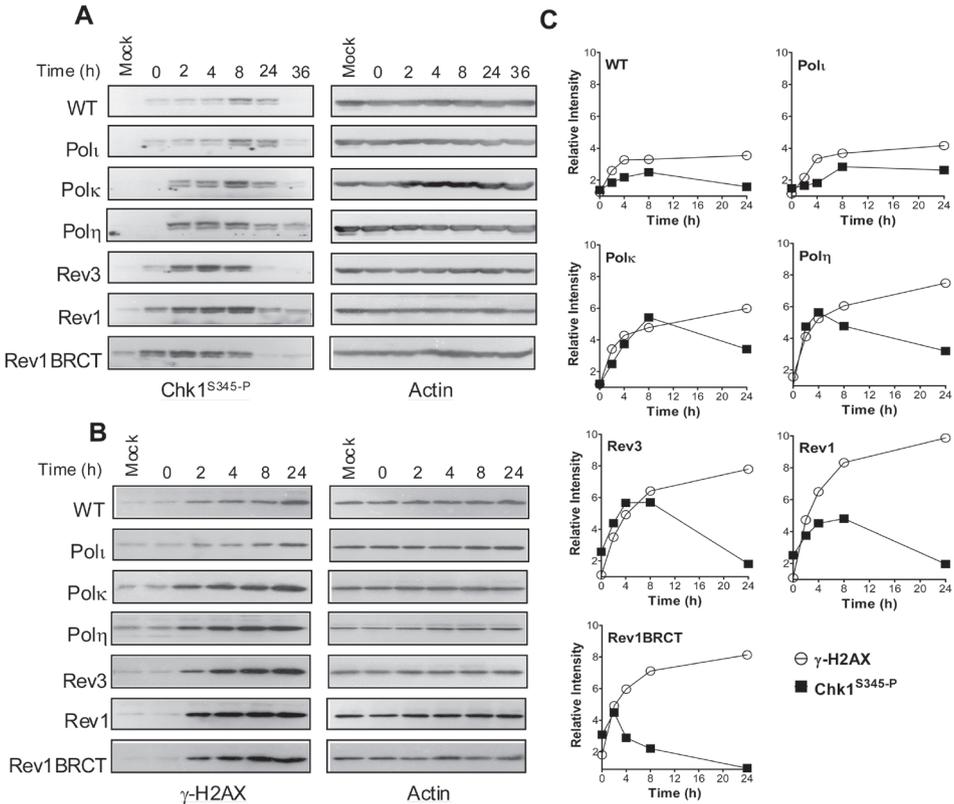
We also performed ADU assays with the cell lines after treatment with 4-HNE. Compared with immediately after treatment (Fig. 2D), at 2h after treatment significantly reduced replication fork progression was observed in MEF lines defective for Pol $\eta$ , Polt, and Rev1 (Fig. 2E). Only in Pol $\eta$ - 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 $\eta$ , 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<sup>S345-P</sup>) following exposure to 0.5 $\mu$ M BPDE or 15 $\mu$ M 4-HNE (Figs. 3A and C). Within 8h after exposure to BPDE, strong induction of Chk1<sup>S345-P</sup> 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 $\eta$ - or Polk-deficient MEF lines, corresponding with the reduced sensitivity and replication fork blockage. BPDE-induced Chk1<sup>S345-P</sup> 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<sup>S345-P</sup> following BPDE treatment (Figs. 3A and C). In contrast to BPDE, exposure to 4-HNE did not result in the induction of Chk1<sup>S345-P</sup> 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 $\gamma$ -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 ( $\gamma$ -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  $\gamma$ -H2AX in the different mutant MEF lines, upon treatment with either BPDE or 4-HNE. The highest induction of  $\gamma$ -H2AX was found in BPDE-exposed MEF lines defective in Rev1, Rev1 BRCT, or Rev3. Also, Pol $\eta$ -defective MEFs exhibited a significant induction of  $\gamma$ -H2AX (Figs. 3B and C). In these cell lines, levels  $\gamma$ -H2AX increased rapidly within 8h after BPDE treatment and continued to increase more slowly at later time points. Thus, the induction of  $\gamma$ -H2AX was correlated with the level of sensitivity and inhibition of



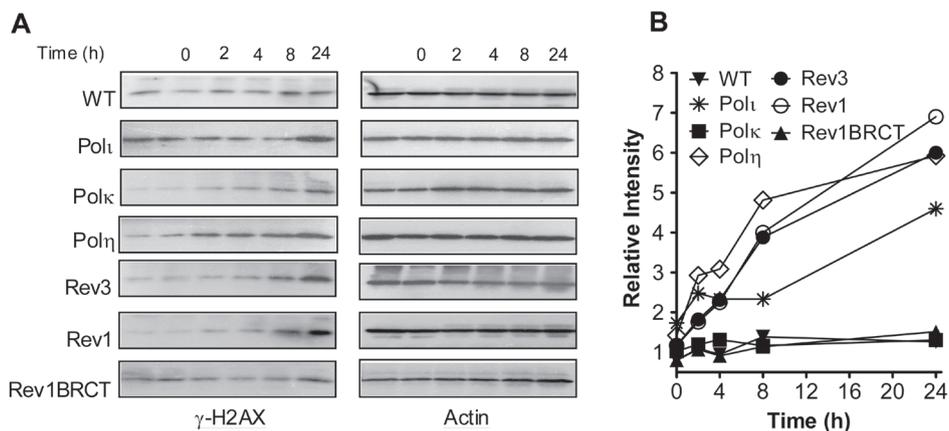
**Figure 3 | Effect of BPDE on DNA damage responses of TLS-deficient MEFs.** Western blot analysis of whole cell extracts prepared at different times after exposure of cells to 0.5 $\mu$ M BPDE for 15 min. 0h: cell extract prepared immediately after treatment. Blots representing expression of Chk1<sup>S345-P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

replication fork progression in the different TLS Pol-mutant cell lines. Compared with the other MEF lines, WT- and Polt-defective cells induced much less  $\gamma$ -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  $\gamma$ -H2AX formation also correlates with that of Chk1<sup>S345-P</sup>, although the latter peaks earlier than the formation of  $\gamma$ -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  $\gamma$ -H2AX in cells defective in Rev1, Rev3, Pol $\eta$ , or Polt (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  $\gamma$ -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  $\gamma$ -H2AX that can be visualized by Western blotting.

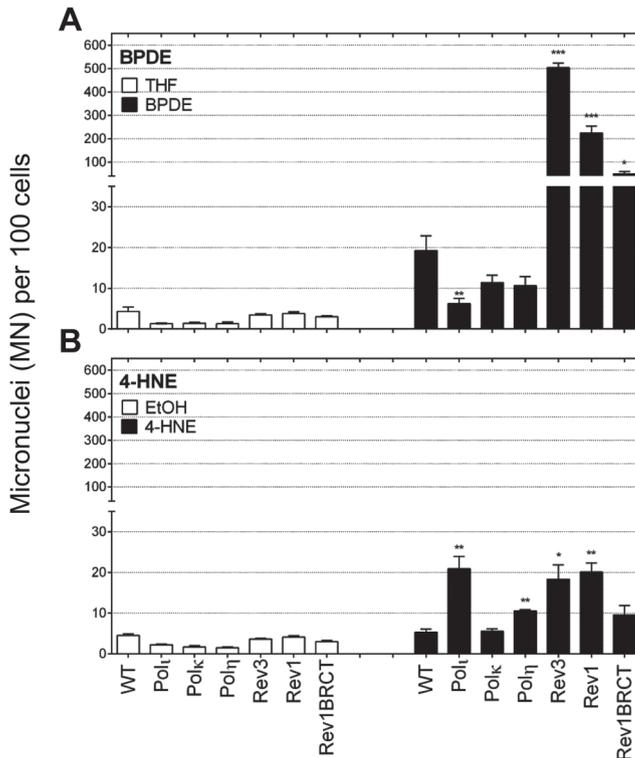
### Induction of Genome Instability after BPDE or 4-HNE Treatment

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 $\mu$ M 4-HNE for 2h. 0h, cell extract prepared immediately after treatment. Blots representing expression of  $\gamma$ -H2AX (left panel) and  $\beta$ -actin (right panel) (A). Quantification of  $\gamma$ -H2AX ( $n=2$ ) relative to the loading control  $\beta$ -actin (B).

BPDE (0.05 $\mu$ M) or 4-HNE (10 and 20 $\mu$ 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 Pol $\iota$ -,  $\eta$ -, and  $\kappa$ -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-,  $\eta$ -, 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 $\zeta$  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 $\eta$  is involved in TLS of both 4-HNE- and BPDE-induced DNA damage. Possibly, Pol $\eta$  mediates TLS opposite 4-HNE-induced DNA adducts at adenine (Schaur, 2003) because 4-HNE-induced DNA adducts at cytosine and guanine are no substrates for nucleotide incorporation by Pol $\eta$  *in vitro* and *in vivo* (Wolffe *et al.*, 2006; Yang *et al.*, 2003). Exposure of mammalian cells to BPDE results in the relocalization of Pol $\eta$  to nuclear foci and BPDE-induced adducts at guanines form substrates for Pol $\eta$ -mediated

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

In our studies, Pol $\kappa$ -deficient MEFs displayed only moderate phenotypes in response to BPDE. However, Pol $\kappa$  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 $\eta$  and Pol $\kappa$  prevents the persistent accumulation of stalled replication forks in the Pol $\kappa$ -deficient cells, whereas Chk1<sup>S345-P</sup>,  $\gamma$ -H2AX, and MN may result from a transient defect in TLS at BPDE adducts. Alternatively, phosphorylation of Chk1 and formation of  $\gamma$ -H2AX may reflect persistence of ssDNA gaps that are not filled by Pol $\kappa$  during nucleotide excision repair of helix distorting DNA lesions in G1 phase cells (Ogi *et al.*, 2010). Although Pol $\kappa$ -defective MEFs displayed a mild sensitivity to 4-HNE (Fig. 1B), 4-HNE-induced DNA lesions did not provoke detectable DDR, indicating that Pol $\kappa$  is not important in TLS of 4-HNE-induced DNA adducts in mammalian cells.

The function of Pol $\iota$  in TLS and DDR is not well understood. It was reported that Pol $\iota$  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 $\eta$  (Wang *et al.*, 2007). We now find that Pol $\iota$ -deficient MEFs are extremely sensitive to 4-HNE, strongly suggesting that Pol $\iota$  is important for efficient bypass of 4-HNE-induced DNA damage. In support, 4-HNE-dG adducts are excellent substrates for Pol $\iota$ -mediated TLS *in vitro* (Wolfe *et al.*, 2006). The induction of MN in Pol $\iota$ -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  $\gamma$ -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 $\eta$  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,  $5 \times 10^4$  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,  $5 \times 10^4$  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 [<sup>3</sup>H]thymidine (2 μCi/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 [<sup>3</sup>H]thymidine (2 μCi/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<sub>2</sub>HPO<sub>4</sub>. 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<sub>2</sub>HPO<sub>4</sub> pH 6.8, and the dsDNA was eluted with 0.25M K<sub>2</sub>HPO<sub>4</sub> pH 6.8. The radioactivity in both eluates was determined by liquid scintillation counting (Perkin-Elmer).

**Western blotting.** Per 9 cm dish 10<sup>6</sup> 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<sup>6</sup> 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 μg/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).

## FUNDING

This work was supported by a PhD scholarship to PT under the project Strategic Frontier Research (MOE 0509(3)/16715, CHE-SFR4) from Office of the Higher Education Commission, Ministry of Education, Thailand.

## ACKNOWLEDGEMENTS

We thank Mark Drost for his critical comments on the manuscript.

## REFERENCES

- Bi X**, Slater DM, Ohmori H, Vaziri C (2005). DNA polymerase kappa is specifically required for recovery from the benzo[a]pyrene-dihydrodiol epoxide (BPDE)-induced S-phase checkpoint. *J Biol Chem.* **280**: 22343-22355
- Cheng SC**, Hilton BD, Roman JM, Dipple A (1989). DNA adducts from carcinogenic and noncarcinogenic enantiomers of benzo[a]pyrene dihydrodiol epoxide. *Chem Res Toxicol.* **2**: 334-340
- de Feraudy S**, Revet I, Bezrookove V, Feeney L, Cleaver JE (2010). A minority of foci or pan-nuclear apoptotic staining of gammaH2AX in the S phase after UV damage contain DNA double-strand breaks. *Proc Natl Acad Sci U S A.* **107**: 6870-6875
- de Groote FH**, Jansen JG, Masuda Y, Shah DM, Kamiya K, de Wind N, Siegal G (2011). The Rev1 translesion synthesis polymerase has multiple distinct DNA binding modes. *DNA Repair (Amst).* **10**: 915-925
- Delbos F**, De Smet A, Faili A, Aoufouchi S, Weill JC, Reynaud CA (2005). Contribution of DNA polymerase eta to immunoglobulin gene hypermutation in the mouse. *J Exp Med.* **201**: 1191-1196
- Eckl PM** (2003). Genotoxicity of HNE. *Mol Aspects Med.* **24**: 161-165
- Fenech M** (1993). The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. *Mutat Res.* **285**: 35-44
- Gu J**, Horikawa Y, Chen M, Dinney CP, Wu X (2008). Benzo(a)pyrene diol epoxide-induced chromosome 9p21 aberrations are associated with increased risk of bladder cancer. *Cancer Epidemiol Biomarkers Prev.* **17**: 2445-2450
- Guo C**, Fischhaber PL, Luk-Paszyc MJ, Masuda Y, Zhou J, Kamiya K, Kisker C, Friedberg EC (2003). Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis. *EMBO J.* **22**: 6621-6630
- Hu W**, Feng Z, Eveleigh J, Iyer G, Pan J, Amin S, Chung FL, Tang MS (2002). The major lipid peroxidation product, trans-4-hydroxy-2-nonenal, preferentially forms DNA adducts at codon 249 of human p53 gene, a unique mutational hotspot in hepatocellular carcinoma. *Carcinogenesis.* **23**: 1781-1789
- Jansen JG**, Langerak P, Tsaalbi-Shtylik A, van den Berk P, Jacobs H, de Wind N (2006). Strand-biased defect in C/G transversions in hypermutating immunoglobulin genes in Rev1-deficient mice. *J Exp Med.* **203**: 319-323
- Jansen JG**, Tsaalbi-Shtylik A, Hendriks G, Gali H, Hendel A, Johansson F, Erixon K, Livneh Z, Mullenders LH, Haracska L *et al* (2009a). Separate domains of Rev1 mediate two modes of DNA damage bypass in mammalian cells. *Mol Cell Biol.* **29**: 3113-3123
- Jansen JG**, Tsaalbi-Shtylik A, Hendriks G, Verspuj J, Gali H, Haracska L, de Wind N (2009b). Mammalian polymerase zeta is essential for post-replication repair of UV-induced DNA lesions. *DNA Repair (Amst).* **8**: 1444-1451
- Jansen JG**, Tsaalbi-Shtylik A, Langerak P, Calleja F, Meijers CM, Jacobs H, de Wind N (2005). The BRCT domain of mammalian Rev1 is involved in regulating DNA translesion synthesis. *Nucleic Acids Res.* **33**: 356-365
- Johansson F**, Lagerqvist A, Erixon K, Jenssen D (2004). A method to monitor replication fork progression in mammalian cells: nucleotide excision repair enhances and homologous recombination delays elongation along damaged DNA. *Nucleic Acids Res.* **32**: e157
- Kanner J** (2007). Dietary advanced lipid oxidation endproducts are risk factors to human health. *Mol Nutr Food Res.* **51**: 1094-1101
- Khan N**, Afaq F, Mukhtar H (2010). Lifestyle as risk factor for cancer: Evidence from human studies. *Cancer Lett.* **293**: 133-143
- Langie SA**, Knaapen AM, Ramaekers CH, Theys J, Brun J, Godschalk RW, van Schooten FJ, Lambin P, Gray DA, Wouters BG *et al* (2007). Formation of lysine 63-linked poly-ubiquitin chains protects human lung cells against benzo[a]pyrene-diol-epoxide-induced mutagenicity. *DNA Repair (Amst).* **6**: 852-862
- LoPachin RM**, Gavin T, Petersen DR, Barber DS (2009). Molecular mechanisms of 4-hydroxy-2-nonenal and acrolein toxicity: nucleophilic targets

and adduct formation. *Chem Res Toxicol.* **22**: 1499-1508

**Mukhopadhyay S**, Clark DR, Watson NB, Zacharias W, McGregor WG (2004). REV1 accumulates in DNA damage-induced nuclear foci in human cells and is implicated in mutagenesis by benzo[a]pyrenediolepoxide. *Nucleic Acids Res.* **32**: 5820-5826

**Nelson JR**, Gibbs PE, Nowicka AM, Hinkle DC, Lawrence CW (2000). Evidence for a second function for *Saccharomyces cerevisiae* Rev1p. *Mol Microbiol.* **37**: 549-554

**Ogi T**, Limsirichaikul S, Overmeer RM, Volker M, Takenaka K, Cloney R, Nakazawa Y, Niimi A, Miki Y, Jaspers NG *et al* (2010). Three DNA polymerases, recruited by different mechanisms, carry out NER repair synthesis in human cells. *Mol Cell.* **37**: 714-727

**Ogi T**, Shinkai Y, Tanaka K, Ohmori H (2002). Polkappa protects mammalian cells against the lethal and mutagenic effects of benzo[a]pyrene. *Proc Natl Acad Sci U S A.* **99**: 15548-15553

**Petta TB**, Nakajima S, Zlatanou A, Despras E, Couve-Privat S, Ishchenko A, Sarasin A, Yasui A, Kannouche P (2008). Human DNA polymerase iota protects cells against oxidative stress. *EMBO J.* **27**: 2883-2895

**Pleasant ED**, Stephens PJ, O'Meara S, McBride DJ, Meynert A, Jones D, Lin ML, Beare D, Lau KW, Greenman C *et al* (2010). A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature.* **463**: 184-190

**Poli G**, Schaur RJ, Siems WG, Leonarduzzi G (2008). 4-hydroxynonenal: a membrane lipid oxidation product of medicinal interest. *Med Res Rev.* **28**: 569-631

**Rogakou EP**, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* **273**: 5858-5868

**Ruggeri B**, DiRado M, Zhang SY, Bauer B, Goodrow T, Klein-Szanto AJ (1993). Benzo[a]pyrene-induced murine skin tumors exhibit frequent and characteristic G to T mutations in the p53 gene. *Proc Natl Acad Sci U S A.* **90**: 1013-1017

**Rybicki BA**, Rundle A, Savera AT, Sankey SS, Tang D (2004). Polycyclic aromatic hydrocarbon-DNA adducts in prostate cancer. *Cancer Res.* **64**: 8854-8859

**Schaur RJ** (2003). Basic aspects of the biochemical reactivity of 4-hydroxynonenal. *Mol Aspects Med.* **24**: 149-159

**Shachar S**, Ziv O, Avkin S, Adar S, Wittschieben J, Reissner T, Chaney S, Friedberg EC, Wang Z, Carell T *et al* (2009). Two-polymerase mechanisms

dictate error-free and error-prone translesion DNA synthesis in mammals. *EMBO J.* **28**: 383-393

**Singh SP**, Janecki AJ, Srivastava SK, Awasthi S, Awasthi YC, Xia SJ, Zimniak P (2002). Membrane association of glutathione S-transferase mGSTA4-4, an enzyme that metabolizes lipid peroxidation products. *J Biol Chem.* **277**: 4232-4239

**Surh J**, Kwon H (2005). Estimation of daily exposure to 4-hydroxy-2-alkenals in Korean foods containing n-3 and n-6 polyunsaturated fatty acids. *Food Addit Contam.* **22**: 701-708

**Wang Y**, Woodgate R, McManus TP, Mead S, McCormick JJ, Maher VM (2007). Evidence that in xeroderma pigmentosum variant cells, which lack DNA polymerase eta, DNA polymerase iota causes the very high frequency and unique spectrum of UV-induced mutations. *Cancer Res.* **67**: 3018-3026

**Ward IM**, Minn K, Chen J (2004). UV-induced ataxia-telangiectasia-mutated and Rad3-related (ATR) activation requires replication stress. *J Biol Chem.* **279**: 9677-9680

**Waters LS**, Minesinger BK, Wiltout ME, D'Souza S, Woodruff RV, Walker GC (2009). Eukaryotic translesion polymerases and their roles and regulation in DNA damage tolerance. *Microbiol Mol Biol Rev.* **73**: 134-154

**Wolfe WT**, Johnson RE, Minko IG, Lloyd RS, Prakash S, Prakash L (2006). Replication past a trans-4-hydroxynonenal minor-groove adduct by the sequential action of human DNA polymerases iota and kappa. *Mol Cell Biol.* **26**: 381-386

**Yang IY**, Hashimoto K, de Wind N, Blair IA, Moriya M (2009). Two distinct translesion synthesis pathways across a lipid peroxidation-derived DNA adduct in mammalian cells. *J Biol Chem.* **284**: 191-198

**Yang IY**, Miller H, Wang Z, Frank EG, Ohmori H, Hanaoka F, Moriya M (2003). Mammalian translesion DNA synthesis across an acrolein-derived deoxyguanosine adduct. Participation of DNA polymerase eta in error-prone synthesis in human cells. *J Biol Chem.* **278**: 13989-13994

**Zhang Y**, Wu X, Guo D, Rechkoblit O, Geacintov NE, Wang Z (2002). Two-step error-prone bypass of the (+)- and (-)-trans-anti-BPDE-N2-dG adducts by human DNA polymerases eta and kappa. *Mutat Res.* **510**: 23-35

**Zhao H**, Piwnica-Worms H (2001). ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol Cell Biol.* **21**: 4129-4139



