Published in final edited form as:

Mutat Res. 2012 March 1; 731(1-2): 85–91. doi:10.1016/j.mrfmmm.2011.11.009.

High Risk of Benzo[α]pyrene-induced Lung Cancer in E160D FEN1 Mutant Mice

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Abstract

Flap endonuclease 1 (FEN1), a member of the Rad2 nuclease family, possesses 5' flap endonuclease (FEN), 5' exonuclease (EXO), and gap-endonuclease (GEN) activities. The multiple, structure-specific nuclease activities of FEN1 allow it to process different intermediate DNA structures during DNA replication and repair. We previously identified a group of FEN1 mutations and single nucleotide polymorphisms that impair FEN1's EXO and GEN activities in human cancer patients. We also established a mouse model carrying the E160D FEN1 mutation, which mimics the mutations seen in humans. FEN1 mutant mice developed spontaneous lung cancer at high frequency at their late life stages. An important unanswered question is whether individuals carrying such FEN1 mutation are more susceptible to tobacco smoke and have an earlier onset of lung cancer. Here, we report our study on E160D mutant mice exposed to benzo[α]pyrene (B[α]P), a major DNA damaging compound found in tobacco smoke. We demonstrate that FEN1 employs its GEN activity to cleave DNA bubble substrates with BP-induced lesions, but the E160D FEN1 mutation abolishes such activity. As a consequence, Mouse cells carrying the E160D mutation display defects in the repair of B[α]P adducts and accumulate DNA double-stranded breaks and chromosomal aberrations upon treatments with B[α]P. Furthermore, more E160D mice than WT mice have an early onset of B[α]P-induced lung adenocarcinoma. All together, our current study suggests that individuals carrying the GEN-deficient FEN1 mutations have high risk to develop lung cancer upon exposure to B[α]P-containing agents such as tobacco smoke.

Keywords

Flap endonuclease 1 (FEN1); Benzo[α]pyrene (B[α]P); Lung cancer; Double-stranded breaks (DSBs); Near-tetraploid aneuploidy

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Conflict of interest statement

No conflicts of interest.

1. Introduction

It is generally recognized that tobacco smoke, which contains more than 60 carcinogens, is the major cause of lung cancer [1, 2]. Benzo[α]pyrene (B[α]P), a five-ring polycyclic aromatic hydrocarbon, is a major DNA damaging compound present in tobacco smoke and an environmental pollutant [3]. In the lung, B[α]P interacts with cytochrome p450 enzymes, producing benzo[α]pyrene-trans-7, 8-dihydrodiol-9, 10-epoxide(anti)(+) (BPDE), the metabolite that interacts with DNA to form adducts [4]. Previous studies indicated that B[α]P adducts are mainly repaired via the nucleotide excision repair (NER), which is mediated by Xeroderma Pigmentosum group G (XPG)- and Excision Repair Cross Complementing group 1- Xeroderma Pigmentosum group F (ERCC1-XPF) [5]. Flap endonuclease 1 (FEN1), an XPG homologue [6], possesses flap endonuclease (FEN), exonuclease (EXO), and gap endonuclease (GEN) activities [7, 8, 9]. FEN1 has been suggested to play a critical role in DNA replication, DNA base excision repair (BER), and rescue of stalled replication forks [10, 11]. Decreased FEN1 activity causes abnormal cell proliferation, genomic instability, and tumorigenesis [12]. However, it is unclear whether FEN1 plays a role in repairing B[α]P-induced DNA lesions.

We have recently identified *Fen1* mutations and single nucleotide polymorphisms (SNPs) in human cancer patients, particularly human lung cancer patients [13]. Most of these *Fen1* mutations displayed deficiency in the EXO and GEN activities [13]. A critical question is whether individuals carrying such mutations or SNPs, which impair FEN1-mediated DNA repair pathways, are more susceptible to the development of tobacco-induced lung cancer. A mouse model carrying the E160D *Fen1* mutation (E160D mice) has been generated to mimic these mutations seen in humans. We observed that E160D mice developed lung cancer spontaneously [14]. More recently, we have showed that E160D mice develop early onset of lung cancer after exposure to the base damaging agent methylnitrosourea [15]. It is unclear whether E160D mice are sensitive to tobacco compounds such as B[α]P and develop B[α]P-induced lung cancer. Here, we report that the E160D FEN1 mutation impairs the cleavage of bubble DNA substrates. Consistent with this observation, nuclear extracts (NEs) from E160D mutant cells display defects in repair of B[α]P-induced DNA lesions and accumulate DNA double-stranded breaks and have high frequency of near-tetraploid aneuploidy. Furthermore, E160D mice are susceptible to the development of early onset of B[α]P-induced lung cancer.

2. Materials and Methods

2.1. Establishment and culturing of MEFs

MEFs were obtained from mice of the 129S1 genetic background. Embryos (E13.5) were isolated and dissected by the treatment with trypsin to produce MEFs. Primary MEFs were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Invitrogen) and penicillin-streptomycin (Invitrogen) [15].

2.2. Cell treatment

B[α]P (50-32-8, Sigma, St. Louis, MO) was dissolved in glyceryl trioctanoate (538-23-8, Sigma). MEFs from the P1 generation (5×10^5 – 2×10^6) were treated with 30 μ M B[α]P for 24 h. Cells were then washed with PBS buffer, and incubated in fresh DMEM for 4 h, and were subjected to γ -H2AX immunofluorescence staining or other cell-based assays or were harvested for Western Blotting analysis.

2.3. Preparation of BPDE-damaged DNA and in vitro BP-damage repair assay [4]

Plasmid pUC18 DNA was propagated in *Escherichia coli* strain DH5 α , then isolated and purified by QIAprep Spin Miniprep Kit (QIAGEN Inc. Valencia, CA). BPDE-damaged DNA substrate was generated in a 50 μ L reaction containing 5 μ g pUC18 DNA, TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), 20% ethanol, and 10 μ M BPDE (National Cancer Institute Chemical Carcinogen Reference Standard Repository, Bethesda, MD). After incubating at 37°C in darkness for 3 h, the reaction was stopped, and the substrate was purified using the QIAquick PCR Purification Kit (QIAGEN). To assay repair of B[α]P-induced DNA adducts, WT or E160D NEs, was incubated with BPDE-damaged DNA substrate in reaction buffer (45 mM HEPES-KOH, pH 7.8, 7.4 mM MgCl₂, 0.9 mM DTT [3483-12-3, Fisher Scientific, Hampton, NH], 0.4 mM EDTA, 2 mM ATP [987-65-5, Sigma], 20 μ M each dATP, dGTP and dTTP, 4 μ M dCTP [Promega, Madison, WI], 40 mM phosphocreatine (disodium salt) [19333-65-4, Sigma], 2.5 μ g creatine phosphokinase [9001-15-4, Sigma], 4% glycerol, 100 μ g/ml bovine serum albumin [9048-46-8, Sigma]), and 1 μ Ci [α -³²P] dCTP (PerkinElmer, Waltham, MA, 3000Ci/mmol). The reaction was incubated at 37°C for 30, 60, 120, or 360 min. QIAquick PCR Purification Kit (QIAGEN) was then used to collect the repair products. Products were digested by *Hind*III and separated by electrophoresis on 1% agarose gel and visualized by radioautography. Ethidium bromide staining was used to as an internal loading control. The band intensity was quantified by Image J. The DNA repair product by the WT NE for 360 min was arbitrarily set as 100%, which was compared to DNA repair products by the WT NE at other time points or by the E160D NE at different time points, in order to calculate the relative DNA repair products.

2.4. In vitro nuclease activity assay on DNA bubble substrates

Two synthetic oligonucleotides were used to prepare the DNA bubble substrate: BRF1 (5'-GTTAAGATAGGTCTGCTTGGGATGTCAAGCAGTCCTAACTGGAAATC TAGCTCTGTGGAGTTGAGGCAGAGTCCTTAAGC-3') and BRF2 (5'-GCTTAAGGACTCTGCCTCAAATCGTCAGGGTTTCTAAAGAAGCCGACGGTAGTC AACGTGCCAAGCAGACCTATCTTAAC-3'). To label the DNA bubble substrate, 10 pmol of the BRF1 oligo was incubated with 15 μ Ci of [α -³²P]-dATP (PerkinElmer) and 400 units of terminal DNA transferase (Roche Applied Science, Mannheim, Germany) at 37°C for 60 min. After heat inactivation of the terminal DNA transferase, 40 pmol of the BRF2 oligo was added to the reaction. Samples were incubated at 70°C for 10 min. and cooled to room temperature. The bubble substrate was precipitated by addition of 3 M NaOAc (20 μ L) and 100% ethanol (1 mL) (-20°C, overnight). The precipitate was then washed with 70% ethanol and air dried [16]. 2 pmol of FEN1 proteins were incubated with 0.5 pmol DNA bubble substrate in a total volume of 10 μ L at 37°C for 5 min, 30 min, and 60 min. Cleavage products were resolved on a 15% denaturing PAGE and visualized by an radioautograph. Bands of DNA substrates or products were quantified by Image J.

2.5. Immunofluorescence microscopy and Western blotting

Immunofluorescence staining and Western blots analysis of γ H2AX were performed following a similar protocol as previously described, using the antibody against γ H2AX (phospho S139) (ab2893, Abcam, Cambridge, MA) [15,17]. β -actin (C4) mouse monoclonal IgG₁ (sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect the β -actin level, which serves as an internal loading control.

2.6. Metaphase spread preparation

Metaphase spreads were prepared as described previously [15]. Mitotic cells were examined and recorded with an AX70 microscope (Olympus Corporation, Shinjuku, Tokyo, Japan)

equipped with a Rentiga EXi (Qimaging, Surrey, BC Canada). Images were analyzed using the ImagePro 6.0 (MediaCybernetics, Bethesda, MD).

3. Results

3.1. The E160D FEN1 mutation causes defects in the repair of BPDE-induced DNA damage

To assay the efficiency of WT and E160D NEs to repair B[α]P-induced DNA lesions, we prepared damaged DNA substrates by treating plasmid pUC18 DNA with BPDE *in vitro*. BPDE, the main metabolite of B[α]P, directly attacks DNA, creates DNA adducts predominantly on the N²-position of guanine, and forms 10S (+)-trans-anti-B[α]P-N²-dG (G*) adducts [18]. To test whether the E160D NEs isolated from the primary MEFs had defects in the repair of B[α]P-induced adducts, we incubated the WT or the E160D NEs with BPDE-damaged DNA substrates. The repair of the B[α]P-induced adducts by the WT and the E160D NEs could be measured by incorporation of [α -³²P] dCTP into the damaged DNA substrate. The WT NEs effectively incorporate [α -³²P] dCTP into the BPDE-damaged DNA substrates but not undamaged DNA substrates (Fig. 1A, B, C & D). However, the repair efficiency by the E160D NEs was considerably less than the WT NEs. At 360 min. the repair efficiency by the E160D NEs was 47% of that by the WT NEs (Fig. 1D). It suggested that the E160D mutation impaired the repair of B[α]P-induced DNA damage.

3.2. E160D FEN1 mutation abolishes FEN1 cleavage of DNA bubble substrate

The B[α]P adducts were mainly repaired via NER [4], in which a DNA bubble structure is formed and the DNA fragment containing the DNA lesions are cleaved by XPG and ERCC1-XPF at the 5' and 3' ends of the structure [19]. We previously observed that FEN1, a functional homologue of XPG, could use its GEN activity to cleave the DNA bubble structure at both the 5' and 3' ends of ssDNA-dsDNA junctions *in vitro*; however, the 5' cleavage product was more than the 3' cleavage product [20]. To determine if the E160D FEN1 mutation impairs its ability to cleave the bubble structure, we incubated purified recombinant WT or E160D proteins with the DNA bubble substrate with or without BPDE damage. WT FEN1 effectively cleaved 40% of the normal substrate and 27% of BPDE-damaged substrate; however, the E160D FEN1 mutant protein failed to significantly cleave either substrate, exhibiting a cleavage percentage of less than 5% for the substrate with or without BPDE damage (Fig. 2 A & B). This finding is consistent with the observation that the E160D NE displayed defects in the repair of BPDE-induced DNA damage.

3.3. E160D retains high frequency of DSBs after B[α]P treatment

We showed that E160D FEN1 mutation caused defects in the cleavage of the bubble structure bearing the B[α]P damage. Because ERCC1/XPF could still cleave the DNA bubble structure, one might anticipate that this could lead to accumulation of unsealed DNA nicks or gaps. The unrepaired DNA nicks might result in collapse of DNA replication forks and formation of DNA double-stranded breaks (DSBs) [21]. In response to DNA DSBs, H2AX is rapidly phosphorylated (γ H2AX) on serine139 (Ser 139) at the site of each nascent DSBs, signaling the presence of damage sites [22]. Therefore, we treated the WT and E160D cells with a single high dose of B[α]P for a short period (30 μ M, 24 h). A previous study showed that such a treatment condition was non-toxic and had a mutagenic effect similar to that was caused by the long-term exposure to low dosage of B[α]P, which could be converted into the active form BPDE within mammalian cells [23]. We then performed immunofluorescence staining for γ H2AX in order to determine whether DSBs accumulation occurred in the E160D MEF cells. In the absence of B[α]P treatment, both WT and E160D cells had low levels of γ H2AX-positive nuclei. However, upon treatment with B[α]P, 18% of WT cells and 37% of E160D cells had γ H2AX-positive nuclei (Fig. 3A & B, $P=0.0011$, two-tailed Fisher's exact test). Western blot analysis confirmed that the B[α]P-treated

E160D cells had higher levels of γ H2AX, and therefore more DSBs, than the B[α]P-treated WT cells (Fig. 3C). This indicated that the E160D cells accumulated more B[α]P-induced DNA damage than the WT cells.

3.4. E160D cells have more B[α]P-induced chromosomal aberrations

The accumulation of DSBs in the genome has been linked to chromosomal breaks and development of aneuploidy [24]. Therefore, we examined whether the E160D FEN1 mutation caused an increase in chromosomal aberrations. In the absence of B[α]P treatment, less than 10% of WT or E160D MEFs cells displayed near-tetraploid aneuploidy. However upon B[α]P treatment, near-tetraploid aneuploidy levels in the E160D cells dramatically increased to nearly 19%, while levels in the WT cells were unchanged (Fig. 4A & B, $**P=0.008$, two-tailed Fisher's exact test). Under normal growth conditions, 0.02 and 0.05 chromosomal break per cell were observed among WT and E160D MEFs. In the presence of B[α]P, 0.07% and 0.2 chromosome break per cell were found in WT and E160D MEFs (Fig. 4C & D, $*P=0.0299$, two-tailed Fisher's exact test). Taken together, these data suggested that FEN1-deficient cells were at greater risk for chromosomal aberrations after B[α]P exposure.

3.5. E160D mice have higher incidence of developing lung tumors than do WT mice after B[α]P induction

The observation that B[α]P induced more chromosomal aberrations in E160D MEF cells than WT MEF cells suggested that E160D mice might be more susceptible to the development of B[α]P-induced lung cancer. To test this hypothesis, we treated WT (n=51) and E160D (n=52) mice (6–8 weeks) with B[α]P (100 mg/kg). By 8 months of age, 64% of the WT and 93% of the E160D mice had developed lung tumors (Fig. 5A). Furthermore, 76% of the WT mice developed only one lung tumor, but 81% of E160D mice developed two or more tumors and 10% grew whole lung tumors (tumors spread throughout the lung, Fig. 5B, boxed). In addition, average lung tumor sizes of E160D mice were considerably larger than those of WT mice (Fig. 5C). 14% of the WT mice had tiny tumors (average size under 1 mm³), nearly 79% had tumors with an average size of 1–9 mm³, and only 7% had tumors at an average size of about 10–20 mm³. However, 16% of E160D mice had tumors under 1 mm³, 43% had tumors in the range of 1–9 mm³, 12% had an average tumor size of 10–20 mm³, and 29% had tumors with an average size greater than 20 mm³.

4. Discussion

Cancer is thought to involve the accumulation of multiple forms of genome instabilities, including DNA mutations and chromosomal aberrations [25]. DNA constantly encounters endogenous and exogenous insults, leading to varying forms of lesions, which, if not properly repaired, may result in genome instabilities and cancer [26, 27]. Epidemiological studies have long suggested that environmental insults, such as B[α]P, and/or genetic variations in DNA repair genes and other related genes are linked to the development of cancer [3, 28]. However, the mechanism by which B[α]P lesions are repaired remained unclear. Here we show that the E160D mutation in FEN1, which is a versatile nuclease involved in various DNA repair pathways [8, 29], have significantly enhanced initiation and progression of B[α]P-induced cancer. This suggests that subtle genetic variations in DNA repair genes, such as in *Fen1*, can cause individuals to become more susceptible to environmental insults and aggravate the development of cancer [15].

The current study also reveals how *Fen1* mutations impair DNA repair and promote genome instabilities and cancer. The E160D FEN1 mutation, which eliminates the EXO and GEN activities but not the FEN activity [14], significantly reduced the ability to repair B[α]P

adducts (Fig. 1). Previous studies demonstrated that removal of DNA adducts is mainly completed by NER, in which ERCC1-XPF and XPG nucleases create incisions in the DNA bubble structure at ssDNA-dsDNA junctions, at the 3' and 5' ends, respectively [30, 31]. Mutations in the ERCC1-XPF and/or XPG nuclease dramatically decreased the ability to repair B[a]P-induced DNA damage *in vitro* and *in vivo* [18]. FEN1, as a functional homologue of XPG, was initially thought to backup the function of XPG in NER pathways. However, subsequent studies found that FEN1 has a very weak activity to cleave bubble structure, raising questions about the role of FEN1 in NER [32]. More recently, we found that the interaction between FEN1 and Werner syndrome protein (WRN) can increase FEN1's GEN activity to cleave the bubble structure by more than 50-fold, suggesting that the GEN activity of the FEN1/WRN complex may be important in the direct cleavage of DNA lesions or removal of stalled replication forks [20]. Our current study provides further evidence that the GEN activity of FEN1 plays a substantial role in the removal of the DNA lesions such as B[a]P adducts. Defective in GEN activity, E160D FEN1 mutant displayed decreased repair of B[a]P-induced damage. Furthermore, purified FEN1 protein from E160D mutants showed markedly decreased cleavage of BPDE-damaged bubble substrates, compared to WT FEN1. Failure to process the intermediate resulted from the XPF-cleaved DNA bubble structures causes accumulation of DNA single-stranded breaks (SSBs), which if not repaired can collapse DNA replication forks and formation DSBs. This is consistent with our observation that the B[a]P-treated E160D cells accumulate more DNA DSBs than the B[a]P-treated WT cells. On the other hand, because the E160D cells are defects in cleavage of the bubble structure, mimicking DNA replication forks, and the bubble substrate bearing BPDE-induced DNA damage, it is unclear about specific role of FEN1 in repair of BPDE-induced DNA damage. Future studies are needed to clarify this issue.

The increase in B[a]P-induced DNA strand breaks and chromosomal aberrations observed in E160D cells and mice may contribute to the development of B[a]P-induced cancer. Numerous studies have found an association between DSBs, genome instability, and cancer predisposition [15, 33, 34]. It is generally accepted that alterations in chromosomes, including chromosome numbers and abnormal structures seen in cancer cells, confer some selective advantage to the evolving tumor [35]. Tetraploidy and aneuploidy are hallmarks of human cancers and have long been implicated as a driving force for cancer initiation and/or progression [36]. More recently, we showed that suppression of formation of tetraploidy and aneuploidy reduced cellular transformation frequency, providing direct experimental evidence for the aneuploidy hypothesis [37]. Chromosomal breaks, which frequently occurred in the B[a]P-treated E160D cells, may cause chromosome deletions or rearrangements and in turn lead to inactivation of tumor suppressor genes or loss-of-heterozygosity, both of which are frequent events in human cancers [34]. A previous study showed that mutant mice carrying a mutation in the Replication Protein A (RPA) developed cancers associated with chromosomal breaks, gross chromosomal rearrangements, and aneuploidy [38]. Consistent with previous studies, our data demonstrate that B[a]P-induced near-tetraploid aneuploidy and tetraploidy, particularly in E160D cells, was accompanied by chromosome breakage. The effects of FEN1 deficiency and B[a]P-treatment on lung tumorigenesis were additive when the tumor incidence in E160D mice is compared to that in WT mice. We chose to count the cancer incidence in relatively young mice following a single intraperitoneal injection of B[a]P while the untreated mice have not yet developed lung cancer at this early stage. This helped eliminate confounding factors, as E160D mice, even without exposure to base-damaging agents, develop autoimmune diseases and cancer at high frequency [14]. Moreover, the lung cancer incidence of the B[a]P-treated E160D mice, which accumulated more chromosomal breaks and aneuploidy, was considerably than that of the B[a]P-treated WT mice plus that of the untreated E160D mice. It suggests that the FEN1 mutation and the B[a]P insult have a synergistic effect in the induction of lung cancer. Therefore, individuals carrying FEN1 mutations that impair this function are likely to be

susceptible to exposure to B[α]P-containing environmental agents such as tobacco smoke. If smoking, these people may have high risk to develop lung cancer.

Highlights

We investigate whether FEN1 mutations cause individuals to be more sensitive to tobacco compounds such as B[α]P. We demonstrate that FEN1 is involved in processing of B[α]P lesions and the GEN activity of FEN1 is important for this process. Individuals carrying FEN1 mutations that impair its GEN activity will have high risk to develop tobacco-induced cancer.

Abbreviations

FEN1	Flap endonuclease 1
FEN	flap endonuclease
EXO	exonuclease
GEN	gap endonuclease
B[α]P or BP	benzo[α]pyrene
NER	nucleotide excision repair
WT	wild-type
BPDE	benzo[α]pyrene-trans-7, 8-dihydrodiol-9, 10-epoxide(anti)(+)
XPG	xeroderma pigmentosum group G
ERCC1-XPF	excision repair cross complementing group 1- xeroderma pigmentosum group F
BER	base excision repair
MEFs	mouse embryonic fibroblasts
NEs	nuclear extract
WRN	Werner syndrome protein
DSBs	double-stranded breaks

Acknowledgments

We thank the microscopy core facility of City of Hope for technical assistance with immunofluorescence staining and imaging of MEF cells. All protocols involving animal use were approved by the Research Animal Care Committee of City of Hope National Medical Center and Beckman Research Institute in compliance with the Public Health Service Policy on Use of Laboratory Animals. This work was supported by NIH grant R01 CA073764 to B.H.S.

References

- Alexandrov K, Rojas M, Satarug S. The critical DNA damage by benzo[α]pyrene in lung tissues of smokers and approaches to preventing its formation. *Toxicol Lett.* 2010; 198:63–68. [PubMed: 20399842]
- Kuper H, Adami HO, Boffetta P. Tobacco use, cancer causation and public health impact. *Journal of Internal Medicine.* 2002; 251:455–466. [PubMed: 12028500]
- Wu X, Zhao H, Suk R, Christiani DC. Genetic susceptibility to tobacco-related cancer. *Oncogene.* 2004; 23:6500–6523. [PubMed: 15322521]

4. Braithwaite E, Wu X, Wang Z. Repair of DNA lesions induced by polycyclic aromatic hydrocarbons in human cell-free extracts: involvement of two excision repair mechanisms in vitro. *Carcinogenesis*. 1998; 19:1239–1246. [PubMed: 9683183]
5. Altieri F, Grillo C, Maceroni M, Chichiarelli S. DNA damage and repair: from molecular mechanisms to health implications. *Antioxid Redox Signal*. 2008; 10:891–937. [PubMed: 18205545]
6. Shen B, Nolan JP, Sklar LA, Park MS. Functional analysis of point mutations in human flap endonuclease-1 active site. *Nucleic Acids Res*. 1997; 25:3332–3338. [PubMed: 9241249]
7. Frank G, Qiu J, Somsouk M, Weng Y, Somsouk L, Nolan JP, Shen B. Partial functional deficiency of E160D flap endonuclease-1 mutant in vitro and in vivo is due to defective cleavage of DNA substrates. *J Biol Chem*. 1998; 273:33064–33072. [PubMed: 9830061]
8. Shen B, Singh P, Liu R, Qiu J, Zheng L, Finger LD, Alas S. Multiple but dissectible functions of FEN-1 nucleases in nucleic acid processing, genome stability and diseases. *Bioessays*. 2005; 27:717–729. [PubMed: 15954100]
9. Tsutakawa SE, Classen S, Chapados BR, Arvai AS, Finger LD, Guenther G, Tomlinson CG, Thompson P, Sarker AH, Shen B, Cooper PK, Grasby JA, Tainer JA. Human flap endonuclease structures, DNA double-base flipping, and a unified understanding of the FEN1 superfamily. *Cell*. 2011; 145:198–211. [PubMed: 21496641]
10. Qiu J, Li X, Frank G, Shen B. Cell cycle-dependent and DNA damage-inducible nuclear localization of FEN-1 nuclease is consistent with its dual functions in DNA replication and repair. *J Biol Chem*. 2001; 276:4901–4908. [PubMed: 11053418]
11. Saharia A, Teasley DC, Duxin JP, Dao B, Chiappinelli KB, Stewart SA. FEN1 ensures telomere stability by facilitating replication fork re-initiation. *J Biol Chem*. 2010; 285:27057–27066. [PubMed: 20551483]
12. Larsen E, Kleppa L, Meza TJ, Meza-Zepeda LA, Rada C, Castellanos CG, Lien GF, Nesse GJ, Neuberger MS, Laerdahl JK, Doughty R William, Klungland A. Early-onset lymphoma and extensive embryonic apoptosis in two domain-specific Fen1 mice mutants. *Cancer Res*. 2008; 68:4571–4579. [PubMed: 18559501]
13. Landi S, Gemignani F, Monnier S, Canzian F. A database of single-nucleotide polymorphisms and a genotyping microarray for genetic epidemiology of lung cancer. *Exp. Lung Res*. 2005; 31:223–258. [PubMed: 15824023]
14. Zheng L, Dai H, Zhou M, Li M, Singh P, Qiu J, Tsark W, Huang Q, Kernstine K, Zhang X, Lin D, Shen B. Fen1 mutations result in autoimmunity, chronic inflammation and cancers. *Nat. Med*. 2007; 13:812–819. [PubMed: 17589521]
15. Xu H, Zheng L, Dai H, Zhou M, Hua Y, Shen B. Chemical-induced cancer incidence and underlying mechanisms in Fen1 mutant mice. *Oncogene*. 2011; 30:1072–1081. [PubMed: 20972458]
16. Zheng L, Li M, Shan J, Krishnamoorthi R, Shen B. Distinct roles of two Mg²⁺ binding sites in regulation of murine flap endonuclease-1 activities. *Biochemistry*. 2002; 41:10323–10331. [PubMed: 12162748]
17. Kurose A, Tanaka T, Huang X, Traganos F, Darzynkiewicz Z. Synchronization in the cell cycle by inhibitors of DNA replication induces histone H2AX phosphorylation: an indication of DNA damage. *Cell Prolif*. 2006; 39:231–240. [PubMed: 16672000]
18. Cai Y, Kropachev K, Xu R, Tang Y, Kolbanovskii M, Kolbanovskii A, Amin S, Patel DJ, Broyde S, Geacintov NE. Distant neighbor base sequence context effects in human nucleotide excision repair of a benzo[α]pyrene-derived DNA lesion. *J Mol Biol*. 2010; 399:397–409. [PubMed: 20399214]
19. Riedl T, Hanaoka F, Egly JM. The comings and goings of nucleotide excision repair factors on damaged DNA. *EMBO J*. 2003; 22:5293–5303. [PubMed: 14517266]
20. Zheng L, Zhou M, Chai Q, Parrish J, Xue D, Patrick SM, Turchi JJ, Yannone SM, Chen D, Shen B. Novel function of the flap endonuclease 1 complex in processing stalled DNA replication forks. *EMBO Rep*. 2005; 6:83–89. [PubMed: 15592449]

21. Pascucci B, Russo MT, Crescenzi M, Bignami M, Dogliotti E. The accumulation of MMS-induced single strand breaks in G1 phase is recombinogenic in DNA polymerase beta defective mammalian cells. *Nucleic Acids Res.* 2005; 33:280–288. [PubMed: 15647510]
22. Matsumoto M, Yaginuma K, Igarashi A, Imura M, Hasegawa M, Iwabuchi K, Date T, Mori T, Ishizaki K, Yamashita K, Inobe M, Matsunaga T. Perturbed gap-filling synthesis in nucleotide excision repair causes histone H2AX phosphorylation in human quiescent cells. *J. Cell Sci.* 2007; 120:1104–1112. [PubMed: 17327276]
23. Chen J, Thilly WG. Mutational spectra vary with exposure conditions: benzo[a]pyrene in human cells. *Mut. Res.* 1996; 357:209–217.
24. Futaki M, Liu JM. Chromosomal breakage syndromes and the BRCA1 genome surveillance complex. *Trends Mol Med.* 2001; 7:560–565. [PubMed: 11733219]
25. Pazhanisamy SK, Jyothi V. Genome organization, instabilities, stem cells, and cancer. *Journal of Stem Cells and Regenerative Medicine.* 2009; 5:11–22.
26. Dai Y, Grant S. New insights into checkpoint kinase 1 in the DNA damage response signaling network. *Clin Cancer Res.* 2010; 16:376–383. [PubMed: 20068082]
27. Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double-strand break repair pathway choice. *Cell Res.* 2008; 18:134–147. [PubMed: 18157161]
28. Schneider J, Classen V, Helmig S. XRCC1 polymorphism and lung cancer risk. *Expert Rev Mol Diagn.* 2008; 8:761–780. [PubMed: 18999926]
29. Liu Y, Kao HI, Bambara RA. Flap endonuclease 1: a central component of DNA metabolism. *Annu Rev Biochem.* 2004; 73:589–615. [PubMed: 15189154]
30. Sarker AH, Tsutakawa SE, Kostek S, Ng C, Shin DS, Peris M, Campeau E, Tainer JA, Nogales E, Cooper PK. Recognition of RNA polymerase II and transcription bubbles by XPG, CSB, and TFIIH: insights for transcription-coupled repair and Cockayne Syndrome. *Mol Cell.* 2005; 20:187–198. [PubMed: 16246722]
31. Tapias A, Auriol J, Forget D, Enzlin JH, Schärer OD, Coin F, Coulombe B, Egly JM. Ordered conformational changes in damaged DNA induced by nucleotide excision repair factors. *J Biol Chem.* 2004; 279:19074–19083. [PubMed: 14981083]
32. Dou H, Mitra S, Hazra TK. Repair of oxidized bases in DNA bubble structures by human DNA glycosylases NEIL1 and NEIL2. *J Biol Chem.* 2003; 278:49679–49684. [PubMed: 14522990]
33. Burma S, Chen BP, Chen DJ. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. *DNA Repair (Amst).* 2006; 5:1042–1048. [PubMed: 16822724]
34. Zheng L, Dai H, Hegde ML, Zhou M, Guo Z, Wu X, Wu J, Su L, Zhong X, Mitra S, Huang Q, Kernstine KH, Pfeifer GP, Shen B. Fen1 mutations that specifically disrupt its interaction with PCNA cause aneuploidy-associated cancer. *Cell Res.* 2011; 21:1052–1067. [PubMed: 21383776]
35. Helleday T, Lo J, van Gent DC, Engelward BP. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst).* 2007; 6:923–935. [PubMed: 17363343]
36. Coleman WB, Tsongalis GJ. Molecular mechanisms of human carcinogenesis. *EXS.* 2006; 96:321–349. [PubMed: 16383025]
37. Herrera LA, Prada D, Andonegui MA, Dueñas-González A. The epigenetic origin of aneuploidy. *Curr Genomics.* 2008; 9:43–50. [PubMed: 19424483]
38. Zheng L, Shen B. Okazaki fragment maturation: nucleases take centre stage. *J Mol Cell Biol.* 2011; 3:23–30. [PubMed: 21278448]

Fig. 1. Repair of BPDE lesions by WT and E160D (ED) nuclear extracts (NEs)

200 ng pUC18 DNA with or without BPDE-induced DNA damage was incubated with (10 μ g) of WT or E160D NEs in the presence of [α - 32 P] dCTP and the other three dNTP. Reactions were carried out at 37°C for 30, 60, 120, and 360 min. (A) The ethidium bromide (EB) stained non-damaged plasmid pUC18 DNA after *Hind*III digestion. (B) The EB stained BPDE-damaged plasmid pUC18 DNA after *Hind*III digestion. (C) Radioautography of repair reactions with non-damaged DNA plasmid. (D) Radioautography of reactions with BPDE-damaged DNA plasmid. BPDE (-): without BPDE treatment; BPDE (+): BPDE treatment; 0: without NEs; WT: wild-type NEs; E160D: E160D NEs. The bottom panels in (C) and (D) are quantification of the repair product (the 32 P-labeled DNA plasmid) normalized with the corresponding input [the EB stained DNA plasmid in (A) and (B)]. Values are mean $\pm$ s.d of three independent assays.

Fig. 2. E160D FEN1 is less efficient at cleaving BPDE bubble substrate

(A) Schematic of the DNA bubble substrate with 3'-end ^{32}P -labeling. Arrow indicates that the efficient cleavage site by FEN1. (B) Purified recombinant WT and E160D FEN1 proteins were incubated with DNA bubble substrates with (right) or without (left) BPDE-induced damage. Reactions were carried out at 37°C, for 5, 30, and 60 min. Bottom panels: quantification of cleavage of DNA bubble substrate with (right) or without (left) BPDE-induced damage. BPDE (-): without BPDE treatment; BPDE (+): BPDE treatment; 0: without FEN1 proteins; WT: wild-type FEN1 protein; E160D: E160D FEN1 protein.

Fig. 3. E160D cells accumulate more B[α]P-induced DSBs than WT cells

MEF cells were either treated with B[α]P (30μM, 24h) or left untreated following a previously published protocol [23]. After washing, the cells were incubated in fresh DMEM for 4 hours. The cells were then subjected to immunofluorescence staining or Western blotting analysis for γH2AX (Ser 139). (A) Immunofluorescence staining of MEF cells. Red, staining for γH2AX; Blue, counterstaining with 4', 6-diamidino-2-phenylindole (DAPI). (B) Percentage of γH2AX-positive nuclei. To exclude false γH2AX-positive nuclei, we arbitrarily scored nuclei with at least 5 γH2AX foci as positive ones. **P=0.0011 (two-tailed Fisher's exact test). (C) Western blot of γH2AX levels in nuclear extracts from B[α]P-

treated and untreated MEF cells. WT, wild-type; ED, E160D mutant; WT/BP, WT with B[α]P treatment; ED/BP, ED with B[α]P treatment.

Fig. 4. B[α]P induces more chromosomal abnormalities in E160D MEF cells than in WT MEF cells

(A) Normal chromosome number (2N=40) and tetraploid chromosome number (4N=80) in B[α]P-treated E160D MEF cells. (B) Percentage of near-tetraploid aneuploid cells in the WT and E160D cells treated or untreated with B[α]P. **P=0.008 (two-tailed Fisher's exact test). (C) Chromosomal breaks in the B[α]P-treated WT and E160D MEF cells. Arrows indicate broken sites of the chromosome. Top right box shows enlarged image of one of the broken chromosomes. (D) Quantification of broken chromosomes in B[α]P-treated and untreated MEF cells (WT and E160D). The number of chromosome breaks were divided by the total number of normal mitotic cells observed. *P=0.0299 (two-tailed Fisher's exact test).

Fig. 5. E160D mice are more susceptible to the development of B[a]P-induced lung cancer
Mice [WT (n=51) and E160D (n=52); 6–8 weeks old] were injected with a single dose of B[a]P (100 mg/kg). All mice were euthanized at 8 months of age to assess the development of lung cancer. (A) Lung cancer frequencies in WT and E160D mice after B[a]P treatment. (B) Numbers of lung tumors in the B[a]P-treated and untreated WT and E160D mice. Four E160D mice, indicated by rectangle, had more than 30 tumors that spread throughout the entire lung (whole lung tumors). *** $P < 0.0001$ (Student's t-test). (C) Sizes of lung tumors in the B[a]P-treated and untreated WT and E160D mice. ** $P = 0.0079$ (Student's t-test).