Germ-line variant of human NTH1 DNA glycosylase induces genomic instability and cellular transformation

Heather A. Galicka, Scott Katheb, Minmin Liua, Susan Robey-Bond, Dawit Kidaneb, Susan S. Wallacea, and Joann B. Sweasyab,1

aDepartment of Microbiology and Molecular Genetics, The Markey Center for Molecular Genetics, University of Vermont, Burlington, VT 05405-0068; and bDepartment of Therapeutic Radiology, Yale University School of Medicine, New Haven, CT 06510

Edited by Philip C. Hanawalt, Stanford University, Stanford, CA, and approved July 16, 2013 (received for review April 9, 2013)

Base excision repair (BER) removes at least 20,000 DNA lesions per human cell per day and is critical for the maintenance of genomic stability. We hypothesize that aberrant BER, resulting from mutations in BER genes, can lead to genomic instability and cancer. The first step in BER is catalyzed by DNA N-glycosylases. One of these, Nth endonuclease III-like (NTH1), removes oxidized pyrimidines from DNA, including thymine glycol. The rs3087468 single nucleotide polymorphism of the NTH1 gene is a G-to-T base substitution that occurs in ~6.2% of the global population and is found in Europeans, Asians, and sub-Saharan Africans. In this study, we functionally characterize the effect of the D239Y variant expressed in immortal but nontransformed human and mouse mammary epithelial cells. We demonstrate that expression of the D239Y variant in cells also expressing wild-type NTH1 leads to genomic instability and cellular transformation as assessed by anchorage-independent growth, focus formation, invasion, and chromosomal aberrations. We also show that cells expressing the D239Y variant are sensitive to ionizing radiation and hydrogen peroxide and accumulate double strand breaks after treatment with these agents. The DNA damage response is also activated in D239Y-expressing cells. In combination, our data suggest that individuals possessing the D239Y variant are at risk for genomic instability and cancer.

DNA repair | mutagenesis | MCF10A breast epithelial cells

The base excision repair (BER) pathway is responsible for the removal of at least 20,000 DNA lesions per cell per day (1), making it critical for the maintenance of genomic stability. The simplest and most common form of BER is short patch BER, which can be initiated by one of several different DNA glycosylases, each having preferences for specific types of lesions (2). Monofunctional DNA glycosylases recognize DNA lesions and catalyze the hydrolysis of the N-glycosyl bond to generate an abasic site. The abasic site is nicked at its 5′ side by the APE1 endonuclease, leaving a 3′OH and a 5′deoxyribose phosphate (dRP). DNA polymerase beta (pol β) fills in the single nucleotide gap and catalyzes removal of the dRP group. Bifunctional glycosylases, which usually recognize oxidative lesions, generate an abasic site and then catalyze its removal via β-elimination to generate a 3′dRP and 5′phosphate. APE1 then catalyzes removal of the 3′dRP leaving a 3′OH, to which pol β can bind and fill in the resulting single nucleotide gap. In both cases, the XRCC1/Ligase IIIα or XRCC1/Ligase I complex catalyzes ligation of the resulting ends. An alternative BER pathway, that does not depend on APE1, is used when the NEIL glycosylases initiate repair (3). NEIL 1, 2, and perhaps 3 catalyze excision of the damaged base via β,δ elimination, leaving a 3′phosphate and a 5′phosphate. The 3′phosphate is removed by polynucleotide kinase, leaving a gap that is most often filled by pol β, followed by ligation.

NTH1 is one of four human bifunctional DNA glycosylases that recognize and remove oxidized pyrimidines and formamidopyrimidines (4). NTH1 is constitutively expressed and recognizes a fairly broad spectrum of oxidized pyrimidines, including 5-hydroxyuracil and thymine glycol (5–8). The rs3087468 single nucleotide polymorphism (SNP) of the NTH1 gene is a G-to-T base substitution that occurs in ~6.2% of the global population and is found in Europeans, Asians, and sub-Saharan Africans (www.ncbi.nlm.nih.gov/SNP/). This nonsynonymous alteration results in substitution of a Tyr codon for an Asp codon (D239Y) within the active site of the protein. Approximately 0.6% of the global population is homozygous for this SNP.

In this study we tested the hypothesis that the D239Y human NTH1 (hNTH1) variant has a functional phenotype that could drive carcinogenesis. We found that the D239Y variant is able to bind to oxidized pyrimidines, but is unable to excise damaged bases from DNA. Expression of the D239Y variant in both human and mouse cells induces genomic instability and cellular transformation, likely as a result of the accumulation of DNA double-strand breaks (DSBs). Our results are consistent with the interpretation that individuals who carry the rs3087468 SNP are at increased risk for cancer.

Results

hNTH1 D239Y Is an Inactive DNA Glycosylase. Both the crystal structures of Escherichia coli (9) and Bacillus stearothermophilus (10) Nth and site-directed mutagenesis of a highly conserved aspartate (9), Asp239 in hNTH1, suggested that Asp239 is the protein’s active site nucleophile. Therefore, we reasoned that alteration of Asp239 to Tyr would result in a significantly less active hNTH1 DNA glycosylase than wild type (WT). To test this hypothesis, we subcloned the WT hNTH1 cDNA into pGEX6P3 and used site-directed mutagenesis to generate the alteration of Asp239 to Tyr. We then overexpressed either WT or D239Y hNTH1 in the E. coli nth nei double mutant that exhibits a high spontaneous mutation frequency due to its inability to remove oxidized bases (11). As shown in Fig. S1, expression of WT hNTH1 in this strain significantly reduces the mutation frequency, whereas expression of the D239Y variant does not. These results suggest that, unlike the WT human protein, the D239Y variant is not able to repair oxidized pyrimidines. To confirm this observation, we purified both WT and D239Y hNTH1 and characterized their activities with DNA glycosylase assays. As shown in Fig. 1, D239Y hNTH1 is unable to excise thymine glycol (Tg) opposite template A and has little, if any, lyase activity, in comparison with the WT protein. We also show that D239Y is unable to excise dihydouracil (DHU) from DNA (Fig. S2). Both D239Y and WT bind with similar affinities to DNA containing Tg (Fig. S3). Interestingly, addition of dH239Y to WT hNTH1 results in decreased overall glycosylase activity, suggesting that D239Y...
and WT compete for binding to damaged bases, in this case Tg (Fig. S4). In combination, these results indicate that D239Y hNTH1 is an inactive DNA glycosylase that has the ability to recognize and bind to Tg and DHU lesions and likely other oxidized pyrimidines.

Expression of D239Y hNTH1 in Human Cells Results in Cellular Transformation. Cells that are not able to remove oxidized bases are likely to exhibit genomic instability that leads to a cancerous phenotype. To test this hypothesis, we subcloned either hemagglutinin (HA)-tagged WT or D239Y hNTH1 cDNA into the pRvYTET vector, prepared retrovirus, and infected MCF10A human breast epithelial cells. Tagging the proteins with the HA epitope enables us to distinguish endogenous from exogenous protein. We selected two independent pools of MCF10A cells expressing either WT or D239Y hNTH1 as described (12, 13). The MCF10A cells are an immortalized but nontransformed cell line that also expresses endogenous WT hNTH1 (Fig. S5). Therefore, cells that express D239Y also express endogenous WT hNTH1. Expression of the protein is under control of doxycycline (Dox); when Dox is present in the growth medium, expression is off and, when absent, the exogenous protein is expressed (13). As shown in the Western blot in Fig. 2A and B, in the absence of Dox, the WT-HA and D239Y-HA NTH1 proteins are expressed in their respective pools at equivalent levels, relative to tubulin. Passage 12 cells grown in the absence of Dox were plated in soft agar as described (12), and colonies were counted after 5 wk. As shown in Fig. 2A and B, cells from two independent pools expressing D239Y hNTH1 formed significantly greater numbers of colonies in soft agar than those expressing WT. Moreover, a significant fraction of the colonies expressing D239Y were substantially larger than those expressing WT, as shown in Fig. 2C. We also expressed either WT-HA or D239Y-HA in C127 mouse mammary epithelial cells, isolated individual stable clones, and characterized focus formation as described (13). Focus formation results from cells losing contact inhibition and growing on top of each other as pictured in Fig. 3D. As shown in Fig. 3A and C, expression of WT hNTH1 in each of three individual clones does not induce focus formation. However, we observed focus formation in each of the three clones of C127 cells that express D239Y (Fig. 3 B and C). We then characterized the invasive index (14) of clones expressing either WT or D239Y. As shown in Fig. 3E, expression of D239Y in the C127 cells results in invasion through the basement membrane, whereas cells expressing WT hNTH1 exhibit a low invasive index. In combination, our results suggest that expression of D239Y
hNTH1 results in cellular transformation. This finding is consistent with the interpretation that individuals who carry the D239Y hNTH1 variant are at increased risk for cancer.

Expression of D239Y hNTH1 Results in Genomic Instability. Deficient excision of oxidized bases could result in an increased mutation frequency that eventually leads to cellular transformation. To determine whether this was the case, we used the λcII mutation assay that we previously described (15). Point mutations and small insertions and deletions are observed in this forward mutation assay. The mutation frequencies of cells expressing D239Y and WT were $1.7 \times 10^{-4}$ and $9.1 \times 10^{-5}$, respectively, and therefore not significantly different from each other. These data suggest that it is unlikely that point mutations, which accumulate in the cells expressing D239Y, lead to cellular transformation. As shown in Fig. 1, WT but not D239Y hNTH1 excises Tg, a replication-blocking lesion (16–19). We speculated that cells expressing D239Y hNTH1 may not remove Tg as efficiently as cells exclusively expressing WT protein, leading to replication blocks that result in chromosomal aberrations. To test this hypothesis, we scored metaphase spreads, prepared as described (12), from MCF10A cells expressing either D239Y or WT hNTH1. As shown in Fig. 4, MCF10A cells expressing D239Y exhibit significantly increased levels of chromatid breaks and fusions compared with cells expressing only WT hNTH1. These results suggest that expression of exogenous D239Y in MCF10A cells results in genomic instability that could lead to cellular transformation.

Cells Expressing D239Y hNTH1 Accumulate DSBs. Chromatid breaks and fusions are likely to arise from a DSB intermediate. Our results showing increased levels of these types of aberrations in cells expressing D239Y suggest that these cells have higher levels of DSBs or that repair of DSBs is slower than in cells expressing WT hNTH1. To determine whether DSBs accumulate in the cells expressing D239Y, we monitored γH2AX levels after treating the cells with H2O2. We surmised that treatment with H2O2 would induce the presence of oxidized bases, including Tg, which would not be excised efficiently in cells expressing the inactive D239Y NTH1 compared with WT cells. Deficient excision of Tg or another unknown replication-blocking lesion could result in collapsed replication forks and DSBs. Pools of MCF10A cells expressing WT or D239Y were synchronized in G1 as described in Experimental Procedures and released into the medium. After 18 h, when cells moved into S phase (Fig. S7), we treated them with H2O2 and monitored γH2AX as a marker for the presence of DSBs; we also stained with propidium iodide (PI) to identify the cell cycle stage. Cells were also allowed to recover for various times, then stained and analyzed to monitor repair of DSBs. As shown in Fig. 5, DSBs accumulate in cells expressing D239Y that are in S and G2/M phases of the cell cycle and continue to be present even after 8 h of recovery, whereas DSBs are more rapidly repaired in cells expressing WT hNTH1.

![Fig. 3. Expression of D239Y induces cellular transformation in mouse C127 epithelial cells. Either WT (A) or D239Y (B) proteins were expressed in mouse C127 cells, and focus formation was monitored as an endpoint. (A) Three individual WT clones, 1 (●), 2 (■), and 3 (▲), were induced (solid lines) or not induced (dashed lines, Inset) to express WT hNTH1. No significant levels of focus formation were observed when Dox was removed (inducing conditions) from the medium to allow expression of the exogenous protein. Western blot under graph shows levels of expression normalized to tubulin. Membranes were probed with antibodies to the hemagglutinin tag (HA tag), and alpha tubulin as a loading control. The ratio of HA tag to tubulin shows a similar expression level among all clones. (B) Three individual D239Y clones, 1 (●), 2 (■), and 3 (▲) were induced (solid lines) or not induced (dashed lines) to express D239Y hNTH1. All three clones induce significant levels of focus formation at various passages when Dox was removed from the medium allowing expression of the exogenous protein, as shown in the Western blot under the graph. (C) Photograph of flasks of C127 cells stained for focus formation and expressing either WT (Left) or D239Y (Right) hNTH1. (D) Photograph of a focus of cells expressing D239Y at passage 12.](https://www.pnas.org/cgi/doi/10.1073/pnas.1306752110)
Expression of D239Y Confers Sensitivity to Ionizing Radiation and Hydrogen Peroxide. The accumulation of DSBs in H₂O₂-treated cells expressing D239Y suggest that these cells could be sensitive to agents that induce oxidative base damage, namely, H₂O₂ and ionizing radiation (IR). To determine whether this was the case, we performed clonogenic survival assays with C127 clones expressing either WT or D239Y hNTH1. As shown in Fig. 6A, cells expressing D239Y hNTH1 were more sensitive than WT to both H₂O₂ (Fig. 6A) and IR (Fig. 6B). In combination with our other results, the finding that expression of D239Y confers sensitivity to H₂O₂ and IR agents suggests that cells expressing this variant do not repair DNA damage as efficiently as WT cells.

Summary. We found that expression of the D239Y hNTH1 germline variant in the presence of WT NTH1 induces genomic instability and cellular transformation. These observations, together with our biochemical data demonstrating that D239Y lacks glycosylase activity yet binds to lesion-containing DNA as well if not better than WT NTH1, strongly suggest that a lesion recognized and bound by WT will be repaired but one recognized and bound by D239Y hNTH1 will not. The genomic instability induced by D239Y likely results from its inability to repair oxidative DNA lesions, such as Tg, that can otherwise block replicative DNA polymerases. If the block is in the lagging strand, replication can reinitiate downstream, resulting in formation of a single-strand gap. The remaining lesion could then be processed by homologous recombination (HR)-mediated repair to restore the genetic information. However, should a functionally redundant glycosylase excise the Tg or other fork-blocking lesion within the context of a single-strand gap, a DSB would result that could be processed by nonhomologous end-joining (NHEJ) leading to cell death or genomic instability. Fork-blocking lesions in the leading strand would lead to replication fork collapse. The collapsed fork could be rescued by regression catalyzed by helicases such as BLM, and restart could occur by HR-mediated repair. Alternatively, excision of the lesion by a functionally redundant glycosylase could again result in the formation of a DSB that could be repaired by HR in a error-free manner, lead to cell death, or lead to genomic instability. Our observation that DSBs accumulate in cells expressing D239Y, even after 8 h of recovery, suggests that fork-blocking lesions

These results suggest that the presence of D239Y induces the accumulation of DSBs, perhaps as a consequence of replication fork collapse upon collision with an unexcised Tg or other replication-blocking lesions. This suggestion is supported by results showing that cells expressing D239Y have increased levels of γH2AX and phosphorylated CHK1 compared with cells expressing WT hNTH1 (Fig. 5 B and C). Importantly, cells expressing D239Y that were not treated with H₂O₂ also express increased levels of Chk1 as they traverse S phase (Fig. 5C, lanes 7, 9, and 11). These results show that the DNA damage response is activated even in D239Y-expressing cells that are not treated with a DNA-damaging agent, indicating that the cells expressing D239Y do not rapidly repair certain types of endogenous oxidative DNA damage. This damage could lead to genomic instability and cellular transformation.

Fig. 4. Expression of D239Y hNTH1 induces chromosomal aberrations. Metaphase spreads were prepared from early passage MCF10A pools expressing either WT or D239Y as described in Experimental Procedures. (A) Column graph showing that cells expressing D239Y had significantly increased levels of fusions and chromosome breaks. (B) Arrows point to examples of fusions observed in cells expressing D239Y. (C) Arrows point to examples of chromosome breaks in cells expressing D239Y hNTH1.

Expression of D239Y hNTH1 in the presence of WT NTH1 induces genomic instability and cellular transformation. These observations, together with our biochemical data demonstrating that D239Y lacks glycosylase activity yet binds to lesion-containing DNA as well if not better than WT NTH1, strongly suggest that a lesion recognized and bound by WT will be repaired but one recognized and bound by D239Y hNTH1 will not. The genomic instability induced by D239Y likely results from its inability to repair oxidative DNA lesions, such as Tg, that can otherwise block replicative DNA polymerases. If the block is in the lagging strand, replication can reinitiate downstream, resulting in formation of a single-strand gap. The remaining lesion could then be processed by homologous recombination (HR)-mediated repair to restore the genetic information. However, should a functionally redundant glycosylase excise the Tg or other fork-blocking lesion within the context of a single-strand gap, a DSB would result that could be processed by nonhomologous end-joining (NHEJ) leading to cell death or genomic instability. Fork-blocking lesions in the leading strand would lead to replication fork collapse. The collapsed fork could be rescued by regression catalyzed by helicases such as BLM, and restart could occur by HR-mediated repair. Alternatively, excision of the lesion by a functionally redundant glycosylase could again result in the formation of a DSB that could be repaired by HR in a error-free manner, lead to cell death, or lead to genomic instability. Our observation that DSBs accumulate in cells expressing D239Y, even after 8 h of recovery, suggests that fork-blocking lesions

These results suggest that the presence of D239Y induces the accumulation of DSBs, perhaps as a consequence of replication fork collapse upon collision with an unexcised Tg or other replication-blocking lesions. This suggestion is supported by results showing that cells expressing D239Y have increased levels of γH2AX and phosphorylated CHK1 compared with cells expressing WT hNTH1 (Fig. 5 B and C). Importantly, cells expressing D239Y that were not treated with H₂O₂ also express increased levels of Chk1 as they traverse S phase (Fig. 5C, lanes 7, 9, and 11). These results show that the DNA damage response is activated even in D239Y-expressing cells that are not treated with a DNA-damaging agent, indicating that the cells expressing D239Y do not rapidly repair certain types of endogenous oxidative DNA damage. This damage could lead to genomic instability and cellular transformation.

Fig. 4. Expression of D239Y hNTH1 induces chromosomal aberrations. Metaphase spreads were prepared from early passage MCF10A pools expressing either WT or D239Y as described in Experimental Procedures. (A) Column graph showing that cells expressing D239Y had significantly increased levels of fusions and chromosome breaks. (B) Arrows point to examples of fusions observed in cells expressing D239Y. (C) Arrows point to examples of chromosome breaks in cells expressing D239Y hNTH1.

These results suggest that the presence of D239Y induces the accumulation of DSBs, perhaps as a consequence of replication fork collapse upon collision with an unexcised Tg or other replication-blocking lesions. This suggestion is supported by results showing that cells expressing D239Y have increased levels of γH2AX and phosphorylated CHK1 compared with cells expressing WT hNTH1 (Fig. 5 B and C). Importantly, cells expressing D239Y that were not treated with H₂O₂ also express increased levels of Chk1 as they traverse S phase (Fig. 5C, lanes 7, 9, and 11). These results show that the DNA damage response is activated even in D239Y-expressing cells that are not treated with a DNA-damaging agent, indicating that the cells expressing D239Y do not rapidly repair certain types of endogenous oxidative DNA damage. This damage could lead to genomic instability and cellular transformation.

These results suggest that the presence of D239Y induces the accumulation of DSBs, perhaps as a consequence of replication fork collapse upon collision with an unexcised Tg or other replication-blocking lesions. This suggestion is supported by results showing that cells expressing D239Y have increased levels of γH2AX and phosphorylated CHK1 compared with cells expressing WT hNTH1 (Fig. 5 B and C). Importantly, cells expressing D239Y that were not treated with H₂O₂ also express increased levels of Chk1 as they traverse S phase (Fig. 5C, lanes 7, 9, and 11). These results show that the DNA damage response is activated even in D239Y-expressing cells that are not treated with a DNA-damaging agent, indicating that the cells expressing D239Y do not rapidly repair certain types of endogenous oxidative DNA damage. This damage could lead to genomic instability and cellular transformation.

Fig. 4. Expression of D239Y hNTH1 induces chromosomal aberrations. Metaphase spreads were prepared from early passage MCF10A pools expressing either WT or D239Y as described in Experimental Procedures. (A) Column graph showing that cells expressing D239Y had significantly increased levels of fusions and chromosome breaks. (B) Arrows point to examples of fusions observed in cells expressing D239Y. (C) Arrows point to examples of chromosome breaks in cells expressing D239Y hNTH1.

These results suggest that the presence of D239Y induces the accumulation of DSBs, perhaps as a consequence of replication fork collapse upon collision with an unexcised Tg or other replication-blocking lesions. This suggestion is supported by results showing that cells expressing D239Y have increased levels of γH2AX and phosphorylated CHK1 compared with cells expressing WT hNTH1 (Fig. 5 B and C). Importantly, cells expressing D239Y that were not treated with H₂O₂ also express increased levels of Chk1 as they traverse S phase (Fig. 5C, lanes 7, 9, and 11). These results show that the DNA damage response is activated even in D239Y-expressing cells that are not treated with a DNA-damaging agent, indicating that the cells expressing D239Y do not rapidly repair certain types of endogenous oxidative DNA damage. This damage could lead to genomic instability and cellular transformation.

These results suggest that the presence of D239Y induces the accumulation of DSBs, perhaps as a consequence of replication fork collapse upon collision with an unexcised Tg or other replication-blocking lesions. This suggestion is supported by results showing that cells expressing D239Y have increased levels of γH2AX and phosphorylated CHK1 compared with cells expressing WT hNTH1 (Fig. 5 B and C). Importantly, cells expressing D239Y that were not treated with H₂O₂ also express increased levels of Chk1 as they traverse S phase (Fig. 5C, lanes 7, 9, and 11). These results show that the DNA damage response is activated even in D239Y-expressing cells that are not treated with a DNA-damaging agent, indicating that the cells expressing D239Y do not rapidly repair certain types of endogenous oxidative DNA damage. This damage could lead to genomic instability and cellular transformation.
accumulate over time and that some of these are likely acted upon by redundant glycosylases, leading to DSBRs and replication fork collapse. Should the DSB be repaired in an error-prone manner via NHEJ, genomic instability will result leading to cellular transformation. Taken together, our results suggest that individuals who carry D239Y likely accumulate chromosomal changes over time, as BER acts on endogenous oxidative lesions. Because genomic instability leads to cancer, our results suggest that individuals who carry the D239Y germ-line variant are at increased risk for cancer. Should these individuals be exposed to DNA-damaging agents, chromosomal abnormalities may accumulate even more rapidly, leading to an earlier onset of cancer.

Experimental Procedures

Cells and Growth Media. MCF10A cells are immortalized, nontransformed epithelial cells derived from human mammary tissue (ATCC). These cells were maintained in DMEM/F12 medium (Corning Cellgro) supplemented with 5% (vol/vol) horse serum, 1% (vol/vol) penicillin-streptomycin (Invitrogen), epidermal growth factor (20 ng/mL; Peprotech), hydrocortisone (0.5 mg/mL), and cholera toxin (100 ng/mL; Sigma-Aldrich). C127 is a non-transformed clonal line derived from a mammary tumor of an RIII mouse (20). These cells and the GP2-293 virus packaging cell line (Clontech) used for retrovirus preparation were maintained in DMEM (Corning Cellgro) supplemented with 10% (vol/vol) FBS and 1% (wt/vol) penicillin-streptomycin (20). These cells and the GP2-293 virus packaging cell line (Clontech) used for retrovirus preparation were maintained in DMEM (Corning Cellgro) supplemented with 10% (vol/vol) FBS and 1% (wt/vol) penicillin-streptomycin (Invitrogen). All cell lines were grown at 37 °C in a 5% CO2 humidified incubator.

Expression of hNTH1. Focus formation assays with the C127 cells were carried out as described (12). Anchorage-independent growth assays with the MCF10A cells were performed as described (12). See SI Text for additional details. Cell invasion assays were carried out using Biocoat Control Inserts (BD Pharmagen no. 354578) and GFR Matrigel Basement Membrane Matrix Invasion Chambers (BD Pharmagen no. 354483) essentially as described (14).

Chromosomal Aberrations. Metaphase spreads were prepared and chromosomal aberrations were analyzed as described (12). See SI Text for additional details.

Flow Cytometry. MCF10A cells expressing WT or NTH1 D239Y were seeded at 8 × 10^5 cells per 10-cm dish, allowed to attach overnight, and synchronized by 24 h of serum and growth factor deprivation. Complete medium was added back and cells incubated at 37 °C/5% CO2 for a further 18 h to reach S phase. Plates were treated with or without 30 mM H2O2 in serum-free medium in the cold for 30 min, then changed back to warm complete medium and allowed to recover for 1 h, 4 h, and 8 h after treatment. Cells were then trypsinized and rinsed with PBS, pelleted and fixed in 2% formalin on ice for 20 min, pelleted again and resuspended by adding 70% ice-cold ethanol dropwise while vortexing. Cells were fixed overnight at −20 °C. The cells were incubated with primary phospho-h2A.X antibody (Millipore 05–636) 1:500 overnight at 4 °C. Following the incubation, cells were washed twice with PBS and incubated with anti-mouse secondary antibody conjugated to FITC 1:500 for 1 h at room temperature. Cells were washed twice with PBS and resuspended in 500 μL of PiR/Nase staining buffer (BD Pharmingen). Fluorescence was measured by flow cytometry using the BD LSRII analytical cytometer and analyzed using FlowJo 8.8.7 software.

Clonogenic Survival. Cells were trypsinized and resuspended in serum-free medium on ice at 2 × 10^4 cells per 2 mL for H2O2 treatment or 5 mL for X-irradiation. Cells were then treated with 0, 2 mM, 4 mM, or 8 mM H2O2 for 30 min on ice, or with 0, 2 Gy, 4 Gy, or 8 Gy X-rays, followed by serial dilution in 10-cm dishes with DMEM/10% FBS. Cells were grown for 12 days before staining with 0.25% crystal violet in 80% methanol. Colonies were scored by eye at 10x magnification. Only colonies with more than 50 cells were counted, and all experiments were repeated at least twice.

ACKNOWLEDGMENTS. We thank the Vermont Cancer Center DNA Analysis Core for sequencing all of the clones and Megan Hess for technical assistance. This work was supported by National Institutes of Health Grant P01 CA098993 awarded by the National Cancer Institute.

The human NTH (hNTH) sequence was amplified by the PCR from a hNTH1 construct in pGEX-6P-3 and cloned into the NdeI, XhoI restriction sites in pET30a. The hNTH D239Y variant was made from the hNTH WT pET 30a construct using the QuickChange XL kit (Stratagene). The hNTH WT and hNTH D239Y variant were expressed from Rosetta 2 Escherichia coli cells via autoinduction and the cells were lysed by sonication in 20 mM Tris (pH 8.0), 0.5 mM NaCl, 10 mM imidazole, 10% glycerol, and 5 mM β-mercaptoethanol. The protein was loaded onto a HisTrap HP, nickel column (GE Healthcare) and eluted with a 10–500 mM imidazole gradient. The fractions containing protein were dia lyzed overnight into 20 mM Tris (pH 8.0), 200 mM NaCl, 10% glycerol, and 5 mM β-mercaptoethanol and this was diluted with an equal volume of 20 mM Tris (pH 8.0), 10% glycerol, and 5 mM β-mercaptoethanol resulting in a final NaCl concentration of 100 mM. The protein was loaded onto a S-Sepharose fast flow column (GE Healthcare) and eluted with a 100–1,000 mM NaCl gradient. The fractions containing protein were collected and dialyzed into storage buffer [50 mM Tris (pH 8.0), 200 mM NaCl, 0.005% Triton-X, 1 mM DTT, 50% glycerol] and stored at –20°C.

To construct pools or clones the WT or variant construct was cotransfected with the pVSV-G plasmid (10 μg of each) into GP2-293 cells using standard calcium phosphate transfection and cells were grown for 72 h. The medium containing low titer virus was removed and cells were grown in medium containing hygromycin B (200 μg/mL; Calbiochem) for 10 d to select for spontaneous integrants that had incorporated the retroviral constructs into their genome. These cells were then transfected with pVSV-G (10 μg) and grown as above to produce high titer virus-containing medium. To infect C127 and MCF10A, cells were grown to ∼30% confluence and incubated overnight with fresh retrovirus medium in the presence of 4 μg/mL polybrene (Sigma).

To infect C127 and MCF10A, cells were grown to ∼30% confluence and incubated overnight with fresh retrovirus medium in the presence of 4 μg/mL polybrene (Sigma). For selection of pools, cells were split 1:3 the day after infection and cells with the integrated construct were selected with 200 μg/mL hygromycin B for C127 cells and 15 μg/mL hygromycin B for MCF10A cells. For generation of stable clones, C127 cells were split at several dilutions following infection and selected with 200 μg/mL hygromycin B. Single cell clones were grown and selected using cloning rings. Clonal cell lines were propagated in the presence of 100 μg/mL hygromycin B. Cells were passed in parallel in the presence or absence of polybrene. For selection, pools of cells were split 1:3 the day after infection and cells with the integrated construct were selected with 200 μg/mL hygromycin B for C127 cells and 15 μg/mL hygromycin B for MCF10A cells. For generation of stable clones, C127 cells were split at several dilutions following infection and selected with 200 μg/mL hygromycin B. Single cell clones were grown and selected using cloning rings. Clonal cell lines were propagated in the presence of 100 μg/mL hygromycin B. Cells were passed in parallel in the presence or absence of 2 μg/mL doxycycline. Expression of exogenous HA-tagged hNTH1 was verified by Western blot.

Glycosylase Assays. Glycosylase assays were carried out with 25 nM substrate, thymine glycol (Tg):A, and alkaline phosphatase (AP): A and 0.25, 2.5, and 25 nM hNTH WT or hNTH D239Y variant in 25 mM Tris (pH 8.0), 250 mM potassium glutamate and 1 mM DTT. The assays were allowed to proceed for 60 min at 37°C. The AP:A reactions were terminated by the addition of an equal volume of formamide stop solution (98% formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanole). The reactions containing Tg:A were terminated by the addition of an equal volume of formamide stop solution. The samples were separated on a 12% (wt/vol) polyacrylamide sequencing gel, transferred to Whatman 3MM paper, dried, exposed to a phosphor imager screen and imaged with an isotope imaging system (Molecular Imaging System, Bio-Rad). Electrophoretic mobility shift assays (EMSAs) were performed in the same buffer as the glycosylase assays but at room temperature for 60 min. The substrate, furan:G, was held constant at 100 nM and the concentration of hNTH1 WT and D239Y was varied from 10 to 3,000 nM. Samples were separated on a 10% native polyacrylamide gel (29:1 acrylamide:bisacrylamide) transferred to Whatman 3MM paper, dried, exposed to a phosphor imager screen and quantitated with an isotope imaging system (Molecular Imaging System, Bio-Rad). The Kd modeling was done in Graph Pad Prism using the one-site specific binding with Hill slope algorithm. The competition assay was carried out with 25 nM Tg:A, and a total of 2.5 nM glycosylase, hNTH1 WT and three different ratios of hNTH1 WT with hNTH D239Y variant (3:1, 1:1, and 1:3) were used. The reaction buffer was 25 mM Tris (pH 8.0), 150 mM potassium glutamate, and 1 mM DTT. The assays were allowed to proceed for 4 min at 37°C. The reactions were terminated by adding NaOH to a final concentration of 0.4 N, heating at 95°C for 5 min, and then adding an equal volume of formamide stop solution (98% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanole).

Western Blotting to Assess Expression. Expression of exogenous HA-tagged NTH1 was verified by Western blot. Cells were passed in parallel in the presence or absence of tetracycline. Approximately 80–90% confluent cells were harvested by scraping in modified RIPA buffer on ice [50 mM Tris (pH 8.0), 0.1% SDS, 0.5% Na Deoxycholate, 1% Nonidet P-40, 150 mM NaCl]. lysates were boiled for 10 min with 2× SDS loading buffer and run on a 12% acrylamide SDS/PAGE gel. Proteins were transferred to nitrocellulose membrane using a Bio-Rad Transblot wet transfer apparatus and probed using anti-HA tag antibody (Abcam no. 9110). DNA damage was assessed by Western blot using anti-phospho-CHK1 (Cell Signaling no. 2348) at 1/500 and anti-γH2AX (phospho S139; Millipore no. 05–636) at 1/250 dilution. Covance anti-tubulin (MMS-489R) at 1/1,000 dilution and Sigma anti-actin (A2066) at 1/1,000 dilution were used as loading controls.

Anchorage-Independent Growth Assays. A total of 1 × 10⁴ cells per mL were mixed into 1:1 0.7% agar (Affymetrix ultrapure)/2× complete MCF10a medium at 42°C. One milliliter of the mixture was layered on each of three wells of a six-well plate, in which 2 mL per well pfi 1:1 10% agar/2× complete MCF10a medium had been allowed to solidify at room temperature. The top layer was allowed to solidify at room temperature again, and the plates were incubated at 37°C/5% CO₂ for 5 wk, feeding twice weekly with 250 μL of 0.7% agar/2× media. All colonies with more than 50 cells were counted under a phase contrast inverted microscope.

Genomic Instability Analysis. Four 10-cm dishes were seeded with 10⁶ MCF10A cells each per cell line and grown at 37°C in 5% CO₂ overnight. The cells were fed fresh medium containing 100 ng/mL colcemid (Invitrogen) and incubated for 3 h before harvesting by mitotic shakeoff. Cells were centrifuged, washed twice with 1× PBS, resuspended dropwise in Hypo (75 mM KCl, Invitrogen) and incubated at 37°C for 30 min. Cells were then...
fixed by gradual resuspension in and 15 min incubation in Carnoy’s Fixative (75% methanol, 25% acetic acid). Finally, cells were dropped onto microscope slides, dried, and stained with Dapi Prolong Gold Anti-fade (Invitrogen). Well spread metaphases were identified under 100× objective (Olympus BX50 Light Microscope with QImaging Retiga 2000R digital camera and software). Metaphase spreads were deidentified and scored for eye for chromosomal fusions, breaks, acentromeric chromosomes, and fragments.

**Lambda (λ) cf Assay.** This assay is a forward mutation assay that mainly detects point mutations and small insertions and deletions and has been described (1, 2). Briefly, the C127 cells harbor multiple copies of the λ genome as described (2). After passaging the cells expressing either WT or D239Y hNTH1 for 7 d, genomic DNA was isolated, packaged into λ particles, and plated for λ cII mutants as described (1). The mutation frequency was calculated by dividing the number of λ cII mutants by the total number of plaques.

**Invasion Assay.** Biocoat Control Inserts (BD Pharmagen no. 354578) and GFR Matrigel Basement Membrane Matrix Invasion Chambers (BD Pharmagen no. 354483) were hydrated in serum-free medium (SFM) for 2 h; this was aspirated, and 0.5 mL of DMEM/10% FBS was added to the outer well. Cells were grown in SFM for 24 h before trypsinization and dilution to 5 × 10⁴ cells per mL of SFM, and 0.5 mL of this was added to each insert. Cells were grown at 37 °C/5% CO₂ for 16 h, and inserts were washed with PBS and fixed in 70% methanol for 20 min. They were then rinsed with PBS, and nonmigrating cells were scraped from the upper surface of membrane with a cotton swab. Inserts were rinsed with propidium iodide (20 μg/mL) and RNase A (200 μg/mL) in PBS for 15 min, rinsed in water, and air dried. The membrane was carefully cut from the insert with a scalpel and mounted on a glass microscope slide in Aqua-Poly/Mount (Polysciences). The slides were evaluated with a Compucyte laser scanning cytometer. In each case, the entire insert mounted on the slide was scanned with a ×20 objective lens with the use of an argon ion laser with an orange/red fluorescence detector. The threshold contour was set based on the orange/red fluorescence intensity, with minimum area of 10 μm² required to trigger an event.

**Mutagenesis Assays in E. coli.** The strains were constructed and the experiments were performed as described (3). The spontaneous mutation rates to rifamycin resistance were calculated using Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) method (4).


**Fig. S2.** D239Y hNTH1 is not able to excise dihydrouracil. In vitro glycosylase assays were performed as described in Experimental Procedures and SI Text. DNA glycosylase/lyase activities of hNTH1 WT versus hNTH1 D239Y. Double-stranded substrate dihydrouracil (DHU):G (50 nM) bifunctional assay: lane 1, no enzyme control; lane 2, incubated with hNTH WT (10 nM); lanes 3 and 4, incubated with hNTH1 D239Y (100 and 500 nM, respectively). Double-stranded substrate AP:G (50 nM) lyase assay: lane 5, no enzyme control; lane 6, incubated with hNTH WT (10 nM); lanes 7 and 8, incubated with hNTH1 D239Y (100 and 500 nM, respectively). The following reactions were terminated with NaOH, heated and followed by the addition of formamide loading dye. DHU:G (50 nM) glycosylase assay: lane 9, no enzyme control; lane 10, incubated with hNTH WT (10 nM); lanes 11 and 12, incubated with hNTH1 D239Y (100 and 500 nM, respectively); lane 13, AP:G (50 nM).

**Fig. S3.** Determination of the apparent dissociation constants of hNTH WT and hNTH D239Y bound to DNA containing a tetrahydrofuran opposite a G. The fraction of hNTH WT and D239Y variant bound to a DNA duplex containing a tetrahydrofuran as a function of enzyme concentration is shown. Error bars represent the SD from three separate experiments.
Fig. S4. Decrease in DNA glycosylase activity of WT hNTH1 when mixed with D239Y. Glycosylase assays were carried out with hNTH1 WT alone and NTH1 WT mixed with various concentrations of NTH1 D239Y such that the total concentration of glycosylase was 2.5 nM. Reactions were terminated at 4 min. The data were normalized to NTH1 WT where the fraction of product is set to 1.0. The data were collected in triplicate and the error bars represent the SD.

Fig. S5. MCF10A cells express NTH1. Western blot of MCF10A extracts prepared from cells expressing either WT or D239Y NTH1 under inducible conditions. Lane 1, cells with WT NTH1 grown in the presence of Dox (noninducing conditions). Lane 2, WT NTH1 cells grown in the absence of Dox (inducing conditions). Lanes 3 and 4, D239Y cells grown in the presence and absence of Dox, respectively. Notice that endogenous NTH1 is expressed in lanes 1 and 3. The blot was probed with antiserum raised against NTH1, and these are the upper bands, labeled NTH1 on the left. The blot was stripped and reprobed with antiserum against the HA tag, and these are the middle bands. Alpha-tubulin was used as a loading control.

<table>
<thead>
<tr>
<th>Ratio HA/Tubulin</th>
<th>NTH1/tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.06</td>
<td>1.0</td>
</tr>
<tr>
<td>1.0</td>
<td>4.6</td>
</tr>
<tr>
<td>0.02</td>
<td>0.45</td>
</tr>
<tr>
<td>0.31</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Fig. S6. Cells expressing D239Y exhibit a higher invasive index. The invasive index was determined as described in Experimental Procedures. OFF is in the presence of Dox when expression is off, and ON is in the absence of Dox when expression is on. There is no significant difference between the invasive index of the WT OFF and ON. There is a significant difference between the OFF and ON invasive indices of both D239Y clones: D239Y-1 ($P = 0.015$) and D239Y-3 ($P = 0.03$).
Fig. S7. Flow cytometry of MCF10A cells stained with propidium iodide. (A) Unsynchronized cells without plasmid. (B and C) pRVYtet-hNTH-WT (B) and pRVYtet-hNTH-D239Y (C) cells synchronized by serum and growth factor deprivation for 24 h, then grown in complete medium for 18 h before harvesting.