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Three Human Pol ι Variants with Impaired Polymerase Activity Fail to Rescue H_2O_2 Sensitivity in *POLI*-Deficient Cells

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Abstract

Human Y-family DNA polymerase (pol) ι is involved in translesion DNA synthesis (TLS) and base excision repair (BER) of oxidative DNA damage. Genetic variations may alter the function of pol ι and affect cellular susceptibility to oxidative genotoxic agents, but their effects remain unclear. We investigated the impacts of ten human missense germline variations on pol ι function by biochemical and cell-based assays. Both polymerase and deoxyribose phosphate (dRP) lyase activities were determined utilizing recombinant pol ι (residues 1−445) proteins. The K209Q, K228I, and Q386R variants showed 4- to 53-fold decreases in specificity constants (k_{cat}/K_m) for dCTP insertion opposite G and 8-oxo-7,8-dihydroguanine compared to the wild-type. The R126C and K345E variants showed wild-type-like polymerase activity, although these two variants (as well as the R209Q, K228I, and Q386R variants) showed greater than 6-fold decreases in dRP lyase activity compared to the wild-type. A CRISPR/Cas9-mediated POLI knockout conferred higher sensitivity to H_2O_2 in human embryonic kidney (HEK293) cells. Exogenous expression of the full-length wild-type, R126C, and K345E variants fully rescued the H_2O_2 sensitivity in *POLI*-deficient cells, while full-length R209Q, K228I, and Q386R variants did not rescue the sensitivity. Our results indicate that the R126C and K345E variants (having wild-type-like polymerase activity, albeit impaired in dRP lyase activity) could fully rescue the H_2O_2 sensitivity in *POLI*-deficient cells, while the R209Q, K228I, and Q386R variants—all impaired in polymerase and dRP lyase activity—failed to rescue the sensitivity, indicating the relative importance of TLS-related polymerase function of pol ι rather than its BER-related dRP lyase function in protection from oxidative stress. The possibility exists that the hypoactive pol ι variants increase the individual susceptibility to oxidative genotoxic agents.

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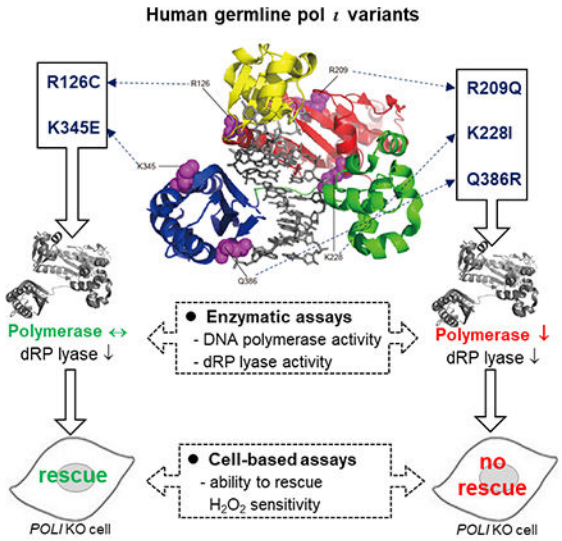
Supporting Information

Analysis of human pol ι (1-445) wild-type and variant proteins by SDS-PAGE (Figure S1), abilities of each pol ι variants to rescue H_2O_2 sensitivity in *POLI*-KO HEK293 cells (Figure S2), correlation plots and coefficients of the relative IC_{50} H_2O_2 values against the relative dCTP insertion efficiencies opposite G or 8-oxoG and relative dRP lyase activities by all thirteen studied pols ι (Figure S3), and analysis of chromatin binding of ectopically expressed wild-type pol ι and four dysfunctional variant proteins in *POLI*-KO cells following H_2O_2 treatment (Figure S4). The Supporting Information is available free of charge at http://pubs.acs.org.

The authors declare no competing financial interest.

Graphical Abstract



Keywords

DNA polymerase ι; genetic variation; DNA damage; translesion DNA synthesis; oxidative stress

INTRODUCTION

Human DNA polymerase (pol) ι, a member of the pol Y-family, is known to be involved in DNA damage tolerance by performing translesion DNA synthesis (TLS) opposite a variety of DNA lesions such as 8-oxo-7,8-dihydroG (8-oxoG), UV-induced photolesions, O^6- and N^2-alkyl and aralkyl adducts, abasic sites, and interstrand DNA cross-links.[1–6] Pol ι distinctively prefers Mn^{2+} over Mg^{2+} as the metal for efficient polymerase catalysis and optimal polymerase active site geometry.[7, 8] In addition to the polymerase activity, pol ι possesses an intrinsic 5′-deoxyribose phosphate (dRP) lyase activity and has been suggested to protect cells against oxidative stress possibly by base excision repair (BER) of oxidative DNA damage.[9, 10] A possible role of Pol ι in mutation and cancer has been supported by multiple lines of evidence. Pol ι deficiency has been suggested to promote UV-induced skin tumorigenesis and urethane-induced lung tumorigenesis in several knock-out mouse models.[11–13] At present, no human disease has been genetically linked to pol ι defeciency, but several single nucleotide variations (SNVs) located in the thumb domain and the ubiquitin binding motifs have been related to melanoma, lung, and prostate cancers.[14–16] In these circumstances, we can speculate that the adequate catalytic function of pol ι is required to protect cells from genotoxic stress in individuals. Therefore, human functional germline pol ι variations that alter its polymerase and/or dRP lyase activity are of special importance, in that they can change the TLS and BER functions of pol ι in cells and consequently modify the susceptibility to genotoxic agents, particularly oxidative agents, in genetically predisposed individuals.

The human *POLI* gene encodes two pol ι proteins, which have identical enzymatic properties under optimal conditions: a long form (740 residues) and a short form (715 residues) that lacks the first 25 amino acids but is abundant in cells.[17] Similar efficiencies are also observed in both the recombinant pol ι (residues 1-445) and pol ι (residues 26-445) proteins from the pre-steady-state kinetic analysis of nucleotide insertion in the presence of 0.15 mM Mn^{2+}.[8] To date, a total of at least 585 non-synonymous germline SNVs of the human *POLI* gene have been listed in the Ensembl genome browser (http://www.ensembl.org). We previously identified and characterized the catalytically polymerase-defective R96G variant from biochemical and structural studies,[8, 18] but the functional effects of other numerous non-synonymous *POLI* SNVs remain unclear. Furthermore, the effects of human germline *POLI* SNVs on dRP lyase activity of pol ι have not been reported yet. Not all non-synonymous SNVs cause the pol ι protein to become dysfunctional, but they can be predicted to be deleterious or not by *in silico* algorithms such as SIFT[19] and Polyphen[20]. However, these prediction algorithms are far less than perfect as shown in our previous studies with human Y-family pols[18, 21–23] and thus biochemical and cellular functional assays are desired to identify and validate the dysfunctional variants.

In this study, we investigated the effects of ten germline *POLI* SNVs on pol ι function at the molecular and cellular levels using biochemical and cell-based assays, especially in terms of protection against oxidative stress. We selected ten missense germline pol ι variants that might be worthy of attention in aspects of the coding change, position, population frequency, and the functional prediction. All studied variants are predicted to be deleterious on protein function by SIFT[19] and PolyPhen[20] and could influence a certain subset of people carrying these variants if functional. First, we performed a series of biochemical experiments: polymerase activity assays ("standing-start" primer extensions and steady-state kinetics for nucleotide incorporation opposite G and 8-oxoG), dRP lyase activity assays, and DNA binding assays using recombinant pol ι (1-445) proteins to evaluate two different enzymatic (DNA polymerase and dRP lyase) activities of pol ι variants. Second, we developed and performed *POLI*-knockout (KO) cell-based complementation assays to evaluate the cellular activities of pol ι variants to rescue H_2O_2 sensitivity in *POLI*-KO cells. Here we report that three variants with impaired polymerase activity failed to rescue H_2O_2 sensitivity in *POLI*-KO cells, while two variants having wild-type-like polymerase activity even with impaired dRP lyase activity could still rescue that sensitivity. These observations are discussed in the context of understanding the functional consequences of pol ι variants and their relationships in terms of polymerase activity, dRP lyase activity, and cellular oxidative sensitivity.

EXPERIMENTAL PROCEDURES

DNA Substrates.

DNA oligonucleotides were obtained from Bioneer (Daejeon, Korea). DNA substrates were prepared for polymerase and binding assays as described,[18] using the 24-mer (5′–GCC TCG AGC CAG CCG CAG ACG CAG–3′), 36-mer (3′–CGG AGC TCG GTC GGC GTC TGC GTC XCT CCT GCG GCT–5′; X = G, 8-oxoG), and 18-FAM-mer (5′–(FAM)-AGC CAG CCG CAG ACG CAG–3′; FAM = 6-carboxyfluorescein). DNA substrates containing

a 5′-dRP group were prepared for dRP lyase assays using the uracil-containing 32-mer (5′–CTG AGC AGT CGC ACA UGT AGT ATC TCT GTG AC–3′) and its complementary oligonucleotide as described.[24]

Selection of Potentially Dysfunctional Human *POLI* Gene Variations.

Ten known human germline *POLI* gene variations were selected (Table 1 and Figure 1) that are likely to alter enzyme function, using the Ensembl genome browser (http://www.ensembl.org), based on three criteria: i) non-synonymous coding change, ii) location in the catalytic core, and iii) deleterious predictions by SIFT[19] and Polyphen.[20]

Expression Vector Construction and Protein Purification.

Mutations for variants were introduced into an *Escherichia coli* expression vector pBG101-*POLI*$^{1\text{-}445}$ for the pol ι core (amino acids 1-445)[18] by site-directed mutagenesis and verified by sequencing. Recombinant pol ι (1-445) proteins were expressed as the N-terminally GST-tagged form in *E. coli* and were purified in the PreScission protease-cleaved, GST-free form to homogeneity (Figure S1, Supporting Information) as described.[18]

Mammalian Expression Vector Construction, Cell Culture, and Transfection.

The full-length *POLI* (long form) coding sequence[4] was subcloned into the mammalian expression vector pcDNA3.1(+). Mutations for variants were introduced into the vector by site-directed mutagenesis and verified by sequencing. Human embryonic kidney (HEK) 293 cells were cultured at 37 °C in a 5% CO_2 atmosphere (v/v) in Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) fetal bovine serum. All transfections were performed using Lipofectamine 3000 according to the manufacturer's instructions.

DNA Polymerase Reactions and Steady-State Kinetic Analysis.

DNA polymerase reactions and steady-state kinetic analysis were performed as described.[18] Reaction mixtures (8 μL) contained 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 10% glycerol (v/v), 100 nM DNA substrate, dNTPs, and 0.15 mM $MnCl_2$ at 37 °C. Reaction products were resolved by 8 M urea-16% polyacrylamide gel electrophoresis (PAGE) and analyzed with a Bio-Rad Personal Molecular Imager (Hercules, CA) as described.[18]

dRP Lyase Activity Assay.

The dRP lyase assay was performed as described with minor modification.[24] The reaction mixture contained 50 mM HEPES (pH 7.5), 20 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 100 nM DNA substrate, and 250 nM pol ι. Reaction products were stabilized with $NaBH_4$ (340 mM final concentration) on ice for 30 min, ethanol-precipitated, resolved by 8 M urea-16% PAGE, and analyzed with a Bio-Rad Personal Molecular Imager.

Fluorescence Polarization.

The fluorescence polarization assay was performed using a BioTek Synergy NEO plate reader (Winooski, VT) and $K_{d,DNA}$ (equilibrium dissociation constant for DNA binding) values were estimated, as described.[18]

Generation of *POLI*-KO Cell Line.

The CRISPR/Cas9-mediated *POLI*-KO HEK293 cell line was generated as described.[25] The guide RNA (5′–CACCGGCCATGGAACTGGCGGACGT–3′) targeting the exon 1 was designed using a CRISPR design tool (http://crispr.mit.edu). Cells were transduced with the gRNA-encoding lentiviruses produced by LentiCRISPRV2 (Addgene). The knockout was confirmed by DNA sequencing of genomic PCR amplicons as well as immunoblotting from puromycin-selected single cell clones.

Western Blotting.

Cells were lysed in ice-cold RIPA lysis buffer, centrifuged, and protein concentration of the supernatant was determined by Bradford assay. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked with 5% skim milk (v/v), and incubated with primary antibodies against pol ι (Abcam, Cambridge, UK) and β-actin (Genetex, Irvine, CA) followed by horseradish peroxidase-conjugated secondary antibody (Genetex). The protein bands were detected with the Bio-Rad Clarity Western ECL substrate according to the supplier's instructions.

Cell Viability Assay.

Cells were seeded at 1.0×10^4 cells/well on 96-well plates, cultured overnight, and treated with hydrogen peroxide (H_2O_2) for 24 h or with cisplatin for 48 h. After treatment, cell viability was measured by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Statistical Analysis.

Statistical comparisons were performed by two-tailed Student's t-test, one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, or Pearson's correlation test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Ten *POLI* Variations on DNA Polymerase Activity of Pol ι.

To evaluate the alterations in DNA polymerase activity of ten pol ι variants, we performed "standing-start" primer extensions with wild-type pol ι (1-445) and variants using 24-mer/36-mer duplexes containing a G or 8-oxoG at template position 25 with all four dNTPs and 0.15 mM $MnCl_2$ as described.[18] To effectively observe the alterations of polymerase activity in pol ι variants, we utilized the optimum metal ion condition (i.e. 0.15 mM Mn^{2+}), which achieves more ideal octahedral coordination geometry and yields much more optimal polymerase efficiency (about three orders of magnitude higher) than Mg^{2+} (2 mM),[8] in polymerase assays. The R209Q, K228I, R385C, and Q386R variants generated extension products opposite G and 8-oxoG to a considerably less extent than wild-type, while the D285G, P390R, and V433M variants yielded somewhat more products (Figure 2). These results are consistent with the steady-state kinetic data for single nucleotide insertion opposite G and 8-oxoG (Table 2). The R209Q, K228I, and Q386R variants showed 4- to 53-fold reductions in k_{cat}/K_m values (specificity constant, a measure of efficiency) for dCTP

insertion compared to wild-type, while the D285G, P390R, and V433M variants showed 2- to 4-fold increases in those values. The misinsertion frequencies (a measure of fidelity) of ten variants with dTTP and dGTP, respectively opposite G and 8-oxoG, did not differ much from those of the wild-type.

Effects of Ten *POLI* Variations on dRP Lyase Activity of Pol ι.

To evaluate the alterations in dRP lyase activity of ten pol ι variants, we measured the dRP lyase activities of wild-type pol ι (1-445) and variants utilizing 5′-dRP-containing DNA substrates (Figure 3a and b). A graph comparing quantitated dRP lyase activities of wild-type and ten variants is presented (Figure 3c). Interestingly, the R126C and K345E variants—having wild-type-like polymerase activity (Figure 2 and Table 2)—as well as the hypoactive R209Q, K228I, and Q386R variants, all showed major (> 6-fold) reductions in dRP lyase activity compared to wild-type. The K228I variant almost abolished dRP lyase activity (230-fold reduction), while the short form wild-type (Δ1-25), D285G, and P390R variants showed 2- to 3-fold increases compared to the wild-type (long form).

Effects of Seven *POLI* Variations on DNA Substrate Binding of Pol ι.

In order to evaluate the changes in DNA binding affinities of ten pol ι variants, we performed fluorescence polarization experiments. The K228I variant had a $K_{d,DNA}$ value 4-fold higher than wild-type, while the D285G variant had a 3-fold lower $K_{d,DNA}$ value (Table 3), indicating a considerable decrease or increase in the DNA binding affinity of pol ι, respectively, by K228I and D285G substitution, which might partly explain their alterations in the catalytic activity. It was also observed that the R126C variant showed an about 2-fold increase in the $K_{d,DNA}$ of pol ι, indicating a slight decrease in the DNA binding affinity of pol ι by R126C substitution. However, the other seven variants had similar $K_{d,DNA}$ values to the wild-type, indicating that those variations did not substantially change the DNA binding affinity of pol ι.

Abilities of Ten Pol ι Variants to Rescue Sensitivity of *POLI*-KO Cells to H_2O_2.

We developed and performed *POLI*-KO cell-based assays to evaluate the ability of each pol ι variant to rescue the H_2O_2-sensitive phenotype in *POLI*-KO cells. First, the *POLI*-KO HEK293 cell line was generated using a CRISPR/Cas9 system and verified by DNA sequencing and immunoblotting (Figure 4a and b). Second, we confirmed the distinctive phenotype of *POLI*-KO cells, i.e., the increased sensitivity to H_2O_2, compared to wild-type cells by CCK8 cell viability assays, as similarly reported previously with *POLI*-knockdown MRC5 cells,[10] which was contrasted with no increased cisplatin sensitivity (Figure 4c). Third, to validate this complementation assay we confirmed that the exogenous introduction of wild type (a long form) pol ι can fully rescue the H_2O_2 sensitivity to the wild-type cell level in *POLI*-KO cells (Figure 4d). This rescue capability was also shown with a short form (Δ1-25) of wild-type pol ι, which appears to be an abundant form in cells (Fig 4d, right panel) and has a similar polymerase efficiency to a long form under optimal conditions,[8, 17] but not with the catalytically defective R96G variant (Figure 4d).[8] These features were also clearly demonstrated by comparisons of their relative IC_{50} H_2O_2 values (Figure 4e). The IC_{50} indexes have been shown to be useful in evalutating chemical genotoxicity in cell sensitivity-based assays with human *POLK* KO cells[26] and *Polh*/*i*/*k* triple-KO mouse

embryonic fibroblasts.[27] Exogenous expression of a long form or a short form (Δ1-25) of wild-type pol ι, but not that of the R96G variant, significantly improved IC_{50} H_2O_2 values to the wild-type cell level in *POLI*-KO HEK293 cells (Figure 4e), where the levels of those expressed pol ι enzymes were similar to the endogenous level in the wild-type HEK293 cells (Figure 4d, right panel). Lastly, we applied this complementation assay to evaluate the abilities of each of the ten variants to rescue the H_2O_2-sensitive phenotype in *POLI*-KO cells (Figure 4f–g, and Figure S2, Supporting Information). The R209Q, K228I, and Q386R variants (impaired in polymerase activity) could not rescue the cells, with no significant improvements in IC_{50} H_2O_2 values. In contrast, six variants with wild-type-like or slightly increased polymerase activity (including two R126C and K345E variants severely impaired in dRP lyase activity) fully rescued the cells, with significant improvements in IC_{50} H_2O_2 values to the wild-type cell level, indicating the relative importance of polymerase activity. The R385C variant, having slightly reduced (2- to 3-fold) catalytic activity (Table 2 and Figure 3), partially rescued the cells with a partial (47%) improvement in IC_{50} H_2O_2. Under our experimental conditions, the levels of pol ι expression with vectors encoding wild-type pol ι and all studied variants in transfected *POLI*-KO HEK293 cells were similar to or only slightly (≤1.6-fold) different from the endogenous level of pol ι expression in wild-type HEK293 cells (Figure 4f, upper panel), and thus we conclude that these slight variations do not compromise our interpretations of the results.

Abilities of D59A and K85G Pol ι Mutants to Rescue Sensitivity of *POLI*-KO Cells to H_2O_2.

We performed *POLI*-KO cell-based rescue assays with two different types of pol ι mutants —a dRP lyase-specific defective mutant (K85G)[24] and a polymerase-specific defective mutant (D59A)[28]—to evaluate whether polymerase activity or dRP lyase activity would be required to rescue the H_2O_2-sensitive phenotype in *POLI*-KO cells. As shown in cell viability curves as well as IC_{50} values in Figure 5, the K85G mutant fully rescued H_2O_2 sensitivity in *POLI*-KO cells, but the D59A mutant failed to rescue the H_2O_2-sensitivite phenotype, indicating that polymerase activity of pol ι rather than its dRP lyase activity might be required for H_2O_2 reistance in cells.

DISCUSSION

In this study we systemically evaluated the functional characteristics of ten germline missense variants of human pol ι utilizing both biochemical and cell-based functional assays. We developed a cell-based complementation assay based on the ability of *POLI* gene to rescue H_2O_2 sensitivity in *POLI*-KO cells and used this to define whether and how much each germline variant can restore the H_2O_2 sensitivity. Our results clearly indicate that ectopic expression of the R209Q, K228I, and Q386R variants (impaired in both polymerase and dRP lyase activities) failed to rescue H_2O_2 sensitivity in *POLI*-KO cells, while the R126C and K345E variants (impaired in dRP lyase activity but possessing wild-type-like polymerase activity) could fully rescue the cells from H_2O_2 sensitivity, implying that intact polymerase activity might be sufficient for pol ι to protect cells from oxidative stress.

We further evaluated whether the polymerase and/or dRP lyase activity of pol ι are associated with cellular ability to restore H_2O_2 sensitivity in *POLI*-KO cells by analyzing

our results with two forms of wild-type pol ι and eleven variants, using Pearson's correlation test. A significant correlation was observed only between the relative IC_{50} H_2O_2 values and the relative polymerase efficiencies opposite G ($r = 0.57$, $p = 0.04$) or 8-oxoG ($r = 0.71$, $p = 0.007$) but was not observed between the relative IC_{50} H_2O_2 values and the relative dRP lyase activities ($r = 0.52$, $p = 0.07$) (Figure S3, Supporting Information), indicating that the cellular ability of the pol ι variant to rescue the H_2O_2-sensitive phenotype of *POLI*-KO cells relates largely to polymerase activity of the complemented pol ι variant rather than to its dRP lyase activity. Moreover, our observation that only the dRP lyase-defective K85G mutant but not the polymerase-defective D59A mutant could rescue the H_2O_2-sensitive phenotype in *POLI*-KO cells (Figure 5) further suggest that polymerase activity of pol ι rather than its dRP lyase activity might be needed for protecting certain cells from oxidative stress. Our view is somewhat different from a previous conclusion that put an emphasis on the BER-related dRP lyase function of pol ι for cellular protection from oxidative stress.[10] However, our findings may be supported by the inherent enzymatic property of pol ι to perform fairly efficient TLS opposite oxidative DNA lesions such as 8-oxoG, 5-hydroxycytosine, 5-hydroxyuracil, and abasic sites.[1, 5, 18, 29] It might also be plausible that pol ι participates in BER of oxidative DNA damage through its robust polymerase activity at a one-nucleotide gap.[30] Pol ι is recruited to sites of oxidized chromatin in association with XRCC1 following H_2O_2 treatment, reflecting a certain BER role against oxidative stress.[10] In the same line, we also found that ectopically expressed wild-type pol ι proteins were increasingly bound to chromatin in *POLI*-KO cells after H_2O_2 treatment, and these properties were not interfered with four dysfunctional R209Q, K228I, R385C, and Q386R variants (Figure S4, Supporting Information). Together, these results suggest that the polymerase function of pol ι is one of the significant determinants of cellular sensitivity to oxidative genotoxic agent in human cells.

To our knowledge, this is the first study analyzing functional alterations of human germline pol ι variants in both biochemical and cellular aspects, using *in vitro* polymerase, dRP lyase, DNA binding assays, and *POLI*-KO cell-based complementation assays. The ten pol ι variants analyzed in this work can be classified into three groups based on their rescue abilities (Figure 4). The first type is the defective variants (R209Q, K228I, and Q386R), which failed to rescue cells and were severely impaired in polymerase/dRP-lyase activity. K228I caused very severe catalytic defects with reduced DNA binding (Tables 2 and 3, Figure 3), comparable to the defective R96G variant identified earlier[18] and indicating a crucial role of Lys-228 in the palm domain for DNA binding and catalytic activities. These results agree with the earlier structural inference that Lys-228 likely affects DNA binding affinity and overall polymerase structure.[31] The second type is the partially defective variant (R385C), which only partially rescued cells and was slightly impaired in polymerase/dRP-lyase activity (Table 2 and Figure 3). The last type is the functionally retained variants (R126C, D285G, D314G, K345E, P390R, and V433M), which fully rescued cells and possessed wild-type-like or slightly increased polymerase activity but with or without impaired dRP activity (Table 2 and Figure 3). It is noticeable that R126C and K345E substitutions compromise only dRP lyase activity without interference with polymerase activity. A recent study suggests a role of the N-terminal region involving Gln-84 and Lys-85 residues in the dRP lyase activity of pol ι, possibly by stabilizing

protein/DNA conformations required for cleavage of dRP group.[24] We note that Arg-126 and Lys-345 residues are located not very far from Gln-84 and Lys-85 in the pol ι catalytic core structure,[8] implying some potential dRP lyase-specific roles of these residues. The substitutions that remove a positive charge with/without adding a negative charge at the Arg-126 or Lys-345 position might perhaps destabilize protein/DNA conformations and interfere with dRP cleavage reaction. It is relevant to mention in this context that that R126C substitution causes a slight reduction in the DNA binding affinity (Table 3), which might somewhat destabilize protein/DNA interaction for dRP lyase reaction. Interestingly, an "over-rescue" phenotype (exceeding the wild-type state) was not observed with exogenous expression of three slightly (2- to 4-fold) hyperactive variants, D285G, P390R, and V433M (Figure 4), indicating that the protective effect of pol ι is probably saturated at endogenous pol ι levels in cells. In the same line, we also found that 3-fold overexpression of pol ι was unable to decrease the H_2O_2 sensitivity in wild-type HEK293 cells (data not shown). Although all ten studied variants were predicted to be deleterious by both SIFT and PolyPhen algorithms, only four variants were at least partially dysfunctional, indicating the low concordance between the actual variant effects and the *in silico* predicted effects, as also previously revealed with three human Y-family DNA polymerases,[18, 21, 23] which further necessitates functional validation assays.

In conclusion, our results suggest that three human missense germline *POLI* variations may impair the catalytic activity of pol ι opposite G, 8-oxoG, and 5′dRP and may deprive pol ι of its cellular ability to protect from oxidative stress, which in turn may be implicated as predisposing factors for oxidative stress susceptibility in the genetically affected individuals. Our findings also suggest a significant role of the TLS-related polymerase function of pol ι rather than its BER-related dRP lyase function in protection from oxidative stress in cells. However, we cannot rule out a role of dRP lyase activity of pol ι for protection from certain oxidative stress in certain tissues or cell types, in that the actual role of pol ι would be dependent on the condition of oxidative stress, and the expressed levels of pol ι and other translesion polymerases, which are different in various types of tissues. Our *in vitro* knockout cell-based functional approaches may enable high-throughput screening for novel functional variants, and the combination with biochemical approaches can provide detailed insight into sequence-function relationships and their translational possibilities as genetic markers determining the susceptibility of an individual to specific genotoxic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

dRP	deoxyribose phosphate

EV	empty vector
IC_{50}	concentration that induces 50% inhibition of cell viability
KO	knockout
8-oxoG	8-oxo-7,8-dihydroG
pol	DNA polymerase
TLS	translesion synthesis
WT	wild-type

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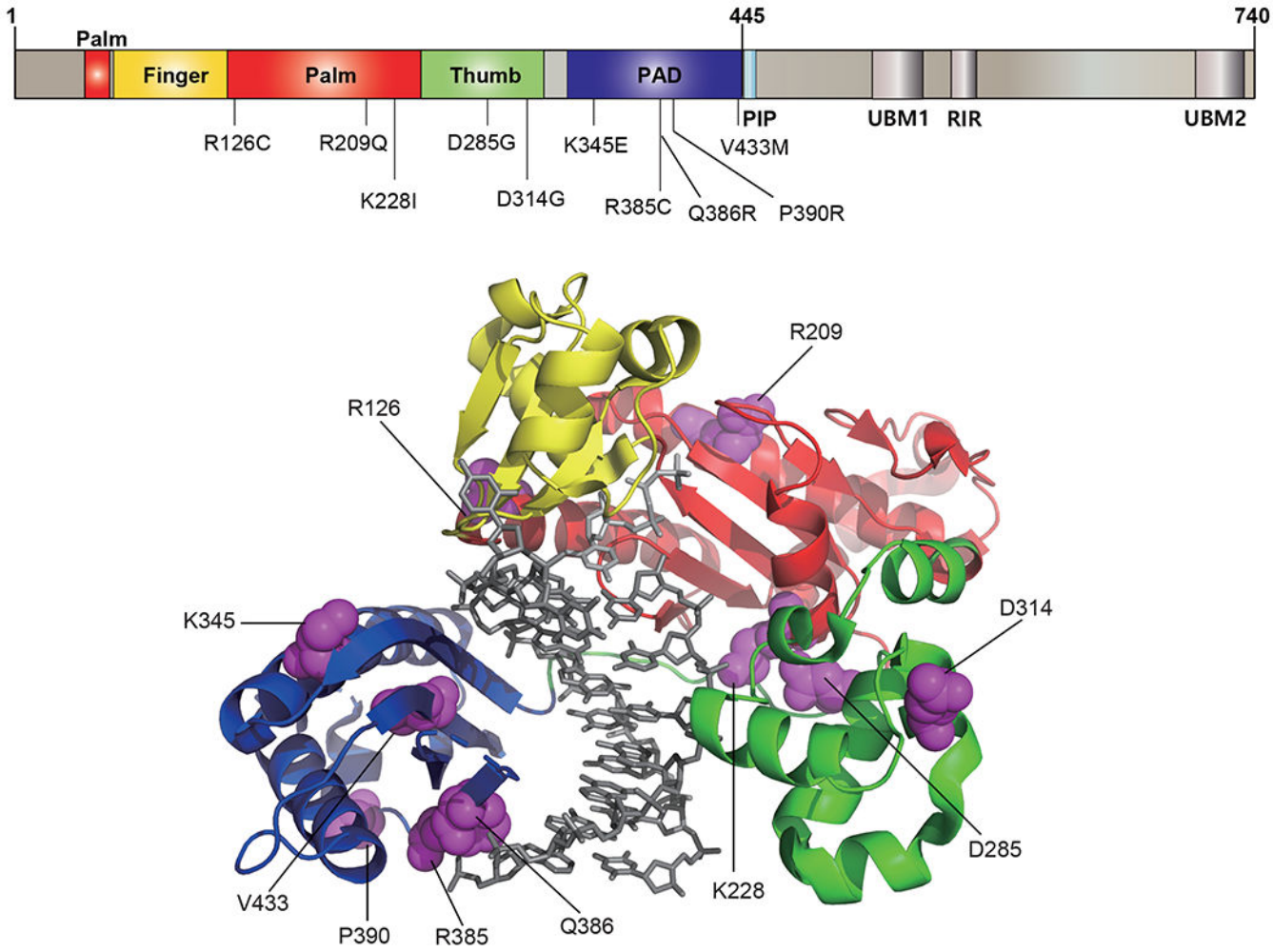

Figure 1. Locations of *POLI* genetic variations.

The structure of human pol ι (PDB code, 5KT3) bound to primer/template DNA and incoming dCTP in the presence of Mn^{2+} is shown using PyMOL (http://www.pymol.org). Pol ι (26-445) and primer/template/dCTP are shown in cartoon ribbons and gray sticks, respectively. The fingers, palm, thumb, and PAD domains are colored yellow, red, green, and blue, respectively. The ten variant residues are indicated in the upper schematic diagram of pol ι domains and are shown as purple spheres in the structure.

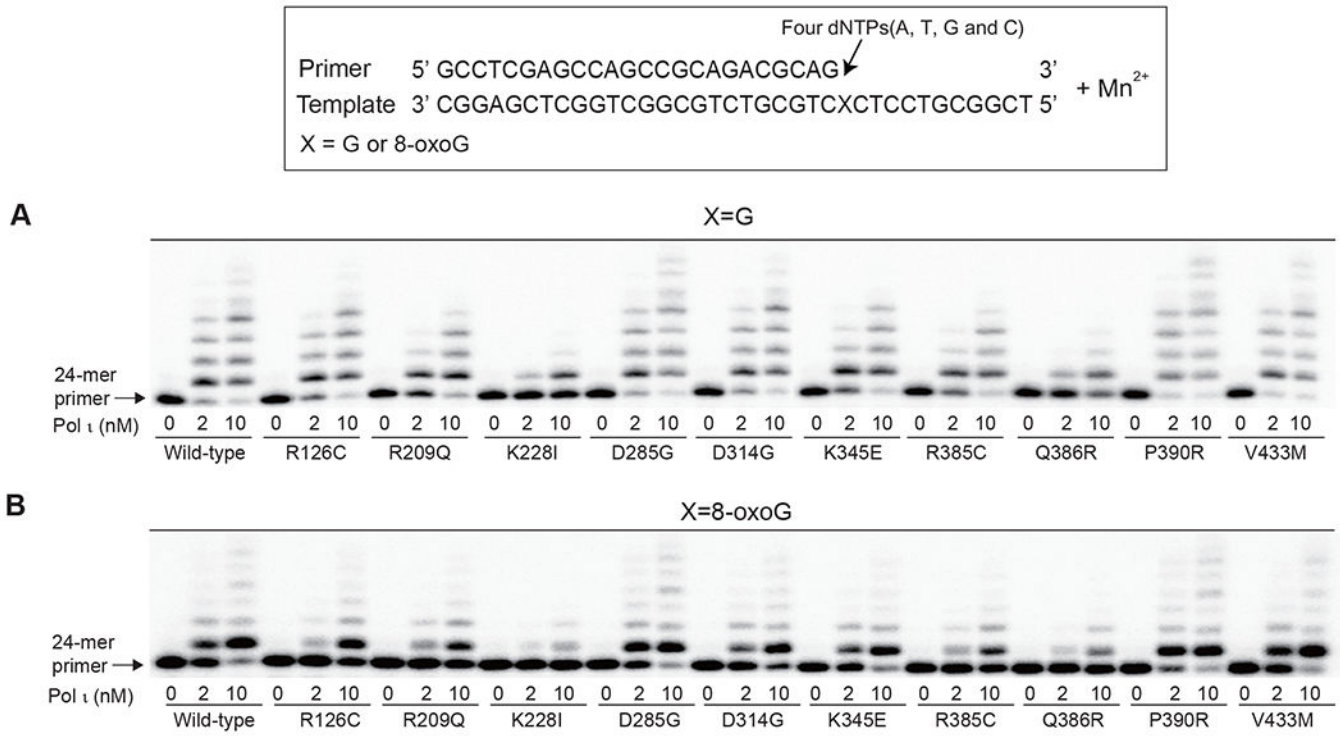

Figure 2. Extension of primers opposite G and 8-oxoG by human pol ι (1-445) wild-type and variants.

Reactions were done in the presence of 0.15 mM $MnCl_2$ for 15 min with 100 nM 24-mer/36-G (or 8-oxoG)-mer DNA substrate, all four dNTPs (50 μM each), and the indicated concentrations of pol ι. The reaction products were analyzed by denaturing gel electrophoresis and phosphorimaging. (a) Extension opposite G. (b) Extension opposite 8-oxoG.

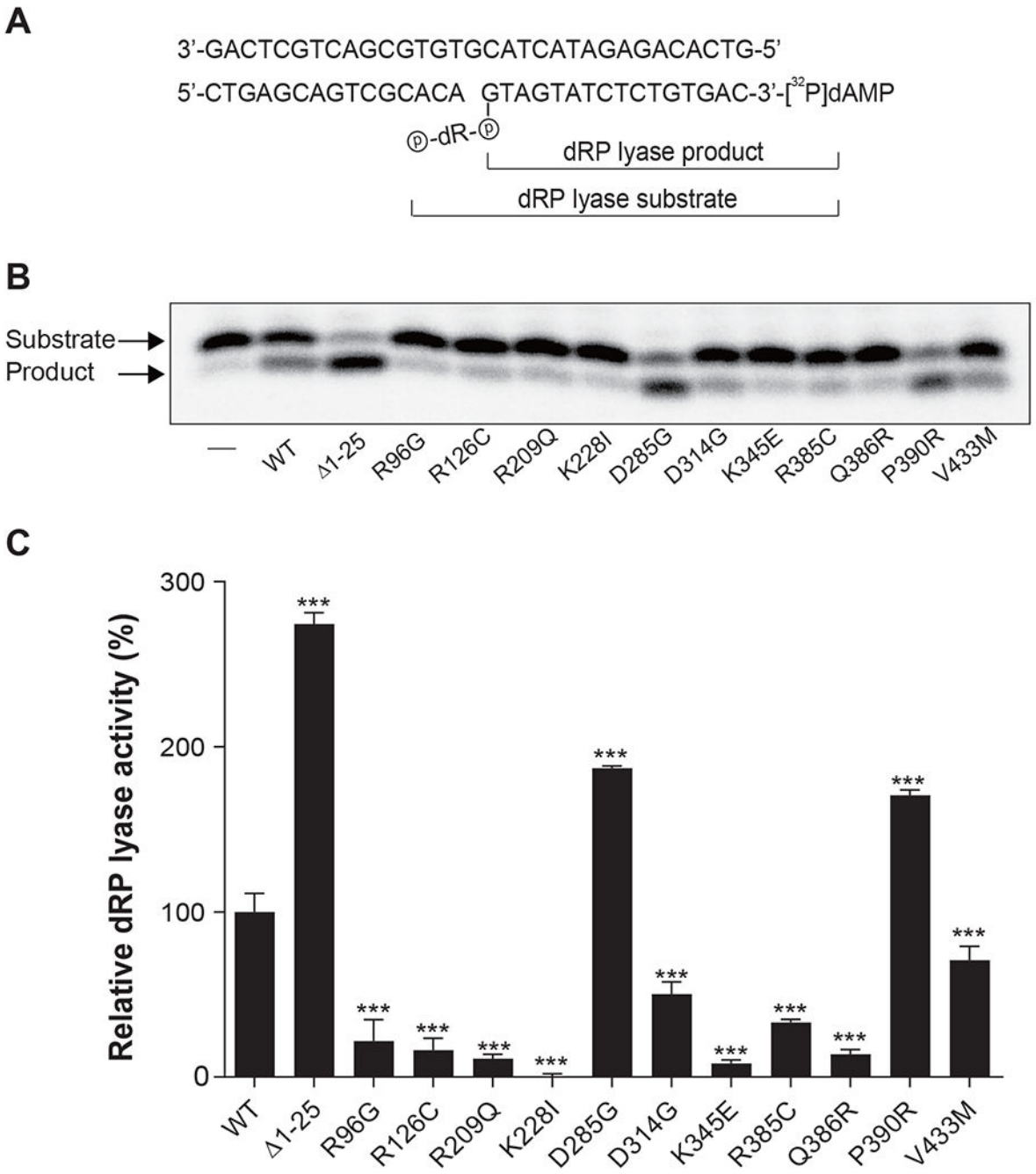

Figure 3. dRP lyase activities of human pol ι (1-445) wild-type and variants.

(a) The single-nucleotide gapped DNA substrate with a 5′-dRP moiety and the expected product. (b) A representative phosphorimage of a gel displaying the dRP lyase activities of pol ι (1-445) wild-type and variants. The positions of the substrate and the product are indicated. The (–) lane indicates no enzyme addition. (c) The dRP lyase activities of pol ι (1-445) variants relative to that of wild-type (set to 100%). The results are shown as means ± SEM (n = 3). ***$P < 0.001$ vs. wild-type pol ι (Student's *t*-test).

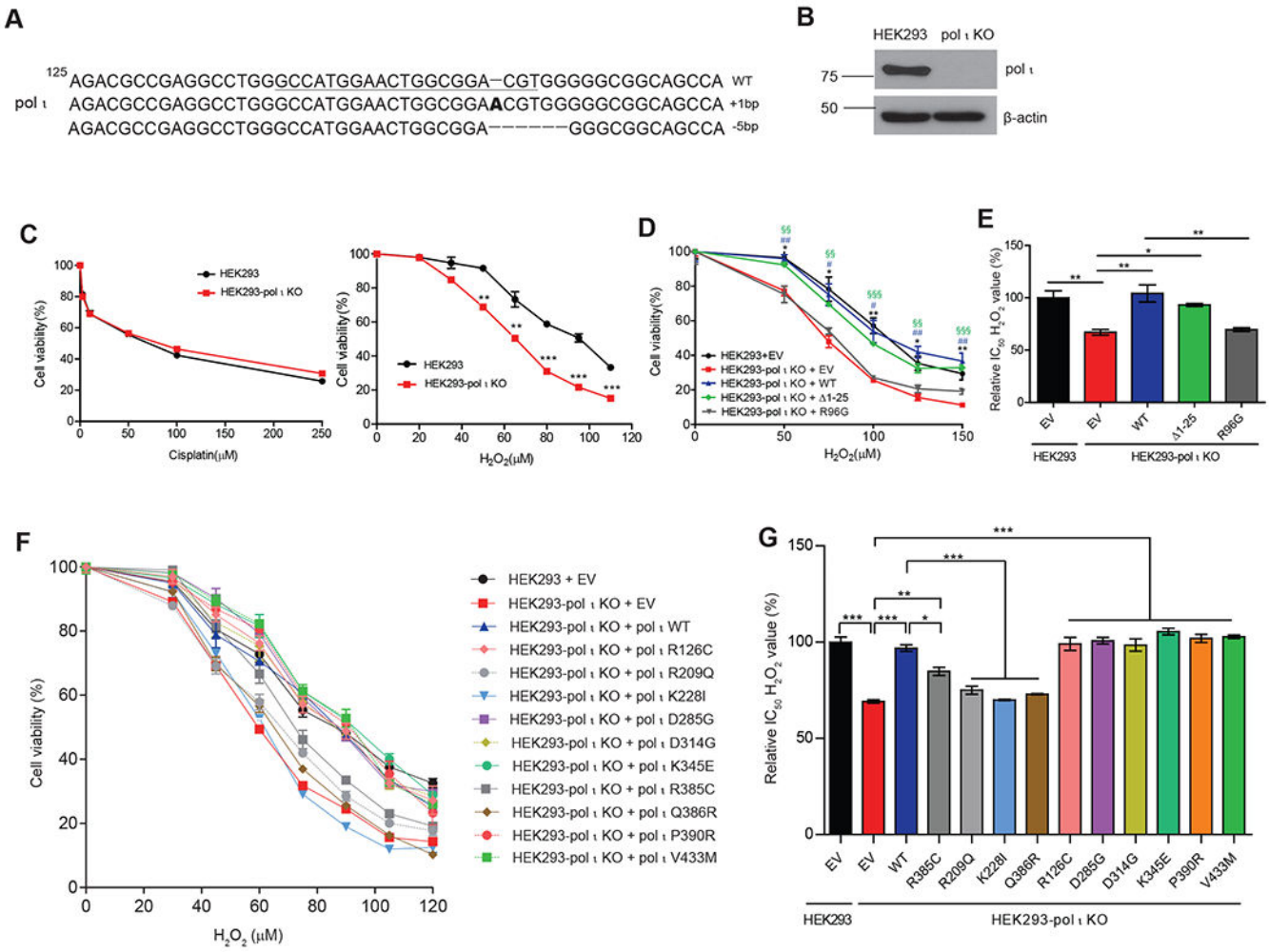

Figure 4. Effects of *POLI* genetic variations on H_2O_2 sensitivity in *POLI*-knockout cells.

(a) Genomic DNA sequences of the CRISPR/Cas9-mediated *POLI*-KO HEK293 cell line. The 20-bp target sequence is underlined. Two mutant alleles with a 1-bp addition and a 5-bp deletion, both of which result in frameshifts at codon 30, were verified by DNA sequencing of genomic PCR amplicons from *POLI*-KO cells. Nucleotide numbers are relative to the transcription start site. (b) Immunoblots confirming the lack of pol ι expression in *POLI*-KO cells. (c) Increased sensitivity of *POLI*-KO cells to H_2O_2 but not to cisplatin. Wild-type and *POLI*-KO cells were exposed to H_2O_2 or cisplatin at the indicated concentrations, after which cell viability was determined. Results are shown as means ± SEM from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. wild-type cells (Student's *t*-test). (d) H_2O_2 sensitivity of *POLI*-KO cells rescued by complementation with either a long or short form of wild-type pol ι but not by the R96G variant. Cells were transfected with vectors encoding full-length pol ι— a long (WT) or short (Δ1-25) wild-type or R96G variant—were incubated for 48 h before H_2O_2 treatment. Results are shown as means ± SEM from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, between empty vector (EV)-transfected *POLI*-KO and wild-type cells; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, between EV- and WT-transfected *POLI*-KO cells; $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$, between EV- and Δ1-25-transfected *POLI*-KO

cells (ANOVA with Tukey's test). On the right panel, Western blots of cell lysates (20 μg) from wild-type and *POLI*-KO cells containing the indicated vectors are shown. #, Numbers below the lanes indicate densitometry results for the pol ι/β-actin ratio normalized to that (set to 1) in EV-transfected wild-type HEK293 cells. (e) Relative IC_{50} H_2O_2 values for wild-type cells and *POLI*-KO cells expressing a long (WT) or short (Δ1-25) wild-type or R96G pol ι. IC_{50} values calculated (Figure 4d) were normalized to wild-type cells. Results are presented as means ± SEM from three independent experiments. ***$P < 0.001$ (ANOVA with Tukey's test). (f) Abilities of ten pol ι variants to rescue H_2O_2 sensitivity in *POLI*-KO cells. Wild-type cells and *POLI*-KO cells transfected with indicated vectors were assessed for H_2O_2 sensitivity. The statistical significance of individual data points for each variant is shown in Figure S2 (Supporting Information). On the upper panel, Western blots of cell lysates (20 μg) from wild-type and *POLI*-KO cells containing the indicated vectors are shown. #, Numbers below the lanes represent densitometry results for the pol ι/β-actin ratio. (g) Relative IC_{50} H_2O_2 values for wild-type cells and *POLI*-KO cells expressing different pol ι variants. IC_{50} values calculated from Figure 4f were normalized to wild-type cells. Results are shown as means ± SEM from three independent experiments. ***$P < 0.001$ (ANOVA with Tukey's test).

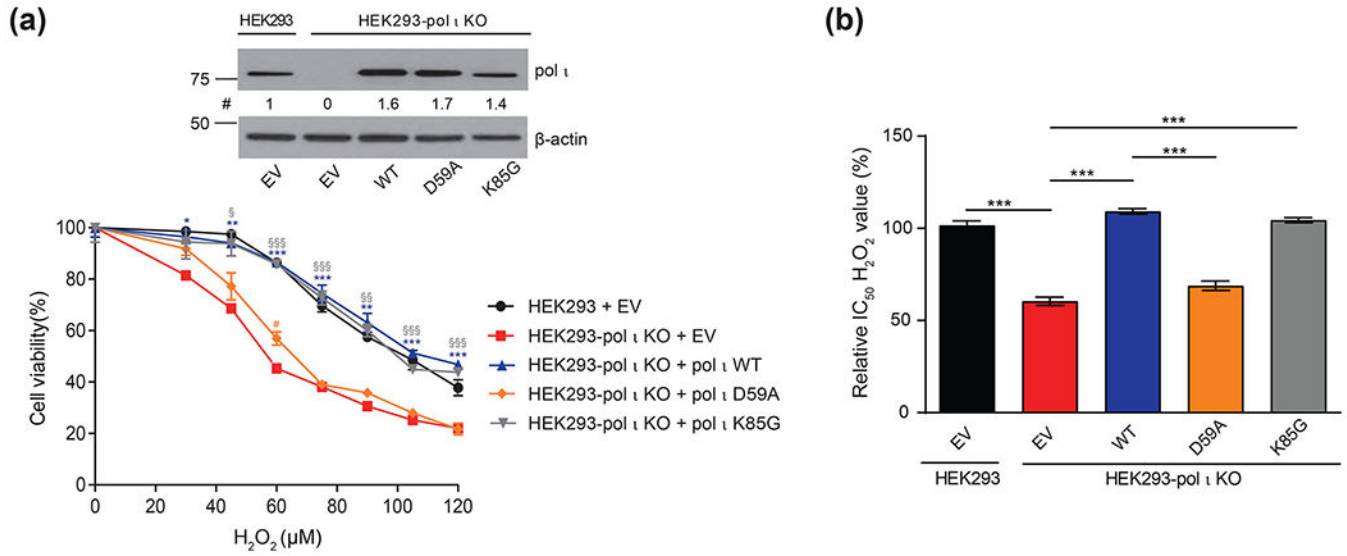

Figure 5. Effects of D59A and K85G *POLI* mutants on H_2O_2 sensitivity in *POLI*-knockout cells.

(a) H_2O_2 sensitivity of *POLI*-KO cells rescued by complementation only with K85G pol ι mutant but not with the D59A mutant. Results are shown as means ± SEM from three independent experiments. $^{**}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, between empty vector (EV)- and WT-transfected *POLI*-KO cells; $^{\#}P < 0.05$ between EV- and D59A-transfected *POLI*-KO cells; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$, between EV- and K85G-transfected *POLI*-KO cells (ANOVA with Tukey's test). On the upper panel, Western blots of cell lysates (20 μg) from wild-type and *POLI*-KO cells containing the indicated vectors are shown. #, Numbers below the lanes represent densitometry results for the pol ι/β-actin ratio. (b) Relative IC_{50} H_2O_2 values for wild-type cells and *POLI*-KO cells containing indicated vectors. IC_{50} values calculated (Figure 5a) were normalized to wild-type cells. Results are presented as means ± SEM from three independent experiments. $^{***}P < 0.001$ (ANOVA with Tukey's test).

Table 1.

Human *POLI* gene variations studied

rs ID[a]	Nucleotide change	Amino acid change	Protein domain	Minor allele frequency	Prediction[b]	
					SIFT	PolyPhen-2
rs369815747	c.376C>T	R126C	Palm	0.0001[c]	deleterious	probably damaging
rs370713519	c.626G>A	R209Q	Palm	0.0001[c]	deleterious	probably damaging
rs200234897	c.683A>T	K228I	Palm	0.0002[d]	deleterious	probably damaging
rs200692792	c.854A>G	D285G	Thumb	0.0002[d]	deleterious	probably damaging
rs199780326	c.941A>G	D314G	Thumb	0.0002[d]	deleterious	probably damaging
rs371749193	c.1033A>G	K345E	PAD	0.0001[c]	deleterious	possibly damaging
rs371876172	c.1153C>T	R385C	PAD	0.0003[c]	deleterious	probably damaging
rs376491192	c.1157A>G	Q386R	PAD	0.0003[c]	deleterious	probably damaging
rs200852409	c.1169C>G	P390R	PAD	0.0002[d]	deleterious	probably damaging
rs372676343	c.1297G>A	V433M	PAD	0.0001[c]	deleterious	probably damaging

[a] Reference SNP identification number provided by dbSNP.

[b] Possible functional effects of missense variations are predicted *in silico* using SIFT and PolyPhen-2.

[c] From the NHLBI ESP6500 project.

[d] From 1000 Genomes project.

Table 2.

Steady-state kinetic parameters for dNTP incorporation opposite G and 8-oxoG by human wild-type pol ι (1-445) and variants

Template base	pol ι (1-445)	dNTP	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} $μM^{-1}$)	f_{ins} [a]	Relative efficiency[b]
G	wild-type	C	0.89 ± 0.13	0.069 ± 0.003	0.078	1	1
		T	0.88 ± 0.26	0.050 ± 0.004	0.057	0.73	
	R126C	C	4.8 ± 0.4	0.29 ± 0.01	0.060	1	0.77
		T	1.7 ± 0.3	0.013 ± 0.001	0.0076	0.13	
	R209Q	C	5.0 ± 0.6	0.059 ± 0.002	0.012	1	0.15
		T	12 ± 2	0.038 ± 0.002	0.0032	0.27	
	K228I	C	19 ± 3	0.028 ± 0.001	0.0015	1	0.019
		T	10 ± 3	0.022 ± 0.002	0.0022	1.5	
	D285G	C	0.22 ± 0.06	0.048 ± 0.003	0.22	1	2.8
		T	0.69 ± 0.05	0.077 ± 0.002	0.11	0.31	
	D314G	C	2.0 ± 0.3	0.091 ± 0.004	0.046	1	0.59
		T	3.1 ± 0.9	0.12 ± 0.01	0.039	0.85	
	K345E	C	0.94 ± 0.10	0.069 ± 0.002	0.073	1	0.94
		T	1.1 ± 0.1	0.025 ± 0.001	0.023	0.32	
	R385C	C	1.9 ± 0.4	0.057± 0.003	0.030	1	0.38
		T	2.8 ± 0.4	0.022 ± 0.001	0.0079	0.26	
	Q386R	C	1.7 ± 0.2	0.017 ± 0.001	0.010	1	0.13
		T	2.2 ± 0.2	0.027 ± 0.001	0.012	1.2	
	P390R	C	0.65 ± 0.13	0.18 ± 0.01	0.28	1	3.6
		T	1.3 ± 0.3	0.24 ± 0.01	0.18	0.64	
	V433M	C	1.0 ± 0.1	0.20 ± 0.01	0.20	1	2.6
		T	1.4 ± 0.1	0.093 ± 0.002	0.066	0.33	
8-oxoG	wild-type	C	2.9 ± 0.4	0.083 ± 0.003	0.029	0.38	1
		G	0.74 ± 0.06	0.057 ± 0.001	0.077	1	
	R126C	C	3.5 ± 0.5	0.078 ± 0.004	0.022	0.44	0.76
		G	0.86 ± 0.16	0.043 ± 0.002	0.050	1	
	R209Q	C	9.6 ± 3.1	0.061 ± 0.006	0.0064	0.58	0.22
		G	1.5 ± 0.4	0.017 ± 0.001	0.011	1	
	K228I	C	5.0 ± 0.8	0.019 ± 0.001	0.0038	1.0	0.13
		G	1.9 ± 0.3	0.0071 ± 0.0003	0.0037	1	
	D285G	C	1.7 ± 0.3	0.10 ± 0.01	0.059	0.26	2.0
		G	0.35 ± 0.14	0.080 ± 0.008	0.23	1	
	D314G	C	3.9 ± 0.9	0.092 ± 0.006	0.024	0.4	0.83
		G	0.84 ± 0.15	0.050 ± 0.002	0.060	1	
	K345E	C	2.2 ± 0.3	0.069 ± 0.002	0.031	0.66	1
		G	0.43 ± 0.15	0.020 ± 0.002	0.047	1	
	R385C	C	2.4 ± 0.5	0.031 ± 0.001	0.013	0.33	0.45

Template base	pol ι (1-445)	dNTP	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} $μM^{-1}$)	f_{ins} [a]	Relative efficiency[b]
		G	0.74 ± 0.13	0.029 ± 0.001	0.039	1	
	Q386R	C	4.1 ± 0.7	0.032 ± 0.001	0.0078	0.33	0.27
		G	1.2 ± 0.1	0.029 ± 0.001	0.024	1	
	P390R	C	3.8 ± 0.2	0.21 ± 0.002	0.055	0.28	1.9
		G	0.38 ± 0.09	0.077 ± 0.004	0.20	1	
	V433M	C	2.0 ± 0.8	0.14 ± 0.01	0.070	0.29	2.4
		G	0.13 ± 0.07	0.54 ± 0.004	0.24	1	

[a] Misinsertion frequency, calculated by dividing k_{cat}/K_m for each dNTP insertion by k_{cat}/K_m for dCTP insertion opposite template.

[b] Relative efficiency, calculated by dividing k_{cat}/K_m of each pol ι(1-445) for dCTP insertion opposite template by the k_{cat}/K_m of wild-type ι(1-445).

Table 3.

Equilibrium dissociation constants (K_d) of human wild-type pol ι (1-445) and variants for binding DNA substrate

pol ι (1-445)	K_d (nM)
wild-type	120 ± 20
R126C	200 ± 70
R209Q	99 ± 22
K228I	430 ± 110
D285G	39 ± 4
D314G	80 ± 20
K345E	130 ± 20
R385C	150 ± 50
Q386R	170 ± 30
P390R	120 ± 10
V433M	130 ± 40