Glycogen Synthase Kinase 3 (GSK-3)-mediated Phosphorylation of Uracil N-Glycosylase 2 (UNG2) Facilitates the Repair of Floxuridine-induced DNA Lesions and Promotes Cell Survival*

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Uracil N-glycosylase 2 (UNG2), the nuclear isoform of UNG, catalyzes the removal of uracil or 5-fluorouracil lesions that accumulate in DNA following treatment with the anticancer agents 5-fluorouracil and 5-fluorodeoxyuridine (floxuridine), a 5-fluorouracil metabolite. By repairing these DNA lesions before they can cause cell death, UNG2 promotes cancer cell survival and is therefore critically involved in tumor resistance to these agents. However, the mechanisms by which UNG2 is regulated remain unclear. Several phosphorylation sites within the N-terminal regulatory domain of UNG2 have been identified, although the effects of these modifications on UNG2 function have not been fully explored, nor have the identities of the kinases involved been determined. Here we show that glycogen synthase kinase 3 (GSK-3) interacts with and phosphorylates UNG2 at Thr60 and that Thr60 phosphorylation requires a Ser64 priming phosphorylation event. We also show that mutating Thr60 or Ser64 to Ala increases the half-life of UNG2, reduces the rate of in vitro uracil excision, and slows UNG2 dissociation from chromatin after DNA replication. Using an UNG2-deficient ovarian cancer cell line that is hypersensitive to floxuridine, we show that GSK-3 phosphorylation facilitates UNG2-dependent repair of floxuridine-induced DNA lesions and promotes tumor cell survival following exposure to this agent. These data suggest that GSK-3 regulates UNG2 and promotes DNA damage repair.

Uracil N-glycosylase (UNG)2 is a member of the uracil DNA glycosylase family of enzymes, which initiate base excision repair (BER) of uracil that results from deamination of cytosine or the misincorporation of uracil or other thymidine analogs during DNA replication (1–4). Importantly, UNG is the main glycosylase that removes uracil and 5-fluorouracil lesions that accumulate in DNA following treatment of cancers with the closely related fluoropyrimidine anticancer agents 5-fluorouracil and 5-fluorodeoxyuridine (floxuridine) (5–7). Consistent with this observation, we and others have shown previously that UNG protects cells from death induced by agents that cause incorporation of uracil or 5-fluorouracil during DNA replication, likely by removing these lesions before they can cause cell death (4, 6–8).

Mammalian cells express two isoforms of UNG: UNG1, a mitochondrial isoform, and UNG2, a nuclear isoform. Both isoforms have identical C-terminal catalytic domains but differ in the N-terminal regulatory domain because of differential promoter usage and alternative splicing (9). These isoforms are expressed highly during late G1/early S phase of the cell cycle and are reduced in late S phase through proteasome-mediated degradation (10, 11).

UNG initiates BER by detecting and excising the damaged base, leaving an apurinic/apyrimidinic endonuclease (12) to cleave the DNA backbone 5′ to the damaged base. The single-stranded nick is then repaired through the subsequent activity of a DNA polymerase and DNA ligase. Although BER can be reconstituted with five or fewer enzymes in vitro (13–16), additional proteins enhance the efficiency of repair in cells through protein-protein interactions or posttranslational modification of BER enzymes (13). Indeed, UNG2 not only interacts with the core BER machinery via its N-terminal regulatory domain, but it also interacts with replication protein A and proliferating cell nuclear antigen (PCNA), two complexes that help orchestrate DNA replication and repair reactions in the cell (17).

The N-terminal regulatory domain of UNG2 is also phosphorylated on multiple sites that likely regulate the function(s) of the glycosylase (18, 19). Three major phosphorylation sites, Ser23, Thr60, and Ser64, conform to cyclin-dependent kinase consensus sites (Ser-Pro and Thr-Pro) and can be phosphorylated by CDK1 and CDK2 in vitro. Conversion of all three sites to phosphomimicking aspartate residues caused a reduction in replication protein A interaction (18). Additionally, phosphor-
A SFB-UNG

IP: SFB-UNG

IB: S-tag

B GSK-3β-HA

IP: HA

32P-phospho-GSK-3

32P-phospho-UNG2

C S-UNG

IP: S-UNG

IB: S-tag

D SFB-tagged wild-type UNG2

IP: SFB

IB: S-UNG

Input

IB: S-tag

Results

Inhibition of GSK-3 Reduces Phosphorylation of UNG2 at Thr\textsuperscript{60}—UNG2 harbors an N-terminal domain containing several serine and threonine residues shown previously to be phosphorylated in cells, including Thr\textsuperscript{60} and Ser\textsuperscript{64}. Although Ser\textsuperscript{64} conforms to a cyclin-dependent kinase consensus site, and cyclin-dependent kinases have been implicated in UNG2 phosphorylation at Ser\textsuperscript{64}, the kinase that phosphorylates Thr\textsuperscript{60} has not been identified. Interestingly, when we performed a mass spectrometric analysis to identify UNG2 binding partners, we observed that two GSK-3 isoforms, GSK-3α and GSK-3β, co-precipitated with UNG2, thus raising the possibility that GSK-3 phosphorylates UNG2. This association was confirmed by showing that wild-type UNG2, tagged with the SFB triple epitope tag, co-precipitated with endogenous GSK-3α (Fig. 1A). Interestingly, mutation of Thr\textsuperscript{60} or Ser\textsuperscript{64} (the putative GSK-3 phosphorylation site and priming site, respectively) to alanine disrupted GSK-3 association with UNG2.

To determine whether GSK-3 is capable of phosphorylating UNG2 at Thr\textsuperscript{60} in vitro, we immunoprecipitated HA-tagged GSK-3β alone or with HA-tagged wild-type UNG2 or UNG2 T60A (Fig. 1B). In the sample containing only GSK-3β, 32P-labeled autophosphorylated GSK-3β was observed. In the samples in which GSK-3β was co-immunoprecipitated with wild-type and mutant UNG2, we observed 32P-labeled wild-type UNG2 (but not GSK-3β autophosphorylation). In contrast, UNG2 T60A was poorly phosphorylated by GSK-3β, demonstrating that Thr\textsuperscript{60} is a major GSK-3 phosphorylation site in UNG2.

To evaluate UNG2 Thr\textsuperscript{60} phosphorylation in cells, we created expression vectors for UNG2 with an S epitope tag appended, in which the threonine and serine residues in the range of Thr\textsuperscript{60}-Ser\textsuperscript{64} were mutated to alanines (S-UNG2 T60A, UNG2 S63A, and S-UNG2 S64A), as well as a vector with Ser\textsuperscript{64} mutated to aspartate (S-UNG2 S64D) as a potential phosphomimetic mutation. We screened a panel of commercially avail-
able phosphospecific antibodies to identify one that recognized phosphorylated wild-type UNG2 but not the UNG2 T60A mutant. S-tagged wild-type and mutant UNG2s were expressed in K562 cells, pulled down with S protein-agarose, and analyzed by Western blotting. The phosphospecific antibody detected a band corresponding to phosphorylated UNG2 in cells expressing wild-type UNG2 and UNG2 S63A but not in cells expressing UNG2 T60A (Fig. 1C). Importantly, no band was detected in UNG2 S64A, suggesting that Thr^{60} phosphorylation depends on priming Ser^{64} phosphorylation. This possibility is further supported by the observation that Thr^{60} phosphorylation is restored by the phosphomimetic mutation that converts Ser^{64} to aspartate (Fig. 1C).

To determine whether the phosphorylation of UNG2 at Thr^{60} was dependent on GSK-3 activity, cells expressing wild-type S-UNG2 were treated with the GSK-3 inhibitor LY2090314 for 90 min prior to lysis, and S-UNG2 was pulled down with S protein-agarose. Treatment of cells with LY2090314 caused complete loss of the Thr^{60} phosphorylation signal from the 5-fluorouracil-substituted fragment was lost (Fig. 1D). Importantly, although wild-type UNG2 was more than 60% degraded within 1 h of cycloheximide treatment, both UNG2 T60A and UNG2 S64A persisted significantly longer (Fig. 3A). Tubulin was included as a loading control. Although wild-type UNG2 was more than 60% degraded within 1 h of cycloheximide treatment, both UNG2 T60A and UNG2 S64A persisted significantly longer (Fig. 3B). These results are consistent with previous reports suggesting that the region surrounding Thr^{60} constitutes a phosphodegron and that Thr^{60} is phosphorylated prior to proteasome-mediated degradation (18).

UNG2 Mutants Exhibit Reduced Enzyme Activity in Uracil-substituted but Not 5-Fluorouracil-substituted DNA—To determine whether UNG2 mutants exhibited altered glycosylase activity in vitro, we developed an assay to assess UNG activity in cell lysates. Using PCR, we generated three small 750-bp DNA fragments: a fragment in which all thymidines were substituted with 5-fluorouracil (synthesized with 5'-fluoro-2'-deUTP plus other dNTPs), a fragment in which all thymidines were substituted with uracil (synthesized with dUTP plus other dNTPs), and a control fragment (synthesized with dTTP plus other dNTPs).

To analyze cleavage of these substrates, we prepared lysates from OVCAR-8ΔUNG cells transfected with empty vector (EV) or stably expressing equal levels of HA-tagged wild-type UNG2 (WT), UNG2 T60A, or UNG2 S64A (Fig. 4A). To demonstrate that 5-fluorouracil substrates were specifically cleaved by UNG2, the 5'-fluorouracil substrate and dTTP control substrate fragments were incubated with or without cell lysates from OVCAR-8ΔUNG cells stably expressing HA-tagged wild-type UNG2 and analyzed by agarose gel electrophoresis. The signal from the 5-fluorouracil-substituted fragment was lost during the incubation, whereas the signal from the control fragment was not affected, demonstrating that 5-fluorouracil lesions are required for substrate degradation (Fig. 4B).

To determine whether the UNG2 phosphomutants have an altered ability to cleave 5-fluorouracil- and uracil-substituted substrates, the substrate fragments were incubated with the cell lysates and analyzed by agarose gel electrophoresis. The DNA substrates were not degraded in EV lysates but were degraded in lysates containing either wild-type or mutant UNG2 (Fig. 4, C and D), demonstrating that UNG2 is required for cleavage of the substrate. Analysis of the UNG2 mutants showed that UNG2 T60A and UNG2 S64A cleaved 5-fluorouracil-substituted DNA as effectively as wild-type UNG2 (Fig. 4C). However, both UNG2 mutants degraded the uracil-substituted DNA substrate at a significantly reduced rate relative to the wild type (Fig. 4D). These results demonstrate that blocking low level of UNG2 relative to unmodified OVCAR-8 parental cells, this amount of UNG2 fully rescued sensitivity to 5-fluorouracil (Fig. 9).
phosphorylation of Thr<sup>60</sup> and Ser<sup>64</sup> does not affect UNG2 activity in cell lysates toward 5-fluorouracil lesions but does reduce the efficiency of uracil lesion repair.

**UNG2 Mutants Exhibit Unaltered Subcellular Localization**—We used two approaches to assess whether UNG2 Thr<sup>60</sup> or Ser<sup>64</sup> phosphorylation affected subcellular localization. In one approach, OVCA-8ΔUNG cells stably expressing HA-tagged wild-type UNG2, UNG2 T60A, or UNG2 S64A were immunostained for the HA tag (Fig. 5A), and in the second approach, EGFP-tagged wild-type UNG2, UNG2 T60A, and UNG2 S64A were expressed in OVCA-8 cells (Fig. 5B). In both cases, the mutant UNG2s localized to the nucleus with the same distribution as wild-type UNG2.

**UNG2 T60A Is Retained on Chromatin Longer Following Replication**—We next examined the interaction of UNG2 with chromatin using isolation of proteins on nascent DNA (iPOND) (37). OVCA-8ΔUNG cells stably expressing HA-tagged wild-type UNG2 or UNG2 T60A were pulsed with EdU...
for 1 h, followed by 1–5 h of thymidine chase prior to cell fixation and lysis. EdU-containing DNA was labeled with biotin azide and pulled down with streptavidin-agarose to enrich for chromatin-associated proteins. Proteins associated with EdU-labeled DNA were subsequently analyzed by Western blotting (Fig. 6A). Samples were also analyzed for chromatin-associated PCNA, a replication fork-associated protein, and histone H3, a persistently chromatin-bound protein, as controls.

After 1 h of EdU pulse, both wild-type UNG2 and T60A UNG2 were associated with newly synthesized DNA at equal levels (Fig. 6A). Wild-type UNG2 was rapidly lost from EdU-labeled tracts of DNA following 1 h of thymidine chase, with ~50% lost in 1 h and 70% lost in 5 h. In contrast, UNG2 T60A remained associated far more persistently, with at least 50% of UNG2 T60A still associated after 5 h of chase (Fig. 6B). These results indicate that GSK-3 regulates UNG2 interaction with chromatin and also suggest that PCNA, which interacts with UNG2, is not required for UNG2 association with chromatin following replication.

UNG2 T60A and S64A Phosphomutants Exhibit Slowed Resolution of Floxuridine-induced DNA Damage—Given that UNG2 T60A and UNG2 S64A removed uracil more slowly from DNA in cell lysates, we next asked whether they lead to increased DNA damage in cells. To address this question, we treated OVCAR-8 cells stably expressing HA-tagged wild-type UNG2 (WT, clone 2a-3), UNG2 T60A (clone A5), or UNG2 S64 (clone 2a-3) with 100 nM floxuridine and stained for Ser(\(\text{P}\))\(^\text{139}\) H2Ax to assess the level of damage induced in each cell line. To assess the progress of DNA repair, cells were incubated for an additional 24 h in medium without floxuridine following the 24-h floxuridine treatment. After 24 h of floxuridine exposure, the wild-type UNG2, UNG2 T60A, and UNG2 S64A cells accumulated similar amounts of phospho-H2Ax (Fig. 7A, center panel); however, after the 24-h washout period, the mutant cells showed far higher levels of unresolved DNA damage compared with cells expressing wild-type UNG2 (Fig. 7A, bottom panel). These results suggest that the UNG2 phosphomutants have a defect in the repair of floxuridine-induced DNA damage. Consistent with these findings, cells treated with both floxuridine and the GSK-3 inhibitor LY2090314 exhibited similar levels of phospho-H2Ax after 24 h (Fig. 7B, center panel) but retained elevated phospho-H2Ax levels 24 h after floxuridine was removed in the continued presence of LY2090314 compared with cells cultured in vehicle only (Fig. 7B, bottom panel).

GSK-3 Phosphorylation of UNG2 Regulates the Cytotoxicity of Floxuridine—Based on the observations that UNG2 T60A and UNG2 S64A have DNA repair defects and that UNG2 deficiency greatly sensitizes OVCAR-8 cells to floxuridine (6), we hypothesized that disrupting GSK-3-mediated phosphorylation of UNG2 would sensitize cells to floxuridine. To assess this possibility, we used two approaches. First, we asked whether disabling GSK-3 affected floxuridine cytotoxicity. Second, we examined how mutating Thr\(^{60}\), the GSK-3 phosphorylation site, and Ser\(^{64}\), the priming phosphorylation site, affected sensitivity to floxuridine.

Cells were treated for 24 h with 100 nM floxuridine and stained for Ser(\(\text{P}\))\(^\text{139}\) H2Ax to assess the level of damage induced in each cell line. To assess the progress of DNA repair, cells were incubated for an additional 24 h in medium without floxuridine following the 24-h floxuridine treatment. After 24 h of floxuridine exposure, the wild-type UNG2, UNG2 T60A, and UNG2 S64A cells accumulated similar amounts of phospho-H2Ax (Fig. 7A, center panel); however, after the 24-h washout period, the mutant cells showed far higher levels of unresolved DNA damage compared with cells expressing wild-type UNG2 (Fig. 7A, bottom panel). These results suggest that the UNG2 phosphomutants have a defect in the repair of floxuridine-induced DNA damage. Consistent with these findings, cells treated with both floxuridine and the GSK-3 inhibitor LY2090314 exhibited similar levels of phospho-H2Ax after 24 h (Fig. 7B, center panel) but retained elevated phospho-H2Ax levels 24 h after floxuridine was removed in the continued presence of LY2090314 compared with cells cultured in vehicle only (Fig. 7B, bottom panel).

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To assess the effect of disabling GSK-3, OVCAR-8 cells were transfected with siRNAs against GSK-3\(_{\text{x}}\), GSK-3\(_{\beta}\), or a combi-
nation and treated with floxuridine for 24 h. Then we assessed proliferation using clonogenic assays. We observed that loss of GSK-3β/H9251 greatly sensitized the cells to floxuridine, loss of GSK-3α/H9252 partially sensitized, and simultaneous knockdown of both isoforms robustly sensitized to floxuridine (Fig. 8A).

Similarly, we evaluated whether pharmacological inhibition of GSK-3 affected floxuridine sensitivity. Consistent with the GSK-3 knockdown studies, the GSK-3 inhibitors LY2090314 and CHIR99021 induced similar levels of sensitivity to floxuridine in OVCAR-8 (Fig. 8B) and HeLa cells (Fig. 8C).

We next evaluated how mutating the UNG2 GSK-3 phosphorylation site Thr60 as well as the Ser64 priming site affected sensitivity to floxuridine. OVCAR-8/UNG cells stably expressing HA-tagged WT UNG2 (clone 2a-3), UNG2 T60A (clone A5), or UNG2 S64A (clone 2a-3) were exposed to 100 nM floxuridine for 24 h, washed, and incubated in drug-free medium for a further 24 h. Cells were stained with an antibody against Ser(P)139-H2Ax and evaluated by flow cytometry. Samples were harvested with no drug treatment (top panel), at the end of the 24 h-floxuridine treatment (center panel), and 24 h after floxuridine removal (bottom panel). Black, wild-type UNG2 cells; red, UNG2 T60A cells; blue, UNG2 S64A cells. B, OVCAR-8 cells were exposed to 100 nM floxuridine in the presence of DMSO (black), 30 nm LY2090314 (red), or 100 nm LY2090314 (blue) for 24 h. Cells were then washed to remove floxuridine and cultured for an additional 24 h in the presence of the indicated concentrations of LY2090314. Samples were harvested with no floxuridine (top panel), at the end of the 24-hour floxuridine plus LY2090314 treatment (center panel), and 24 h after floxuridine removal (bottom panel). The histograms are representative of three independent experiments.

FIGURE 6. UNG2 T60A is retained on DNA longer following DNA replication. A, OVCAR-8/UNG cells expressing HA-tagged WT UNG2 (clone 2a-3) or UNG2 T60A (clone A5) were pulsed with EdU for 1 h, followed by thymidine chase for 1, 3, or 5 h. Proteins were cross-linked to DNA with formaldehyde, and EdU-labeled DNA was pulled down with streptavidin-agarose. Control (Ctrl) samples were prepared with unlabeled DNA. Pulldowns were resolved by SDS-PAGE and analyzed by Western blotting for the HA tag to detect UNG2, histone H3 as a loading control, and PCNA as a replication fork-associated protein. B, pulled down HA-tagged proteins were quantified using ImageJ and normalized against the HA tag on the input blot. Black bars represent the mean ± S.D. of four independent experiments (*, p < 0.05; **, p < 0.10; paired t test).

FIGURE 7. UNG2 phosphomutants exhibit slower resolution of the DNA damage marker following treatment with floxuridine. A, OVCAR-8/UNG cells stably expressing HA-tagged WT UNG2 (clone 2a-3), UNG2 T60A (clone A5), or UNG2 S64A (clone 2a-3) were exposed to 100 nM floxuridine for 24 h, washed, and incubated in drug-free medium for a further 24 h. Cells were stained with an antibody against Ser(P)139-H2Ax and evaluated by flow cytometry. Samples were harvested with no drug treatment (top panel), at the end of the 24 h-floxuridine treatment (center panel), and 24 h after floxuridine removal (bottom panel). Black, wild-type UNG2 cells; red, UNG2 T60A cells; blue, UNG2 S64A cells. B, OVCAR-8 cells were exposed to 100 nM floxuridine in the presence of DMSO (black), 30 nm LY2090314 (red), or 100 nm LY2090314 (blue) for 24 h. Cells were then washed to remove floxuridine and cultured for an additional 24 h in the presence of the indicated concentrations of LY2090314. Samples were harvested with no floxuridine (top panel), at the end of the 24-hour floxuridine plus LY2090314 treatment (center panel), and 24 h after floxuridine removal (bottom panel). The histograms are representative of three independent experiments.
Thr$^{60}$ regulates the ability of UNG2 to repair floxuridine-induced DNA lesions that lead to cell death.

**Discussion**

Previous studies have found that the N-terminal domain of UNG2 is phosphorylated on multiple sites (18, 19). Several of these sites, which are Ser/Thr-Pro cyclin-dependent kinase consensus motifs, were indeed shown to be phosphorylated by cyclin-dependent kinases, including Ser$^{64}$. Notably, however, these studies did not identify the kinase responsible for Thr$^{60}$ phosphorylation, nor did they address the effect of this phosphorylation on UNG2 function. The evidence presented here provides multiple insights into the role of UNG2 and Thr$^{60}$ phosphorylation in ovarian cancer cells treated with floxuridine.

The role of UNG2 in cells treated with agents that cause incorporation of uracil and uracil analogs into DNA remains unclear. Although UNG2 is the major uracil glycosylase responsible for the removal of uracil and 5-fluorouracil from genomic DNA (3–7, 38), multiple studies have found that depletion of UNG2 does not affect the cytotoxicity of 5-fluorouracil, floxuridine, or thymidylate synthase inhibitors, all of which promote uracil incorporation (39–42). In contrast, other studies have shown that UNG deficiency sensitizes cells to thymidylate synthase inhibitors (4, 8), that UNG levels correlated with sensitivity to thymidylate synthase inhibitors (43), and that siRNA-mediated UNG depletion sensitized cells to floxuridine (6), suggesting that UNG-mediated repair removes toxic lesions. Consistent with these later findings, our results, which use UNG knockout cells and UNG2-rescued cells, provide strong genetic evidence that UNG deficiency markedly sensitizes OVCAR-8 cells to floxuridine. These results support a model in which UNG-initiated repair of uracil and/or 5-fluorouracil lesions facilitates the survival of cells exposed to floxuridine. Moreover, our results also clearly demonstrate that UNG2, the nuclear isoform, is sufficient to rescue the floxuridine sensitivity of UNG knockout cells, suggesting that the incorporation of uracil and uracil derivatives into mitochondrial DNA has limited toxicity, at least under the conditions used here.

Although GSK-3 regulates multiple signaling pathways by phosphorylating key pathway intermediates (21, 22), to our knowledge, the kinase has not been implicated previously in directly regulating DNA repair. Here we demonstrate that GSK-3 can directly regulate BER by showing that pharmacological inhibiting the kinase blocks phosphorylation of Thr$^{60}$. Additionally, we show that a priming phosphorylation at Ser$^{64}$, which has been shown to depend on cyclin-dependent kinase activity, is required for phosphorylation of Thr$^{60}$. Finally, we found that depletion GSK-3 isoforms and inhibition of GSK-3 with small molecules sensitizes cells to floxuridine in a manner similar to disabling UNG2. Taken together, these observations suggest that GSK-3 regulates UNG2-initiated BER, which removes toxic lesions and promotes cell survival.

The role of UNG2 phosphorylation in regulating BER is not fully understood. A previous study showed that phosphoryla-
tion of Thr<sup>60</sup> and Ser<sup>64</sup> created a phosphodegron, leading to proteasomal degradation of UNG2 (18). Consistent with that report, and with the known role of GSK-3 in creating phosphodegrons (21–25), we found that Thr<sup>60</sup> and Ser<sup>64</sup> destabilized UNG2. However, our results show that these phosphorylations independent experiments; mean data sets for parental OVCAR-8 cells (UNG trans-WT-OVCAR8) and OVCAR-8<sup>26882</sup> led to floxuridine FIGURE 9. Cells expressing UNG2 T60A and UNG2 S64A are more sensitive to floxuridine versus wild-type UNG2. Parental OVCAR-8 cells (WT-OVCAR8) and OVCAR-8 ΔUNG cells transfected with empty vector (pcDNA3 1a-2 and 1a-6) or stably expressing HA-tagged wild-type UNG2 (UNG2 2a-3 and 2a-8), UNG2 T60A (T60A A5 and B5), or UNG2 S64A (UNG2 S64A 2a-2 and 2a-3) were tested for sensitivity to floxuridine by clonogenic assay (two independent clones for wild-type UNG2 and each UNG2 mutant). All cell lines were analyzed in parallel in the same experiment. However, for clarity of presentation, the T60A clones and the S64A clones were plotted separately versus the same data sets for parental OVCAR-8 cells (WT-OVCAR8) and OVCAR-8 ΔUNG transfected with empty vector or wild-type UNG2. Data are representative of three independent experiments; mean ± S.D. of 3 replicates/dose.

**Experimental Procedures**

**Materials**—Reagents were obtained as follows: LY2090314 (MedChem Express, Monmouth Junction, NJ); PEG4 carboxamide-6-azidohexyl biotin (biotin azide), ethynyl-deoxyuridine (EdU), SuperSignal Pico West (Thermo Scientific, Waltham, MA); dATP, dGTP, and dCTP (Roche); 5′-fluoro-2′-dUTP (TriLink Biotechnologies, San Diego, CA); dUTP (Promega Corp., Madison, WI); [<sup>32</sup>P]ATP (PerkinElmer Life Sciences, Boston, MA), and CHIR99021 (Selleck Chemicals, Houston, TX). All other reagents were purchased from Sigma. Antibodies were obtained from the following sources: mouse anti-β-actin (Sigma, A 5441, clone AC-15), rabbit anti-GSK-3α (Epitomics, 1742-1), goat anti-GSK-3β (L-17, Santa Cruz Biotechnology, sc-8257), mouse anti-HA.11 epitope tag (Covance, MMS-101R, lot 1), rabbit anti-histone H3 (EPR16987, Abcam, ab176842, lot GR206289-3), rabbit anti-phospho-PLK1 binding motif (ST<sup>T/P</sup>, D73F6, Cell Signaling Technology, Danvers, MA, 5243S), mouse anti-Ser(P)<sup>399</sup>-histone H2Ax (Millipore, 05-636, clone JBW301, lot JBC1881392), mouse anti-proliferating cell nuclear antigen (PC10, Santa Cruz Biotechnology, sc-56), mouse anti-S tag (44), rabbit anti-α-tubulin (Cell Signaling Technology, 2144), rabbit anti-UNG (Epitomics, 3130-1, lot YH030808C), HRP-linked anti-mouse immunoglobulin G and HRP-linked anti-rodent immunoglobulin G (Cell Signaling Technology), HRP-linked anti-rodent immunoglobulin G (Santa Cruz Biotechnology, sc-2354, lot K2009), and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, PA).

**Cell Lines and Culture**—OVCAR-8 cells were a gift from D. Scudiero (NCI, National Institutes of Health), and HeLa and K562 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 8% fetal bovine serum (Sigma) at 37 °C in 5% CO<sub>2</sub>.

**CRISPR/Cas9 UNG Mutant Cells**—The UNG gene was targeted in OVCAR-8 cells using CRISPR/Cas9 as described previously (45). A 455-bp gBlock (IDT, Coralville, IA) containing a U6 promoter, the UNG targeting sequence (TTTCCCGAAC-TCCCCGGCTGT), a guide RNA scaffold, and a transcription termination site was PCR-amplified using the primers 5′-TGTACAA AAAAAAGCAGCCTTTAAG-3′ and 5′-TAAT GCCAATCTTGTACAGAAAG-3′. The UNG targeting sequence was designed to target a region in the N terminus to introduce frameshift mutations that would prevent production of a protein with a catalytic domain in both the UNG1 and UNG2 isoforms. The PCR product was purified using the High Pure PCR product purification kit (Roche) and co-transfected into OVCAR-8 cells with pcDNA3.3-TOPO-hCas9 (Addgene plasmid 41815) by electroporation as described previously (46). Individual clones were isolated and assayed by Western blotting to identify clones that lacked UNG1 and UNG2 expression.

Purified genomic DNA from these clones was amplified with primers flanking the intended Cas9 cut site (5′-GGCCATTC-CAGGCAAGAG-3′ and 5′-CCGGCCAGCTCCACATTTA-3′), and the PCR fragments were Sanger-sequenced to identify genomic alterations in the UNG gene. OVCAR-8ΔUNG clone H2, which was used for the studies here, contained an identical 19-bp deletion in both UNG alleles. This deletion introduces a frameshift immediately following Trp<sup>98</sup> in UNG2 and following Trp<sup>98</sup> in UNG1 so that both proteins are prematurely truncated nine amino acids later and do not contain a catalytic domain.

Cell lines stably expressing UNG2 mutants were created by transfecting OVCAR-8ΔUNG cells with an empty vector or a vector expressing HA-tagged UNG2, UNG2 T60A, or UNG2
S64A and selecting with G418 at 0.75 μg/ml. Two independent clones were chosen for each line, and expression of UNG2-HA was verified by Western blotting. Both clones for each cell line were used in clonogenic assays. In other assays using a single clone, the following clones were used: pcDNA3 clone 1a-2; wild-type UNG2, clone 2a-3; UNG2 T60A, clone A5; and UNG2 S64A, clone 2a-3.

Plasmids—UNG2 was amplified using primers bearing the appropriate restriction sites and cloned into pcDNA3-HA to append the HA tag to the C terminus, pRES-EGFP to append the SFB tag to the N terminus, and pSPN vectors to append the S tag to the N terminus of UNG2. UNG2 T60A, UNG2 S63A, UNG2 S64A, and UNG2 S64D mutants were created by site-directed mutagenesis. The HA-tagged GSK-3β expression plasmid was from Jim Woodgett (Addgene plasmid 14753) (47).

Immunoprecipitation and Western Blotting—Western blotting analyses were performed as described previously (48). Equal amounts of protein were separated by SDS-PAGE, transferred to an Immobilon-P membrane (Millipore), and blotted for the indicated antigens. For cycloheximide experiments, 150,000 cells/well were plated on 6-well tissue culture clusters, allowed to adhere overnight, treated with 80 μg/ml cycloheximide for the indicated times, washed with PBS, and lysed directly in SDS-PAGE buffer prior to Western blotting analysis. For immunoprecipitation experiments, cells were transfected with the indicated plasmids and lysed in 1 ml of 30 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 5 mM MgCl₂, with 1 mM Na₃VO₄, 10 mM 2-glycerophosphate, 20 mM microcystin-LR, 5 μg/ml pepstatin, 5 μg/ml aprotinin, and 10 μg/ml leupeptin. For each sample, 0.9 ml of lysate was incubated with 15 μl of packed volume S protein-agarose beads (Millipore) or streptavidin-agarose beads for 2 h. Beads were washed three times in lysis buffer without inhibitors, and proteins were eluted in SDS-PAGE sample buffer by heating to 95 °C for 10 min.

UNG2 Activity in Cell Lysates—The substrate for the UNG2 activity assay was prepared through PCR. A plasmid encoding uracil substrate, 3 μg of cell lysate was used. At the indicated time points, reactions were placed on ice, quenched with DNA binding buffer, purified using the High Pure PCR product purification kit, and analyzed on 1% agarose gels stained with ethidium bromide. Images were obtained using Quantity One 1D analysis software and analyzed using ImageJ.

Immunofluorescence and Flow Cytometry—For flow cytometry analysis, 500,000 cells were plated on 10-cm tissue culture dishes, allowed to adhere for 4 h, exposed to floxuridine with or without LY2090314 for 24 h, washed with PBS, and incubated in medium without floxuridine for 24 h. At the indicated points, cells were harvested with trypsin-EDTA (Mediatech), washed in PBS, and fixed as described previously (48). Cells were stained with 2 μg/ml anti-Ser(P)139-H2Ax antibody followed by staining with 1:50 dilution of FITC-conjugated anti-mouse antibody and analyzed by flow cytometry. For fluorescence microscopy, cells expressing wild-type or mutant HA-tagged UNG2 or EGFP-UNG2 were seeded at a density of 50,000 cells/well on 8-well Nunc Lab-Tek II chamber slides (Thermo) and allowed to adhere overnight. Slides were washed twice with PBS, fixed with 4% formaldehyde for 20 min at room temperature, and permeabilized with 0.25% Triton X-100 in PBS. For the anti-HA stain, slides were then incubated with anti-HA tag antibody for 1 h, followed by staining with FITC-conjugated anti-mouse secondary antibody. DNA was stained for 5 min with 10 μg/ml Hoechst 33342 (Thermo) in 100 mM Tris-Cl (pH 7.4) and 50% glycerol, and slides were mounted with SlowFade Gold antifade reagent (Invitrogen). Slides were analyzed for FITC or EGFP and Hoechst at 20 °C using an LSM 510 inverted laser-scanning confocal microscope (Carl Zeiss Microscopy) with a ×100 oil immersion objective. Images were acquired and processed using Zeiss Efficient Navigation software.

iPOND—iPOND was performed as described previously with a few modifications (37). Briefly, 5 × 10⁶ cells were seeded on 15-cm dishes, grown overnight, pulsed with 10 μM Edu for 1 h, and incubated in label-free medium for 1–5 h. Cells were cross-linked with formaldehyde, washed with PBS, and permeabilized with 0.25% Triton X-100 in PBS. Cells were washed, and Edu-containing DNA was labeled by incubating each sample for 2 h at room temperature with 1 ml of 10 μM biotin azide, 10 mM ascorbic acid, and 2 mM CuSO₄ in PBS or in buffer with no biotin azide for a control sample. Samples were treated with streptavidin-agarose, washed with 25 mM Tris-Cl (pH 8.0) and 0.5% SDS, and sonicated to fragment DNA. Biotin-labeled DNA was pulled down with streptavidin-agarose, washed with 25 mM Tris-Cl (pH 8.0), and 0.1% SDS, followed by 1x NaCl three times each, and boiled 25 min in 2× SDS-PAGE sample buffer to elute proteins from beads and reverse cross-links. Proteins were analyzed by Western blotting for the indicated antigens.

Transfections—Transfections of cells with small inhibitory RNAs and plasmids were performed by electroporation as described previously (46). Sequences of siRNAs used for transfection were as follows: GSK-3α-2, 5′-GGAGCAACAAUCC-GAGAGAUG UU-3′; GSK-3β-1, 5′-AGAAAGAUUUGCA GGACAAUU-U-3′.

Clonogenic Assays—For clonogenic assays, OVCAR-8 and HeLa cells were plated at 300 cells/well on 6-well tissue culture
clusters, allowed to adhere for 4–6 h, exposed to flurouridine continuously for 7 days or for 24 h, washed with medium, and grown in drug-free medium for 6 days. Colonies were stained with Coomassie Brilliant Blue and counted manually. For clonogenic assays using GSK-3 inhibitors, percent colony formation was normalized against vehicle-treated cells. For assays using siRNA-transfected cells or mutant UNG2-expressing cells, percent colony formation was normalized against the vehicle-treated control for the given siRNA or cell line.

Kinase Assays—K562 cells were transfected with the indicated plasmids and lysed in 1 ml of 30 mM Tris–Cl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 5 mM MgCl₂ with 1 mM Na₂VO₄, 10 mM 2-glycerophosphate, 20 mM microcystin-LR, 5 μg/ml pepstatin, 5 μg/ml aprotinin, and 10 μg/ml leupeptin. For each sample, 0.9 ml of lysate was incubated with 2 μg of anti-HA-tag antibody and 20 μl of protein G-Sepharose beads (Thermo) for 3 h. Beads were washed three times in lysis buffer and twice in kinase buffer containing 40 mM HEPES (pH 7.6), 0.5 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, and 2 mM magnesium acetate. For the kinase assay, 20 μl of kinase buffer containing 20 μCi [32P]ATP and 1 μM cold ATP was added to beads and incubated at 30°C. After 12 min, 7 μl of 4× SDS-PAGE sample buffer was added, and samples were boiled to halt reactions, separated by SDS-PAGE, and analyzed for [32P] radioactivity.

Author Contributions—C. A. B. conducted most of the experiments, analyzed the results, and wrote most of the paper. C. J. H. conducted the experiments shown in Figs. 1B and 9 and produced the cell lines expressing UNG2 S64A-HA. S. M. H. produced the cell lines expressing WT UNG2-HA. C. R. J. conducted some of the experiments shown in Fig 2. L. M. K. conceived the idea for the project, produced the cell lines expressing UNG2 T60A-HA, and helped write the paper.

References
