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Genomically incorporated 5-fluorouracil that escapes UNG-initiated base excision repair blocks DNA replication and activates homologous recombination

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Running Title: UNG depletion sensitizes to floxuridine

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Nonstandard abbreviations: BER, base excision repair; BrdU, bromodeoxyuridine; 5-FU, 5-fluorouracil; FdUrd, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridine monophosphate; FdUTP, 5-fluorodeoxyuridine triphosphate; HR, homologous recombination; PBS, phosphate-buffered saline; siRNA, small inhibitory RNA; ssDNA, single-stranded DNA; UDG, uracil DNA glycosylase

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ABSTRACT

5-fluorouracil (5-FU) and its metabolite 5-fluorodeoxyuridine (FdUrd; floxuridine) are chemotherapy agents that are converted to FdUMP and FdUTP. FdUMP inhibits thymidylate synthase and causes the accumulation of uracil in the genome, whereas FdUTP is incorporated by DNA polymerases as 5-FU in the genome; however, it remains unclear how either genomically incorporated U or 5-FU contribute to killing. We show that depletion of the uracil DNA glycosylase UNG sensitizes tumor cells to FdUrd. Furthermore, we show that UNG depletion does not sensitize cells to the thymidylate synthase inhibitor (raltitrexed), which induces uracil but not 5-FU accumulation, thus indicating that genomically incorporated 5-FU plays a major role in the anti-neoplastic effects of FdUrd. We also show that 5-FU metabolites do not block the first round of DNA synthesis but instead arrest cells at the G1/S border when cells again attempt replication and activate homologous recombination (HR). This arrest is not due to 5-FU lesions blocking DNA polymerase δ, but instead depends, in part, on the glycosylase TDG. Consistent with the activation of HR repair, disruption of HR sensitized cells to FdUrd, especially when UNG was disabled. These results show that 5-FU lesions that escape UNG repair activate HR, which promotes cell survival.

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INTRODUCTION

5-Fluorouracil (5-FU), one of the most widely used anticancer agents, and its closely related metabolite FdUrd (which is also an FDA-approved drug) have activity against an array of solid tumors, including colorectal neoplasms (Reviewed in (Longley et al., 2003)). 5-FU, which is converted to FdUrd in the cell, is metabolized to two active metabolites that affect DNA metabolism: FdUMP and FdUTP (Reviewed in (Wyatt and Wilson, 2009)). FdUTP is a substrate for DNA polymerases and can be incorporated into DNA. In contrast, FdUMP inhibits thymidylate synthase, which ultimately causes dTTP depletion and a corresponding massive increase in the levels of dUTP, which is then incorporated into DNA during replication. Notably, despite this deep understanding of the effects of 5-FU and FdUrd on nucleotide and DNA metabolism, the DNA repair pathways mobilized by the lesions inflicted by 5-FU metabolites and how they cause cancer cell cytotoxicity remain obscure.

In experimental systems using purified proteins and synthetic DNA substrates, both lesions are substrates for the known uracil DNA glycosylases (UDG): UNG1 (which is localized in the mitochondria), UNG2 (which is localized in the nucleus), TDG, SMUG1, and MBD4 (An et al., 2007; Baker et al., 2002; Kunz et al., 2009; Mauro et al., 1993; Petronzelli et al., 2000; Pettersen et al., 2011; Turner et al., 2004), which excise the aberrant base, an event that initiates repair by the base excision repair (BER) pathway (Kim and Wilson, 2012; Wyatt and Wilson, 2009). In contrast, studies in a variety of cell lines and animal models have variably implicated individual UDGs in sensitivity to 5-FU and its metabolites. For example, a recent study demonstrated that UNG, but not the other UDGs, removes the vast majority of the uracil and 5-FU from the

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genomic DNA of cells (Pettersen et al., 2011), suggesting that this glycosylase might play a key role in the toxicity of 5-FU and FdUrd. Surprisingly, however, disrupting UNG did not affect (An et al., 2007; Andersen et al., 2005; Grogan et al., 2011; Kemmerich et al., 2012; Kunz et al., 2009; Nagaria et al., 2012; Pettersen et al., 2011) or slightly increased resistance (Fischer et al., 2006) to 5-FU or FdUrd in human, mouse, and chicken models. Similarly, studies of TDG, MBD4, and SMUG1 have also revealed discrepant results. Genetic deletion of *TDG* or *MBD4* conferred resistance to 5-FU and FdUrd in mouse models (Cortellino et al., 2003; Kunz et al., 2009; Sansom et al., 2003), whereas analyses of SMUG1 have shown modestly increased (An et al., 2007) or unchanged (Kemmerich et al., 2012) sensitivity to 5-FU in *SMUG1* mouse knockout models, with no change in (Pettersen et al., 2011) or limited sensitization (Nagaria et al., 2012) to 5-FU in SMUG1-depleted human cancer cells. Nonetheless, despite these discordant results with the UDGs, studies of downstream BER components (APE1, XRCC1, and poly[ADP-ribose] polymerase) indicate that BER protects cells from 5-FU and FdUrd (Geng et al., 2011; Guikema et al., 2011; Huehls et al., 2011; McNeill et al., 2009), thereby indicating that BER plays an important role in facilitating the survival of cells exposed to these agents.

Given these divergent findings, it therefore remains unclear what, if any, role UDGs play in tumors treated with 5-FU or FdUrd. To gain insight into this question, we systematically depleted each UDG from colon and ovarian cancer cell lines. These cell lines were used because 5-FU and FdUrd are used to treat hepatic colon cancer metastases (Longley et al., 2003), and, based on our previous findings (Huehls et al., 2011; Huehls et al., 2012), FdUrd is in clinical trials in combination with a poly(ADP-

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ribose) polymerase inhibitor in ovarian cancer (ClinicalTrials.gov Identifier NCT01749397). We found that depletion of UNG—and only UNG—remarkably sensitized these cell lines to FdUrd. Our finding that UNG plays a critical role in cells exposed to FdUrd afforded us a unique opportunity to 1) show that genomically incorporated 5-FU is a key determinant of cytotoxicity, 2) determine how the lesions repaired by UNG affect DNA replication, and 3) show that lesions not repaired by UNG activate HR repair.

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MATERIALS AND METHODS

Cell lines and culture. OVCAR-8 and HT29 cells were cultured at 37°C in 5% CO_2 in RPMI-1640 (Mediatech, Manassas, VA) containing 8 or 10% fetal bovine serum or 8% dialyzed fetal bovine serum (Atlanta Biologicals, Atlanta, GA and Sigma, St. Louis, MO). For clonogenic assays, media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Mediatech). OVCAR-8 cells were a gift from D. Scudierio (National Cancer Institute), and HT29 were obtained from American Type Culture Collection (Manassas, VA). Cell lines were expanded and cryopreserved upon receipt and reinitiated from these stocks every three months.

Materials. Reagents were from the following sources: FdUrd (Bedford Laboratories, Bedford, OH), Slowfade Gold (Invitrogen, Waltham, MA), EM-grade paraformaldehyde (Electron Microscopy Systems, Hatfield, PA), SuperSignal Pico West (Thermo Scientific, Waltham, MA), and all other reagents were from Sigma. Antibodies against the listed antigens were as follows: UNG (Abcam, Cambridge, MA); MBD4 and RPA32 (Bethyl, Montgomery, TX); RAD51 for immunofluorescent staining (Calbiochem, Billerica, MA); FANCD2 (GeneTex, Irvine, CA); Rad18 (Novus, Littleton, CO); 5-bromodeoxyuridine (Becton Dickinson, Franklin Lakes, NJ); ERCC1, RAD51 for Western blotting, and MSH2 (Epitomics, Cambridge, MA); SMUG1 (N-19) for immunoprecipitation and SMUG1 (H-11) for Western blotting (Santa Cruz, Santa Cruz, CA); Ku80, HRP-linked anti-mouse immunoglobulin G, and HRP-linked anti-rabbit IgG (Cell Signaling, Danvers, MA); FITC-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch, Westgrove, PA).

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Cell transfections and siRNAs. Transfection of small inhibitory (si)RNAs was performed as previously described (Huehls et al., 2011). Sequences of siRNAs were as follows:

UNG-1, 5'-CUACAGACAUAGAGGAUUU-3';

UNG-2, 5'-GCAGUUGUGUCCUGGCUAA-3';

UNG-3, 5'-GCAGAAGAAAGAAAGCAUU-3';

SMUG1-1, 5'-GAUUAUGAGCUUACAGAAA-3';

SMUG1-3, 5'-UCACAGGGACCAAGACAAA-3';

TDG-4, 5'-GAAGAUGGCUGUUAAGGAAUU-3';

TDG-6, 5'-CCAUAAGAUUCCAGACACAUU-3';

MBD4-1, 5'-GGGAAAGAGUUGUGAAGCA-3';

MBD4-2, 5'-GGAAAGAGUGGGAGAAGAU-3';

MBD4-3, 5'-GGAAAGAGUUGUGAAGCAA-3';

MSH2-1, 5'-GAUCCUAAUCUCAGUGAAU-3' (Geng et al., 2011);

ERCC1-1, 5'-UAUGCCAUCUCACAGCCUC-3' (Youn et al., 2004);

KU80-1, 5'-GCGAGUAACCAGCUCAUAA-3' (Nimura et al., 2007);

RAD51, ON-TARGETplus SMARTpool-human RAD51 (GE Healthcare Life Sciences, Pittsburgh, PA) or RAD51-4, CTTAAGTGCTGCAGCCTAA;

MLH1, GGAAGAUUCUGAUGUGGAA (Geng et al., 2011);

Rad18-1, 5'-GCUCUCUGAUCGUGAUUUA-3' (Geng et al., 2011);

FANCD2-1, 5'-GGUCAGAGCUGUAUUAUUC-3' (Wagner and Karnitz, 2009);

and luciferase 5'-CUUACGCUGAGUACUUCGA-3' (Elbashir et al., 2001).

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Cell lysis, immunoblotting, and clonogenic assays. Cell lysis, immunoblotting, and clonogenic assays were performed as previously described (Wagner and Karnitz, 2009). Equal amounts of protein were separated by SDS-PAGE, transferred to Immobilon-P, and blotted for indicated antigens. To detect SMUG1, cells were lysed in 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2), 2 mM EDTA, 1 mM Na_3VO_4, 10 mM 2-glycerophosphate, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, and 20 nM microcystin-LR, and equal amounts of protein were immunoprecipitated using 1 μg anti-SMUG1 (Santa Cruz, N-19) and 15 μL packed protein G Sepharose. Washed precipitates were separated by SDS-PAGE and immunoblotted with anti-SMUG1 immunoglobulin G (Santa Cruz, H-11).

For clonogenic assays, HT-29 (300 cells per well) and OVCAR-8 (250 cells per well) cells were plated, allowed to adhere 4-6 h, exposed to FdUrd for 24 h, washed, replenished with media, and incubated at 37°C for 7-10 days. Cells were stained with Coomassie Brilliant Blue, and colonies (≥50 cells) were enumerated manually. For clonogenic assays using cells transfected with siRNA, percent survivals at each drug concentration were normalized to the vehicle-treated control for the given siRNA.

Cell cycle analysis. Cell cycle analysis with propidium iodide was performed as described (Wagner and Karnitz, 2009). For BrdU analysis, 750,000 cells were plated in 100-mm tissue culture dishes, incubated overnight, pulsed for 20 min with 10 μM BrdU, washed, replenished with fresh media, and treated with indicated concentrations of FdUrd. At specified time points, cells were released with trypsin, washed with ice-cold

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phosphate-buffered saline (PBS), resuspended in PBS, and fixed with drop-wise addition of 95% ethanol to a final concentration of 70%. For staining, DNA was denatured in 2 N HCl at 37˚C for 30 min, quenched with 0.1 M sodium borate, incubated with anti-BrdU antibody (1:10) in PBS containing 0.5% Tween-20 and 0.5% BSA at room temperature overnight, washed with PBS with 0.5% Tween-20 and 0.5% BSA, incubated with anti-mouse immunoglobulin G conjugated to fluorescein isothiocyanate (1:50) in PBS containing 0.5% Tween-20 and 0.2% goat serum for 30 min at room temperature, stained with propidium iodide as previously described, and analyzed by flow cytometry.

Immunostaining. To detect RPA foci, cells were extracted in 25 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose, and 0.5% Triton X-100 for 5 min on ice. Extracted cells were then fixed with 4% paraformaldehyde in PBS, washed with PBS containing 0.5% Triton X-100, blocked in PBS containing 3% bovine serum albumin, incubated with anti-RPA32 antibody (1:4000) in PBS containing 1% bovine serum albumin at room temperature for 2 h. To detect RAD51 foci, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.25% Triton X-100 in PBS, blocked in PBS containing 3% bovine serum albumin, 0.05% Triton X-100, and 0.04% sodium azide, and incubated with anti-RAD51 antibody (1:250) in blocking buffer for 2 h at room temperature. Following primary antibody staining, cells were washed, incubated with donkey anti-rabbit immunoglobulin G coupled to fluorescein isothiocyanate (1:200) in blocking buffer for 1 h, washed, and stained with bisbenzimide H 33258. Slides were

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mounted with Slowfade Gold and analyzed using an LSM 510 confocal microscope (Zeiss, Oberkochen, Germany).

DNA polymerase δ assays. Four-subunit DNA polymerase δ holoenzyme, PCNA, and replication factor C complex were prepared as described (Zhou et al., 2012). The following template oligonucleotides (where b = biotin) were synthesized by Midland Certified Reagents and purified by polyacrylamide gel electrophoresis by the manufacturer: unmodified, b-5'-CGACAACCTGAATCTAAGCGCTCTCCTAGTTTACGAAGTCGACCTGCAGAAATTCACTGG-3'-b; single 5-FU lesion, b-5'-CGACAACCTGAATCTAAGCGCTCTCCTAGTT-5FU-ACGAAGTCGACCTGCAGAAATTCACTGG-3'-b; double 5-FU lesion, b-5'-CGACAACCTGAATCTAAGCGCTCTCCTAGT-5FU-5FU-ACGAAGTCGACCTGCAGAAATTCACTGG-3'-b. The primer, 5'-CCAGTGAATTTCTGCAGGTCG-3', from Integrated DNA Technologies, was 5′-end-labeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences, Waltham, MA) using T4 polynucleotide kinase (New England Bioloabs, Ipswich, MA). The primer was annealed to the templates and reactions were incubated and processed as described (Meng et al., 2009). The final reaction conditions were 30 nM primer-template DNA, 3 nM polymerase δ complex, 10 nM replication factor C, and 40 nM PCNA in 50 mM Tris, pH 7.5, 125 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 0.1 mg/ml BSA, 1 mM ATP, and 0.1 mM dNTP.

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RESULTS

Depletion of UNG, but not other UDGs, sensitizes ovarian and colon cancer cells to FdUrd. Studies have demonstrated disparate effects of disabling different UDGs on the cytotoxicity of 5-FU (or FdUrd) in multiple model systems, many of which were not human tumors or were not derived from human tumors or tumors that are treated with 5-FU or FdUrd. Thus, it remains unclear which, if any, UDG affects the toxicity of 5-FU metabolites in human tumors. To that end, we identified multiple, independent siRNAs that effectively depleted UNG, SMUG1, TDG and MBD4 (Fig. 1) and examined how they affected the sensitivity of HT29 colon cancer and OVCAR-8 ovarian cancer cells to the 5-FU metabolite FdUrd by clonogenic survival. Strikingly, depletion of UNG2 and UNG1 (Fig. 1A and 1B), which are derived from the same gene, with multiple, unique siRNAs profoundly sensitized both HT29 (Fig. 1A) and OVCAR-8 (Fig. 1B) to FdUrd. In contrast, depletion of TDG (Fig. 1C and 1D) and MBD4 (Fig. 1e and 1F) had no effect on the sensitivity of either cell line to FdUrd. Similarly, SMUG1 siRNAs, which depleted SMUG1 from HT29 cells (Fig. 1G), did not affect the sensitivity of HT29 (Fig. 1G) or OVCAR-8 cells (Fig. 1H) to FdUrd. Because we could not detect SMUG1 in OVCAR8 cells (data not shown), we instead treated them with 5-hydroxymethyldeoxyuridine, which is more toxic in cells with functional SMUG1 (Boorstein et al., 1992; Boorstein et al., 2001; Horton et al., 2003; Mi et al., 2001). Consistent with SMUG1 depletion, the SMUG1 siRNA-transfected cells were less sensitive to 5-hydroxymethyldeoxyuridine (Fig. 1H, inset).

To address whether UNG affects the sensitivity of other cancer cell lines, we depleted UNG with siRNA in cell lines of diverse origin. UNG depletion sensitized cell

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lines derived from ovarian cancer (OVCAR-5, Fig. 2A), colon cancer (SW480, Fig. 2C; HCT8, Fig. 2D), pancreatic (HUPT3, Fig. 2E), and cervical (HeLa, Fig. 2F) cancers. Only A2780 (Fig. 2B) cells, which are derived from ovarian cancer, were not sensitized by UNG depletion. These observations indicated that UNG may be important for determining sensitivity to FdUrd in a variety of cell types.

UNG does not alter the toxicity of genomic uracil introduced by thymidylate synthase inhibition. FdUrd is converted to two metabolites, FdUTP and FdUMP, that cause the accumulation of genomic 5-FU and uracil, both of which are substrates of UNG. It remains unclear, however, whether genomically incorporated uracil, FU, or both contribute to the killing of human cancer cells. To assess whether uracil incorporation was toxic in these cells, we treated control and UNG-depleted cells with raltitrexed and FdUrd. The comparison of these two agents is informative because raltitrexed is a thymidylate synthase inhibitor that causes the selective accumulation of uracil (but not 5-FU) in the DNA. In contrast, FdUrd not only inhibits thymidylate synthase (causing uracil incorporation) but also generates genomic 5-FU (due to incorporation of FdUTP). Although depletion of UNG sensitized cells to FdUrd, it did not affect the antiproliferative effects of raltitrexed (Fig. 3), consistent with previous studies using this and similar agents (Grogan et al., 2011; Luo et al., 2008; Welsh et al., 2003). These studies therefore demonstrate that UNG-mediated removal of uracil does not affect cytotoxicity. Furthermore, because UNG depletion markedly sensitizes to FdUrd, these results suggest that genomic 5-FU, which is removed by UNG (Pettersen et al., 2011), is a major cause of cytotoxicity in FdUrd-treated cells.

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UNG removes lesions that block DNA replication. To further explore how UNG depletion affects cellular responses to FdUrd, we examined cell cycle progression in control and UNG-depleted cells exposed to this agent. For these studies we used a low concentration of FdUrd (100 nM), the approximate IC_{50} for cells depleted of UNG (see Fig. 1), to minimize cell death during these experiments. In the first set of studies, we examined how FdUrd affected progression through S phase. OVCAR-8 cells were pulsed with BrdU to label S-phase cells, washed, treated with FdUrd, and the fate of the BrdU-labeled cells was analyzed at 3, 6, 12, and 24 h. This concentration of FdUrd caused only a modest S-phase delay (26% vs. 20% at 6 h; and 32% vs. 17% at 12 h) that was not enhanced by UNG depletion (Fig. 4A), thus demonstrating that UNG-mediated removal of 5-FU does not affect progression through this first S phase. Notably, however, the BrdU-labeled cells did accumulate in G1/S after 24-h exposure to FdUrd, with 46% vs. 32% in control (luciferase) cells and 44% vs. 35% in UNG-depleted cells. These observations suggest that genomically incorporated 5-FU affects progression into the subsequent S phase.

Because we saw an unexpected accumulation of cells in G1/S after 24 of FdUrd treatment, we next explored how these arrested cells moved through S phase following removal of the FdUrd. For these experiments, control and UNG-depleted cells were exposed to 100 nM FdUrd for 24 h, which again caused accumulation of the cells in G1/S (Fig. 4B). The cells were then washed to remove FdUrd and cell cycle profiles were obtained up to 24 h post wash off. Following removal of the FdUrd, the control cells synchronously moved through S phase, were in G2/M by 12 h, and had reentered

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G1 and S by 24 h (Fig. 4B). In contrast, the UNG-depleted cells moved much more slowly through S phase, with a large fraction still in S phase at 12 h. Even at 24 h, a large portion of these cells were still in G2/M, while a smaller portion had reentered G1 and S. Taken together, these results indicate that a low concentration of FdUrd primarily disrupts cell cycle progression only when cells attempt reentry into the subsequent S phase. Significantly, because the progression through this subsequent S phase was far slower in UNG-depleted cells, these results suggest that genomically incorporated 5-FU directly blocks cell cycle progression or is converted into lesions that are more toxic than 5-FU when UNG-mediated repair is disrupted.

UNG depletion enhances FdUrd-induced replication fork stalling and RPA and RAD51 foci formation. The observations that FdUrd-treated cells accumulate in G1/S after a 24-h exposure to FdUrd, and that subsequent progression through S phase is slowed when UNG is depleted, suggested that the lesions that were not repaired by UNG block DNA replication. A consequence of inhibiting DNA polymerase progression is the accumulation of single-stranded (ss)DNA, which is initially coated by RPA, a ssDNA-binding protein. If, however, the replication block persists, RAD51 is then loaded by the HR machinery in a process that facilitates restarting stalled replication forks (Petermann and Helleday, 2010; Petermann et al., 2011). We therefore examined the effects of FdUrd on RPA and RAD51 foci formation in control and UNG-depleted cells (Fig. 4C and 4D). For these experiments, cells were treated with FdUrd for 24 h, washed and further cultured. Consistent with the idea that FdUrd is blocking replication fork progression in the second S phase, both control and UNG-depleted cells showed

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increased levels of RPA foci at the end of the 24-h FdUrd exposure (time = 0), with even greater levels 8 h after FdUrd was washed off (Fig. 4C). Interestingly, however, 24 h after FdUrd was removed, the number of RPA foci in the UNG-depleted cells remained elevated, whereas the number of RPA foci had returned to near-background levels in control cells. Similarly, the number of cells with RAD51 foci, a marker for the activation of HR, was significantly increased in UNG-depleted cells 24 h after removal of FdUrd (Fig. 4D). Collectively, these results demonstrate that the lesions induced by FdUrd (and that can subsequently be repaired by UNG), block replication and activate the HR repair pathway.

5-FU in the template strand does not block DNA polymerase δ replication. To gain insight into why FdUrd caused cells to arrest in the subsequent round of replication, we first asked whether 5-FU lesions in the template strand could block the progression of DNA polymerase *δ*. For these studies we used synthetic oligonucleotide substrates (with 5' and 3' ends blocked with biotin) containing one or two 5-FU lesions annealed to a ^{32}P-labelled primer (Fig. 5). The annealed primer-template was then incubated with recombinant four-subunit DNA polymerase δ, PCNA, and the replication factor C clamp loader under conditions that support DNA synthesis (Zhou et al., 2012). As shown in Fig. 4, neither a single 5-FU lesion nor a pair of lesions slowed progression of DNA polymerase δ in this system, thus suggesting that genomically incorporated 5-FU does not impede S-phase DNA replication by directly blocking this replicative DNA polymerase.

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TDG contributes to activation of HR repair when FdUrd-induced lesions escape UNG-mediated repair. Given that 5-FU lesions did not directly block DNA replication, we next asked whether FdUrd-induced lesions that escape UNG-mediated repair might be recognized by another uracil glycosylase, which could produce lesions that activate HR repair. To test this idea, we examined how co-depletion of UNG with each of the other uracil glycosylases TDG, SMUG1, and MBD4 affected RAD51 foci formation induced by FdUrd. We found that MBD4 depletion (Fig. 6A) and SMUG1 depletion (as assessed by decreased sensitivity to 5-hydroxymethyldeoxyuridine [Fig. 6B]) did not reduce FdUrd-induced RAD51 foci formation in UNG-depleted cells (Fig. 6C). In contrast, TDG depletion (Fig. 6A) attenuated RAD51 recruitment when UNG was depleted (Fig. 6C). These findings suggest that FdUrd-induced lesions that escape UNG-mediated repair are acted upon by TDG, which leads to lesions that then block cell cycle progression and activate HR repair.

HR repair, but not other DNA repair pathways, protects against FdUrd toxicity in UNG-depleted cells.

Our studies so far show that UNG removes lesions that activate HR repair, thus raising the possibility that HR participates in the repair of these lesions and subsequent cell survival. To address this possibility, we co-depleted UNG and RAD51 from OVCAR-8 cells. Additionally, to address whether the other major DNA repair pathways could repair FdUrd-induced damage that escapes UNG repair, we also co-depleted UNG and MSH2 (mismatch repair), Rad18 (translesion synthesis), FANCD2 (Fanconi Anemia interstrand crosslink repair), ERCC1 (nucleotide excision repair), or KU80

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(nonhomologous end joining). The cytotoxicity of FdUrd was not affected by depletion of MSH2 (Fig. 7A), Rad18 (Fig. 7B), FANCD2 (Fig. 7C), ERCC1 (Fig. 7D), or KU80 (Fig. 7E) alone or when each of these was co-depleted with UNG. In stark contrast, depletion of RAD51 increased sensitivity to FdUrd (Fig. 7F), consistent with our previous results (Huehls et al., 2012). More importantly, co-depletion of RAD51 and UNG made cells remarkably sensitive to FdUrd. Taken together, these results indicate that both BER repair and HR play key roles in repairing lesions induced by FdUrd.

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DISCUSSION

The mechanism(s) by which 5-FU metabolites kill cells remain unclear. The studies reported here employed FdUrd because this agent is the subject of a current clinical trial at our institution, and because this agent's effects are primarily caused by disrupting DNA metabolism. In contrast, 5-FU not only disrupts DNA metabolism after it is converted into intracellular FdUrd, but also disturbs RNA metabolism (after conversion into 5-FUTP) (Longley et al., 2003), an effect that may contribute to 5-FU's efficacy in human tumors but that would complicate the mechanistic studies described here.

Because previous studies found widely disparate effects of modulating the functions of various UDGs in different model systems (An et al., 2007; Andersen et al., 2005; Cortellino et al., 2003; Fischer et al., 2006; Grogan et al., 2011; Kemmerich et al., 2012; Kunz et al., 2009; Nagaria et al., 2012; Pettersen et al., 2011; Sansom et al., 2003), we performed a systematic analysis in multiple human cell lines derived from tumors that are treated with and respond to 5-FU or FdUrd. In these studies, using multiple siRNAs targeted to each UDG, we showed that UNG, and only UNG, played a role in preventing cell death following exposure to FdUrd. This result was consistent with the elegant studies of Pettersen *et al*, who showed that UNG removed the vast majority of genomically incorporated uracil and 5-FU (Pettersen et al., 2011). Collectively, these studies demonstrate that UNG plays a critical role in repairing the damage inflicted by 5-FU metabolites, at least in these human tumor cell lines, and possibly in patient tumors treated with these agents.

The present results demonstrate that 5-FU incorporation has a major role in cytotoxicity. However, it has long been known that FdUMP-mediated thymidylate

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synthase inhibition, which disrupts dNTP supplies and slows DNA replication, also plays a role in 5-FU and FdUrd cytotoxicity (Wyatt and Wilson, 2009). Consistent with these findings, many studies have shown that tumor thymidylate synthase levels correlate with responses to 5-FU and FdUrd (Wilson et al., 2014). However, not all of these analyses have found a correlation, suggesting that other factors may affect sensitivity. As we show here, UNG levels may be an additional biomarker for responses to 5-FU and FdUrd.

The finding that UNG removes 5-FU and uracil from genomic DNA (Pettersen et al., 2011), coupled with the present studies showing that UNG depletion sensitized cells to FdUrd, allowed us to ask how failure to remove and/or repair these genomically incorporated lesions affected tumor cells. We found that UNG depletion did not alter the rate at which cells replicated their DNA in the presence FdUrd, demonstrating that 5-FU incorporation does not disrupt replication during the first S phase. Nevertheless, as cells then attempted to replicate their DNA again, there was an increased accumulation in the subsequent G1/S phase and a markedly hindered progression through S phase.

Our results also demonstrated that HR plays a critical role in cells exposed to FdUrd. While previous studies showed that HR promotes survival when thymidylate synthase is inhibited, likely due to disrupted DNA replication (Rytelewski et al., 2013; Yang et al., 2008), the present studies demonstrated that 5-FU incorporated into DNA also activated HR. We also showed that activation of HR repair is, in part, dependent on TDG. This finding suggests that TDG creates more toxic lesions when UNG is disabled, a result consistent with the findings of Kunz et al, who showed that 1) TDG-mediated excision of 5-FU from DNA generated persistent DNA breaks, and 2) $TDG^{-/-}$ mouse cells

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were less sensitive to 5-FU (Kunz et al., 2009). Additionally, we showed that activation of HR contributed to cell survival, especially when UNG was depleted. Taken together, these results suggest the following model for how 5-FU that is incorporated into DNA affects tumor cells. During DNA replication in the presence of FdUrd (or 5-FU), 5-F-dUTP is incorporated by DNA polymerases, causing the accumulation of 5-FU into genomic DNA. If this lesion is removed by UNG (and then repaired by BER), the cell continues to proliferate. If, however, the 5-FU is not removed, TDG creates lesions, such as abasic sites, single-strand DNA breaks and double-stranded DNA breaks (Kunz et al., 2009; Sjolund et al., 2014) that slow replication and activate HR repair. HR, but no other DNA repair pathways (including the translesion synthesis pathway), then promotes cell survival by facilitating recovery from replication arrest and/or the repair of the double-stranded DNA breaks that accumulate as a result of replication fork stalling (Chandramouly et al., 2011; Heyer et al., 2008; Petermann and Helleday, 2010; Roy et al., 2012).

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Huehls, Huntoon, Joshi, Baehr, Wagner, Wang, Lee, Karnitz

Conducted experiments: Huehls, Huntoon, Joshi, Baehr, Wagner, Wang, Karnitz

Contributed new reagents or analytic tools: Huehls, Huntoon, Joshi, Baehr, Wagner, Wang, Karnitz

Performed data analysis: Huehls, Huntoon, Joshi, Baehr, Wagner, Wang, Lee, Karnitz

Wrote or contributed to the writing of the manuscript: Huehls, Karnitz

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Depletion of UNG, but not other UDGs, sensitizes colon and ovarian cancer cells to FdUrd. HT29 colon **(A, C, E, G)** and OVCAR-8 ovarian cancer **(B, D, F, H)** cells were transfected with control (Luc) or siRNAs targeting the indicated glycosylases. 48 h after the last transfection, tyrpsinized cells were processed to quantitate UDG levels and analyzed by clonogenic assay. For clonogenic assays, 250 (OVCAR-8) or 300 (HT29) cells were plated, allowed to adhere for 4-6 h, exposed to FdUrd for 24 h, washed, and cultured 7-10 days until colonies formed. Colonies were stained with Coomassie Blue and manually counted. To detect UNG **(A, B)**, TDG **(C, D)**, and MBD4 **(E, F)**, lysates were immunoblotted with antibodies to the listed antigens. UNG1 is variably detected using this antibody. *, denotes non-specific band. To detect SMUG1 **(G)**, HT29 cells were lysed and SMUG1 was immunoprecipitated with anti-SMUG (N-19) bound to immunoglobulin G and immunoblotted with anti-SMUG (H-11) immunoglobulin G. To demonstrate SMUG1 depletion in OVCAR8 cells, cells were plated, allowed to adhere 4-6 h, exposed to 5-hydroxymethyldeoxyuridine (HmdUrd) for 24 h, washed, and allowed to grow for 7-10 days. Colonies were stained with Coomassie Blue and counted. Results for a representative experiment that has been performed three times are shown.

Figure 2. UNG depletion sensitizes multiple cell lines to FdUrd. OVCAR-5 **(A)**, A2780 **(B)**, SW480 **(C)**,HCT8 **(D)**, HupT3 **(E)**, and HeLa **(F)** cells were transfected with control (Luc) or UNG-3 siRNA. 48 hours after the last transfection, cells were analyzed

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for UNG expression by immunoblotting or plated, allowed to adhere for 4 – 6 hours, exposed to FdUrd for 24 h, washed, and cultured 7 – 14 days until colonies formed. Colonies were stained with Coomassie Blue and counted. Results for representative experiments, which have been repeated at least three times, are shown.

Figure 3. UNG depletion sensitizes cells to FdUrd but not the thymidylate synthase inhibitor raltitrexed. OVCAR-8 cells were transfected with control (Luc) or UNG-3 siRNA. 48 h after the last transfection, cells were analyzed for UNG expression by immunoblotting (inset) for the indicated antigens or plated in media containing 8% dialyzed serum, allowed to adhere for 4-6 h, exposed to FdUrd for 24 h or continuously to raltitrexed, washed, and cultured 10-12 days until colonies formed. Colonies were stained with Coomassie Blue and counted. *, denotes non-specific band. Results for a representative experiment that has been performed three times are shown.

Figure 4. 5-FU metabolites induce UNG-repairable DNA lesions that block subsequent DNA replication and cause accumulation of single-stranded DNA that accumulates RPA and RAD51. OVCAR-8 cells were transfected with control (Luc) or UNG-3 siRNA and analyzed for UNG expression by immunoblotting or used experiments 48 h later. **(A)** Cells were pulsed with BrdU for 20 min, washed, exposed to vehicle or 0.1 μM FdUrd, and harvested at the indicated times. Fixed cells were then stained with anti-BrdU antibody and propidium iodide and analyzed by flow microfluorimety. **(B-D)** Cells were treated with vehicle or 0.1 μM FdUrd for 24 h, washed to remove FdUrd, harvested at the indicated times after removal of FdUrd, stained with

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propidium iodide, and analyzed by flow microfluorimetry **(B)**, fixed and stained with anti-RPA32 antibodies **(C)**, or stained with anti-RAD51 antibodies **(D)**. Results for a representative experiment that has been performed three times are shown. Arrows in **(B)** indicate G1 and G2 cells.

Figure 5. 5-FU lesions do not slow progression of DNA polymerase δ in vitro. Primers labeled with ^{32}P on the 5' end were annealed to templates that contained no, one, or two 5-FU substitutions and were biotinylated on the 5' and 3' ends. ^{32}P-labeled, streptavidin-blocked primer-template complexes were incubated with four-subunit DNA polymerase δ, PCNA, and replication factor C for the indicated times and analyzed by urea denaturing gel electrophoresis. S indicates the position of the primer substrate, P indicates position of product. Results for a representative experiment that has been performed three times are shown.

Figure 6. TDG depletion reduces activation of HR in the absence of UNG. OVCAR-8 cells were transfected with control luciferase (Luc), UNG-3, MBD4-1, and TDG-4, SMUG1-3 siRNAs as indicated. **(A-C)** Forty-eight hours later, cells were analyzed for the indicated antigens **(A)**, analyzed for sensitivity to 5-hydroxymethyldeoxyuridine (HmdUrd) **(B)**, and treated with vehicle or 100 nM FdUrd for 24 hours, washed, cultured for an additional 24 hours, stained for RAD51, and analyzed by fluorescent microscopy **(C)**. (A) and (B) show data from a representative experiment. (C) shows averages from 3 independent experiments. *, denotes non-specific band.

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Figure 7. HR, but not other DNA repair pathways, participates in the repair of lesions that are escape UNG repair and block subsequent DNA replication.

OVCAR-8 cells were transfected with control (Luc), UNG-3 siRNA, or UNG-3 plus MSH2 **(A)**, RAD18 **(B)**, FANCD2 **(C)**, ERCC1 **(D)**, Ku80 **(E)**, or RAD51 **(F)** siRNAs. 48 h after the last transfection, expression of the indicated antigens was assessed by immunoblotting and the cells were analyzed by clonogenic assay. For the clonogenic assays, 250 cells were plated, allowed to adhere for 4-6 h, exposed to FdUrd for 24 h, washed, and cultured 7-10 days until colonies formed. Colonies were stained with Coomassie Blue and counted.

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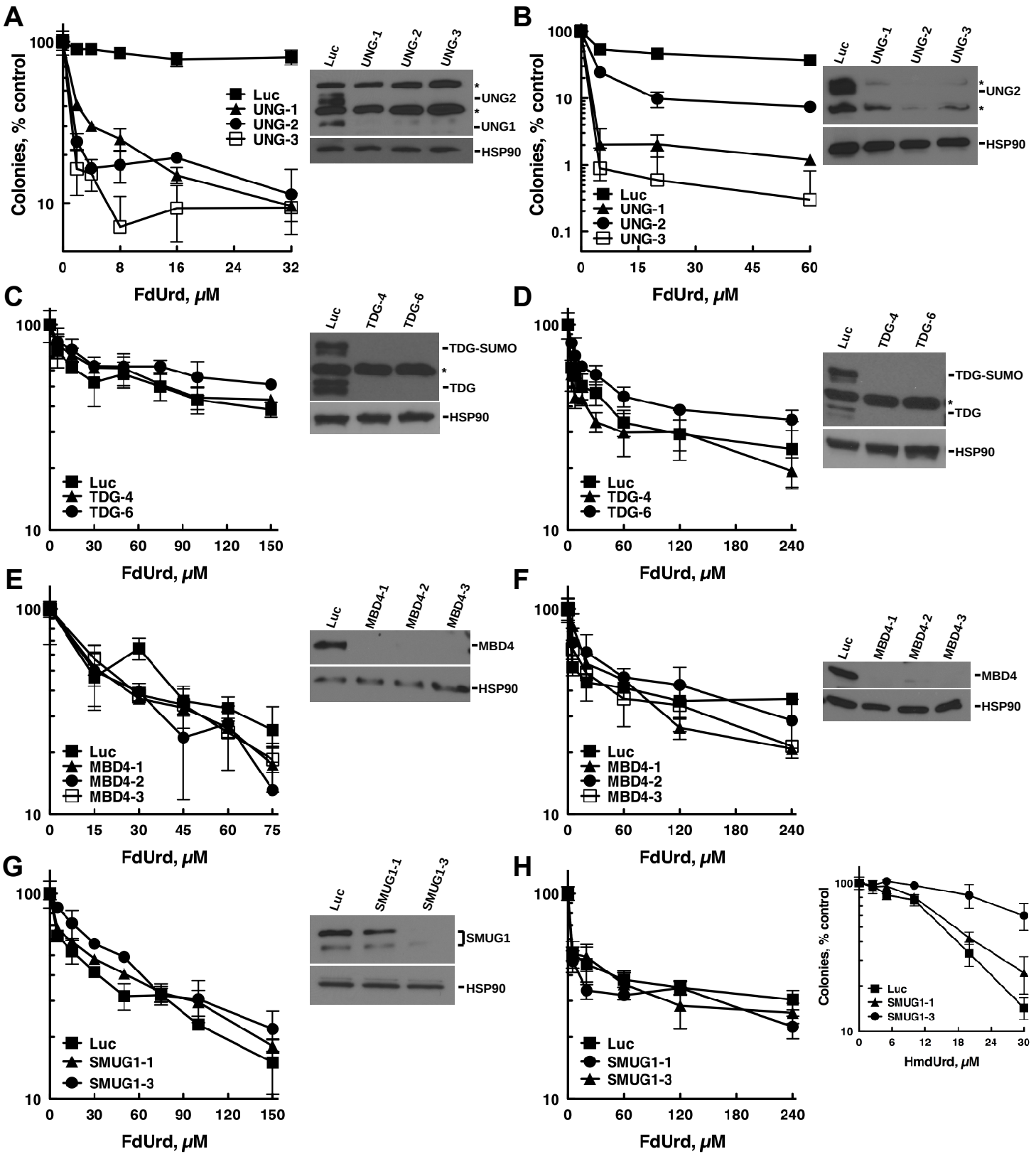

Figure 1

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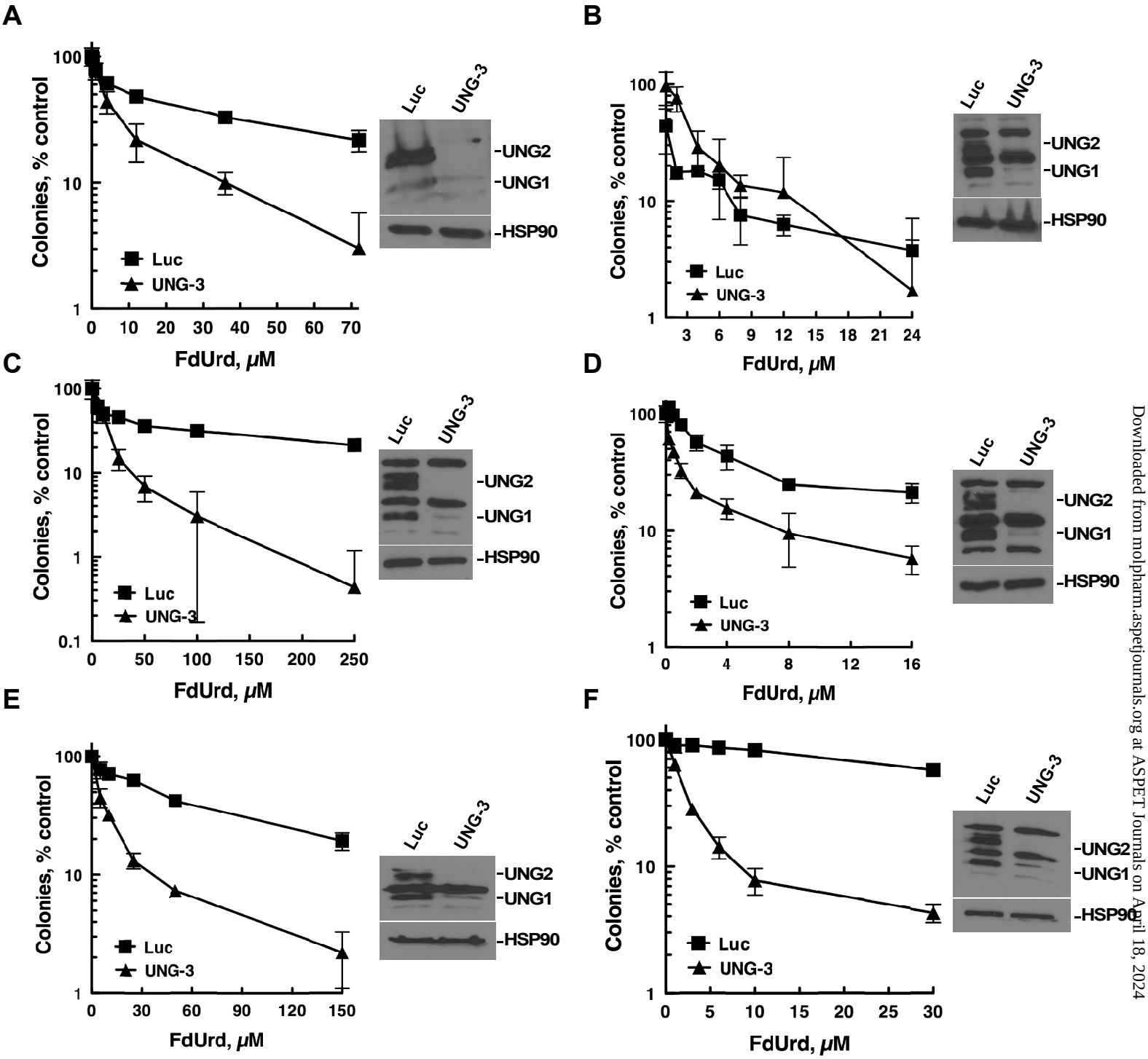

Figure 2

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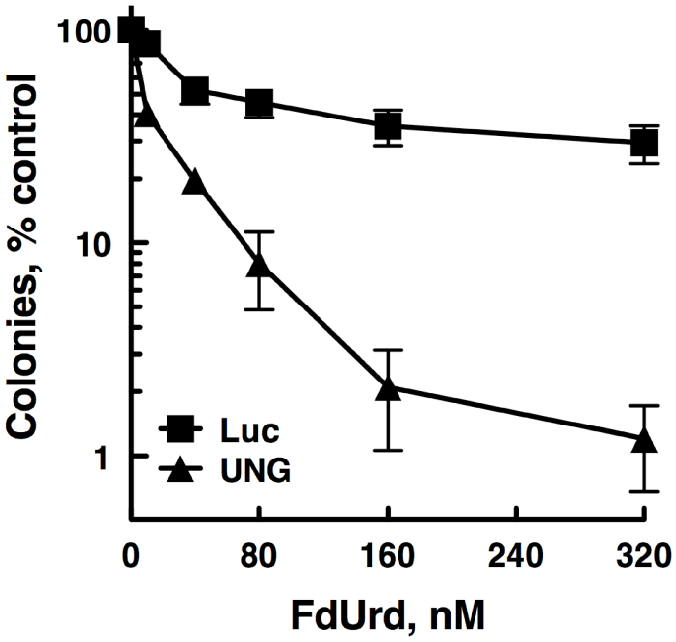

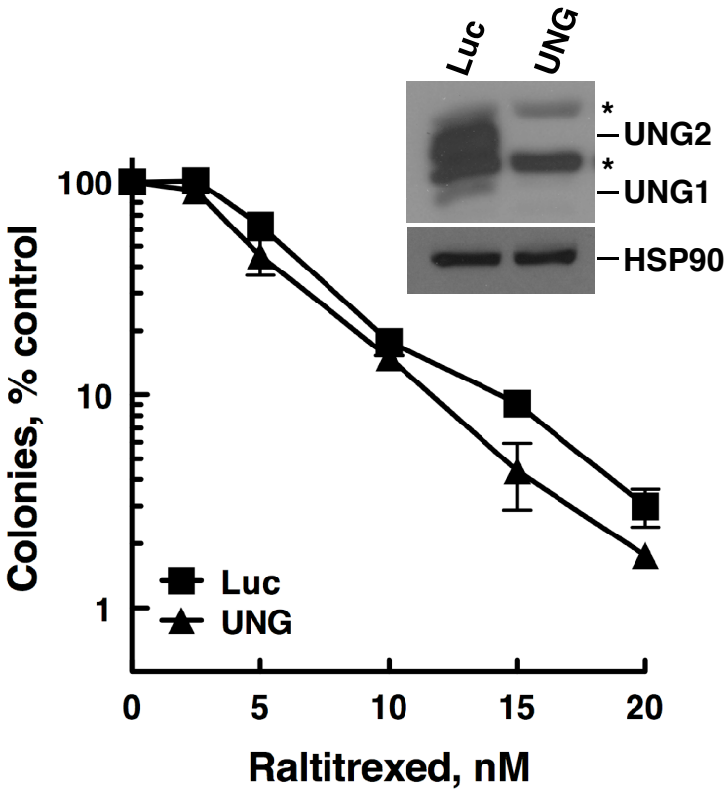

Figure 3

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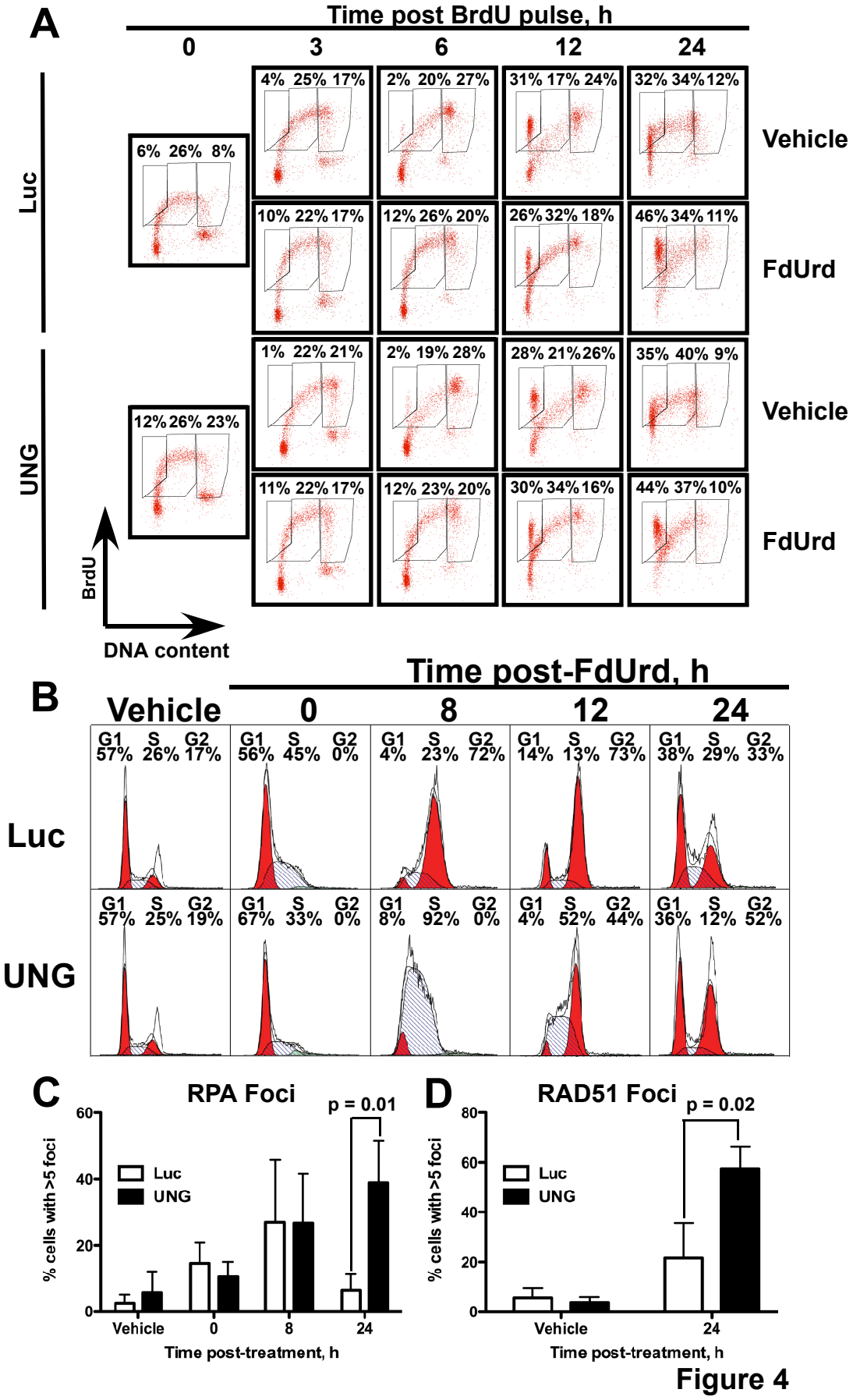

Figure 4

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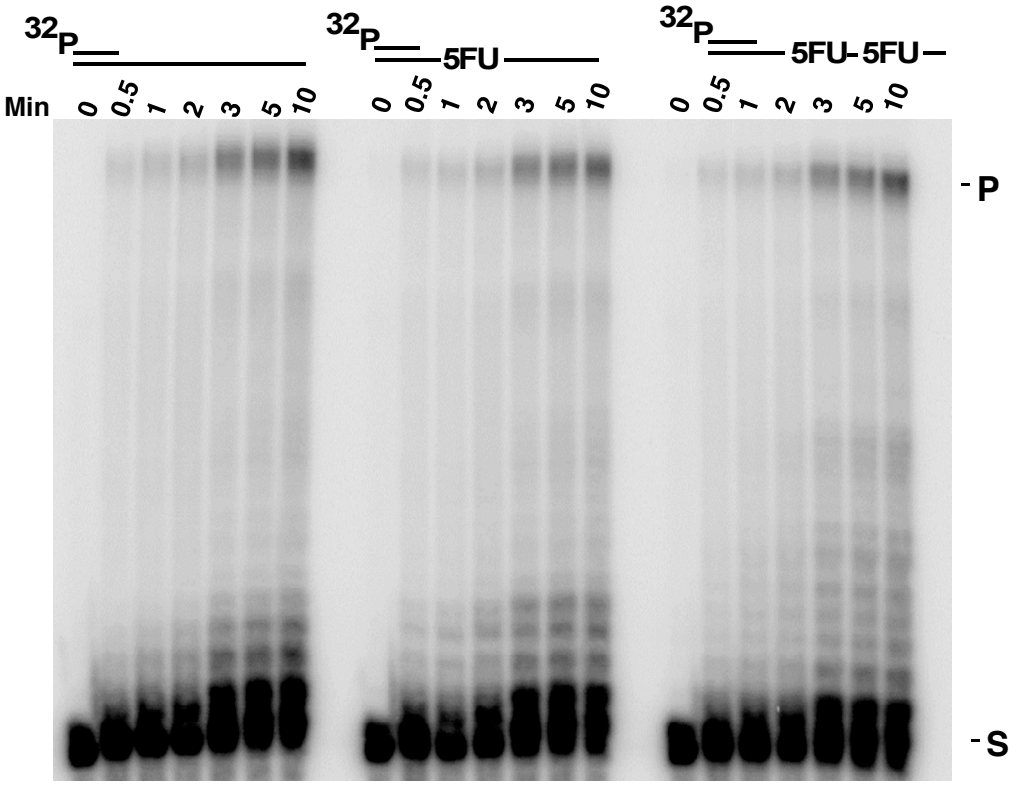

Figure 5

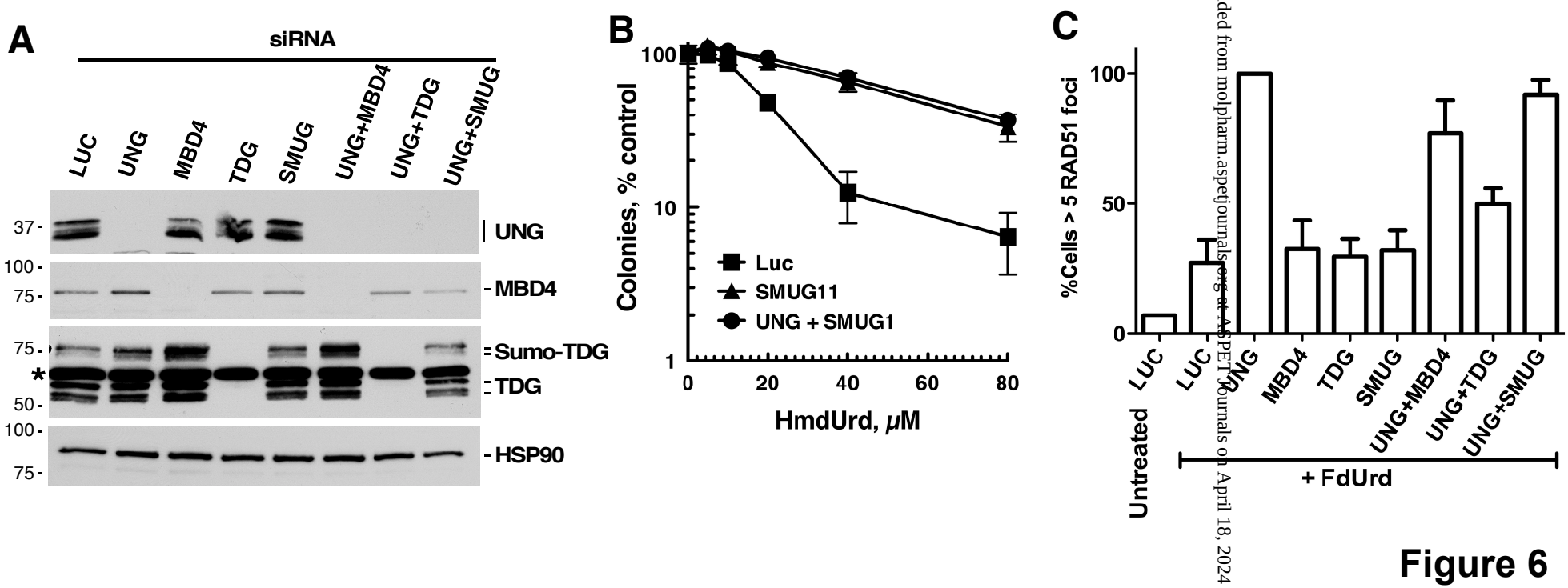

Figure 6

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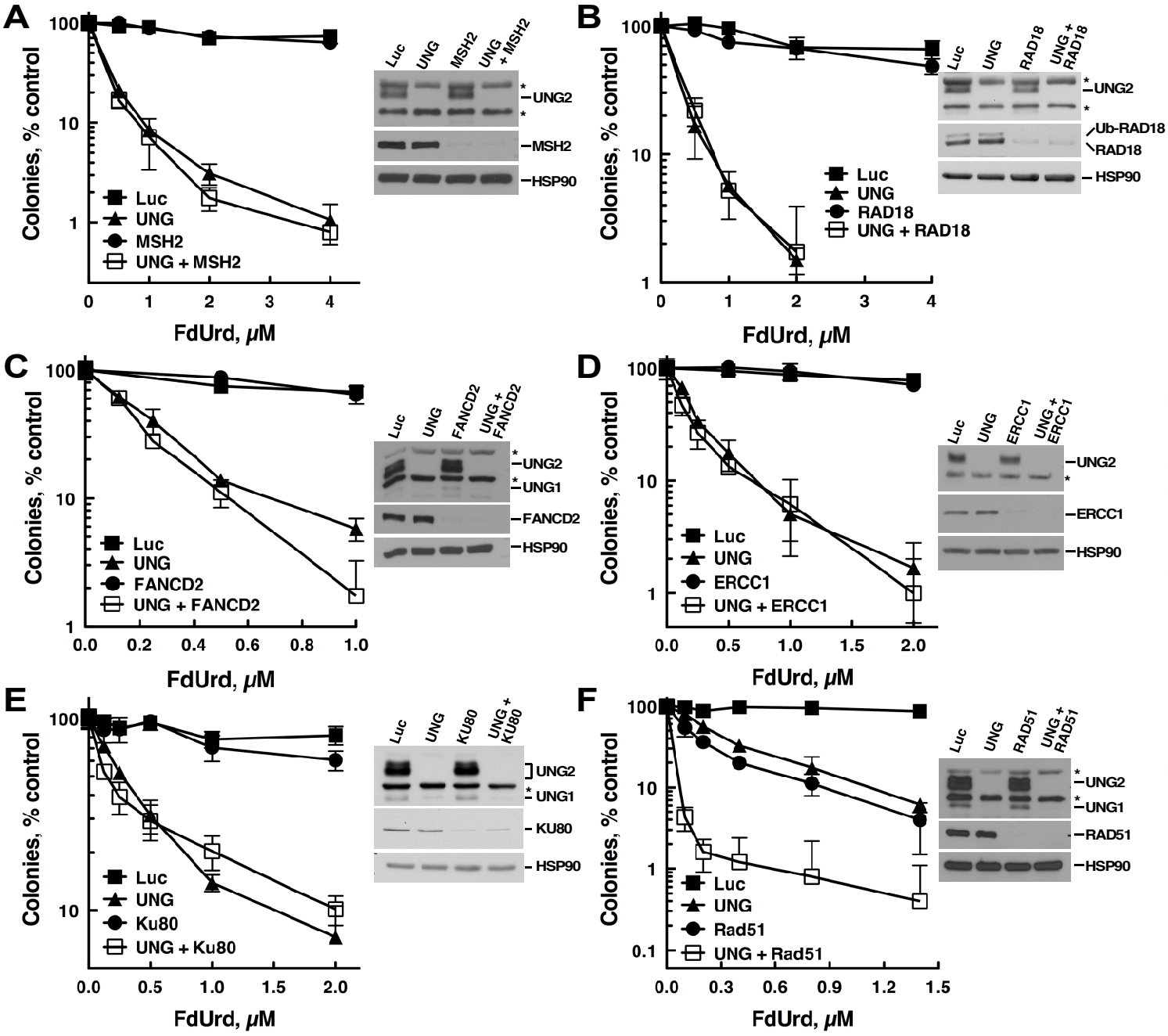

Figure 7