Deregulated redox metabolism in cancer leads to oxidative damage to cellular components including deoxyribonucleoside triphosphates (dNTPs). Targeting dNTP pool sanitizing enzymes, such as MTH1, is a highly promising anticancer strategy. The MTH2 protein, known as NUDT15, is described as the second human homologue of bacterial MutT with 8-oxo-dGTPase activity. We present the first NUDT15 crystal structure and demonstrate that NUDT15 prefers other nucleotide substrates over 8-oxo-dGTP. Key structural features are identified that explain different substrate preferences for NUDT15 and MTH1. We find that depletion of NUDT15 has no effect on incorporation of 8-oxo-dGTP into DNA and does not impact cancer cell survival in cell lines tested. NUDT17 and NUDT18 were also profiled and found to have far less activity than MTH1 against oxidized nucleotides. We show that NUDT15 is not a biologically relevant 8-oxo-dGTPase, and that MTH1 is the most prominent sanitizer of the cellular dNTP pool known to date.
Despite advances in targeted therapies and personalized medicine, DNA damaging treatments such as radio- and chemotherapy remain the primary anticancer treatment modality. These treatments induce damage indiscriminately and often result in dose-limiting side effects that reduce patients' quality of life. Because of the unspecific nature of DNA damaging agents, advanced metastasized cancers are rarely cured and cancer remains one of the most common causes of death.

Altered metabolic and redox activities in cancer cells increase the production of reactive oxygen species that can cause DNA damage. While bases in DNA are protected by the double helix and nucleosome packing, free deoxyribonucleoside triphosphates (dNTPs) are unprotected and therefore more easily damaged. Thus, for cancer cells with deregulated reactive oxygen species, nucleotide pool sanitizing enzymes are critically important, as damaged dNTPs can be incorporated into the DNA thus inducing mutations and DNA damage.

First discovered in Escherichia coli, the NUDIX protein MutT was identified as a sanitizer of the oxidized dNTP pool. MutT selectively hydrolyses 8-oxo-dGTP to 8-oxo-dGMP, thus preventing 8-oxo-guanine from being incorporated into DNA and subsequent A:T→C:G transition mutations. In this way, MutT serves as a 'house-keeping' enzyme that maintains DNA integrity by preventing 8-oxo-dG-induced mutations. The NUDIX hydrolase superfamily is widespread, found in >250 species in eukaryotes, bacteria and archaea. Members of this family consist mainly of pyrophosphohydrolases that act upon nucleoside diphosphates linked to other moieties, X (NUDIX). The human genome has 24 NUDIX hydrolase genes and at least 5 pseudogenes that display considerable substrate diversity. The substrate diversity of NUDIX family proteins is to be expected given that the identifying sequence motif encompasses the metal binding and catalytic site, whereas substrate recognition and selectivity are carried out by variable regions within the superfamily. Multiple functional human homologues of MutT have been suggested, including NUDT1 (MutT homologue 1, MTH1; ref. 9), NUDT15 (MutT homologue 2, MTH2; refs 10,11) and NUDT18 (MutT homologue 3, MTH3; ref. 12). NUDT17 is a close familial relative of MTH1 whose activity has yet to be explored.

The MTH1 protein is the best characterized of the suggested MutT homologues. We and others have recently demonstrated that MTH1 is critical for the survival of cancer cells where oxidative damage to dNTPs is prominent. Interestingly, MTH1 is not essential in non-transformed cells, and MTH1−/− knockout mice are viable with a similar lifespan as wild-type mice. For these reasons, MTH1 inhibition has shown great potential as a novel strategy to treat cancer—a concept coined 'cancer phenotypic lethality'. Despite the promise of first-in-class MTH1 inhibitors, there is a concern that NUDT15 or other dNTP sanitizing enzymes may compensate for lost MTH1 activity. Hence, we sought to investigate other enzymes that sanitize the dNTP pool and determine whether these enzymes might be targeted to induce cancer phenotypic lethality or overcome resistance to MTH1 inhibitors.

NUDT15 is reported to convert 8-oxo-dGTP and 8-oxo-dGDP to 8-oxo-dGMP, whereas NUDT18 is known to hydrolyse 8-oxo-dGDP to 8-oxo-dGMP. The activity of NUDT15 towards 8-oxo-dGTP has led to the assumption that it may contribute to sanitizing the oxidized dNTP pool. While NUDT15 loss has been shown to increase mutation frequency 1.5-fold upon exposure to 8-oxo-dGTP, the in vitro activity of NUDT15 against 8-oxo-dGTP is much lower than the activity of MTH1 towards 8-oxo-dGTP or 2-OH-dATP. Despite rather strong claims supporting NUDT15 as an 8-oxo-dGTP hydrolase, extensive profiling of human NUDT15 is noticeably lacking within the scientific literature.

Here, we solve the first crystal structure of NUDT15 and elucidate major structural differences compared with MTH1, especially in the enzymatic pocket. We demonstrate that NUDT15 has considerably lower activity to oxidized guanine species than to the undamaged nucleotides. Furthermore, knockdown of NUDT15 does not influence the level of 8-oxo-dG incorporation into DNA or cancer cell survival, suggesting that the enzyme does not sanitize 8-oxo-dGTP in cells. We also test NUDT17 and NUDT18 for their activity against a panel of oxidized and canonical NUDIX substrates and show that these enzymes have poor activity against oxidized dNTPs compared with MTH1. Altogether, we conclude that MTH1 is the major sanitizer of the oxidized dNTP pool with little or no overlapping functions of NUDT15, NUDT17 and NUDT18.

**Results**

MTH1 is a major sanitizer of 8-oxo-dGTP and 2-OH-dATP. The MTH1 protein is a well-studied NUDIX protein that hydrolyses oxidized adenosine and guanosine triphosphates, thus preventing their incorporation into DNA. Similar to MTH1, other NUDIX proteins have proposed roles as dNTP pool sanitizers and may function in concert with MTH1 or may act as compensatory enzymes if the activity of MTH1 is lost. In the search for potential anticancer targets that sanitize the oxidized nucleotide pool, we investigated other MutT homologues with reported activity towards oxidized nucleotides (NUDT15 and NUDT18). NUDT17 was also analysed as it is closely related to the human MutT homologues. These enzymes were purified and screened for activity against oxidized and canonical nucleoside triphosphates and diphosphates (substrate abbreviations found in Supplementary Table 1) using a malachite green-based assay (Fig. 1a and Supplementary Fig. 1). While MTH1 demonstrated strong activity towards 8-oxo-dGTP, 2-OH-ATP and 2-OH-dATP, none of the other proposed dNTP pool sanitizing enzymes showed appreciable activity with oxidized nucleotides.

Of these enzymes, NUDT15 showed the second highest activity against 8-oxo-dGTP (Fig. 1a), but had much stronger activity towards 2'-deoxyguanosine-5'-triphosphate (dGTP) (Fig. 1a–c). To more thoroughly compare the activity of NUDT15 and MTH1, kinetic analysis of each enzyme using 8-oxo-dGTP and dGTP as substrates was conducted. Each protein was incubated with dGTP (0–400 μM) or 8-oxo-dGTP (0–100 μM) in assay buffer at pH 7.5, pyrophosphate formation was measured and initial rates were calculated (Fig. 1b). Kinetic parameters were determined using the Michaelis–Menten equation and are displayed in Fig. 1c. While MTH1 prefers 8-oxo-dGTP to dGTP ($k_{cat}/K_m = 840,200$ and $19,100 \text{ M}^{-1} \text{s}^{-1}$, respectively), NUDT15 had an opposite selectivity profile ($k_{cat}/K_m = 3,600$ and $32,400 \text{ M}^{-1} \text{s}^{-1}$ towards 8-oxo-dGTP and dGTP, respectively). MTH1 is a more efficient catalyst of 8-oxo-dGTP hydrolysis compared with NUDT15, which is reflected in a considerably higher $k_{cat}$ value (36-fold higher), and an ~44-fold lower $K_m$ value for this substrate. MTH1 displays a slightly higher $k_{cat}$ value compared with NUDT15 towards dGTP, but NUDT15 has a lower $K_m$ value resulting in a similar catalytic efficiency of dGTP hydrolysis for the two enzymes. High-performance liquid chromatography (HPLC) experiments confirmed that NUDT15 had minimal activity against 8-oxo-dGTP, whereas MTH1 and NUDT15 had nearly equal activity against dGTP (Fig. 1d). Thus, our data show that NUDT15 has a much lower activity towards 8-oxo-dGTP than MTH1 and prefers dGTP over 8-oxo-dGTP. This was not the case for NUDT17 and NUDT18, which show higher
activity with oxidized nucleotides compared with the corresponding canonical nucleotides (Supplementary Fig. 1b). This activity, however, is very low. Taken together, these data suggest that while other NUDIX enzymes show some biochemical activity against oxidized nucleoside diphosphates and triphosphates, the activity of these enzymes are very modest in comparison with the activity of MTH1 towards 8-oxo-dGTP and 2-OH-dATP.

The structure of NUDT15 is similar but distinct from MTH1. Although NUDT15 is similar in sequence to MTH1, we have demonstrated that NUDT15 has a remarkably low activity towards 8-oxo-dGTP. To gain insight into the substrate preference of NUDT15, we determined the structure of the human NUDT15 protein (Fig. 2). NUDT15 was crystallized in sitting drop vapour diffusion experiments at 18°C after 2 days in the conditions described in the Methods section. Crystals were analysed using the beamline 14.1 at Bessy, Germany, at 100 K. The structure was solved in a P1 space group at a resolution of 1.8 Å. The overall structure of the NUDT15 monomer follows a typical NUDIX fold with an α-helix, β-sheet, α-helix arrangement, where the helices reside on opposite sides of a mixed β-sheet (Fig. 2a,c). NUDT15 also has multiple small regions of 3_{10}-helices. The NUDIX box (GX_{5}EX_{7}REUXEEXGU) is located in the first α-helix relative to the N terminus and contains residues responsible for magnesium coordination and substrate hydrolysis. Four magnesium ions are present in the NUDIX box region (Fig. 2d and Supplementary Fig. 3). Three out of four magnesium ions exhibited octahedral coordination: Mg1 coordinates Glu63 and 4 water molecules; Mg2 coordinates Glu67, Glu63, Glu67 and 4 water molecules; and Mg3 coordinates Glu63 and 5 water molecules; and Mg4 coordinates Glu67, the carbonyl oxygen of Glu47 and 4 water molecules. The fourth magnesium ion, Mg4, has trigonal bipyramidal coordination (equatorial coordination angles that average 119.6°) and coordinates five water molecules, two of which are bridged with Mg1 and one is bridged with Mg2. While the presence of four magnesium ions may not be physiologically relevant, the structural differences between MTH1 and NUDT15 may have profound impact on the biological roles of these enzymes.
NUDT15 substrate pocket poorly accommodates 8-oxo-dGTP. While NUDT15 and MTH1 share considerable sequence homology (Fig. 3a) and are similar in overall structure (Fig. 3b), there are pronounced differences within the substrate binding sites that impact substrate recognition. Structural superimposition of NUDT15 and MTH1 reveals a change in the orientation of the second α-helix relative to the N terminus (helix α2) that forms the base of the binding pocket. This divergence leads to a 5.7Å shift at the C-terminal end of the helix9, resulting in a considerably shallower putative binding pocket for NUDT15 (Fig. 3c). The compositions of the individual binding pockets are also markedly different. In MTH1, Asp119 and Asp120 make up the base of the binding pocket and form hydrogen bonds with 8-oxo-dGTP (Fig. 3d). In NUDT15, Trp136 and Gly137 occupy equivalent positions but are unable to form the same hydrogen bonding network. Residue Asn33 in MTH1, which is important for substrate recognition9, is partially conserved with Gln44 in the NUDT15 structure. However, Gln44 of NUDT15 is not in the same orientation and instead forms hydrogen bonds with residues of the adjacent β7 and β2 beta sheets. MTH1 residue Trp117 is involved in π-stacking interactions with the substrate and is functionally conserved by residue Phe135 in NUDT15. The active site differences are highlighted in Fig. 3d, illustrating that NUDT15 and MTH1 have distinct binding site features that confer distinct substrate preferences.

NUDT15 and MTH1 have distinct biological roles. Since NUDT15 had only limited activity against 8-oxo-dGTP in vitro, we analysed whether removal of NUDT15 from cells would increase the 8-oxo-dG content of DNA, induce any DNA damage markers or impact cancer cell survival. Interestingly, NUDT15 depletion using short interfering RNA (siRNA) had no effect on the survival of several cancer cell lines (U2OS, NTUB1/p, A375, H460, MCF7 and SW480; Fig. 4a,b and Supplementary Fig. 4a,b). This is in strong contrast to MTH1, whose depletion results in significantly impaired cancer cell survival (Fig. 4a,b).

Furthermore, NUDT15 siRNA depletion did not influence the survival of MTH1 siRNA-depleted cells, indicating no additive or synergistic effect of combined knockdown (Fig. 4b). In addition, no alterations in cell cycle distribution could be detected after NUDT15 knockdown (Supplementary Fig. 4c,d), and the NUDT15 expression showed no fluctuations throughout the cell cycle (Supplementary Fig. 4e). It is established that MTH1 siRNA depletion results in an increase in DNA damage markers13. Here we confirm that MTH1 siRNA depletion induces RPA and 53BP1 foci formation, but find no such effect after NUDT15 depletion (Fig. 4c,d). Altogether, these data suggest that NUDT15 is not required for prevention of 8-oxo-dGTP incorporation into DNA. To test this directly, we determined the presence of 8-oxo-dG in DNA using the modified comet assay after depleting NUDT15 by siRNA and compared the effect with MTH1 knockdown. In line with our findings that NUDT15 has a poor 8-oxo-dGTPase activity, no increase of 8-oxo-dG was observed in DNA following depletion of NUDT15 by siRNA as was observed when depleting MTH1 (Fig. 4e,f). Even though siRNA depletion does not lead to complete removal of the protein, these data further corroborate that NUDT15 has a limited role in sanitizing the dNTP pool from 8-oxo-dGTP in cells, and that the cellular function is distinct from MTH1.

Distinct activity profiles of MTH1 and NUDT15. As NUDT15 does not have a significant role in cellular 8-oxo-dGTP hydrolysis, we tested the activity of NUDT15 against a panel of potential substrates in vitro. Substrate hydrolysis was assessed using a malachite green assay (Fig. 5a) and HPLC (Fig. 5b). Strong correlation was observed between the two analysis techniques. To our knowledge, there have not been any reports of post-translational modifications of these enzymes, and we have not found any indications of such. However, the assays were...
Figure 3 | Structural comparison and sequence alignment of NUDT15 and MTH1. (a) Sequence alignment of human NUDT15 with human MTH1 (29% overall sequence identity). The secondary structure of NUDT15 is displayed above the sequence alignment and that of MTH1 below the alignment. Sequence similarity is represented by yellow boxes, strict sequence identity in red boxes, beta sheets (β) as arrows, alpha-helices (α) and 3_10-helices (η) as squiggles. (b) Superimposition of MTH1 (purple) and NUDT15 (chain A, cyan) with the NUDT15 Mg²⁺ coordination is shown. Major structural deviation is observed in helix α2 that affects the depth of the putative binding pocket. (c) Comparison of NUDT15 and MTH1 binding pocket depth. Cut view of surface representation highlights the variation in binding pocket depth of MTH1 (purple) and NUDT15 (cyan). (d) Comparison of NUDT15 and MTH1 binding pocket composition. MTH1 helix α2 residues W117, P118, D119, D120 and N33 (grey) are involved in 8-oxo-dG (green) binding. NUDT15 helix α2 residues F135, W136, G137, L138 and Q44 (grey) form the bottom of the putative binding pocket and are unable to make the equivalent hydrogen bonding network observed in MTH1 binding to 8-oxo-dGMP.

carried out using recombinant, purified enzymes, and differences from the in vivo activity cannot be ruled out.

Importantly, when comparing the activity of NUDT15 to MTH1, it is clear that NUDT15 has a distinct selectivity profile from MTH1 (Fig. 5c). Unlike MTH1, NUDT15 was not active towards adenosine nucleotides. Of the substrates tested, low activity was observed towards dCTP, dTTP and 8-oxo-dGTP. The strongest activity was observed against dGTP, 6-thio-dGTP, 6-thio-GTP and dUTP. Two independent studies have recently identified NUDT15 as a pharmacogenetic determinant of thiopurine sensitivity20,21. The particular mutation of NUDT15 leading to sensitivity to thiopurine therapy (Arg139Cys) is located in helix α2, which forms the base of the substrate binding pocket (Fig. 5d). This mutation could lead to the formation of a disulfide bond with the adjacent cysteine residue (Cys140) that may impact the structure of the enzyme active site. Such structural perturbation would likely reduce NUDT15 hydrolysis of 6-thio-dGTP in the cell, causing increased sensitivity to thiopurine treatment.

Discussion
Since the MTH1 protein is a validated target for anticancer treatments13,22, it is critically important to explore other proposed dNTP pool sanitation enzymes and assess their relevance as possible cancer therapeutic targets. Also, these enzymes may greatly influence the efficiency of MTH1 inhibitors in the clinic. Here we profiled enzymes suggested to sanitize the oxidized dNTP pool. Although our data confirm that NUDT15 and NUDT18 have the ability to hydrolyse 8-oxo-dGTP or 8-oxo-dGDP in biochemical assays, this activity was extremely weak when compared with MTH1 and is not likely to have physiological relevance. Of the proposed MutT homologues, NUDT15 had the second highest activity towards 8-oxo-dGTP, but displayed much higher activity towards canonical dGTP. In fact, NUDT15 displays a nearly 10-fold preference for dGTP rather than 8-oxo-dGTP as a substrate ($k_{cat}/K_m = 32,400 \text{M}^{-1}\text{s}^{-1}$ for dGTP and $3,600 \text{M}^{-1}\text{s}^{-1}$ for 8-oxo-dGTP). This is in stark contrast to MTH1 where the $k_{cat}/K_m$ is over 40 times greater for 8-oxo-dGTP than for dGTP. This may be especially relevant in a biological setting where the amount of 8-oxo-dGTP is expected to be only a small fraction of the total dGTP pool. The kinetic values obtained in our experiments are in agreement with those previously published17,19 for MTH1 activity. Our experiments were conducted at the physiological pH of 7.5 in which we detected a reaction rate of 8-oxo-dGTP hydrolysis by MTH1 (8.6 s⁻¹) in between the published $k_{cat}$ values at pH 7.2 (2.1 s⁻¹) and pH 8.0 (12.3 s⁻¹) while the $K_m$ value is similar to that previously reported17. At pH 8, Fujikawa et al., observe a 13.5-fold preference of 8-oxdGTP over dGTP as substrate for MTH1. This preference for 8-oxo-dGTP seems to be more pronounced at pH 7.5 based on the results presented here. No kinetic parameters of human NUDT15 for any substrate have been reported previously. However, Takagi et al.12 estimate that MTH1 is ~40-fold more active with 8-oxo-dGTP than NUDT15, which is in agreement with our results.
Furthermore, while MTH1 depletion led to marked reduction in clonogenic survival and increased levels of 8-oxo-dG in DNA, siRNA knockdown of NUDT15 had no effect on cell cycle progression, clonogenic survival, 8-oxo-dG content of DNA or DNA damage. Altogether, these data call into question the importance of NUDT15 in 8-oxo-dGTP sanitation and suggest a non-vital role of NUDT15 in this realm.

The poor activity of NUDT15 towards 8-oxo-dGTP can be explained by studying the crystal structure. While NUDT15 and MTH1 share a similar overall fold, a shift in NUDT15 helix α2

Figure 4 | Effect of NUDT15 knockdown on clonogenic survival, DNA damage responses and 8-oxo-dG levels in DNA. (a) Western blot showing knockdown of MTH1 or NUDT15 in U2OS cells 96 h after transfection. (b) U2OS cells depleted of MTH1, NUDT15 or both by siRNA were seeded for clonogenic survival. Colonies were stained after 10 days with methylene blue and colonies were counted by eye; (n = 2). (c) Representative immunostainings. After NUDT15, MTH1 or combination of both RPA foci, 53BP1 foci and DAPI (4,6-diamidine-2-phenylindole) were visualized using immunofluorescence. (d) Quantification of 53BP1- and RPA-positive cells (>5 foci per cell) from c; (n = 2). (e) Quantification of the tail moment after MTH1 and NUDT15 knockdown; (n = 2). (f) Representative pictures of comets formed after MTH1 or NUDT15 depletion by siRNA. Lysed cells were treated with OGG1 or buffer control. UT, untransfected; NT, non-targeting siRNA control. Data shown as average ± s.d.
Our data support a role for NUDT15 in thiopurine metabolism, interactions of 8-oxo-dGTP compared with dGTP. Modification results in electronic changes that strengthen the identification as a sensitivity factor in thiopurine therapy. While especially interesting given that a NUDT15 mutation was recently demonstrated the importance of Asp119 and Trp117 in 8-oxo-guanine metabolism, we analysed its activity against a panel of potential NUDIX family substrates. We show that NUDT15 is structurally distinct from MTH1, explaining the different substrate profile of NUDT15. We show that NUDT15 has a nonessential role in the survival of cancer cells but may be a mediator of thiopurine therapeutic effectiveness.

**Methods**

Expression and purification of recombinant NUDIX proteins. The bacterial expression construct pNIC28hNUDT15 was a kind gift from the Structural Genome Consortium (Stockholm, Sweden). cDNAs encoding NUDT17 and NUDT18 were optimized for expression in *E. coli*, purchased from GeneART (Life Technologies) and subcloned into pET28a (+) (Novagen). The constructs were verified by sequencing. His-tagged MTH1, NUDT15, NUDT17 and NUDT18 were expressed in *E. coli* BL21 (DE3) TIR pRARE2 cells at 18°C overnight after induction with 0.5 mM isopropyl-β-D-thiogalactoside. Cultures were grown in a LEX system, harvested and lysed using immobilized metal ion affinity chromatography (IMAC) lysis buffer (100 mM HEPES (pH 8.0), 500 mM NaCl, 10 mM imidazole, 10% glycerol and 0.5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)), and pulsed sonication was performed. His-tagged proteins were affinity purified using HiTrap HP (GE Healthcare) followed by gel filtration using HiLoad 16/60 Superdex 75 (GE Healthcare) in gel filtration buffer (20 mM HEPES (pH 7.5), 300 mM NaCl, 10% glycerol and 0.5 mM TCEP). The His-tag was removed by TEV protease or thrombin digestion and separated using a Ni-NTA column or gel filtration using HiLoad 16/60 Superdex 75 (GE Healthcare). Purity of protein preparations was examined on SDS–polyacrylamide gel electrophoresis gel followed by Coomassie staining and concentration was determined using NanoDrop. The correct mass of the protein preparations was confirmed using mass spectrometry analysis.
Crystalization and structure determination. Full-length NUDT15
(20 mg ml \(^{-1}\)) was crystallized in the presence of x-ray crystallography (0.2 mg ml \(^{-1}\)) in 20 mM HEPES-Na \((pH 7.5), 300 mM NaCl, 10\% (v/v) glycerol and 2 mM TCEP. The flowthrough of the concentration step was used as buffer for SAXS measurements. Samples were centrifuged immediately before measurement. Data were collected at beamline ID29 at ESRF. The position of the beamstop was determined towards a panel of nucleotide substrates using 75 nM NUDT15 and 8-oxo-dGTP. Enzymatic activity was assayed by coupling the reaction to an excess of bovine alkaline phosphatase (Sigma-Aldrich) and measuring the absorbance at 500 nm in duplicates. The column was then washed with 100% B over 10 min followed by from 50 to 70% B over 10 min were used. The column was then washed with 100% B for 4 min and re-equilibrated to 100 (% A for 4 min. Absorbance detection at 260 nm was used to quantify the amount of substrates at the time points indicated. Substrate consumption was measured by HPLC after 30 min incubation at 37 \(^{\circ}\)C in assay buffer (0.1 M Tris-acetate \((pH 8.0), 40 mM NaCl, 10 mM MgAc, 1 mM dithiothreitol and 0.005% Tween-20) with 10 mM NUDT15 protein. Enzymatic activity was assayed by adding 10 µl of triluoroacetic acid to 30 µl of the reaction mixture. All samples were then diluted with HPLC buffer A (40 µl), which was loaded to the HPLC system. Per cent hydrolysis was calculated by subtracting the peak areas at time 30 min from the area at time 0 and then dividing this by the area at time 0.

Cell culture. U2OS, A373, HCT116, HEK293T, HeLa, H460, MCF7 and SW480 cells were maintained in a humidified incubator at 5\% CO\(_2\) and 37 \(^{\circ}\)C. Cell lines were cultured in the following media supplemented with 10\% fetal bovine serum and penicillin/streptomycin: RPMI and McCoy's: U2OS, HCT116. The cell lines were regularly checked for mycoplasma with MycoAlert Mycoplasma Detection Kit (Lonza). U2OS, HEK293T, HCT116, H460, MCF7 and HeLa were obtained from American Type Culture Collection, SW480 from Walter Bodmer (University of Oxford) and HCT116 from University of Gothenburg and Sahlgrenska University Hospital. None of the used cell lines were listed on ICLAC or were known to be cross-contaminated.

siRNA transfection. Cells were seeded to reach 50\% confluence at the day of transfection. After 24h, 10 nM siRNA was transfected using INTERFERin (Polyplus Transfection), as described by the manufacturer using a final concentration of 10 nM siRNA. As negative control, the ALLstars negative control siRNA was used. The following oligonucleotide sense-sequences were used: MTH1 siRNA no. 5'-GAGCAGACGUACUGUUU-3', MTH1 siRNA no. 3'-CCAGCAGAGCUACUGUUU-3' and for NUDT15 a pooled mix of 50\% GGAUGUGCUACUGAUATTT-3' and 50\% CUCAGUGUAAUCCUUUT-3'. Target sequences of siRNAs used in Supplementary Fig. 4 are as follows:

Qiagen 4: 5'-CAGCAGTACTTCTTCTCAAA-3'
Qiagen 6: 5'-CCTAAGAGGCTTTCAGAAAG-3'
Qiagen 7: 5'-CCUGGUUGUAUGUAATTT-3'
Qiagen 8: 5'-CCAUCAUUUAUCUGMATA-3'
Ambion 1: 5'-GGAGUUGAUUCAUA-3'
Ambion 2: 5'-GAAGCAGAUUACAUAAAUATT-3'
Ambion 3: 5'-CUCAGUGUAAUCCUUUTT-3'
Western blotting. Brieﬂy, protein lysates in lithium dodecyl sulfate (LDS) loading buffer and Precision Plus Protein Dual Color Standards were separated on NuPage 4–15\% gradient gels with MOPS running buffer (Life Technologies), transferred to nitrocellulose membranes and blocked with 5\% bovine serum albumin (BSA) in 1× TBS-T (Life Technologies). ECL was used to detect the primary antibodies in 5\% BSA/TBS-T with agitation at 4 °C overnight followed by incubation with secondary antibodies diluted in 5\% BSA/TBS-T for 1 h at room temperature. The antibody against actin (dilution factor 1:10,000; ab6276; mouse monoclonal) was obtained from Abcam. NUDT15 (dilution factor 1:500; SC4533; rabbit), cyclin D1 (dilution factor 1:1,000; SC198; rabbit) antibodies were bought from Santa Cruz Biotechnology, and MTH1 (dilution factor 1:1,000; NB100-109; rabbit polyclonal) from Novus Biologicals. Secondary antibodies against rabbit and mouse were obtained from LI-COR Biosciences. Protein bands were visualized with an Odyssey FC Imaging System and analysed with Image Studio Software (LI-COR Biosciences). The un cropped images of western blots are provided in Supplementary Fig. 5.

Clonogenic survival. Cells were transfected by siRNA as described above, and after 2 days 500 cells were trypsinized, counted and re-seeded into 10-cm Petri dishes. After 10 days, colonies were stained with 4\% methylene blue in MeOH and counted positive if cell number exceeded 50 cells.
Immunofluorescence. Cells were seeded on glass coverslips in 12-well formats 24 h after siRNA transfection. Ninety-six hours after transfection, cells were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Before antibody staining, cells were blocked with 3% BSA in PBS for 45 min. Antibodies used were as follows: RPA (dilution factor 1:500; Cell Signalling; 2,208; rat) and 33BP1 (dilution factor 1:500; Abcam; ab36823; rabbit polyclonal). Alexa-conjugated secondary antibodies were diluted 1:500 and incubated for 2 h. Slides were counterstained with 4,6-diamidino-2-phenylindole. Quantification was done by manual counting of positive cells in the microscope (n=400 cells per sample). Images were taken with a Zeiss LSM 780 with a ×63 oil objective. For 5-ethyl-2 deoxyuridine (EdU) analysis, U2OS cells were seeded into 96-well plates. After 96 h of transfection, 10 μg/ml 1-EdU was added for 20 min as indicated by the manufacturer’s instructions (Invitrogen). Plates were fixed and stained as indicated above. Images were acquired using the Operetta (PerkinElmer) and analysed using the Harmony software.

Modified comet assay. After 5 days of siRNA transfection, U2OS cells were harvested by trypsinization, washed with 1× PBS and resuspended to 1×10^6 cells per ml in 1× PBS. Cell suspension was diluted 1:5 in 1.2% low-melting agarose (37°C). A volume of 10 μl of cell suspension was spread on agarose-coated fully frosted slides (Thermo Fisher Scientific) with the help of a coverslip. Cells were lysed overnight at 4°C using a buffer containing 10 mM Tris (pH 10), 1.5 M NaCl, 0.1 M EDTA, 10% dimethylsulfoxide and 1% Triton X-100. After washing the slides three times with enzyme buffer (40 mM HEPES (pH 8.0), 0.1 M KCl, 0.5 mM EDTA and 0.2 mg ml^-1 BSA), DNA was exposed to buffer alone or OGG1 (2.0 μg ml^-1) for 45 min at 37°C. Slides were washed in enzyme buffer, followed by incubation in alkaline electrophoresis buffer (40 mM HEPES (pH 8.0), 0.1 M KCl, 0.5 mM EDTA and 0.2 mg ml^-1 BSA) for 30 min. The gel electrophoresis condition used was 300 mA, 23 V for 30 min in electrophoresis buffer. After neutralisation (0.4 M NaOH), DNA was exposed using SybrGold dye (Invitrogen) and images were taken with a ×10 objective of a Zeiss LSM 780 confocal microscope. Comets were analysed using CometScore software and tail moment was calculated as per cent DNA in the tail multiplied by the tail length.

Propidium iodine staining. Seventy-two hours after siRNA treatment, cells were lysed and DNA was stained with a buffer containing 50 μg ml^-1 propidium iodine, 20 mM Tris (pH 8.0), 100 mM NaCl, 0.1% NP40 and 20 μg ml^-1 RNase. After 1 h incubation in the dark, DNA content was analysed on a Navios flow cytometer from Beckman Coulter.

Thymidine block. U2OS cells were blocked with a double thymidine block. Thymidine (2 mM; Sigma) was added for 18 h followed by a 9 h period with media. For the second block, 2 mM thymidine was added for 17 h. Cells were released by washing with PBS and addition of media. Western blot samples were taken at the indicated time points.

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Author contributions
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Additional information
Accession codes: The structure and structure factors of NUDT15 have been deposited in the Protein Data Bank with the ID 5BON.

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