Systematic characterization of deubiquitylating enzymes for roles in maintaining genome integrity

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DNA double-strand breaks (DSBs) are perhaps the most toxic of all DNA lesions, with defects in the DNA-damage response to DSBs being associated with various human diseases. Although it is known that DSB repair pathways are tightly regulated by ubiquitylation, we do not yet have a comprehensive understanding of how deubiquitylating enzymes (DUBs) function in DSB responses. Here, by carrying out a multidimensional screening strategy for human DUBs, we identify several with hitherto unknown links to DSB repair, the G2/M DNA-damage checkpoint and genome-integrity maintenance. Phylogenetic analyses reveal functional clustering within certain DUB subgroups, suggesting evolutionally conserved functions and/or related modes of action. Furthermore, we establish that the DUB UCHL5 regulates DSB resection and repair by homologous recombination through protecting its interactor, NFRKB, from degradation. Collectively, our findings extend the list of DUBs promoting the maintenance of genome integrity, and highlight their potential as therapeutic targets for cancer.

Genomic DNA in all organisms is exposed to various endogenously generated and exogenous DNA damaging agents, including ultraviolet light, reactive oxygen species, ionizing radiation (IR) and chemotherapeutic medicines. These agents generate DNA lesions that threaten genome integrity by compromising normal DNA-based processes such as replication, transcription and cell division. To mitigate the deleterious effects of DNA lesions, specialized DNA repair mechanisms have evolved, whose loss or deregulation causes cancer and various hereditary diseases\(^1\). In eukaryotic cells, DNA double-strand breaks (DSBs), perhaps the most toxic DNA lesions, are mainly repaired by homologous recombination (HR) or non-homologous end joining (NHEJ; ref. 2). DSBs also trigger intracellular signalling processes termed the DNA-damage response (DDR), which includes cell-cycle checkpoint arrest responses. It is well known that DSB repair and associated events are tightly regulated by post-translational protein modifications. For instance, protein phosphorylation plays key roles in DSB repair and DDR signalling through the actions of protein kinases such as DNA-PK, ATM, ATR, CHK1 and CHK2 (refs 3–5). Furthermore, it has become apparent that ubiquitylation—the covalent attachment of the 76 amino-acid residue ubiquitin to target molecules—also plays important roles in controlling DSB repair and DDR processes\(^6\). Ubiquitylation is a sequential enzymatic reaction mediated by E1, E2 and E3 enzymes, resulting in mono- or poly-ubiquitylation involving the use of seven lysine residues (Lys 6, Lys 11, Lys 27, Lys 29, Lys 33, Lys 48 and Lys 63) on ubiquitin as well as the ubiquitin amino terminus\(^7\). The classical function of ubiquitylation, mainly through Lys 48 linked chains, is to target substrates to proteasome-dependent degradation. However, it is now clear that other types of polyubiquitylation as well as monoubiquitylation also play other prevalent roles through regulating protein interactions, activity and subcellular localization\(^8\).

In many instances, ubiquitylation is regulated by its removal through the actions of specific deubiquitylating enzymes (DUBs), some of which also play key roles in ubiquitin-precursor processing\(^9\). The human genome encodes 94 potential DUBs that can be divided into five subfamilies on the basis of the sequences and structural features of their catalytic domains: ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs), Machado–Joseph disease enzymes (MJDs) and JAB1/MPN/MOV34 metalloenzymes (JAMMs; ref. 10). Although various DUBs have been connected to DDR processes\(^11–18\), an important challenge is to achieve a more comprehensive understanding of DUB functions in this context. Here, to address this challenge, we have systematically characterized human DUBs for roles in DSB repair, the DNA-damage induced G2/M cell cycle checkpoint and the overt maintenance of genome integrity.
addition to identifying many DUBs with DDR roles, this work also establishes that one, UCHL5, promotes DSB end resection and HR through regulating the stability of the NFRKB protein (nuclear factor related to κB-binding protein) that is a subunit of the chromatin remodelling complex INO80.

### RESULTS

#### Primary screens for DUBs promoting the DDR or the G2/M DNA-damage checkpoint

To identify DUBs with DSB-responsive roles, we carried out systematic screens employing three different techniques (Fig. 1). First, after cloning the coding regions for 71 of the 94 human DUBs into vectors to express them fused to green fluorescent protein (GFP) in human U2OS cells, we used live-cell imaging to examine each GFP–DUB fusion for recruitment to DNA-damage sites generated by laser micro-irradiation, a technique commonly used to measure DSB responses (Supplementary Table 1; ref. 19). In a parallel primary screen, we employed 90 short interfering RNA (siRNA) pools to individually deplete each of the corresponding DUBs in human U2OS cells. Ensuing DUB-depleted cells and control cells were transfected into cells stably expressing red fluorescent protein (RFP)-fused 53BP1, then localization of GFP–DUBs to sites of DNA damage induced by laser micro-irradiation was examined. In parallel, each DUB was depleted by siRNA pools and subjected to immunoblotting analysis for DDR or G2/M checkpoint markers. In the secondary screen, 44 DUBs obtained from the primary screen were subjected to a neutral comet assay after depletion of each DUB by siRNA pools.

This work identified 17 DUBs, whose depletion caused persistence of phosphorylated H3 Ser 10 after DNA-damage induction (Fig. 2a) to a degree greater than twice (green) or three times (red) the s.d. of the siRNA control. This thereby suggested these DUBs as playing roles in cell-cycle progression or G2/M checkpoint control (for the identities of these DUBs, see Supplementary Table 1). We note that this aspect of our work identified several DUBs (CSN5, also known as COP5, USP19 and USP37) that had been previously linked to the G2/M checkpoint, thereby providing validation of our screening methodology. Moreover, our DNA-damage localization and DDR signalling screens collectively identified 44 DUBs as candidates for affecting the DDR following DSB induction (Fig. 1 and Supplementary Table 1).

#### Identifying DUBs involved in DSB repair and/or genome integrity maintenance

To further examine whether the 44 DDR-DUB candidates we identified were involved in maintaining genome stability and/or DSB repair, we used siRNA pools to deplete them, then used neutral comet assays (Fig. 1 and Methods) to directly measure DSBs in cells that had or had not been exposed to phleomycin. Importantly, by employing siRNA-mediated depletion of BRCA1 and XRCC4, which play important roles in HR and NHEJ, respectively, we established that defects in either of these DSB repair pathways can be detected by the comet assay (Fig. 2b). This work indicated that depleting each of ten DUBs (USP44, PSMD14, USP26, MYSM1, OTUD6B, USP5, USP49, JOSD1, USPL1 and USP1) resulted in DSB induction even without exposing cells to phleomycin. Importantly, by employing siRNA-mediated depletion of BRCA1 and XRCC4, which play important roles in HR and NHEJ, respectively, we established that defects in either of these DSB repair pathways can be detected by the comet assay (Fig. 2b). This work indicated that depleting each of ten DUBs (USP44, PSMD14, USP26, MYSM1, OTUD6B, USP5, USP49, JOSD1, USPL1 and USP1) resulted in DSB induction even without exposing cells to phleomycin (Fig. 2c) and Supplementary Table 1), suggesting functions for these DUBs in replication and/or repairing endogenously generated DNA damage that can become converted to DSBs during replication. Moreover, these comet-assay screens identified 23 DUBs whose depletion resulted in a DSB repair defect greater than twice the s.d. of the siRNA control (Fig. 2b and also see Figs 3a and 4a). These included USP1, USP3, USP5, USP7, USP11,

- **Figure 1** Screen to identify DUBs connected to DSB repair or the DNA damage G2/M checkpoint. Schematic representation of the screen for human DUBs involved in DSB responses. In the primary screen, GFP-fused DUB constructs were transfected into cells stably expressing red fluorescent protein (RFP)-fused 53BP1, then localization of GFP–DUBs to sites of DNA damage induced by laser micro-irradiation was examined. In parallel, each DUB was depleted by siRNA pools and subjected to immunoblotting analysis for DDR or G2/M checkpoint markers. In the secondary screen, 44 DUBs obtained from the primary screen were subjected to a neutral comet assay after depletion of each DUB by siRNA pools.
Figure 2 Classification of screen results. (a) Results of screen for DUBs involved in the G2/M DNA-damage checkpoint. The ratio of signal intensity of phosphorylated histone H3 Ser 10 (H3 pSer 10) normalized to total histone H3 level before and after damage (40 μg·ml⁻¹ phleomycin, 2 h) is plotted. Each DUB is numbered in ascending H3 pSer 10 level order, with the names of the corresponding DUBs provided in Supplementary Table 1. Data shown are the single experiment carried out for each DUB depletion and the mean of ten experiments for siRNA control. Numbers 74–90, which are green or red, are CSN6, STAMBP, USP6, HINL1, USP8, EIF3S3, USP52, UCHL1, CSN5, USP20, USP49a, USP19, USPL1, PSMD14, USP29, UCHL3 and USP37, respectively. (b) Results of DSB repair secondary screen with indicated DUB siRNAs. Repair efficiencies were determined by the tail moment ratio of recovered (2 h after phleomycin removal) to damaged (immediately following treatment) cells. Data show the means of two (DUB, XRCC4 and BRCA1 depletions) or seven (siRNA control depletions) biologically independent experiments. (a,b) Two and three times the s.d. of the siRNA control are indicated by the horizontal blue lines, and bar colour (green and red, respectively). Depletion of XRCC4 and BRCA1 are supplied as positive controls. (c) Tail moments (arbitrary units, a.u.) of cells transfected with indicated siRNAs without exogenous DNA damage were plotted. Data show the means of two (DUB depletions) or seven (siRNA control) biologically independent experiments. One s.d. of the siRNA control is indicated by the horizontal blue line, and red bars.
BAP1 and BRCC3 (BRCC36), which have previously suggested DDR connections\textsuperscript{11-18}, thereby indicating that our screen identified DUBs with positive roles in DSB repair. On the other hand, we also found 13 DUBs that were recruited to DNA-damage sites but gave only marginal DSB repair defects on siRNA depletion, including OTUB1, a negative regulator of the UBE2N (UBC13) ubiquitin E2 enzyme\textsuperscript{24}, USP44, which is reported to antagonize RNF168-dependent ubiquitylation\textsuperscript{25}, and OTUB2, which functions in repair-pathway choice\textsuperscript{26} (Figs 2b, 3a and 4a), suggesting that some of the other DUBs we identified might be negative DDR regulators and/or be involved in repair-pathway choice. Interestingly, phylogenetic analysis of the DUBs that we identified with associations to DSB repair (Fig. 3b) or G2/M checkpoint control (Fig. 3c) revealed clustering in certain DUB subgroups (for instance, in Fig. 3b, the entire UCH subclass and those containing USP5 and USP13, USP11 and USP15, or BRCC3 and CSN5). This suggests that these DUBs might have overlapping functions and/or related modes of action in DSB responses.

To extend our analyses further, we selected the ten highest scoring DUBs from the DSB repair secondary screen and assessed the impacts of their depletion with individual siRNAs in neutral comet assays (Fig. 4a,b). active individual siRNAs were deconvoluted from the siRNA pools through assessing their target DUBs by immunoblotting; data not shown). This work identified six DUBs (USP7, USP13, USP15, USP20, CSN5 and UCHL5) whose depletion yielded significant and reproducible DSB repair defects in neutral comet assays (Fig. 4b). Moreover, we carried out clonogenic survival assays to establish the effect of depleting these DUBs on cellular sensitivity to IR. This revealed that depletion of each of these DUBs resulted in significant IR...
Figure 4 Verification of screen results. (a) Three-dimensional scatter plot of screen results. Localization to DNA-damage sites is divided into three categories on the x-axis (no effect, recruited or excluded). Immunoblotting screen results were scored on the basis of the numbers of altered phosphorylation signals (0, 1, 2 and 3). For the DSB repair assay, tail moment ratio is plotted on the z-axis. DUBs are coloured on the basis of comet assay DNA repair defects, as indicated by the bar on the left. (b) Neutral comet assays with two individual siRNAs targeting indicated DUBs. Data show the means of two (DUB depletions) or three (control siRNA) biologically independent experiments. The blue line indicates twice the s.d. of the siRNA control. DUBs that scored positive in all three screens are shown in blue. (c) Clonogenic survival assays with individual siRNAs targeting top hit DUBs from the screen. Data represent the individual results of two biologically independent experiments (solid lines and dashed lines).
Figure 5 UCHL5 promotes HR repair. (a) Left: clonogenic survival assays with IR. Depletions of XRCC4 (XRCC4 siRNA) and BRCA1 (BRCA1 siRNA) are positive controls. Data show the means of four biologically independent experiments. The error bars indicate s.e.m. Right: depletion efficiency of UCHL5 with siRNAs targeting the coding sequence (no. 1 and no. 2) or 3' UTR. Tubulin is shown as a loading control. (b, c) U2OS cells or U2OS cells carrying a direct repeat GFP reporter transfected with the indicated siRNAs were processed for NHEJ (b) or HR (c) repair assays; Ligase IV depletion (LigIV siRNA) and CtIP depletion (CtIP siRNA) are respective positive controls. Data show the means of two (b) or three (c) biologically independent experiments, respectively. *P values are indicated by asterisks (**P < 0.005; ***P < 0.001). The error bars indicate s.e.m. (d) GFP or GFP–UCHL5 (wild-type, WT, or deubiquitylase dead, DD) expressing U2OS cells transfected with the indicated siRNAs were processed for neutral comet assays. Data represent the means of two biologically independent experiments. (e) U2OS cells stably expressing RFP–53BP1 were transiently transfected with plasmids encoding GFP–UCHL5 (WT or DD) and subjected to laser micro-irradiation. Images were taken before (undamaged, UD) or 15 min after irradiation (damaged, D). Localization of endogenous UCHL5 to the site of DNA damage was not examined owing to a lack of a suitable antibody. Arrows indicate irradiated areas. Scale bars, 10 μm.

hypersensitivity, demonstrating that our screen effectively identified DUBs with positive roles in DSB repair (Figs 4a–c and 5a; note that USP11 depletion, which we had found to result in a DSB repair defect in the comet assay, also caused mild IR hypersensitivity, consistent with a previous report linking it to the DDR; ref. 17). Although it will be worthwhile pursuing DDR functions for all of these DUBs as well as others identified as putative DDR regulators by our screens, we focused our further analyses on UCHL5 (ubiquitin C-terminal hydrolase L5; also known as UCH37). This protein was prioritized because it was the only DUB other than the deneddylase CSN5 that was positive in all three of our screening assays (localization, DDR signalling and DSB repair) and because we found that deleting each member of the human UCH DUB family resulted in DSB repair defects (Figs 3b and 4a).

UCHL5 promotes HR and extensive DNA-end resection

As shown in Fig. 5a, we found that UCHL5 depletion rendered cells hypersensitive to IR in clonogenic survival assays, producing sensitivity similar to that caused by depleting BRCA1, a well-characterized HR factor. On further investigation, we observed no effect of UCHL5 depletion on NHEJ as assessed by random plasmid integration (Fig. 5b). By contrast, depleting UCHL5 with three distinct siRNAs led to significant impairments in HR repair efficiencies as measured by a chromosomal DSB-induced gene-conversion assay...
Figure 6 UCHL5 contributes to resection by regulating EXO1 recruitment. (a–c) Cells transfected with indicated siRNAs were treated with camptothecin (CPT, 1 μM, 1 h) then subjected to immunoblotting with indicated antibodies (a) or quantitative resection assay with anti-BrdU antibody (means of three biologically independent experiments TC = U ± s.e.m., * P < 0.05) (b) or anti-RPA antibody (data represent the means of two biologically independent experiments) (c). (d,e) GFP or GFP–UCHL5 (WT or DD) expressing U2OS cells transfected with indicated siRNAs were treated or mock treated with 1 μM CPT for 1 h and analysed by immunoblotting as indicated. (f) Intensity of GFP–CtIP at DNA damage sites relative to the unirradiated area was quantified 15 min after irradiation. Data show the means of three biologically independent experiments with error bars indicating s.e.m. (g) Kinetics of GFP–EXO1 accumulation at DNA-damage sites was assessed in cells transfected with indicated siRNAs. The signal intensity of GFP–EXO1 at DNA-damage sites relative to the unirradiated area was quantified. Data show the means of three biologically independent experiments with error bars indicating s.e.m.

system (Fig. 5c; as shown in Supplementary Fig. 1a, this was not associated with potentially confounding changes in cell-cycle profiles). Furthermore, in accordance with an HR defect, depletion of UCHL5 resulted in hypersensitivity to camptothecin, which stabilizes topoisomerase I cleavage complexes, leading to DNA-replication-dependent DSBs in S-phase that are repaired by HR-mediated mechanisms (Supplementary Fig. 1b). To determine whether these defects were indeed due to UCHL5 depletion, we established stable cell lines expressing GFP alone or GFP-tagged UCHL5 (GFP–UCHL5; Supplementary Fig. 1c). These cells were treated with a control siRNA, an siRNA targeting both endogenous UCHL5 and the GFP–UCHL5 construct, or an siRNA against the UCHL5 3′ untranslated region (3′UTR) to target endogenous UCHL5 but not GFP–UCHL5 (Fig. 5d). Importantly, the effects of endogenous UCHL5 depletion on phleomycin-induced DSB repair were rescued by expressing wild-type GFP–UCHL5 but not by expressing a GFP–UCHL5 construct.
(deubiquitylase dead; DD; ref. 27) lacking deubiquitylase activity because Cys 88 was replaced by alanine (Fig. 5d and Supplementary Fig. 1c). This was despite GFP–UCHL5 DD being recruited to sites of damage as efficiently as wild-type GFP–UCHL5 (Fig. 5e). Taken together, these data supported a model in which UCHL5 is recruited to sites of DNA damage, where its deubiquitylase activity then promotes DSB repair.

To gain insights into how UCHL5 promotes DSB repair, we examined the effect of its depletion on DDR signalling following camptothecin treatment. This revealed that UCHL5 depletion impaired phosphorylation of replication protein A subunit 2 (RPA2) on Ser 4/Ser 8 (Fig. 6a). As RPA2 Ser 4/Ser 8 phosphorylation defects correlate with compromised DNA-end resection28–30, we assessed single-stranded DNA (ssDNA) production after camptothecin treatment. This revealed that ssDNA focus formation was reduced, although still detectable, on UCHL5 depletion (Supplementary Fig. 1d). To quantify ssDNA formation, we established a flow-cytometry-based method to measure the signal intensity of native anti-BrdU (5-bromo-2’-deoxyuridine) staining in cells after camptothecin treatment (Methods). Thus, we found that UCHL5 depletion reduced resection of camptothecin-induced DSBs to a similar extent as did depleting the end-resection factors EXO1 or BLM (Fig. 6b). Furthermore, whereas UCHL5 depletion did not abolish camptothecin-induced RPA focus production (Supplementary Fig. 1e,f), the intensities of RPA on ssDNA were reduced (Fig. 6c). UCHL5 depletion also reduced camptothecin-induced focus formation by the key HR factor RAD51, a phenotype rescued by wild-type UCHL5 but not by catalytically inactive UCHL5 (Supplementary Fig. 1g and data not shown). Furthermore, the camptothecin-induced RPA2 phosphorylation defect caused by UCHL5 depletion was rescued by wild-type but not catalytically inactive UCHL5 (Fig. 6d,e). Collectively, these data suggested that UCHL5 is dispensable for initiation of DNA-end resection but is important for the full resection process.

To define which step(s) leading to DNA-end resection was affected by UCHL5, we examined the DNA-damage recruitment of various factors linked to resection. Although depleting UCHL5 had no marked effect on accumulation of GFP–ChIP (also known as RBBP8, retinoblastoma binding protein 8) at damage sites (Fig. 6f and Supplementary Fig. 1h), it significantly impaired GFP–EXO1 recruitment (Fig. 6g). These observations implied that UCHL5 functions before EXO1 but after ChIP to promote resection and ensuing HR. To examine whether UCHL5 regulates extensive resection pathways involving BLM and possibly DNA2, we carried out assays in cells depleted for various resection factors. As additive effects were observed when UCHL5 was co-depleted with either EXO1 or BLM (Supplementary Fig. 1i), this suggested that UCHL5 affects both EXO1- and BLM-dependent resection processes.

**UCHL5 affects HR repair apart from its function as a proteasome component**

Because UCHL5 is a subunit of the proteasome 19S regulatory particle lid(37,38–32), and as proteasome inhibition causes defective DSB-induced RPA2 phosphorylation33,34, we considered whether UCHL5 depletion might affect resection and HR by causing general proteasome dysfunction. However, we found that, whereas treating cells with the proteasome inhibitor MG132 resulted in significant accumulation of ubiquitylated proteins, this did not occur on UCHL5 depletion (Supplementary Fig. 2a). Moreover, although 53BP1 (also known as TP53BP1, tumour protein p53 binding protein 1) focus formation after IR was inhibited by MG132 treatment or depletion of the DDR ubiquitin E3 ligase RNF8 (refs 35–37), it was not affected by UCHL5 depletion (Supplementary Fig. 2b). Furthermore, although MG132 treatment strongly inhibited camptothecin-induced DNA-end resection (Supplementary Fig. 2c), depletion of hRPN13 (ADRM1), which recruits UCHL5 to the proteasome and enhances *in vitro* UCHL5 deubiquitylating activity27, had only marginal effects on resection and accumulation of ubiquitylated proteins (Supplementary Fig. 2d,e), suggesting that the role of UCHL5 in resection is distinct from its proteasomal function30,31. Although these findings did not exclude a possible DSB repair function of UCHL5 in association with the proteasome, they suggested that UCHL5 might affect resection and HR through other mechanisms.

**UCHL5 aids resection by protecting NFRKB from proteasomal degradation**

Previous work has established that UCHL5 is a component of both the proteasome and the INO80 chromatin remodelling complex, these complexes being mediated through UCHL5 interactions with hRPN13 and NFRKB, respectively (Supplementary Fig. 3a; refs 30,31,38). We found that UCHL5 depletion reduced the steady-state level of NFRKB but not hRPN13 (Fig. 7a and data not shown). Also time-course studies in cells treated with cycloheximide, to prevent *de novo* protein synthesis, revealed that UCHL5 depletion reduced NFRKB protein half-life (Fig. 7b). UCHL5 depletion did not, however, reduce NFRKB messenger RNA levels, or protein levels of other INO80-complex subunits with suggested roles in DSB repair (Supplementary Fig. 3b,c; refs 39–42). Furthermore, NFRKB reduction caused by UCHL5 depletion was rescued in cells expressing GFP–UCHL5 and was prevented by MG132 treatment (Fig. 7c and Supplementary Fig. 3d). During these studies we observed that, when cells were incubated with MG132, NFRKB in the chromatin fraction was modified in a manner enhanced by UCHL5 depletion, suggestive of ubiquitylation (Supplementary Fig. 3d). In addition, immunoprecipitation and western blotting studies established that GFP–NFRKB but not GFP alone was conjugated with ubiquitin moieties in the chromatin fraction (Fig. 7d; for controls, see Supplementary Fig. 3e,f). Also, we found that purified UCHL5 could act to remove ubiquitylations on NFRKB and/or associated proteins *in vitro* (Supplementary Fig. 3g). Taken together, these findings suggested that UCHL5 removes ubiquitin chains to protect NFRKB from proteasomal degradation.

Consistent with a model in which the effects of UCHL5 on DSB repair reflect its stabilization of NFRKB, we found that, as with UCHL5 depletion, NFRKB depletion reduced DNA-end resection in a manner complemented by a GFP–NFRKB expression construct that was resistant to a 3’UTR-targeting siRNA (Fig. 7e and Supplementary Fig. 4a). In addition, as observed for UCHL5 depletion, NFRKB depletion reduced the intensity but not the proportion of cells with camptothecin-induced RPA foci (Supplementary Fig. 4b–d). Furthermore, NFRKB depletion decreased HR efficiency in cells as examined by a modified ‘traffic light reporter system’ (Methods), RAD51 focus formation, IR resistance and camptothecin-induced
UCHL5 regulates resection by protecting NFRKB from proteasomal degradation. (a) Cells transfected with indicated siRNAs were processed for immunoblotting with indicated antibodies. The arrow indicates the position of NFRKB. (b) Left: cells transfected with indicated siRNAs were incubated with 100 μg/ml cycloheximide (CHX) for various times and processed for immunoblotting with indicated antibodies. Right: quantification of data shown on left. Data show the means of two biologically independent experiments. (c) GFP or GFP–UCHL5 expressing cells transfected with indicated siRNAs were analysed by immunoblotting as indicated. (d) GFP and GFP–NFRKB expressing cells were mock transfected or transiently transfected with an expression plasmid of haemagglutinin-tagged ubiquitin (HA–Ub). Chromatin fractions were immunoprecipitated with an anti-GFP antibody followed by immunoblotting. Brackets indicate ubiquitylated NFRKB. For inputs, see Supplementary Fig. 3f. (e) GFP or GFP–NFRKB stably expressing cells transfected with indicated siRNAs were subjected to quantitative resection assays. Data show the means of two biologically independent experiments. (f) Modified ‘traffic light reporter system’ based HR assay with indicated siRNAs. Data show the means of two biologically independent experiments. (g) U2OS cells transfected with indicated siRNAs were subjected to clonogenic survival assays after IR. Data show the means of two biologically independent experiments. (h) Cells transfected with individual or indicated combinations of siRNAs were processed for quantitative resection assays. Data show the means of two biologically independent experiments. (i) U2OS cells transfected with indicated siRNAs were subjected to quantitative resection assays. Data show the means of two biologically independent experiments. (j) U2OS cells were processed for quantitative resection assays. Data show the means of two biologically independent experiments. (k) U2OS cells were incubated with CHX (100 μg/ml) and or MG132 (10 μM) for 1 h before camptothecin (CPT) treatment (1 μM, 1 h). Nucleoplasmic fractions were subjected to immunoblotting with indicated antibodies. Relative protein levels of NFRKB are indicated with normalization by HDAC1 levels. (l) After incubating GFP or GFP–NFRKB expressing cells with 10 μM MG132, nucleoplasmic (Nu) and chromatin (Ch) fractions were immunoprecipitated with anti-GFP antibody followed by immunoblotting. For inputs, see Supplementary Fig. 4l.
RPA2 Ser 4/Ser 8 phosphorylation (Fig. 7f,g and Supplementary Fig. 4e,f), without markedly affecting cell cycle profiles or levels of the HR proteins XRCC3 and RAD54B (ref. 41; Supplementary Fig. 4g,h). In accord with these findings, co-depleting NFRKB and UCHL5 had effects on resection similar to those of their individual depletions (Fig. 7h and Supplementary Fig. 4i). Importantly, we found that depleting INO80 chromatin remodelling complex core subunits INO80, YY1 or RUVBL1 (which are responsible for in vitro nucleosome remodelling activity of the complex42) also resulted in defective resection, HR repair efficiency, IR resistance and RPA2 phosphorylation (Fig. 7i,f,g and Supplementary Fig. 4j). Also, we found that NFRKB depletion or YY1 depletion reduced the recruitment of EXO1 to DNA-damage sites, but had no discernible effect on GFP-CtIP recruitment (Supplementary Fig. 4k and data not shown). These findings therefore suggested that NFRKB contributes to DNA-end resection as a part of the INO80 chromatin remodelling complex, and support a model in which the functions of UCHL5 in DSB resection and repair are specifically connected to its role in stabilizing NFRKB. During the course of our studies, we observed that NFRKB protein levels were reduced somewhat on camptothecin treatment, in a manner that was largely prevented by proteasome inhibition (Fig. 7j), suggesting that, although it promotes resection and HR (Fig. 7e,f), NFRKB may undergo proteasome-dependent degradation/turnover after DSB induction. Interestingly, immunoprecipitation studies from MG132-treated cells revealed that NFRKB interacted with UCHL5 less in the chromatin fraction than in the nucleoplasm, despite its interactions with INO80 being essentially equivalent in these two fractions (Fig. 7k; for input fractions, see Supplementary Fig. 4l). Collectively, these results suggested that ubiquitylation and degradation/turnover of NFRKB are regulated by dynamic, chromatin-compartment-specific interactions with UCHL5 that are affected by DNA-damage induction, perhaps as a mechanism to prevent excessive ssDNA formation at DNA damage sites.

**DISCUSSION**

Through carrying out focused, multifaceted systematic functional screening, we have identified DUBs that are recruited to or excluded from DNA-damage regions, as well as DUBs whose depletion affects G2/M checkpoint control, DSB induction, DSB repair and/or DSB-induced DDR signalling. In addition to identifying DUBs with already established links to DDR processes, our findings have indicated DDR functions for DUBs that had not hitherto been connected to such events. This work thus provides a resource that will be of value in future studies to define DDR and potentially other functions for DUBs and their targets. Highlighting this potential, by studying one DUB arising from our screens, UCHL5, we have established that it functions to modulate the stability of the NFRKB component of the INO80 complex to promote HR through enhancing the key process of DNA-end resection, downstream of CtIP and at the level of EXO1 recruitment. Whereas an involvement of the INO80 complex in DSB repair has been reported in yeast44, and although studies in mammalian cells have connected the INO80 complex to resection39,40,42,43, it was not known how this occurs and whether the INO80 complex directly contributes to resection rather than affecting it indirectly through its roles in transcription. We have revealed that UCHL5 and NFRKB, non-essential for the in vitro nucleosome sliding activity of the INO80 complex44 and not conserved in yeast, enhance resection by regulating the recruitment of the resection factor EXO1. This suggests that UCHL5 and NFRKB have acquired INO80-related functions in higher eukaryotes to promote and control resection in the context of higher-order chromatin or other chromatin features distinct from those found in simpler organisms. It will therefore be worthwhile exploring whether UCHL5 and INO80 control resection and HR in more compact regions of chromatin that may be recalcitrant to HR processes46. Given that UCHL5 depletion also resulted in moderately reduced phosphorylation of γH2AX in addition to CHK1 in our immunoblotting screens, it will also be interesting to investigate whether UCHL5 and the INO80 complex have roles in the DDR in addition to their HR-related functions. Finally, we note that developing small-molecule inhibitors of UCHL5 or other DUBs highlighted by our screens as having DDR functions might provide opportunities for therapeutic targeting of cancers exhibiting high levels of DNA damage or that have underlying defects in DDR processes or chromatin components.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

R.N. designed experiments through discussion with P.W., J.T., C.I.S., Y.G. and S.P.J.; P.W., R.N., M.J.C. and S.U. cloned human DUBs. R.N., P.W., C.I.S. and J.T. carried out the screens. P.W. and C.I.S. carried out cell cycle analyses. R.N. carried out most of the other studies with P.W. or S.U. assistance. R.N. and S.P.J. wrote the paper. All other authors, especially P.W., commented and suggested revisions for the paper.

**COMPETING FINANCIAL INTERESTS**

S.P.J. is a founder and shareholder of MISSION Therapeutics Ltd., which is developing small-molecule inhibitors of UCHL5 or other DUBs in addition to their HR-related functions. Finally, we note that developing small-molecule inhibitors of UCHL5 or other DUBs highlighted by our screens as having DDR functions might provide opportunities for therapeutic targeting of cancers exhibiting high levels of DNA damage or that have underlying defects in DDR processes or chromatin components.

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**AUTHOR CONTRIBUTIONS**

R.N. designed experiments through discussion with P.W., J.T., C.I.S., Y.G. and S.P.J.; P.W., R.N., M.J.C. and S.U. cloned human DUBs. R.N., P.W., C.I.S. and J.T. carried out the screens. P.W. and C.I.S. carried out cell cycle analyses. R.N. carried out most of the other studies with P.W. or S.U. assistance. R.N. and S.P.J. wrote the paper. All other authors, especially P.W., commented and suggested revisions for the paper.

**COMPETING FINANCIAL INTERESTS**

S.P.J. is a founder and shareholder of MISSION Therapeutics Ltd., which is developing DUB inhibitors for therapeutic applications. The other authors declare no competing financial interests.

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METHODS

Cell lines and cell culture. All cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere. U2OS cell lines stably expressing GFP (ref. 47), GFP–33BP1, RFP–33BP1 (ref. 48), GFP–UCHL1 (wild type and DD), GFP–NFRKB and GFP–CIP2A (pH 6.8, 3 mM MgCl₂, 1 mM EDTA, 0.5% Triton X-100, 0.3 mM sucrose and 30 mM NaCl) for 24 h before fixing with paraformaldehyde for 15 min at room temperature and then permeabilized by incubating with 0.2% Triton X-100 in PBS for 5 min at room temperature. To examine RPA2 foci formation, cells were transfected with control siRNA or in U2OS cells carrying a modified ‘trac light’ DSB-induced gene-conversion assay system with transient expression of reagent treatment.

Repeat–GFP and U2OS cells carrying modified traffic light reporter based HR assay were cultured with DMEEM containing FBS, penicillin, streptomycin, 1-glutamine and 1 μg ml⁻¹ of puromycin (Sigma-Aldrich).

Live cell imaging based screening. U2OS cells stably expressing RFP–33BP1 in 35 mm glass-bottom dishes (WPIco-dish) were transfected with 1 μg of expression plasmids coding each GFP–DUB with Fagene 6 and further cultured for 48 h in the presence of 10 μM 5-bromo-2-deoxyuridine (BrdU). On the day of analysis, the media was replaced with phenol red-free DMEEM (Sigma-Aldrich) supplemented with 10% FBS, penicillin, streptomycin and 25 mM HEPES buffer (pH 7.0–7.6, Sigma-Aldrich). DNA damage was induced by irradiating cells through a UPlanSapo x60/1.35 oil objective lens with an ultraviolet-A laser (405 nm) using a DX1 confocal microscope (Olympus) equipped with a 37°C heating stage (Bild). The laser output was set at 400 μW with 50 scans of 10 ms per pixel. Up to 1 h after damage induction, images were taken and analysed using FluoView 1000 software (Olympus).

Immunoblotting-based screen. U2OS cells were transfected with 30 nM of control siRNA (ABStars Negative Control, QIAGEN) or an siRNA pool of four siRNAs targeting each DUB (QIAGEN) over two days using HiPerfect (QIAGEN). Seventy-two hours after the initial siRNA transfection, the cells were treated with 40 μg ml⁻¹ of phleomycin for 2 h or mock treated. After phleomycin removal, the cells were further cultured for 6 h. For immunoblotting analysis, cell extracts were prepared with Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulphate and 5% β-mercaptoethanol) at each time and the protein concentration was determined by using the absorbance at 280 nm using a NanoDrop (Thermo Scientific) with a bovine serum albumin protein standard (Thermo Scientific).

Neutral comet assay. Seventy-two hours after 30 nM siRNA transfection, the cells were incubated with 40 μg ml⁻¹ phleomycin for 2 h or mock incubated. Following phleomycin treatment, cells were washed twice with PBS and cultured for a further 2 h. The cells were subsequently washed twice with PBS (–) (Gibco) and collected by trypsinization. Approximately 5 × 10⁴ cells in 10 μl of PBS (–) were mixed with 90 μl of LMAgarose ( Trevigen), placed on GelBond Film (Lonza), covered with a 22 mm cover slide (VWR International) and left at 4°C for 1 h. On removal of the cover slide, the cells were lysed with lysis solution (Trevigen) at 4°C for 1 h. Following a wash with TBE (90 mM Tris borate (pH 8.3) and 2 mM EDTA), the samples were subjected to electrophoresis at 35 V, for 7 min in TBE. After washing the TBE, samples were fixed with 70% ethanol for 5 min at room temperature and dried overnight. The nuclei were stained with SYBR Green I (Invitrogen) in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA for 5 min at 4°C. Images were taken with an IX71 fluorescence microscope (Olympus) with Cell F software (Olympus). Tail moments were measured using CometScore software (TriTek). The means of tail moment of at least 30 cells or 30 cells were measured for each sample with mRNA pools or for the assay with the individual siRNAs, respectively. Efficiency of DSB repair was determined as the tail moment ratio between 2 h after phleomycin removal and immediately after treatment.

Clonogenic survival assay. Clonogenic viability was examined using a colony formation assay. Briefly, 48 h after initial transfection with siRNAs, cells were seeded in six-well plates and treated with acute IR or various doses of camptothecin for 1 h on the following day. Colonies were stained with crystal violet solution (2% crystal violet (Sigma-Aldrich) in 10% ethanol) 10–13 days after DNA damaging reagent treatment.

NHEJ and HR repair assay. NHEJ and HR repair assay was carried out as previously described. Briefly, NHEJ repair efficiency was examined by random plasmid integration. HR repair efficiency was investigated by a chromosomal DSB-induced gene-conversion assay system with transient expression of 1-Scel restriction enzyme in U2OS cells carrying direct-repeat GFP reporter as previously described or in U2OS cells carrying a modified ‘trac light’ reporter system that was modified from the published protocol (J. Y. F. et al. personal communication).

Immunofluorescent staining. For the purpose of ssDNA detection, cells were fixed immediately with 0.8% formaldehyde for 20 min at room temperature. After permeabilizing with 0.25% triton X-100 in PBS, cells were incubated with primary antibodies at 4°C overnight. Secondary antibodies were used with a 1:200 dilution and a 1:200 dilution of Dylight-488 coupled with Alexa-647 (Life Technology) in the dark for 30 min. Samples were washed twice with PBS and stained for 10 min with 3 μg ml⁻¹ DAPI solution. The nuclei were counted using an anti-DNA antibody conjugated with Alexa Fluor 594 and anti-mouse IgG (Vector Laboratories). Neural cell lines and cell culture. All cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere. U2OS cell lines stably expressing GFP (ref. 47), GFP–33BP1, RFP–33BP1 (ref. 48), GFP–UCHL1 (wild type and DD), GFP–NFRKB and GFP–CIP2A (pH 6.8, 3 mM MgCl₂, 1 mM EDTA, 0.5% Triton X-100, 0.3 mM sucrose and 30 mM NaCl) were used for 24 h before 1 μM of camptothecin treatment for 1 h. Following camptothecin treatment, cells were collected by trypsinization, washed twice with PBS and fixed with 70% ethanol at −20°C overnight. After fixation, cells were washed twice with PBS for 5 min at 4°C. The cells were resuspended with nuclear extraction buffer (20 mM HEPES (pH 7.4), 10 mM MgCl₂, 1 mM EDTA, 25% glycerol, 0.1% Triton X-100) containing 300 mM NaCl, 1 μl of proteinase inhibitor cocktail, and 1 μl of 50 mM phenylmethylsulphonyl fluoride (PMSF, Sigma-Aldrich). Cells were washed twice with ice-cold PBS and incubated with an appropriate volume of CSK buffer for 1 h on ice with occasional mixing. Soluble fractions were collected by centrifugation at 20,000 g for 10 min at 4°C. The residual pellet fraction was washed twice and resuspended with the same buffer, followed by sonication. For immunoblotting-based screens, after washing with ice-cold PBS, cells were lysed with Laemmli buffer and boiled for 5 min at 95°C. For cellular fractionation, cells were collected with ice-cold PBS, washed in 0.5 M of camptothecin treatment for 1 h. Following camptothecin treatment, cells were collected by trypsinization, washed twice with PBS and fixed with 70% ethanol at −20°C overnight. After fixation, cells were washed twice with PBS for 5 min at 4°C. The cells were resuspended with nuclear extraction buffer (20 mM HEPES (pH 7.4), 10 mM MgCl₂, 1 mM EDTA, 25% glycerol, 0.1% Triton X-100) containing 300 mM NaCl, 1 μl of proteinase inhibitor cocktail, and 1 μl of 50 mM phenylmethylsulphonyl fluoride (PMSF, Sigma-Aldrich). The cytoplasmic fraction was isolated by low speed centrifugation (1,500 g, 5 min at 4°C). The residual pellet was washed once with ice-cold buffer and resuspended with nuclear extraction buffer containing 0.5 M of NaCl, solubilized by sonication and saved as a chromatin fraction. The protein concentration of cell extracts was determined with the Coomassie protein assay reagent (Thermo Scientific).

Quantitative DNA-end resection assay. For direct detection of ssDNA formation in the context of DNA-end resection, cells were incubated with 30 μM of BrdU for 24 h before 1 μM of camptothecin treatment for 1 h. Following camptothecin treatment, cells were collected by trypsinization, washed twice with PBS and fixed with 70% ethanol at −20°C overnight. After fixation, cells were washed twice with PBS for 5 min at 4°C. After incubating with blocking buffer (5% FBS, 0.1% Triton X-100 in PBS) for 30 min, cells were incubated for 1 h with anti-BrdU antibody and anti-cyclin A antibody in blocking buffer under non-denaturing conditions to detect only ssDNA with anti-BrdU antibody. The cells were further incubated with anti-rabbit IgG antibody conjugated with Alexa Fluor 594 and anti-mouse IgG antibody conjugated with Alexa Fluor 488 (Life Technology) in the dark for 1 h. The cells were suspended in 1 μl of DAPI solution and processed with LSRII Fortessa (BD Biosciences). RPA loading onto ssDNA generated through DNA-end resection during HR was quantitatively measured as previously described by using an anti-cyclin A antibody instead of an anti-human histone H2A antibody. The anti-BrdU or anti-RPA antibody signal intensities were obtained from a subpopulation of cells that was positive for anti-cyclin A antibody staining using FlowJo software (TreeStar). After subtraction of the camptothecin non-treated background signal, the mean intensity of the anti-BrdU and anti-RPA antibody staining of each sample was normalized to that seen immediately after camptothecin treatment with siRNA control.

Immunoprecipitation. Nucleoplasm fractions, chromatin fractions or soluble fractions from cell extracts prepared with CKS buffer were immunoprecipitated with an anti-GFP antibody coupled to agarose beads (GFP-Trap, ChromoTek) or anti-HA antibody coupled to agarose beads (EZview Red anti-HA affinity gel, Sigma-Aldrich) by rotating overnight at 4°C. The beads were washed six times with their respective cell extraction buffer and bound proteins were eluted by boiling at 95°C for 10 min with 1 × Laemmli SDS buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10%
glycerol, 0.002% bromophenol blue). For the detection of ubiquitylated proteins, beads were washed twice with the buffer used for cell extract preparation, three times with same buffer containing 500 mM NaCl and once with the original buffer, followed by elution as described above.

**Cell cycle profile analysis.** U2OS cells were incubated for 30 min with 10 µM of BrdU, harvested by trypsinization and fixed with 70% ethanol overnight at −20 °C. Hereafter, the samples were washed twice with PBS containing 0.1% Tween 20. The samples were then incubated with denaturing solution (5 M HCl and 0.5% Triton X-100) at 37 °C for 20 min and neutralized with 0.1 M Na2B4O7. After blocking buffer incubation, samples were incubated with anti-BrdU antibody and an Alexa Fluor 488 conjugated secondary antibody. Finally, the samples were incubated with PI buffer (10 µg ml⁻¹ propidium iodide (Invitrogen), 250 µg ml⁻¹ RNase A (Invitrogen)) for 20 min at 37 °C and analysed with LSRFortessa.

**In vivo ubiquitylation assay.** The HA-Ub expression vector was transiently transfected in appropriate cell lines. Forty-eight hours after transfection, fractionated cell extracts were prepared and subjected to immunoprecipitation as described above.

**In vitro deubiquitylation assay.** Stable cell lines expressing GFP-NFRKB were transiently transfected with a plasmid coding HA-Ub, and 48 h after transfection 2 mg of nucleoplasm extracts were prepared as described above. GFP-NFRKB was immunoprecipitated with anti-GFP antibody conjugated beads in an identical way to that described in the immunoprecipitation section. Furthermore, the beads were washed twice with deubiquitylation buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1 x protease inhibitor cocktail, 1 x phosphatase inhibitor cocktail, and 0.25 mM PMSF). The deubiquitylation reaction was carried out in a total volume of 50 µl deubiquitylation buffer by incubating immunoprecipitated GFP-NFRKB (approximately 125 ng) with 100 or 200 ng of recombinant GST-UCHL5 (Abnova) for 1 h at 37 °C. The reaction was terminated by adding 12.5 µl of 4 x SDS sample buffer and subsequent boiling at 95 °C for 10 min. The supernatant was subjected to immunoblotting analysis.

**Quantitative PCR with reverse transcription.** Total mRNA was isolated from U2OS cells transfected with siRNAs using an RNasey kit (QIAGEN), and residual genomic DNA was digested with a TURBO DNA-free kit (Life Technologies). Total mRNAs were reverse transcribed into complementary DNA using a SuperScript III First-Strand Synthesis System (Life Technologies) with an oligo-dT primer. Quantitative PCR was carried out using Fast SYBR Green Master Mix (Life Technologies) and a StepOnePlus Real-Time PCR System (Life Technologies) with glyceraldehyde-3-phosphate dehydrogenase targeting primers (5'-CTGACCAGCTTCTTTGTG-3', 5'-GCAGCGAATACGACCAAATC-3') and NFRKB targeting primers (5'-GATTGGGCGCAATTCCCATC-3', 5'-CACACCTGCGACCTGAACA-3').

**Live cell imaging with laser micro-irradiation.** U2OS cells stably expressing GFP-CtIP or both GFP-EXO1 and mKO2-hGeminin were incubated with 10 µM of BrdU for 24 h before laser micro-irradiation. For quantitative analysis of accumulation to sites of damage, cells were irradiated with reduced laser output (200 µW) to avoid excess generation of ssDNA. For GFP-EXO1 accumulation kinetics, images were taken every 6 s up to 300 s after irradiation with the microscope and software as described for live cell imaging based screening (previous section). For GFP-CtIP, images were taken 15 min after irradiation. Each single experiment contains at least 10 cells and in total at least 30 cells were analysed.

**Ionizing irradiation.** IR was carried out with a Faxitron X-ray machine (Faxitron X-ray Corporation).

**Antibodies and siRNAs.** Antibodies and siRNAs used here are summarized in Supplementary Tables 2 and 3, respectively.

**Statistical and quantitative analysis.** All statistical analysis was done by a standard two-sided Student t-test. For quantitative analysis, the mean was used as a centre value. The experiments shown with representative images were successfully reproduced at least twice.

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Supplementary Figure 1 UCHL5 is dispensable for resection initiation but required for full resection. (a) Cells transfected with the indicated siRNAs were subjected to cell cycle profile analysis. Data show the means of three biologically independent experiments. The error bars indicate standard error of means. (b) Clonogenic survival assay with camptothecin (CPT). Depletion of CtIP (siCtIP) is a positive control. Data show the means of two biologically independent experiments. (c) Stable cell lines expressing GFP or GFP-UCHL5 (WT or DD) were processed for immunoblotting with indicated antibodies. Tubulin is used as a loading control. Protein levels of GFP and GFP-UCHL5 derivatives are indicated below. The modified bands detected with GFP-UCHL5 (DD) are ubiquitylated UCHL5 as revealed by IP-western blotting studies (data not shown). (d, e, f, g) Cells transfected with indicated siRNAs were treated with 1 μM of CPT for 1 h and processed for immunofluorescent staining with indicated antibodies. Nuclei were stained with DAPI. Depletion of CtIP (siCtIP) is a positive control. (f) Quantification of data shown in e. Proportion of cells with >10 RPA foci in γH2AX positive cells were calculated by counting 100 γH2AX positive cells per experiment. Data show the means of two biologically independent experiments. (h) Kinetics of GFP-CtIP accumulation at DNA damage sites was assessed in cells transfected with indicated siRNAs. Signal intensity of GFP-CtIP at DNA damage sites relative to the unirradiated area was quantified. Data show the means of two biologically independent experiments. (i) Cells transfected with the individual or indicated combinations of siRNAs were processed for quantitative resection assays. Data show the means of two biologically independent experiments. Scale bars indicate 10 μm.
Supplementary Figure 2 UCHL5 depletion does not result in general proteasome dysfunction. (a) Cells treated with indicated siRNAs or proteasome inhibitor MG132 (10 μM for 6 h) were processed for immunoblotting with indicated antibodies. (b) U2OS cells stably expressing GFP-53BP1 transfected with indicated siRNAs were mock irradiated (- IR) or irradiated with 5 Gy of IR followed by 1 h incubation (+ IR). Depletion of RNF8 or PSMD14 and MG132 treatment (10 μM for 1 h) were positive controls. Scale bar indicates 10 μm. (c) Quantitative resection assay with MG132 treatment (10 μM for 1 h). Data show the means of two biologically independent experiments. (d) Quantitative resection assay with indicated siRNAs. Data show the means of two biologically independent experiments. (e) Cells treated with indicated siRNAs or MG132 (10 μM for 6 h) were processed for immunoblotting with indicated antibodies.
Supplementary Figure 3 UCHL5 protects NFRKB from proteasomal turnover.

(a) GFP or GFP-UCHL5 (WT or DD) expressing cells were subjected to immunoprecipitation with an anti-GFP antibody, followed by immunoblotting with the indicated antibodies.

(b) Cells transfected with indicated siRNAs were subjected to RT-qPCR for NFRKB mRNA. NFRKB depletion (siNFRKB) is a positive control. Data show the means of two biologically independent experiments.

(c) Cells transfected with indicated siRNAs were processed for immunoblotting with indicated antibodies.

(d) GFP-NFRKB expressing cells transfected with indicated siRNAs were treated or mock treated with MG132 (10 μM for 6 h) and processed for immunoblotting with indicated antibodies. Brackets indicate modified forms of NFRKB. Tubulin and histone H2AX are loading controls.

(e) Stable cell lines expressing GFP or GFP-NFRKB were processed for immunoblotting with indicated antibodies.

(f) Input fractions corresponding to Fig. 7d.

(g) In vitro deubiquitylation assay with recombinant GST-tagged UCHL5 and human cell derived NFRKB modified with HA-tagged ubiquitin.
Supplementary Figure 4 NFRKB depletion results in defective resection similar to UCHL5 depletion. (a) Stable cell lines expressing GFP or GFP-NFRKB were transfected with indicated siRNAs and subjected to immunoblotting analysis with indicated antibodies. (b, c, d) U2OS cells transfected with indicated siRNAs were treated with CPT (1 μM for 1 h) and subjected to immunofluorescent staining with indicated antibodies (b, c) or quantitative DNA-end resection assays with anti-RPA2 antibody. Data show the means of two biologically independent experiments. (d). Scale bar indicates 10 μm. (e) Quantification of data shown in b. Population of cells with >10 RPA foci in γH2AX positive cells were calculated by counting 100 γH2AX positive cells per experiment. Data show the means of two biologically independent experiments. (f) U2OS cells transfected with indicated siRNAs were treated with CPT (1 μM for 1 h) and subjected to immunoblotting analysis with indicated antibodies. (g) Cells transfected with indicated siRNAs were subjected to cell cycle profile analysis. Data show the means of two biologically independent experiments. (h) U2OS cells transfected with indicated siRNAs were processed for immunoblotting with indicated antibodies. (i) U2OS cells transfected with indicated combinations of siRNAs were processed for immunoblotting with indicated antibodies. (j) U2OS cells transfected with indicated combinations of siRNAs were treated with 1 μM of CPT for 1 h and processed for immunoblotting with indicated antibodies. (k) Kinetics of GFP-EXO1 accumulation at DNA damage sites was assessed in cells transfected with indicated siRNAs. Signal intensity of GFP-EXO1 at DNA damage sites relative to the unirradiated area was quantified. Data show the means of two biologically independent experiments. (l) Input fractions corresponding to Fig. 7k. Arrows indicate NFRKB protein band. Tubulin is used as a loading control.
Supplementary Table legends

**Supplementary Table 1 Summary of data from primary screens.**
DUBs, which are classified into five subfamilies (USP, UCH, OTU, MJD and JAMM) based on their catalytic domains, examined in this study are shown with the results of the primary screens: localisation to DNA damage sites, effects on DDR markers, comet tail moment in cells not treated with DNA-damaging agent, and effect on G2/M checkpoint. Subcellular localisations (nucleus and/or cytoplasm) of GFP-fused DUBs are also indicated (> represents greater than). The immunoblotting screen was scored based on numbers of altered phosphorylation signals (0, 1, 2 and 3) of CHK1 (S345), CHK2 (T68) and H2AX (S139). Effects on G2/M DNA-damage checkpoint and increased tail moment without DNA damage are shown. In the case that elevated phosphorylation signal of histone H3 Ser 10 was observed, or increased tail moment was detected upon DUB depletion, these DUBs are marked with +. Numbers (#) on the left column given to DUBs correspond to the order of scores in the G2/M checkpoint defect screen (also see Fig. 2a). Positive results are coloured green or red corresponding to the degree of defects as shown in Figure 2. Not tested: n.t. Tag locations indicate whether the tag was fused to the DUB N- or C-terminus.

**Supplementary Table 2 List of antibodies used in this work.**
Immunoblotting: IB. Immunofluorescent staining: IF. Flow cytometry analysis: FC. The clone number of the antibody was indicated for monoclonal antibodies. Antibody dilutions are also shown for the relevant application(s).

**Supplementary Table 3 List of siRNAs used in this work.**
| Subfamily | # | DUB | Localization to DNA damage sites | Effect on DDR | Increased UD moment | Effect on G2/M checkpoint | Subcellular localization | Tag |
|-----------|---|-----|----------------------------------|--------------|---------------------|--------------------------|--------------------------|-----|
| USP       | 24| USP57 | Recruited                       | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 25| USP56 | Recruited                       | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 26| USP55 | Recruited                       | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 27| USP54 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 28| USP53 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 29| USP52 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 30| USP51 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 31| USP50 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 32| USP49 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 33| USP48 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 34| USP47 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 35| USP46 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 36| USP45 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 37| USP44 | Recruited                       | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 38| USP43 | Recruited                       | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 39| USP42 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 40| USP41 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 41| USP40 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 42| USP39 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 43| USP38 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 44| USP37 | Recruited                       | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 45| USP36 | Recruited                       | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 46| USP35 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 47| USP34 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 48| USP33 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 49| USP32 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 50| USP31 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 51| USP30 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 52| USP29 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 53| USP28 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 54| USP27 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 55| USP26 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 56| USP25 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 57| USP24 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 58| USP23 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 59| USP22 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 60| USP21 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 61| USP20 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 62| USP19 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 63| USP18 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 64| USP17 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 65| USP16 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 66| USP15 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 67| USP14 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 68| USP13 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 69| USP12 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 70| USP11 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 71| USP10 | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 72| USP9  | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 73| USP8  | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 74| USP7  | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 75| USP6  | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 76| USP5  | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 77| USP4  | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 78| USP3  | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 79| USP2  | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |
|           | 80| USP1  | No effect                        | Nucleus      | -                   | -                        | Cytoplasm/Nucleus        | EGFP-N |

**Subfamily: DUB**
- USP
- UCH
- OTU
- MJD
- JAMM

**Effect on DDR:**
- Increased UD moment
- Effect on G2/M checkpoint

**Subcellular localization:**
- Nucleus
- Cytoplasm/Nucleus
- Cytoplasm

**Tag:**
- EGFP-N
- Cytoplasm/Nucleus
- Cytoplasm

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| Antibody target                  | Supplier                  | Catalog Number | Clone number | Application | dilution |
|----------------------------------|---------------------------|----------------|--------------|-------------|----------|
| UCHL5                            | Epitomics                 | 3904-1         | EP4897       | IB          | 1,000    |
| Phospho-Chk1 (S345)              | Cell Signaling Technology | 2348           | 133D3        | IB          | 1,000    |
| Chk1                             | Sant Cruz                 | sc-8408        | G-4          | IB          | 1000     |
| Phospho-Chk2 (T68)               | Cell Signaling Technology | 2661           |              | IB          | 1,000    |
| Chk2                             | Cell Signaling Technology | 2662           |              | IB          | 1,000    |
| Phospho-RPA2 (S4/S6)             | Bethyl Laboratories       | A300-245A      |              | IB/IF       | 10,000/500|
| RPA2                             | Abcam                     | ab2175         | 9H8          | IB/IF       | 1,000/200|
| α-tubulin                        | Sigma-Aldrich             | T9026          | DM1A         | IB          | 5,000    |
| RAD51                            | Calbiochem                | PC130          |              | IF (CPT)    | 1,000    |
| RAD51                            | Sant Cruz                 | sc-8349        |              | IF (IR)     | 50       |
| γH2AX                            | Cell Signaling Technology | 2577           |              | IF          | 100      |
| γH2AX                            | Millipore                 | 05-636         | JBW301       | IB/IF       | 1,000/200|
| H2AX                             | Abcam                     | ab11175        |              | IB          | 5,000    |
| GFP                              | Roche                     | 11814460001    | 7.1 and 13.1 | IB          | 1,000    |
| BrdU                             | GE Healthcare             | RPN20AB        | BU-1         | FC/IF       | 100      |
| NFRKB                            | Bethyl Laboratories       | A301-459A      |              | IB          | 1,000    |
| INO80A                           | Bethyl Laboratories       | A303-371A      |              | IB          | 2,000    |
| YY1                              | Millipore                 | ABE77          |              | IB          | 5,000    |
| RUVBL1                           | Millipore                 | 06-1299        |              | IB          | 1,000    |
| Ubiquitin                        | Cell Signaling Technology | 3936           | P4D1         | IB          | 1,000    |
| Ubiquitin Lys-48 specific        | Millipore                 | 05-1307        | Apu2         | IB          | 500      |
| XRCC3                            | Novus Biologicals         | NB100-180      | 10F1/6       | IB          | 1,000    |
| RAD54B                           | Sant Cruz                 | sc-5852        |              | IB          | 500      |
| USP14                            | Bethyl Laboratories       | A300-919A      |              | IB          | 1,000    |
| Cyclin A                         | Sant Cruz                 | sc-751         |              | FC          | 500      |
| HA                               | Cancer Research UK        | 12CA5          | 12CA5        | IB          | 2,000    |
| HDAC1                            | Cell Signaling Technology | 5356           | 10E2         | IB          | 1,000    |
| Histone H3                       | Abcam                     | ab1791         |              | IB          | 1,000    |
| Phospho-Histone H3 (S10)         | Millipore                 | 06-570         |              | IB          | 1,000    |
| siRNA       | Sequence                        | Supplier       |
|------------|---------------------------------|----------------|
| UCHL5#1    | 5’ CAGCAGUUAAGACACCUAGUA 3’     | QIAGEN         |
| UCHL5#2    | 5’ UCAGAUGUAUCUGCAACUGUA 3’     | QIAGEN         |
| UCHL5-UTR  | 5’ CAGACUUCCUCAGUACUGGA 3’      | QIAGEN         |
| XRCC4      | 5’ AUUUGUUGGUGAACUGGA 3’        | eurofins       |
| LigaseIV   | 5’ AGGAAGUAAUUCUCAAGGAUA 3’     | eurofins       |
| CtIP       | 5’ GCUAAACAGGAAACGGAADC 3’      | eurofins       |
| BRCA1      | 5’ GGAACCUGUCUCCAAAAG 3’        | eurofins       |
| EXO1       | 5’ CAGCCCUAUUCUGGUAUUCUUU 3’    | eurofins       |
| BLM        | 5’ AGCAAGGAUUGUAUUGCA 3’        | eurofins       |
| NFRKB      | 5’ CUGGAAUCGGCUAUAUCGAA 3’      | eurofins       |
| NFRKB-UTR  | 5’ GGGAGGUUGCAUCUUUGU 3’        | QIAGEN         |
| RNF8       | 5’ UGGACAAUUAUGGAAACA 3’        | eurofins       |
| hRpn13     | 5’ GGCUGGUGUAACUUCAGC 3’        | eurofins       |
| USP13#1    | 5’ AGCGAAGAUAUGGAAUAAGAA 3’     | eurofins       |
| USP13#2    | 5’ CACAAGCAGCAAGAUAUUAUU 3’     | eurofins       |
| USP15#1    | 5’ AUGUAAUAUGGUGAUAUGCGA 3’     | eurofins       |
| USP15#2    | 5’ UAGUCUGGAAUUCUUAUAUU 3’      | eurofins       |
| USP7#1     | 5’ CCCAAUAUAUCGCGGCAAA 3’       | eurofins       |
| USP7#2     | 5’ AACCGUCUCAUUGCAUAACA 3’      | eurofins       |
| USP20#1    | 5’ ACCGUCUACUGCCUCAAGAA 3’      | eurofins       |
| USP20#2    | 5’ UGGAGAUGACGCAUGAAGAAA 3’     | eurofins       |
| CSNS#1     | 5’ AAGACAAUUAUCGCGGAA 3’        | eurofins       |
| CSNS#2     | 5’ CUGGACUAAAGAUCACAAUA 3’      | eurofins       |
| EIF3S5#1   | 5’ CAAAIAGACUAGCAGAAIGGA 3’     | eurofins       |
| EIF3S5#2   | 5’ AACGCCCAGAIAGCAUCAAA 3’      | eurofins       |
| UCHL1#1    | 5’ CCAGCCAGCGCAUAAUACGAA 3’     | eurofins       |
| UCHL1#2    | 5’ CUCGCGCGAGAUAUCGAGC 3’       | eurofins       |
| USP49#1    | 5’ AUUGAUAUGCAACAAUGUA 3’       | eurofins       |
| USP49#2    | 5’ CACAUUAUAUCAGCAUAUAU 3’      | eurofins       |
| MYSM1#1    | 5’ CCAGAUGCCUCUUAUCGCUUA 3’     | eurofins       |
| MYSM1#2    | 5’ AUUGGGAUAUGGCAAAAACCAA 3’    | eurofins       |
| Ctrl (Luciferase) | 5’ AACGUAACGCGGAAUACUUCGA 3’ | eurofins       |