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The de-ubiquitylating enzymes USP26 and USP37 regulate homologous recombination by counteracting RAP80

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ABSTRACT

The faithful repair of DNA double-strand breaks (DSBs) is essential to safeguard genome stability. DSBs elicit a signalling cascade involving the E3 ubiquitin ligases RNF8/RNF168 and the ubiquitin-dependent assembly of the BRCA1-Abraxas-RAP80-MERIT40 complex. The association of BRCA1 with ubiquitin conjugates, which occurs in a RAP80-dependent manner, is known to be inhibitory to DSB repair by homologous recombination (HR). However, the precise regulation of this mechanism remains poorly understood. By performing genetic screens, we identified USP26 and USP37 as key de-ubiquitylating enzymes (DUBs) that limit the repressive impact of RNF8/RNF168 on HR. Both DUBs are recruited to bona fide DSBs where they actively remove RNF168-induced ubiquitin conjugates. Depletion of USP26 or USP37 disrupts the execution of HR and this effect is alleviated by the simultaneous depletion of RAP80. In addition, we demonstrate that these DUBs prevent the ubiquitin-dependent sequestration of BRCA1 via the BRCA1-Abraxas-RAP80-MERIT40 complex, allowing BRCA1 to form a complex and cooperate with PALB2-BRCA2-RAD51 in HR. These findings reveal a novel ubiquitin-dependent mechanism that orchestrates the spatial assembly of distinct BRCA1-containing complexes for efficient repair of DSBs by HR.
INTRODUCTION

DNA double-strand breaks (DSBs) pose a considerable threat to the stability of the human genome and their timely repair is essential to safeguard genome stability and counteract tumour development [1]. Cells activate robust signalling pathways in response to DSBs that coordinate cell cycle progression, changes in chromatin structure and DNA repair [2, 3]. Eukaryotic cells primarily utilise homologous recombination (HR) or non-homologous end-joining (NHEJ) to remove DSBs from their genomes.

A key feature of the DNA damage response (DDR) is the rapid assembly of signalling and repair factors in the vicinity of DSBs, by progressively modifying histones and DNA repair enzymes [4, 5]. An initial phosphorylation-dependent cascade of post-translational modifications in DSB-containing chromatin requires the ATM kinase and culminates into the association of MDC1 with phosphorylated histone H2A variant H2AX (γH2AX) [6]. The binding of the RNF8 E3 ubiquitin ligase to MDC1 subsequently initiates a ubiquitylation-dependent cascade, involving the recruitment of the E3 ubiquitin ligase RNF168 in cooperation with the E2 ubiquitin-conjugating enzyme UBC13 [7, 8]. The activity of these enzymes contributes to the ubiquitylation of K13/15 on histone H2A/H2AX [9, 10], as well as the ubiquitin-dependent assembly of 53BP1 [11], RAD18 [12] and the BRCA1-Abraxas-RAP80-MERIT40 (or BRCA1-A) complex [13-16] onto DSB-neighbouring chromatin.

This ubiquitylation cascade is tightly controlled by sophisticated mechanisms that entail chromatin remodelling enzymes [17-19] and additional ubiquitin ligases [20]. Furthermore, it has recently become clear that the removal of ubiquitin by specific de-ubiquitylating enzymes (DUBs) represents an equally important regulatory mechanism in the DDR [21-24]. The human genome contains ~90 potential DUBs that belong to five distinct subfamilies: ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs), Machado-Joseph disease enzymes (MJDs) and JAB1/MPN/MOV34 metalloenzymes (JAMMs). A number of DUBs have been linked to reversing RNF8/RNF168-mediated chromatin ubiquitylation during DNA damage signalling [21, 25] and a recent genetic
screening approach identified many DUBs with potential roles in the DDR [26].

Although the principles underlying the RNF8 signalling pathway are by now well understood, we are only beginning to comprehend how this pathway is linked to the actual repair of DSBs through the major repair pathways NHEJ [27] and HR [28-30]. During HR, the ends of a DSB are resected to expose 3’ single-stranded DNA (ssDNA) overhangs, which are rapidly coated with the ssDNA-binding protein RPA. Following resection, the PALB2 protein is recruited by BRCA1 and subsequently facilitates the assembly of BRCA2 [31, 32]. This, in turn, promotes the exchange of RPA with RAD51, which drives the search for and pairing with a homologous sequence, as well as the exchange of homologous DNA during the final steps of HR [31-33]. BRCA1 is incorporated into distinct multi-protein complexes, including BRCA1-PALB2-BRCA2-RAD51 and BRCA1-Abraxas-RAP80-MERIT40 [34]. Strikingly, while the BRCA1-PALB2-BRCA2-RAD51 complex promotes HR, the BRCA1-Abraxas-RAP80-MERIT40 complex functionally antagonises this repair process by sequestering BRCA1 from HR sites by binding to RNF8/RNF168-ubiquitylated chromatin [16, 35-40]. These findings suggest that distinct BRCA1-containing complexes can differentially affect HR in a manner dependent on DNA damage-induced ubiquitylation. Remarkably, little is known about the involvement of DUBs in regulating BRCA1-dependent HR.

Through genetic screens we identified the de-ubiquitylating enzymes USP26 and USP37 as key factors critical for DSB repair by HR. Mechanistically, we show that by removing RNF168-induced ubiquitin conjugates distal from DSBs, these enzymes prevent the ubiquitin-dependent sequestration of BRCA1 through the BRCA1-Abraxas-RAP80-MERIT40 complex, ultimately allowing BRCA1 to form a complex with PALB2-BRCA2-RAD51 and execute HR. Thus, these enzymes promote HR by limiting the repressive impact of RAP80 on HR. These findings reveal a novel ubiquitin-dependent mechanism that orchestrates the spatial assembly and function of HR complexes at DSBs.
RESULTS

A screen for DUBs reveals novel regulators of 53BP1 and RAD51

The BRCA1 protein is incorporated into distinct multi-protein complexes that are not all competent in promoting HR. While the BRCA1-PALB2-BRCA2-RAD51 complex promotes HR, the BRCA1-Abraxas-RAP80-MERIT40 complex functionally antagonises this repair process by sequestering BRCA1 from HR sites by binding to RNF8/RNF168-ubiquitylated chromatin [16, 35-40]. These findings suggest that distinct BRCA1-containing complexes can differentially affect HR in a manner dependent on RNF8/RNF168 damage-induced ubiquitylation. Although the responsible E3 ubiquitin ligases RNF8 and RNF168 have been characterised [7, 8, 13-15], potential DUBs that play a role in this ubiquitin-dependent regulation of HR remain elusive. In order to identify such proteins, we performed an over-expression screen using a FLAG-tagged cDNA library of ~60 human DUBs (Supplemental Fig. 1A) in human U2OS cells (Fig. 1A). Specifically, we monitored if DUB over-expression simultaneously antagonises the ionising radiation (IR)-induced formation of 53BP1 foci, a read-out for RNF168-mediated ubiquitylation [11], as well as the IR-induced focal accumulation of RAD51, a measure of HR efficiency. Given that 53BP1 directly binds to RNF168-induced ubiquitin conjugates [9, 11], we reasoned that DUBs modulating both these processes are likely to regulate RNF168-mediated HR events.

Imaging-based analysis revealed that most DUBs did not appreciably alter the IR-induced accumulation of 53BP1 or RAD51 (Fig. 1B; black circles – see also Supplemental Fig. 1B,C). However, a subset of DUBs predominantly impinged on 53BP1 accrual (Fig. 1B; green circles), such as the earlier reported enzyme USP44, [21] (Supplemental Fig. 1D), while another set of DUBs mainly impacted RAD51 foci formation (Fig. 1B; blue circles), including the previously described USP1 [41]. Only a small number of DUBs affected both 53BP1 and RAD51 recruitment simultaneously (Fig. 1B; red circles), including USP29, an enzyme linked to H2A de-ubiquitylation [21], and the recently reported HR modulator DUB3 (Supplemental Fig. 1D,E) [42]. The
fact that we identified various published DUBs demonstrates the validity of our screen. Importantly, among the DUBs that suppressed both 53BP1 and RAD51 IRIF formation, USP26 and USP37 emerged as novel candidates (Fig. 1B and Supplemental Fig. 1D,E). We could not distinguish a common pattern in the impact of DUBs on 53BP1 or RAD51 foci formation within USP, UCH, MJD, JAMM, OTU or unclassified DUBs (Supplemental Fig. 1B-E), suggesting this is a unique property of the identified enzymes. Thus, via our screen, we identified USP26 and USP37 as potential novel regulators of 53BP1 and RAD51.

Figure 1. DUB screen for regulators of 53BP1 and RAD51 IRIF formation. (A) Experimental design of the DUB over-expression screens. (B) Bi-dimensional representation of the relative decrease in 53BP1 (x-axis) and RAD51 (y-axis) IRIF formation upon over-expression of FLAG-tagged DUBs. (C) Impact of the expression of wild-type (WT) or catalytic inactive (CI) mCherry-tagged DUBs (red) on 53BP1 (white; left panel) or RAD51 IRIF formation (white; right panel) in mAG-geminin-expressing (images not shown) S/G2 cells. Quantified data are represented as mean ± S.D. (n=3). *, P<0.05, **, P<0.01, ***, P<0.001 (student’s t test). See also Supplementary Figure 1.
USP26 and USP37 reverse RNF168-induced ubiquitylation at DSBs

To validate and extend these findings, we generated mCherry- and GFP-tagged versions of USP26 and USP37. Over-expression of these DUBs did not change the accumulation of γH2AX, MDC1, and RNF8 (Supplemental Fig. 2A), yet abrogated ubiquitin conjugation and all events downstream of it, including the assembly of ubiquitin-binding factors RNF168, RAP80, BRCA1 and 53BP1 after IR in a catalytic-dependent manner (Fig. 1C and Supplemental Fig. 2B). Both DUBs were rapidly recruited to laser-induced DNA damage tracks (Supplemental Fig. 2C). Given the multitude of DNA lesions inflicted by laser micro-irradiation, we additionally used a U2OS cell line in which DSBs are specifically induced by targeting the LacR-tagged FokI nuclease to a genomic locus containing LacO repeats [43]. In line with the results obtained by laser micro-irradiation, both USP26 and USP37 accumulated at bona fide, FokI-induced DSBs marked by γH2AX (Fig. 2A,B). Importantly, USP26 and USP37 were able to remove ubiquitin conjugates at these DSBs in a manner dependent on their catalytic activity, as well as their C-terminal ubiquitin-binding domains (UIMs) (Fig. 2B,C and Supplemental Fig. 2B (FK2 foci), 2D-E). Strikingly, loss of these UIMs did not affect recruitment of the DUBs to FokI-induced DSBs (Supplemental Fig. 2E). This suggests that both DUBs can directly reverse RNF168-induced ubiquitin conjugation. Indeed, when mCherry-LacR-RNF8 or mCherry-LacR-RNF168 was tethered to a LacO array to induce local chromatin ubiquitylation [18, 44, 45], robust accumulation of both DUBs was observed (Fig. 2D), indicating that these DUBs can recognise RNF8/RNF168-induced ubiquitin moieties. Furthermore, we examined if our DUBs affect the ubiquitylation of chromatin substrates by RNF168 immobilised at the LacO array or the subsequent recognition of ubiquitylated-H2A-type histones by 53BP1. Both USP26 and USP37 reduced FK2 (Supplemental Fig. 3A) and 53BP1 (Supplemental Fig. 3B) accumulation at the array, implying that they directly remove RNF168-induced ubiquitylation. RNF168 targets H2A-type histones for ubiquitylation [9, 10]. Interestingly, ectopic expression of USP37 moderately decreased RNF168-induced H2A ubiquitylation [9, 46], while expression of USP26 nearly eliminated such ubiquitylation (Fig. 2E). Thus, our results suggest that USP26 and USP37 are able to bind to chromatin modified by RNF8/RNF168...
and reverse ubiquitylation induced by these E3 ligases at DSBs.

Figure 2. USP26 and USP37 accumulate at DSBs and regulate chromatin ubiquitylation. (A) Recruitment of the indicated GFP fusion proteins to FokI-mCherry-LacR-induced DSBs marked by γH2AX (white) in cells containing a LacO array. (B) As in A, but stained for ubiquitin-FK2 (white). (C) Recruitment of GFP-tagged DUBs lacking their C-termini (ΔC; green) and their impact on ubiquitin-FK2 at FokI-induced DSBs (white). (D) Recruitment of GFP-tagged DUBs upon tethering of the indicated mCherry-LacR fusion proteins in cells containing a LacO array. (E) IP of GFP-H2A under denaturing conditions in the absence or presence of mCherry-RNF168 and the indicated mCherry-tagged DUBs. Quantified data are represented as mean ± S.D. (n=3). *, P<0.05, **, P<0.01, ***, P<0.001 (student’s t test).
Loss of USP26 or USP37 impairs DSB repair

To address if the identified DUBs play a role in HR under physiological conditions, USP26 and USP37 were depleted using independent siRNAs. Immunoblotting (Fig. 3A) and RT-qPCR (Supplemental Fig. 4A) analysis confirmed that the protein and mRNA levels of both DUBs were dramatically reduced. Loss of these DUBs led to a significant increase in IR-induced ubiquitin conjugates (Fig. 3B) and accumulation of the ubiquitin-binding protein 53BP1 (Supplemental Fig. 4B), suggesting that USP26 and USP37 control the levels of DSB-induced ubiquitylation. Surprisingly, however, depletion of either DUB also resulted in a clear reduction of IR-induced PALB2 and RAD51 (Fig. 3C) accumulation, indicating that excessive DSB-induced ubiquitylation disrupts HR. In further agreement with a physiological role of these DUBs in regulating HR, loss of USP26 or USP37 resulted in defective accumulation of the CtIP nuclease and moderately decreased DNA-end resection, assayed by RPA foci formation (Supplemental Fig. 4C).

Flow cytometric analysis of DR-GFP cells confirmed that USP26 or USP37 depletion leads to a significant defect in HR (Fig. 4A). Notably, over-expression of mCherry-tagged RNF8 or RNF168 also strongly inhibited HR (Fig. 4B). Thus, the over-expression of RNF8/168 phenocopied the depletion of USP26/37 as both conditions trigger excessive chromatin ubiquitylation. The effects on HR were not due to alterations in the cell cycle as cell cycle profiles were unchanged under these conditions (Supplemental Fig. 5A,B). Depletion of USP26 or USP37 also rendered cells highly sensitive to a poly(ADP-ribose) polymerase (PARP) inhibitor, which is a hallmark of HR-deficient cells such as those lacking BRCA2 (Fig. 4C) [47].

Having shown that USP26 and USP37 regulate HR, we next sought to address if these enzymes affect the other major DSB repair pathway, non-homologous end-joining (NHEJ). Using the flow cytometry-based EJ5-GFP reporter assay to monitor NHEJ efficiency [48], we found that USP26 or USP37 depletion substantially impaired this repair pathway (Fig. 4D). In line with a general defect in DSB repair, the combined knockdown of USP26 and USP37 led to a delay in the clearance of IR-induced γH2AX foci (Supplemental Fig. 5C), whereas depletion of either DUB rendered cells sensitive to IR (Fig.
Collectively, this work reveals USP26 and USP37 as novel factors that are required for DSB repair.

Figure 3. USP26 or USP37 depletion abrogates the formation of PALB2-RAD51 complex at DSBs. (A) Western blot analysis of GFP-USP26, GFP- or endogenous-USP37 expression in cells treated with the indicated siRNAs. (B) Effect of DUB depletion on ubiquitin-FK2 IRIF formation in time. Note that 0 hr indicates non-irradiated cells. (C) Effect of DUB depletion on PALB2 (white; left panel) or RAD51 (white; right panel) IRIF formation in mAG-geminin-expressing (green) S/G2 cells. Quantified data are represented as mean ± S.E.M (n=3). *, P<0.05, **, P<0.01, ***, P<0.001 (student’s t test).
USP26 and USP37 regulate HR by counteracting RAP80

Figure 4. USP26 or USP37 depletion disrupts HR and NHEJ and sensitises cells to DNA damaging agents. (A) Impact of the indicated siRNAs on HR efficiency measured using the DR-GFP reporter. (B) Impact of the expression of the indicated mCherry-fusion proteins on HR efficiency using the DR-GFP reporter. (C) Clonogenic survival of VH10-SV40 cells that were transfected with the indicated siRNAs and exposed to PARP inhibitor (Olaparib). (D) Impact of the indicated siRNAs on NHEJ efficiency measured using the EJ5-GFP reporter. (E) Clonogenic survival of IR-exposed U2OS cells transfected with the indicated siRNAs. Quantified data are represented as mean ± S.D. (n=3). *, P<0.05, **, P<0.01, ***, P<0.001 (student’s t test).
Loss of USP26 or USP37 impairs HR by antagonising RAP80-dependent sequestration of BRCA1

To gain insight into the mechanism that disrupts HR under conditions of excessive ubiquitylation, we turned our attention to the BRCA1-Abraxas-RAP80-MERIT40 complex, which through RAP80 drives the ubiquitin-dependent recruitment of BRCA1 to RNF8/RNF168-modified chromatin [7, 13-16, 35-37]. It was recently unveiled that RAP80-mediated recruitment of BRCA1 inhibits HR [38, 39], by sequestering BRCA1 from HR sites, thus hampering the formation of a BRCA1-PALB2-BRCA2-RAD51 complex, which is essential for HR [38, 40]. We reasoned that USP26 and USP37 may antagonise the RAP80-dependent sequestration of BRCA1 by removing RNF8/RNF168-mediated ubiquitylation and thereby promote HR. To address this, we established a quantitative, computer-assisted approach to measure BRCA1 foci size. In agreement with an earlier report [38], we found that BRCA1 foci were not reduced in number, but rather were considerably smaller in size following depletion of RAP80 (Fig. 5A). In contrast, depletion of either DUB had the opposite effect, leading to an increase in larger BRCA1 foci without affecting the total number of foci (Fig. 5B,C and Supplemental Fig. 5D). Remarkably, depletion of RAP80 in DUB knock-down cells completely rescued the shift towards larger foci and led to a reappearance of small BRCA1 foci (Fig. 5B,C). To test the functional relevance of these findings, we depleted RAP80 and examined if this would restore HR proficiency in USP26 or USP37-depleted cells. Indeed, we found that defective IR-induced accrual of both PALB2 and RAD51 in USP26- or USP37-depleted cells could be fully rescued by additional depletion of RAP80 (Fig. 6A,B). Similarly, HR efficiency was completely restored upon co-depletion of either DUB and RAP80, as measured in the DR-GFP reporter assay (Fig. 6C). Cell-cycle profiles in these cells remained unchanged ruling out effects of cell cycle misregulation (Supplemental Fig. 5E). Together these results suggest that USP26 and USP37 promote the BRCA1-dependent loading of PALB2 and RAD51 by counteracting the repressive impact of RAP80-dependent BRCA1 sequestration during HR (Fig. 7).
USP26 and USP37 regulate HR by counteracting RAP80

Figure 5. RAP80 depletion restores the formation of small BRCA1 IRIF, indicative of HR centers in USP26 and USP37 knock-down cells. (A) Effect of RAP80 depletion on RAP80 (green) and BRCA1 (white) IRIF formation (left and middle-right panel), endogenous RAP80 expression on Western blot (middle-left panel) and BRCA1 IRIF size (right panel). (B) Effect of DUB and RAP80 depletion on RAP80 (green) and BRCA1 (white) IRIF formation. (C) Histograms of BRCA1 foci size in cells treated with the indicated siRNAs. Green indicates small foci of typical HR size, while red indicates larger foci of the size observed for signalling factors. Quantified data are represented as mean ± S.D. (n=2). *, P<0.05, **, P<0.01, †††, P<0.001 (student’s t test).
Figure 6. HR defects caused by the loss of USP26 and USP37 are reversed upon concomitant RAP80 removal. (A) Effect of DUB and RAP80 depletion on PALB2 (white) foci formation in mAG-geminin expressing (green) S/G2 cells. (B) As in A, but stained for RAD51 (white). (C) Effect of the indicated siRNAs on HR efficiency measured using the DR-GFP reporter. Quantified data are represented as mean ± S.E.M. (n=2). *, P<0.05, **, P<0.01, ***, P<0.001 (student’s t test).
DISCUSSION

DSBs elicit a signalling cascade that is driven by the ubiquitin E3 ligases RNF8 and RNF168. These ligases promote progressive chromatin ubiquitylation, eventually leading to the ubiquitin-dependent assembly of BRCA1, RAD18 and 53BP1 onto damaged chromosomes [7-9, 13-16]. However, while a clear picture of the factors that orchestrate the RNF8/RNF168 signalling pathway has emerged, we are only starting to understand how it is linked to DSBs repair [12, 30, 38, 40, 49-53].

In this study, we identify USP26 and USP37 as novel DUBs that reverse RNF168-mediated ubiquitylation (Fig. 1-2, Supplemental Fig. 1-2), a process known to repress HR by sequestering the BRCA1-Abraxas-RAP80-MERIT40 complex through its ubiquitin-binding subunit RAP80 [38, 40]. By removing RNF168-induced ubiquitin conjugates distal from DSBs, USP26 and USP37 prevent the RAP80-dependent assembly of this BRCA1-containing complex, allowing BRCA1 to function in the BRCA1-PALB2-BRCA2-RAD51 complex during HR (Fig. 7). These findings advance our conceptual understanding of the RNF168-dependent response to DSBs, by revealing pathways that differentially regulate the spatial assembly and function of HR complexes at DSBs.

We propose the following model for RNF168-dependent regulation of HR (Fig. 7): RNF168-induced ubiquitin conjugates spread away from DSBs into more distal chromatin regions [20]. The BRCA1-Abraxas-RAP80-MERIT40 complex through RAP80 interactions associates with the RNF168-induced ubiquitin conjugates in these regions, thereby sequestering BRCA1 from the ssDNA compartment and inhibiting HR [38, 40]. In line with this model, we demonstrate that supra-physiological levels of RNF168 triggered extensive ubiquitylation of H2A (Fig. 2E), concomitant with a substantial reduction in HR efficiency (Fig. 4B). We extend these findings by showing that this phenomenon is actively antagonised by USP26 and USP37. Loss of USP26/USP37 function markedly impairs the assembly of PALB2, RAD51 and efficient HR (Fig. 3C, 4A and 6A-C). However, these defects can be rescued
by the additional loss of RAP80 (Fig. 6A-C). Together, these data suggest that USP26/37 limit the magnitude of the BRCA1-Abraxas-RAP80-MERIT40 complex assembly in DSB-neighbouring chromatin, by actively removing RNF168-mediated H2A ubiquitylation (Fig. 2E and Supplemental Fig. 3). Indeed, depletion of either DUB resulted in an increase in the size of BRCA1 foci, indicative of more extensive spreading of the BRCA1-Abraxas-RAP80-MERIT40 complex from the DSB site, which could be rescued by additional loss of RAP80 (Fig. 5A,B and Supplemental Fig. 5D). This scenario explains how these DUBs limit the repressive impact of RAP80 on HR. An alternative, yet not mutually exclusive scenario, would be in line with recent findings showing that DSB-induced H2A/H2AX ubiquitylation needs to be reversed in the core of IRIF for DNA end-resection to occur [39]. Given that USP26 and USP37 are able to reverse RNF8/168-mediated ubiquitylation and promote HR, these enzymes would be ideal candidates to facilitate such events.

Figure 7. Molecular model for the role of USP26 and USP37 in HR. BRCA1 is sequestered from HR sites through RAP80, which is functionally antagonised by USP26- and USP37-dependent de-ubiquitylation of RNF168-modified chromatin (see discussion for details). Loss of USP26 or USP37 leads to more extensive RNF168-dependent sequestration of BRCA1, thereby preventing BRCA1 to form a complex with PALB2-BRCA2-RAD51 in HR. Additionally, the more extensive spreading of RAP80 upon USP26 or USP37 depletion reduces DNA end-resection, which also impairs HR.
Several other DUBs that affect H2A ubiquitination have been identified as important players in the DDR. For instance, the activity of tumour suppressor BAP1, which de-ubiquitylates H2A at K119, appeared to be critical for efficient HR [42, 54]. However, whether the BAP1-dependent removal of this histone modification is important during HR remains unclear. Similar to USP26 and USP37, two other DUBs, USP3 and USP44, were shown to reverse RNF168-induced chromatin ubiquitylation, thereby controlling the accumulation of BRCA1 and 53BP1 at sites of DNA damage [21, 25, 55]. Future work has to reveal whether these DUBs, similarly to USP26 and USP37, operate to control DSB repair, in particular HR. Unravelling the interplay between different DUBs during HR may uncover how ubiquitin-dependent control of this important DNA damage repair process is orchestrated.

Notably, both USP26 and USP29 are retrogenes of USP37, which likely explains why these DUBs display certain functional similarities. Although USP26 is often considered as testis-specific, we were able to detect USP26 expression in different cell types (U2OS and HEK293, Fig. 3A and Supplemental Fig. 4A), showing that this classification is incorrect. In agreement, it has been shown by extensive proteomic analysis that USP26 is expressed in various human cell-lines and organs [56, 57]. Moreover, knockdown of USP26, similar to that of USP37, confers defects in the signalling and repair of DSBs in human cells, illustrating non-redundant roles for both DUBs in the DSB response.

A striking conclusion from our study is that non-physiological expression of USP26 and USP37 impairs HR. Therefore, it seems likely that the expression of USP26 and USP37 needs to be tightly controlled. Failure to do so might not only correlate with enhanced genomic instability, but also with increased malignant transformation rates. Indeed, a plethora of cancer cell lines appear to have either lost (USP26=1457/USP37=446; COSMIC) or amplified (USP26=309/USP37=359; COSMIC) the expression of these DUBs. In conclusion, we report distinct ubiquitin-dependent pathways that orchestrate the assembly and function of HR complexes at DSBs and identify the factors responsible for these events.
MATERIALS AND METHODS

Cell culture

U2OS, HEK293 and VH10-SV40-immortalised cells were grown in DMEM (Gibco) containing 10% FCS (Bodinco BV). U2OS cells containing an inducible shRNA against endogenous RNF8 and stably expressing FLAG-RNF8, U2OS 2–6-3 cells containing 200 copies of a LacO-containing cassette (~4 Mbp) and U2OS 2-6-3 cells stably expressing an inducible version of FokI-mCherry-LacR fused to the estrogen receptor (ER) and harbouring a destabilisation domain (DD) were previously described [7, 15, 43, 58]. The ViraPower system (Life Science) was used to produce lentivirus using mAG- or mCherry-geminin expression vectors [59]. U2OS cells stably expressing mAG- or mCherry-geminin were made by standard lentiviral transduction, followed by FACS sorting, in order to select homogeneously fluorescent cells.

Plasmids

A collection of cDNAs encoding FLAG-tagged DUBs, originally generated in Wade Harper’s laboratory [60], was obtained from Addgene. An IRES-Puro cassette was amplified by PCR and inserted as an EcoRV-EcoRV fragment into the HpaI site of EGFP-C1 (Addgene). The USP26 and USP37 cDNAs were inserted in EGFP-C1-IRES-Puro. Overlap PCR was used to introduce the inactivation mutations C304S into GFP-USP26 and C350S into GFP-USP37. Wild-type and catalytic inactive versions of USP26 and USP37 were also inserted into mCherry-C1. A BsrGI-BstZ17I fragment encompassing amino acids 1-641 of USP26 was inserted into the BsrGI-Smal site of EGFP-C1 to generate GFP-USP26 lacking the putative C-terminal UIM. Likewise, a BsrGI-SacI fragment encompassing amino acids 1-643 of USP37 was inserted into the BsrGI-Sacl site of EGFP-C1 to generate GFP-USP37 lacking the C-terminal UIM domains. Additional plasmids used are listed in the Supplemental Table.

Transfections and RNAi interference

siRNA and plasmid transfections were performed using Lipofectamine
RNAiMAX (Invitrogen), Lipofectamine 2000 (Invitrogen) and JetPEI (Polyplus Transfection) according to the manufacturer’s instructions. Cells were transfected twice with siRNAs (40 nM) within 24 h and examined further 48 h after the second transfection, unless stated otherwise. siRNA sequences are listed in the Supplemental Table.

**Generation of DSBs**

IR was delivered by a YXlon X-ray generator (YXlon International, 200 KV, 10 mA, dose rate 2 Gy/min).

**Cell survival assay**

VH10-SV40 or U2OS cells were transfected with siRNAs, trypsinised, seeded at low density and exposed to IR. 7 days later cells were washed with 0.9% NaCl and stained with methylene blue. Colonies of more than 10 cells were scored.

**FokI assays**

U2OS 2-6-3 cells expressing inducible FokI-mCherry-LacR [43] were treated with 300 nM 4-OHT and 1 μM Shield-I for 5 hrs. Subsequently, cells were fixed with formaldehyde and immunostained with the indicated antibodies.

**UV-A laser micro-irradiation**

U2OS cells were grown on 18 mm coverslips and sensitised with 10 μM 5′-bromo-2-deoxyuridine (BrdU) for 24 hours as described [18, 44]. For micro-irradiation, the cells were placed in a Chamlide TC-A live-cell imaging chamber that was mounted on the stage of a Leica DM IRBE wide-field microscope stand (Leica, Wetzlar, Germany) integrated with a pulsed nitrogen laser (Micropoint Ablation Laser System; Photonic Instruments, Inc., Belfast, Ireland). The pulsed nitrogen laser (16 Hz, 364 nm) was directly coupled to the epifluorescence path of the microscope and focused through a Leica 40x HCX PLAN APO 1.25-0.75 oil-immersion objective. The growth medium was replaced by CO₂-independent Leibovitz’s L15 medium supplemented with 10% FCS and penicillin-streptomycin and cells
were kept at 37°C. The laser output power was set to 78 to generate strictly localised sub-nuclear DNA damage. Following micro-irradiation, cells were incubated for the indicated time-points at 37°C in Leibovitz’s L15 and subsequently fixed with 4% formaldehyde before immunostaining. Typically, an average of 50 cells was micro-irradiated (2 iterations per pixel) within 10–15 minutes using Andor IQ software.

**Multiphoton laser micro-irradiation**

U2OS cells were grown on 18 mm coverslips. Subsequently, they were placed in a Chamlide CMB magnetic chamber and the growth medium was replaced by CO\text sub$_2$-independent Leibovitz’s L15 medium supplemented with 10% FCS and penicillin-streptomycin. Laser micro-irradiation was carried out on a Leica SP5 confocal microscope equipped with an environmental chamber set to 37°C. DSB-containing tracks (1.5 µm width) were generated with a Mira mode-locked titanium-sapphire (Ti:Sapphire) laser (λ = 800 nm, pulse length = 200 fs, repetition rate = 76 MHz, output power = 80 mW) using a UV-transmitting 63× 1.4 NA oil immersion objective (HCX PL APO; Leica). Confocal images were recorded before and after laser irradiation at 5 or 10 seconds time intervals over a period of 5 - 10 minutes.

**Microscopy analysis**

Images of fixed cells were acquired on a Zeiss AxioImager D2 widefield fluorescence microscope equipped with 40x, 63x and 100x PLAN APO (1.4 NA) oil-immersion objectives (Zeiss) and an HXP 120 metal-halide lamp used for excitation. Fluorescent probes were detected using previously described filters [61]. Images were recorded using ZEN 2012 software and analysed using ImageJ. The average reflects the quantification of 50-150 cells from 3 independent experiments.

**IRIF analysis**

PALB2, RAD51, BRCA1 and RAP80 (except in Supplemental Fig. 2B) IRIF were analyzed in U2OS cells 6hr after 10Gy, RPA and CtIP IRIF were assayed 4hr after 10Gy, whereas conjugated ubiquitin (FK2), γH2AX, MDC1, FLAG-RNF8, RNF168, BRCA1 and RAP80 (at Supplemental Fig.
2B) and 53BP1 IRIF were examined 1hr after 2Gy, unless stated otherwise. IRIF were evaluated in ImageJ, using a custom-built macro that enabled automatic and objective analysis of the foci. Full details of this macro will be published elsewhere. In brief, cell nuclei were detected by thresholding the (median-filtered) DAPI signal, after which touching nuclei were separated by a watershed operation. The foci signal was background-subtracted using a Difference of Gaussians filter. For every nucleus, foci were identified as regions of adjacent pixels satisfying the following criteria: (i) the grey value exceeds the nuclear background signal by a set number of times (typically 2-4x) the median background standard deviation of all nuclei in the image, and is higher than a user-defined absolute minimum value; (ii) the area is larger than a defined area (typically 2 pixels). These parameters were optimised for every experiment by manually comparing the detected foci with the original signal.

Immunofluorescent labelling

Immunofluorescent labelling was carried out as described previously [18, 19]. Briefly, cells were grown on glass coverslips and treated as indicated in the figure legends. Subsequently, cells were washed with PBS, then fixed with 2% formaldehyde for 20 minutes and permeabilised with 0.25% Triton X-100 in PBS for 5 minutes. Cells were rinsed with phosphate-buffered saline (PBS) and then treated with 100 mM glycine in PBS for 10 minutes to block unreacted aldehyde groups. Finally, cells were equilibrated in PBS containing 0.5% BSA and 0.05% Tween 20, and incubated with primary antibodies. Detection was done using goat anti-mouse or goat anti-rabbit IgG coupled to Alexa 488, 555 or 647 (Invitrogen Molecular Probes). Samples were incubated with 0.1 μg/ml DAPI and mounted in Polymount. Primary antibodies and secondary antibodies are listed in the Supplemental Table.

Western blotting

Cell extracts were generated by boiling cell pellets in Laemmli buffer, separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were probed with the antibodies listed in Supplemental Table 1.
followed by protein detection using the Odyssey infrared imaging scanning system (LI-COR Biosciences).

**HR and NHEJ assay**

HEK293 and U2OS cells containing a stably integrated copy of either the DR-GFP of EJ5-GFP reporter were used to measure the repair of I-SceI-induced DSBs by HR or NHEJ, respectively [48, 62]. Briefly, 48 h after siRNA transfection, cells were transfected with the I-SceI expression vector pCBASce and an mCherry expression vector. [62] 48 or 72 h later the fraction of GFP-positive cells among the mCherry-positive cells was determined by FACS on a LSRII flow cytometer (BD Bioscience) using FACSDiva software version 5.0.3. Quantifications were performed using Flowing Software (www.flowingsoftware.com).

**Cell cycle profiling**

For cell cycle analysis cells were fixed in 70% ethanol, followed by DNA staining with 50 µg/ml propidium iodide in the presence of RNase A (0.1 mg/ml). Cell sorting was performed on a LSRII flow cytometer (BD Bioscience) using FACSDiva software (version 5.0.3; BD). Quantifications were performed using Flowing Software.

**RT-qPCR-based gene expression analysis**

RNA isolation, reverse transcription (RT)-based cDNA synthesis and quantitative (q)PCR were carried out as previously described [61]. The primers used are listed in the Supplemental Table.

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Supplementary Figure 1. A DUB over-expression screen reveals novel DSB response regulators. (A) List of FLAG-tagged DUBs expressed in U2OS cells. Enzymes are colour-coded to signify the different DUB families. In yellow Ubiquitin C-terminal Hydrolases (UCH), in green Machado-Josephin Domain-DUBs (MJD), in blue JAMM metalloproteases (JAMM), in red OTUbain domain-DUBs (OTU), in purple unclassified DUBs and in grey Ubiquitin Specific Proteases (USP). (B) Quantification of 53BP1 IRIF formation upon over-expression of the indicated DUBs. Red bars signify hits, i.e. population of cells with >5 53BP1 IRIFs is lower than 15% of total transfected cells. (C) Quantification of RAD51 IRIF formation upon over-expression of the indicated DUBs. Red bars signify hits, i.e. population of cells with >10 RAD51 IRIFs is lower than 15% of total transfected cells. (D) Examples of the impact of the over-expression of the indicated (non-) hits/DUBs (green) on 53BP1 IRIF formation (white). (E) Examples of the impact of the over-expression of the indicated (non-) hits/DUBs (green) on RAD51 IRIF formation (white).
Supplemental Figure 2. USP26 and USP37 accumulate at DSBs and regulate chromatin ubiquitylation. (A) Quantification of the impact of the expression of GFP- or mCherry-tagged DUBs (green or red) on γH2AX, MDC1, FLAG-RNF8 IRIF formation. (B) As in A, but RNF168, FK2, RAP80 and BRCA1 IRIF formation was quantified. (C) Recruitment of mCherry-tagged DUBs (red) to laser-induced DNA damage, marked by γH2AX (green). (D) Schematic representation of the catalytic cysteine residues and UIMs of USP26 and USP37. In yellow: alignment between UIMs 1 - 3 of USP37 and UIM2 of USP26 in different species. Note that USP26 lost two UIMs during evolution. In red: conserved residues important for interaction with ubiquitin. UIM consensus sequence: ΦxxΦxΦS. In light blue: Φ=hydrophobic residues (A, F, G, I, L, P, V). (E) Quantification of the localisation of GFP-DUBs lacking their C-termini (ΔC) at FokI-induced DSBs in cells containing a LacO array. The ΔC mutants lack functional immunosuppressive proteins (A, F, G, I, L, P, V). Quantified data are represented as mean ± S.D. (n=3). * P<0.05, ** P<0.01, *** P<0.001 (student’s t test).
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Supplemental Figure 3. USP26 or USP37 can remove RNF168-induced ubiquitylation.
(A) Effect of GFP-DUB over-expression on conjugated ubiquitin (FK2) (white) upon tethering of mCherry-LacR-RNF168 in cells containing a LacO array. (B) As in (A) only stained for 53BP1 (white). Quantified data are represented as mean ± S.D. (n=3). *, P<0.05, **, P<0.01, ***, P<0.001 (student’s t test).
Supplemental Figure 4. USP26 or USP37 depletion impairs the DSB response. (A) Relative USP26 mRNA expression in U2OS cells treated with the indicated siRNAs. The USP26 transcript was not detectable (ND) by reverse transcriptase (RT)-qPCR in cells treated with siRNAs against USP26 (left panel). Representative agarose gel showing USP26 PCR product amplified from total RNA without (-RT) or with (+RT) reverse transcriptase reaction (middle panel). Relative USP37 mRNA expression in U2OS cells treated with the indicated siRNAs (right panel). (B) Effect of DUB depletion on 53BP1 IRIF formation in time after 2 Gy of IR. (C) Effect of DUB depletion on CtIP and RPA IRIF formation 4hr after 10Gy of IR. Quantified data are represented as mean ± S.D. (n=3). *, P<0.05, **, P<0.01, ***, P<0.001 (student's t test).
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Supplemental Figure 5. USP26 or USP37 depletion does not alter cell-cycle progression.
(A) Cell cycle profiles of cells treated with the indicated siRNAs. (B) Cell cycle profiles of cells over-expressing the indicated GFP fusion proteins. (C) Effect of combined DUB depletion on DSB repair, assayed by clearance of γH2AX foci after 2 Gy of IR. (D) Effect of DUB and RAP80 depletion on RAP80 IRIF formation. (E) Cell cycle profiles of cells treated with the combinations of the indicated siRNAs. Quantified data are represented as mean ± S.D. (n=3), except in (D) where mean ± S.E.M. (n=2).
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### Supplemental Table

| Antibodies |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| **Target protein** | **Host** | **Obtained from** | **Cat. nr.** | **IF** | **WB** |
| 53BP1 | rabbit | Novus Biologicals | NB100-304 | 1:2000 |  |
| BRCA1 | mouse | SantaCruz | sc-6954 | 1:100 |  |
| BRCA1 | rabbit | gift Dr. Daniel Durocher | | 1:1000 |  |
| BRCC36 | rabbit | Abcam | ab62075 | 1:1000 |  |
| FLAG | mouse | Sigma | F1804 | 1:1000 |  |
| GFP | rabbit | Abcam | ab290 | 1:1000 |  |
| GFP | mouse | Roche | #11814460001 | 1:2000 |  |
| γH2AX | mouse | Millipore | JBW301 | 1:2000 |  |
| H2AX | rabbit | Bethyl | A330-082A | 1:10000 |  |
| H2B | rabbit | Millipore | 07-371 | 1:10000 |  |
| HA | mouse | SantaCruz | sc-7392 | 1:500 |  |
| mCherry | mouse | Abcam | ab25096 | 1:1000 |  |
| MDC1 | rabbit | Abcam | ab11771 | 1:1000 |  |
| PALB2 | rabbit | Bethyl | A301-246A | 1:1000 |  |
| PALB2 | rabbit | gift Dr. Bing Xia | | 1:100 |  |
| RAD51 | rabbit | SantaCruz | sc-8349 | 1:100 |  |
| RNF168 | rabbit | Millipore | AB367 | 1:200 |  |
| Tubulin | mouse | Sigma | T6199 | 1:5000 |  |
| Ubiquitin K48 chains | rabbit | Millipore | APU205-1307 | 1:100 |  |
| Ubiquitin K63 chains | rabbit | Millipore | APU305-1308 | 1:1000 |  |
| Ubiquitin-FK2 | mouse | EnzoLifeSciences | BML-PW8810-0500 | 1:100 |  |
| RPA | mouse | ThermoScientific | Ab-1 9H8 | 1:1000 |  |
| CtIP | mouse | gift Dr. Richard Baer | | 1:10 |  |
| RAP80 | rabbit | Bethyl | A300-764 | 1:1000 |  |
| RAP80 | rabbit | Bethyl | A300-763A | 1:500 |  |
| Rabbit-700CW | donkey | Licor | | 1:20000 |  |
| Mouse-800CW | donkey | Licor | | 1:20000 |  |

| siRNAs |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| **Target Protein** | **siRNA name** | **Sequence (5’-3’)** |  |  |  |  |
| Luciferase | Luc | CGUACGCGGAAUACUUCGA |  |  |  |  |
| BRCA2 | BRCA2 | GAAGAAUGCAAGGGUAUAUA |  |  |  |  |
| RNF8 | RNF8-1 | GAGGGCCAUGGGAACCAUUAU |  |  |  |  |
| USP26 | USP26-1 | CCAAAAGCUCUGGAGGAA |  |  |  |  |
| USP26 | USP26-2 | CCAAAAGUUGUAGGAAAA |  |  |  |  |
| USP26 | USP26-3 | CAGAAGAGCGTTAGTATAAA |  |  |  |  |
| USP37 | USP37-1 | CUAACACUGAAGGAGAU |  |  |  |  |
| USP37 | USP37-4 | GAAGAUUACCCAAGGAAA |  |  |  |  |
| RAP80 | RAP80 (Dharmacon SMARTpool L-006995-00-0005) | GTAAATCCCTGGTCCCATT |  |  |  |  |

| qPCR primers |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| **Target gene** | **Gene/Primer** | **Sequence (5’-3’)** |  |  |  |  |
| USP26 | USP26-Forward | AGTGGTTCACGGCATCTGG |  |  |  |  |
| USP26 | USP26-Reverse | CCCGACATATCCGTAAGT |  |  |  |  |
| USP37 | USP37-Forward | GCCCAAAACAACTACAGAGC |  |  |  |  |
| USP37 | USP37-Reverse | TCCCTTTACGCCGCTTACA |  |  |  |  |

| Plasmids |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| **Encoded fusion protein** | **Described in** |  |  |  |  |  |
| GFP-H2A | Luijsterburg et al. Journal of Cell Biology 197: 267-281 (2012) |  |  |  |  |  |
| FokI-LacR-mCherry | Tang et al. Nature Structural & Molecular Biology 20: 317-325 (2013) |  |  |  |  |  |
| mCherry-LacR-RNF168 | Luijsterburg et al. EMBO Journal 31: 2511-2527(2012) |  |  |  |  |  |
| mCherry-LacR-RNF8 | Luijsterburg et al. EMBO Journal 31: 2511-2527(2012) |  |  |  |  |  |
| mCherry-RNF168 | Smeenk et al. Journal of Cell Science 126: 889-903 (2013) |  |  |  |  |  |
| mCherry-RNF8 | Smeenk et al. Journal of Cell Science 126: 889-903 (2013) |  |  |  |  |  |
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