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Genome integrity, repair and replication

TRIP12's role in the governance of DNA polymerase β involvement in DNA damage response and repair

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Abstract

The multitude of DNA lesion types, and the nuclear dynamic context in which they occur, presents a challenge for genome integrity maintenance as this requires the engagement of different DNA repair pathways. Specific "repair controllers" that facilitate DNA repair pathway crosstalk between double-strand break (DSB) repair and base excision repair (BER) and that regulate BER protein engagement at lesion sites have yet to be identified. Here, we find that DNA polymerase β (Polβ), crucial for BER, is ubiquitylated in a BER complex-dependent manner by TRIP12, an E3 ligase that partners with UBR5 to restrain DSB repair signaling. Furthermore, we find that TRIP12, but not UBR5, controls cellular levels and chromatin loading of Polβ. Required for Polβ foci formation, TRIP12 influences Polβ involvement after radiation-induced DNA damage, a process regulated by TRIP12-mediated ubiquitylation of Polβ. Notably, excessive TRIP12-mediated engagement of Polβ affects DSB formation and radiation sensitivity, underscoring its role in promoting precedence for BER over DSB repair. The herein discovered function of TRIP12, in the governance of Polβ-directed BER, supports a role for TRIP12 in assuring BER lesion removal at complex DSB sites to optimize DSB repair at the nexus of DNA repair pathways.

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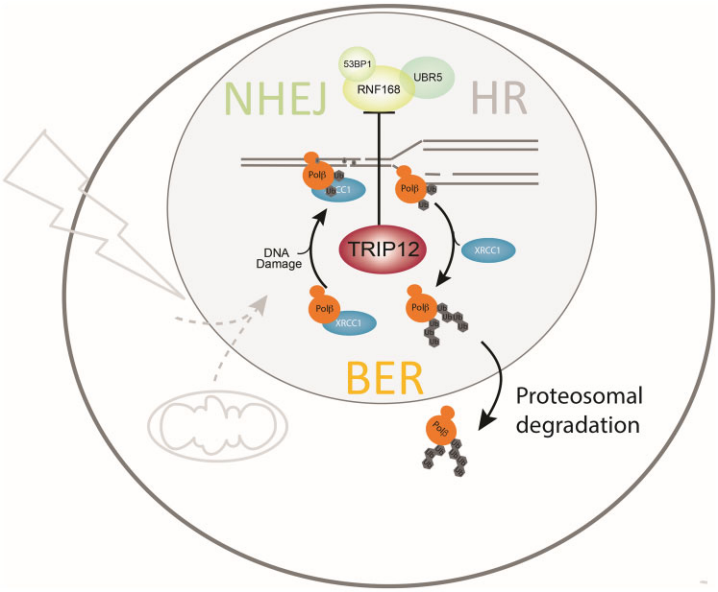
Graphical abstract



Introduction

Genome integrity maintenance is challenged by the multitude of different DNA lesion types and the nuclear dynamic context in which they occur. Resolution of DNA damage caused by exogenous or endogenous sources requires the engagement of different DNA repair pathways. This is typically determined by the type of lesion and the cell cycle phase. DNA double-strand break (DSB) repair pathways are tightly regulated to ensure efficient repair in different cell cycle phases and chromatin conditions [1, 2]. Single-strand breaks (SSBs) and damaged bases are mainly repaired by DNA polymerase β (Polβ)-dependent base excision repair (BER). In a previous study, we found that BER protein complex composition and protein stability change upon exposure to damage such as from radiation and in a cell cycle phase-dependent manner [3].

Complex DNA lesion sites that contain different lesions in close proximity pose particularly difficult repair conditions as they may require the involvement of intrinsically very distinct repair pathways. Thus, repair pathway choice and sequence are crucial to the success of DNA damage repair under such conditions. Nonhomologous end joining (NHEJ) and homologous recombination (HR) are the most prominent DSB repair options, and their interaction and relative contribution have been actively researched in recent years [4, 5]. DSBs, such as those caused by radiation or generated due to DNA crosslinks from metabolic formaldehyde, cisplatin, or similar crosslinkers, can create marked threats to DNA replication and cellular division. These potentially lethal lesions are resolved by DSB repair processes. BER, on the other hand, deals with base lesions or contributes to SSB repair (SSBR), abundantly generated by metabolic processes, reactive oxygen species, deamination, or depurination [6, 7]. Greatly outnumbering DSBs, radiation also causes a multitude of BER-specific lesion types [8, 9]. Of these, oxidative base lesions and SSBs are the most predominant. As such, radiation-induced DNA damage can be very complex in nature, comprised of a mixture of DNA lesions in close proximity requiring both DSB repair and BER.

Different DNA lesion types, when adjacent to each other, require proper spatiotemporal as well as chronological (or sequential) engagement of different repair proteins. In fact, radiation-induced complex DSBs, which also comprise multiple base damages, are the most difficult to repair and hence most detrimental to cellular survival and genomic integrity. The chromatin context in which those lesions occur is also an important factor in DNA damage repair that needs to consider replication, transcription, and different (hetero)chromatic conditions. Even though close links between distinct lesion repair pathways would be expected, no studies exist that demonstrate an inter-pathway collaboration of BER and DSB repair, and few report the involvement of individual BER elements in DSB repair processes [10–12]. Crosstalk mechanisms that enable DNA repair pathway choice at lesions and chromatin sites that are common to both, and the orchestration of protein complex formation thereof, remain elusive.

Chromatin and repair enzyme modifications by ubiquitylation are relevant processes that regulate the DNA damage response (DDR) and DSB repair [13]. An RNF8/RNF168 E3 ubiquitin ligase-coordinated cascade of non-proteolytic ubiquitylation events results in chromatin marks in the vicinity of DSBs. RNF168-dependent chromatin ubiquitylation mediates the accrual of the DSB repair proteins 53BP1 and BRCA1 to the lesion site [14], and additional studies have shown how RNF168-mediated 53BP1 engagement can support the regulation of DSB repair [15–17]. 53BP1 recruitment blocks resection, an important step in HR, to ultimately promote NHEJ. Resection and therefore HR, on the other hand, is facilitated by BRCA1 by counteracting 53BP1 recruitment at such ubiquitylated sites [18]. A recent report further supports a role for RNF168 in facilitating HR through PALB2 loading in a BRCA1-independent manner [19, 20]. Together, this highlights the intricate molecular mechanisms by which chromatin ubiquitylation directs DSB repair pathway choice. Surprisingly, to date no similar processes have been linked directly to BER/SSBR.

A different E3 ubiquitin ligase, TRIP12 (ULF), has also been shown to regulate DDR protein homeostasis. TRIP12 prevents 53BP1 hyperaccumulation by controlling RNF168 residence at break sites [21], thus indicating an involvement of TRIP12 in DSB repair suppression. TRIP12 was also reported to affect PARP inhibitor efficacy [22, 23] and to interact with Ku70 [24], while its expression was found to be regulated by p16 [25, 26]. Despite this and a reported effect on the USP7-regulated stabilization of p53 [27], little is known about TRIP12's overall impact on DNA repair and cellular functions.

Polβ has a crucial function in BER and SSBR. Upon lesion recognition and removal by glycosylases and AP endonuclease 1, Polβ executes end-tailoring and DNA synthesis at the gap [7]. The 5′dRP lyase and nucleotidyl transferase activities allow Polβ, supported by XRCC1, to execute base and single-strand break repair in many cases [28]. The distinctive expression pattern of Polβ, across different tissues and during development, points to processes that tightly regulate Polβ protein levels, suggesting a requirement to assure context-appropriate BER and Polβ-dependent activity [29]. Deregulated expression has indeed been reported to lead to interference in replication processes and defects in Polβ subcellular localization resulting in altered damage response [30–35]. Such findings led us to postulate the existence of molecular and cellular mechanisms that counteract the dangers of such "over"-repair by stringently controlling Polβ levels to exclude Polβ from such disruptive activities. Our past studies revealed elements in the governance of Polβ levels and stability that ultimately also define BER protein complex makeup. We elucidated the regulation of the BER/SSBR protein complex architecture through proteolytic ubiquitylation events and HSP90 binding via its interaction with XRCC1 in response to damage [3]. In this earlier study [3], we showed that separation-of-function mutants of Polβ failed to interact with XRCC1 and were targeted for ubiquitylation and degradation. A knock-in mouse model expressing such a separation-of-function mutant of Polβ confirmed reduced Polβ protein levels *in vivo* [36]. Importantly, we also found that the proliferation status of the cell and the nature of the damaging agent defined BER complex formation and Polβ stability [3]. Solely in proliferating cells and distinctively different from the BER complex formation pattern observed after alkylating agents, radiation exposure led to increased levels of the HSP90-bound XRCC1 [3]. These data led us to hypothesize that BER/SSBR protein complex formation is context dependent and can vary according to cell cycle phase or DNA damage type. Two ubiquitylation sites on Polβ suggested an unidentified regulatory component of BER/SSBR. We, therefore, searched for a ubiquitin ligase that fulfills this role in Polβ regulation and discovered how this new element in BER/SSBR (TRIP12) provides a "repair-controller" function.

Materials and methods

Materials

All materials and supplies are listed in Supplementary Table S1.

Cellular models

Cell line models were developed by lentivirus-mediated transduction of the glioma cell line LN428 to allow expression of the indicated proteins (Flag-Polβ, myc-TRIP12, copGFP-Polβ), as indicated in detail in Supplementary Document S1 and Supplementary Table S1. LN428 cells were chosen here as an extension of our previous work [3], demonstrating that the ubiquitylation of Polβ is dependent on the interaction between Polβ and XRCC1 [3]. In some cases, modified cells were further modified by a second transduction, such as expressing TRIP12-shRNA in cells modified for expression of Flag-Polβ, and using lentiviral vectors with different selection makers (puromycin, geneticin, hygromycin). Flag-Polβ, myc-TRIP12, myc-TRIP12-HECT, and myc-TRIP12-SB vectors were mainly used to assess binding interactions or ubiquitylation assays, as indicated throughout. CopGFP-Polβ-based models were generated for laser-induced micro-irradiation experiments, radiation- or cisplatin-induced foci analysis, and nuclear colocalization analyses. For overexpression models, cell lines were developed with elevated Flag-Polβ expression and respective mutants. Several different TRIP12 shRNA-based vectors were used to establish the cellular role of TRIP12 expression in Polβ stability and radiation response.

Methods

Detailed methods are described in Supplementary Document S1.

In brief, standard cell lysis, chromatin fraction isolation, immunostaining, immunoblotting (IB), and immunofluorescence microscopy procedures were applied using the antibodies and conditions as indicated in the figures and legends and detailed in Supplementary Document S1 and Supplementary Table S1.

For protein complex partner identification and immunoprecipitation (IP), anti-Flag M2 affinity gel was used to immunoprecipitate the individual proteins after cell lysis with Pierce IP lysis buffer and according to the manufacturer's protocols. Twenty-seven individual IP samples were prepared ($n = 9$ per condition) and loaded onto SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gels for label-free differential mass spectrometry (dMS) analysis. Tryptic peptides extracted from each IP product were separated by reverse-phased nano-flow liquid chromatography (EASY-nLC II, Thermo Scientific, San Jose, CA) and analyzed on an LTQ/Orbitrap Velos Elite hybrid mass spectrometer (Thermo Fisher, San Jose, CA). Mass spectrometry data collected for each sample were analyzed using dMS software (Infoclinika, Bellevue, WA). The high-resolution full MS spectra were aligned and the m/z, charge state, retention time, and intensity data for all molecular features detected in the full-scan mass spectra were integrated and matched to protein identification results as detailed in Supplementary Document S1. Student's t-test implemented in MATLAB® was used to determine the statistical significance of the difference in the abundance of identified proteins/features in different IP samples. The abundance values in Flag-Polβ(WT) and Flag-Polβ(TM) samples were normalized to the Polβ level in each sample to calculate the WT/TM ratio used to assess the differential interaction between Flag-Polβ(WT) and Flag-Polβ(TM) samples.

Recombinant proteins (His-tagged ubiquitin, His-Ub) were generated as detailed in Supplementary Table S1 and Supplementary Document S1. To study interactions with TRIP12 or ubiquitylation of Polβ, IPs were performed using anti-TRIP12, anti-Myc, and anti-Polβ (Clone 61) antibodies (Supplementary Table S1) as indicated in the figures

and following standard protocols (Supplementary Document S1). To assess the ubiquitylation activities of TRIP12 and the HECT or substrate binding (SB) domains of TRIP12, myc-TRIP12, myc-HECT, myc-HECT(C2007A), and myc-TRIP12-SB were immunoprecipitated using an anti-myc antibody or Myc-Trap agarose and exposed to *in vitro* ubiquitylation assays (Supplementary Document S1) with or without purified Polβ. Ubiquitylation of purified Polβ was examined after gel electrophoresis and IB with anti-Polβ (Clone 61), anti-ubiquitin, and anti-His-tag antibodies. Autoubiquitylation of the TRIP12-HECT domain was examined after gel electrophoresis and IB as described above with an anti-ubiquitin or anti-His-tag antibody (Supplementary Document S1).

For Polβ stability and degradation evaluation, cells were seeded and treated with 0.2 mM cycloheximide (Cyc) or with Cyc (0.2 mM) and MG132 (25 μM) for the time indicated in the figures.

Radiation sensitivity and H_2O_2-induced cytotoxicity were determined using the colony formation assay as described in Supplementary Document S1. The CometChip assay was used to assess DNA damage and repair kinetics, as outlined in Supplementary Document S1.

Statistical analysis

The statistical analysis procedures and parameters for the different analyses are described in Supplementary Document S1.

In brief, averages and standard deviations (SDs) were calculated from the means (of technical replicates) of multiple independent experiments (n = number of independent experiments as indicated in figure legends), unless stated otherwise. ANOVA was used to test for significant differences, generally compared to controls and as indicated in the figure legends. To enable the evaluation of potential changes in the distribution of the foci number per cell values, foci dot plots are shown and contain pooled foci data from all independent experiments, with a minimum of 50 analyzed cells for each experiment. A non-parametric test (Kruskal–Wallis with Dunn's multiple comparison test) was used if the data did not pass the normality test. To evaluate robustness of the findings across multiple experiments, a second analysis considers the interexperimental variation and compares the means of the individual experiments using ANOVA after calculating the average and SD of the mean foci number per cell values derived from the independent experiments (Supplementary Document S1). Results of both analyses are shown throughout, either in the main or in the supplementary figures.

As the dependence of the individual data points in the survival dose response data precludes ANOVA analyses, nonlinear regressions were computed using $Y = 100/[1 + (X^{\mathrm{HillSlope}})/(\mathrm{IC}_{50}{}^{\mathrm{HillSlope}})]$ (with Y = survival in % and $X = H_2O_2$ concentration) on the normalized H_2O_2 dose response survival data to calculate IC_{50} (half-maximal inhibitory concentration) values for each individual experiment. Similarly, D_{37} (radiation dose permitting 37% cell survival) values were calculated from linear quadratic fits [$Y = \exp(-\alpha^*X - \beta^*X^2)$, with X = radiation dose and Y = surviving fraction] on the survival curves. One-way ANOVA was then used to test for significant differences in these response parameter values as stated in the text. P-values are indicated by asterisks with $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

Results

DDR suppressor TRIP12 is a novel partner of the BER protein Polβ

The majority of Polβ partners with XRCC1 to conduct BER [37]. Yet, XRCC1 and Polβ function independently, in different BER subcomplexes, for yet undefined roles in DNA metabolism [3]. We recapitulated these main cellular BER complexes (herein termed Complex A, comprised of Polβ and XRCC1, and Complex B, XRCC1-devoid complexes) by exploiting a separation-of-function mutant of Polβ that does not bind to XRCC1, the V303-loop mutant Polβ(TM), encoding the Polβ mutations L301R/V303R/V306R (Supplementary Fig. S1A). This approach allowed us to reveal novel partners of Polβ that are not dependent on XRCC1 (Fig. 1A). Using label-free dMS, an unbiased quantitative proteomic method [38, 39], we discovered TRIP12, a DSB repair-related E3 ubiquitin ligase, as a novel Polβ partner. We found TRIP12 to bind preferentially to the Polβ-centered Complex B, suggesting involvement in BER complex regulation (Fig. 1B, Supplementary Fig. S1B and C, and Supplementary Table S2). Polβ(TM) cellular levels drop significantly (Supplementary Fig. S1B and C), as also reported previously [3]. The dMS analyses and IP experiments show that TRIP12 is bound to both Polβ(WT) and Polβ(TM), yet we observed a significantly increased interaction with Polβ(TM) (Fig. 1B and Supplementary Fig. S1C). Other novel partners selectively bound to Polβ(TM) include RFC1, EIF, BCKDHA, SPT16, and SSRP1 (Supplementary Table S2 and Supplementary Fig. S1D). Quantitative dMS and immunoprecipitation/immunoblot (IP/IB) analysis confirms the lack of XRCC1 and known XRCC1-mediated BER factors (LIG3, PARP2, PNKP, Aprataxin) in the Polβ(TM) samples, supporting Polβ specificity of the approach, and points to the dominance of TRIP12 among non-BER proteins in both the transgenic and endogenous setting and in different cell lines (Fig. 1C and D, and Supplementary Fig.1C–E). Domain mapping of the interaction between the TRIP12 E3 ubiquitin ligase active site HECT domain and the remaining N-terminal E3 ubiquitin ligase SB domain (TRIP12-SB) shows Polβ binding at TRIP12-SB and a preferential binding to the TRIP12-HECT and TRIP12-SB domains in the Polβ-centric Complex B as revealed by the interaction with Polβ(TM) (Fig. 1E–G and Supplementary Fig. S1F). This binding is mediated by the C-terminus of Polβ that also binds to XRCC1 and contains the ubiquitylation target sites [K206, K244; mutated to alanine in Polβ(DM)] [3].

TRIP12 ubiquitylates Polβ and targets it for degradation

The interaction of TRIP12 with Polβ and its complex-selective nature suggest a role for TRIP12 as the primary E3 ubiquitin ligase regulating the observed cell cycle and DNA damage type-dependent stability of Polβ [3, 37]. We therefore determined whether TRIP12 ubiquitylates Polβ and whether this TRIP12-mediated ubiquitylation depends on the previously identified lysine residues (K206/K244) [3]. An *in vitro* "on-bead" assay [40–43] was designed to test TRIP12's ability to ubiquitylate purified recombinant Polβ and revealed evidence of prominent (mono- and poly-)ubiquitylated species.

As depicted in the "on-bead" Ubi-assay graphic (Fig. 2A, left), myc-tagged full-length TRIP12, TRIP12-HECT domain (WT or encoding the active site mutant C2007A), TRIP12

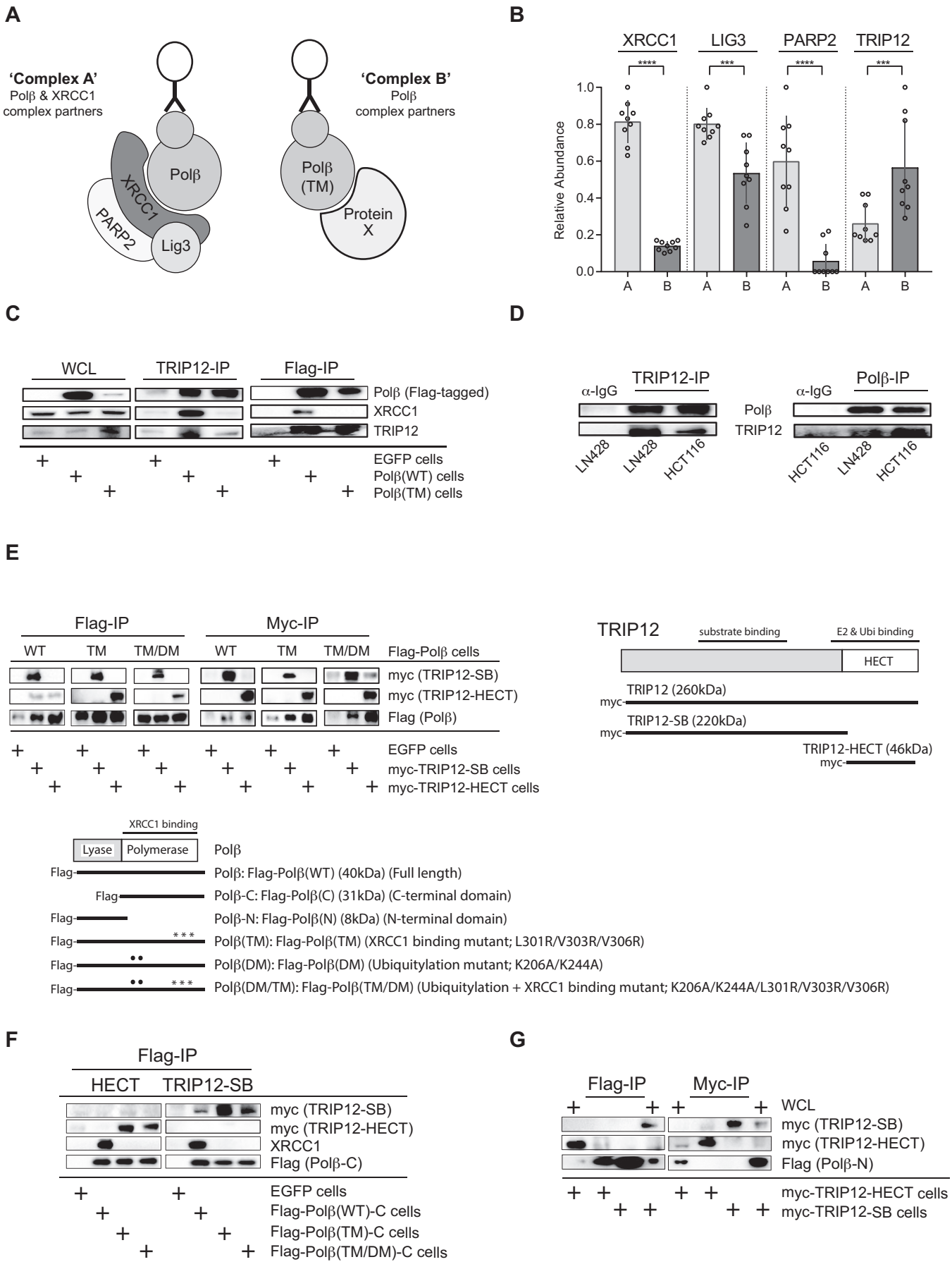


Figure 1. TRIP12 as a novel Polβ partner. (**A**) BER complex compositions and differential partner identification strategy. The graphic depicts the immunoprecipitation scheme used to discover novel interacting BER protein partners. (**B**) TRIP12 is a novel Polβ binding partner with an increased

SB domain (TRIP12-SB), or EGFP (serving as negative control) was expressed in LN428 cells. Captured on beads (FR1 in Supplementary Fig. S2A, left), these myc-tagged proteins were then incubated with recombinant His-tagged ubiquitin (FR2) and recombinant Polβ (WT or the DM mutant, K206A/K244A) (in FR3) to compare TRIP12-mediated ubiquitylation of both Polβ variants (Fig. 2A and B, and Supplementary Fig. S2A). HECT domain E3 ubiquitin ligases, and thus also TRIP12, first form a covalent intermediate (as depicted in FR2) before the ubiquitin is transferred to its substrate (in FR3) [41–43], as we also observed in previous UBE3B "on-bead Ubi-assay" analyses [40]. Here, we found that full-length TRIP12 and the HECT domain are both covalently modified by ubiquitin in this *in vitro* "on-bead" assay (Supplementary Fig. S2A, right). Importantly, Polβ (bound to the myc-tagged protein and beads in FR3, or released in FR4) is ubiquitylated in the presence of full-length TRIP12, or the TRIP12-HECT domain (Fig. 2A, right, and B). This ubiquitylation is impaired when the HECT domain active site is mutated (C2007A) as demonstrated by the lack of larger and modified Polβ forms and greatly reduced mono-ubiquitylated forms (Fig. 2A, right, and B, right).

In vivo evaluation of the ubiquitylation of Polβ was accomplished by expressing HA-tagged ubiquitin in Flag-tagged Polβ (WT and mutants) expressing LN428 cells. IP of Flag-tagged Polβ, followed by immunoblot for HA, confirmed TRIP12-dependent (poly-)ubiquitylation. Consistent with its preference to bind to BER Complex B (Fig. 1), this is increased in the triple mutant Polβ(TM), which does not bind XRCC1 (Fig. 2C and Supplementary Fig. S2C). TRIP12-dependent (poly-)ubiquitylation is abrogated when modifying the potential ubiquitylation target sites in the Polβ(TM/DM) and Polβ(DM) mutants (Fig. 2C and Supplementary Fig. S2B) or when Flag-Polβ is not expressed (Supplementary Fig. S2C, right). Consistent with its proposed function [44], we observed binding of the TRIP12-HECT domain to ubiquitylated Polβ in these IP experiments (Supplementary Fig. S2C).

Loss of XRCC1 binding results in five-fold decreased basal levels of Polβ mutants that do not bind to XRCC1, such as Polβ(TM) (as previously shown in [3]). Importantly, addition of the K206A/K244A mutations, Polβ(TM/DM), however, reverts this instability phenotype [3]. Here, we find that TRIP12 affects Polβ levels, revealing its role in this Polβ degradation route (Fig. 2D). Underlining a complex-specific (and XRCC1-dependent) activity, loss of TRIP12 stabilizes Polβ(TM) most profoundly (Fig. 2D and Supplementary Fig. S2D and E). These comparative expression analyses show that this effect on Polβ protein levels is dependent on the potential ubiquitylation target sites K206/K244 [mutated in Polβ(DM) and Polβ(TM/DM)] (Fig. 2D), despite similarly efficient binding to full-length TRIP12 (Supplementary Fig. S1F). As also shown in our previous report [3], if bound to XRCC1 (Complex A) in undamaged cells, the wild-type version of Polβ, Polβ(WT), is stable and not subjected to proteasomal degradation within a 6-h time frame (Supplementary Fig. S2F, left panel, Flag-Polβ(WT), and G). Conversely, Polβ is rapidly degraded if unable to bind to XRCC1 [3] (Supplementary Fig. S2F, right panel, Flag-Polβ(TM), and G). We further show that this degradation of (XRCC1-free) Polβ is regulated by TRIP12 through the loss of such proteasome-mediated degradation processes in TRIP12-shRNA expressing cells (Supplementary Fig. S2F and G).

TRIP12's impact on base excision repair processes

Supporting its nuclear role, TRIP12 mostly resides in the nucleus and on chromatin (Fig. 3A and B) [45, 46]. We find that, despite overall increased levels, the chromatin-associated fraction of endogenous Polβ is reduced in TRIP12-shRNA expressing cells, suggesting that TRIP12 promotes Polβ chromatin association (Fig. 3B). Furthermore, mutations of the Polβ K206/K244 ubiquitylation sites greatly reduces chromatin, but not nuclear, localization of such transgenic Polβ variants [Polβ(DM) versus Polβ(WT) in Supplementary Fig. S3A] indicating a role for TRIP12-mediated ubiquitylation in this process. This prompted us to investigate TRIP12's role in BER beyond an influence on BER complex-specific Polβ degradation.

At the chromatin level, TRIP12 has been reported to prevent excessive DNA damage signaling and DSB repair protein accumulation to contain the cellular response to DSBs [21]. After confirming that TRIP12 depletion also affects RNF168 levels in our cellular models (Supplementary Fig. S3B), we first questioned whether TRIP12 affects Polβ's primary function in BER or affects BER overall. For this, we tested two BER-specific outcomes: (i) recruitment to sites of laser-induced DNA damage and (ii) response to and repair of oxidative and alkylation DNA damage. Our previous studies showed that Polβ is recruited to sites of laser-induced DNA damage in a manner that depends on PARP1 activation and binding to XRCC1 [3, 5]. To test a potential impact on this BER process, here we compared copGFP-tagged Polβ recruitment parameters in TRIP12-depleted cells to those in parental or scrambled shRNA (SCR)-expressing cells. We find that recruitment or retention of Polβ at laser-induced DNA damage sites is not significantly affected by the presence or absence of TRIP12 (ANOVA $P = .2–.65$; Fig. 3C and Supplementary Fig. S3C). Next, we evaluated whether the reduction of TRIP12 levels affects alkylation or oxidative damage repair as determined by alkaline DNA comet analysis using the CometChip

affinity to the XRCC1-free complex (Complex B). Quantification of selected proteins bound to Complex A or Complex B, as indicated, by label-free dMS. The relative abundances of prototypic peptides with unique amino acid sequences AIGSTSKPQESPK, SEAHTADGISIR, VNNGNTAPEDSSPAK, and LSTQSNSNNIEPAR were used as surrogate measures of XRCC1, LIG3, PARP2, and TRIP12, respectively. Peptide abundance levels were normalized to Polβ in each immunoprecipitated sample and are shown as dots on bar plots with mean and SD. Significant differences between complexes A and B are marked by asterisks ($^{***}P < .001$, $^{****}P < .0001$; ANOVA). (**C**) BER complex-dependent interaction of TRIP12. Interaction of TRIP12 with Flag-Polβ(WT) or Flag-Polβ(TM) was revealed by IP from whole cell lysates (WCLs) and IB as indicated. (**D**) Interaction of endogenous Polβ with TRIP12. IP/IB of endogenous proteins in the indicated cell lines are shown. (**E**) Domain interaction mapping. Flag-Polβ binding to myc-TRIP12 domains as depicted in the scheme shown to the right and determined by IP/IB: myc-TRIP12 (full length TRIP12, with an N-terminal myc-tag), myc-TRIP12-SB (TRIP12 SB domain, amino acids 1–1650, with an N-terminal myc-tag), and myc-TRIP12-HECT (TRIP12-HECT domain, amino acids 1651–2040, with an N-terminal myc-tag). (**F**) Domain interaction mapping of myc-TRIP12 to the C-terminal domain of Polβ wild-type and mutants by IP/IB: Flag-Polβ(WT)-C (amino acids 91–335), Flag-Polβ(TM)-C, XRCC1 binding mutant (amino acids 91–335), and Flag-Polβ(TM/DM)-C, ubiquitylation and XRCC1 binding mutant (amino acids 91–335). (**G**) Domain interaction mapping of myc-TRIP12 to the N-terminal domain of Polβ wild-type and mutants by IP/IB: Flag-Polβ(WT)-N (amino acids 1–90).

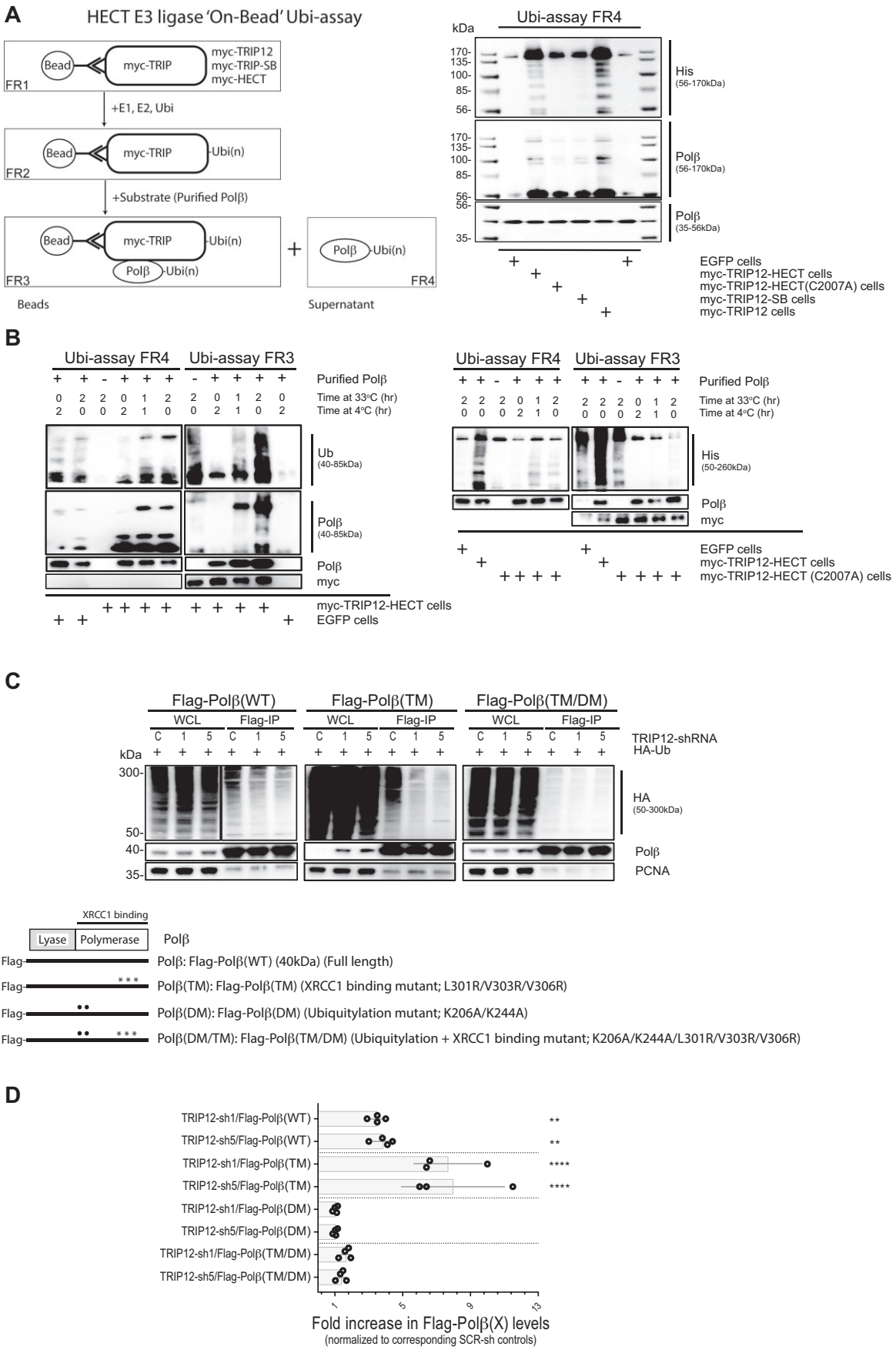


Figure 2. TRIP12's role in Polβ ubiquitylation and degradation. (**A**) *Ex vivo* Polβ ubiquitylation by TRIP12. Polβ ubiquitylation by TRIP12 as identified by immunoblot (right) after the "on-bead" Ubi- assay as outlined in the scheme (left) using recombinant and purified His-ubiquitin, E1/E2, and Polβ together

[47] or survival analyses. We, however, do not find significant changes in damage levels or survival that would support a role for TRIP12 as an essential factor to efficiently execute oxidative or alkylation damage repair (Fig. 3D and E, and Supplementary Fig. S3D). We then evaluated a potential role for TRIP12's involvement in cellular fitness and found that loss of TRIP12 does not affect proliferation or survival (Fig. 3F and G). Overall, TRIP12 does not appear to be essential for canonical BER activities or cell fitness, or its loss is compensated for by other regulators.

TRIP12 facilitates Polβ engagement after radiation

Polβ has also been reported to participate in noncanonical BER-like processes in DSB repair. We previously reported that XRCC1-dependent functions of Polβ differ and depend on damage type and proliferation status, thereby linking BER protein complex makeup to a radiation damage and a replication context [3]. We observed enhanced HSP90/XRCC1 binding after radiation in proliferating cells that consequently promotes the Polβ-centric Complex B (XRCC1-free), herein observed to be preferred by TRIP12 (Fig. 1) [3]. This prompted an assessment of the Polβ response to radiation. Here, we find that after exposing cells to radiation, Polβ has both a rapid and persistent focal appearance in a radiation dose (thus DNA damage load)-dependent manner that indicates involvement in radiation-induced DNA damage repair (Fig. 4A–C and Supplementary Fig. S4A and B) [7]. Colocalization analyses show that this radiation-induced recruitment of Polβ is accompanied by the BER scaffold protein XRCC1 (Fig. 4D). The data show that the majority of Polβ(WT) foci colocalize with XRCC1 foci in control and irradiated cells. Radiation induction levels are comparable (mean of 3.1 versus 2.6 ± 0.8 foci at 5 h) [3]. Radiation-induced changes in mean XRCC1 foci numbers are also similar to those in Polβ foci (1.32-fold with SD = 0.11 versus 1.17-fold with SD = 0.07). As predicted by its strong requirement for XRCC1 interaction, Polβ foci formation depends on its ability to bind XRCC1 (abrogated in copGFP-tagged Polβ(TM) and Polβ(TM/DM) mutants; Fig. 4E and Supplementary Fig. S4C–E).

While there is a small but discernable fraction of Polβ foci that colocalizes with phosphorylated H2AX foci in irradiated cells (Fig. 4F), Polβ foci are not associated with radiation-induced DSB repair markers such as 53BP1 (Fig. 4G and H), thereby largely excluding significant involvement in DSB repair processes. Importantly, TRIP12 promotes Polβ accumulation and foci formation, since TRIP12 depletion lowers baseline foci levels in untreated cells and abrogates Polβ foci induction by irradiation (Fig. 4I–K). TRIP12 depletion affects the formation of large and persistent Polβ foci most profoundly, which appear to be characteristic of radiation exposure (Fig. 4J and K, and Supplementary Fig. S4F). In comparison, cisplatin treatment solely induces small Polβ foci that are, however, equally induced in TRIP12-shRNA expressing cells, indicating a lack of dependence on TRIP12 (Supplementary Fig. S4G and H). Notably, and consistent with its role in Polβ degradation, cytoplasmic levels of copGFP-Polβ were suppressed at 5 and 24 h after radiation (Fig. 4A) but not in cells expressing TRIP12 shRNA (Figs. 4I and J), further indicating radiation-induced TRIP12 activation that also facilitates cytoplasmic Polβ degradation as described above (Fig. 2D and Supplementary Fig. S2F).

TRIP12's role in Polβ recruitment after radiation is surprising when considering its previously reported DDR suppressive function [21] and suggests precedence for BER over DSB repair. The orchestration of repair protein engagement is indeed particularly challenging at complex DNA lesion sites that are typical of radiation exposure [48]. Risks due to a lack of repair coordination are two-fold: strand incision during repair can cause DSBs at counter-posing BER lesions, while base lesions can also hamper DSB repair. Thus, BER and DSB repair processes need to be tightly regulated both locally and temporally. Complex radiation-induced lesions with extensive BER lesions may therefore require a ruling in favor of BER (and before DSB repair) for optimal repair [49]. This led us to propose a DDR controller role for TRIP12 that ultimately prioritizes BER (through the ubiquitylation of Polβ) over DSB repair in the competition for access to complex lesions that may require both DNA repair processes.

Role of Polβ levels in radiation response

Interference in the herein postulated DDR regulatory role could evoke a survival detriment. However, despite a large impact on the nuclear engagement of Polβ, TRIP12 depletion did not affect cellular survival (Fig. 3E–G). Radiation-induced complex lesion sites require highly balanced and coordinated BER/SSBR processes to prevent DSB formation from opposing lesion incisions [50]. While impacting Polβ recruitment after radiation, as shown here, TRIP12 depletion, however, also alleviates constraints on DSB signaling, which in turn facilitates compensatory DSB repair for cellular survival. In line with this, Lukas and colleagues found that depletion of TRIP12 led to an increase in the levels of RNF168 and promoted ubiquitylation near DNA damage sites with little increase in survival after radiation, as compared to controls [21]. In line with these earlier findings, we find that survival after radiation is not affected by TRIP12

with myc-HECT, myc-HECT(C2007A), myc-TRIP12-SB, or myc-TRIP12 expressed in and isolated from LN428 cells. Boxes indicate the fractions analyzed (FR1–FR4). Immunoblot of FR4 (right) indicates that Polβ is ubiquitylated by full-length myc-TRIP12 or the HECT domain but not the substrate-binding domain or the HECT domain with the active site mutation (C2007A). (**B**) TRIP12-HECT-dependent ubiquitylation. Immunoblots as in panel (A), using FR3 and FR4 from the Ubi assay as indicated in panel (A) with myc-HECT (wild-type or C2007A mutant) after different incubation times. (**C**) TRIP12-dependent ubiquitylation of transgenic Polβ in cells. Ubiquitylation of Flag-tagged wild-type Polβ [Flag-Polβ(WT)], XRCC1-binding mutant Polβ(L301R/V303R/V306R) [Flag-Polβ(TM)], and of the ubiquitylation and XRCC1-binding mutant Polβ(L301R/V303R/V306R/K206A/K244A) [Flag-Polβ(TM/DM)] was determined by IP/IB following transfection of HA-ubiquitin and using two different shRNA (sh1 and sh5) to TRIP12. Flag-IP HA antibody blotted lanes show ubiquitylation of pulled down proteins of which the majority are Polβ and Polβ complex-associated proteins. These are compared to IP input (WCL extracts) and probed for expression of PCNA (input loading control), Polβ, and HA (Ub). (**D**) TRIP12 affects cellular Polβ levels. Shown are changes (fold increase) in Polβ isoform levels, as listed [Flag-Polβ(WT), Flag-Polβ(TM), or Flag-Polβ(DM)], in TRIP12-depleted cells (by TRIP12-sh1 or sh5 knockdown) when compared to their respective control-shRNA (SCR) expressing LN428 cells. Bar graph depicts average control-shRNA (SCR) normalized Polβ isoform levels as determined by multiple immunoblots (representative IB in Supplementary Fig. S2D), each indicated by a dot, with SD and $n = 3$–4. Asterisks indicate statistically significant differences ($^{**}P < .01$, $^{****}P < .0001$; ANOVA) to the corresponding control-shRNA results.

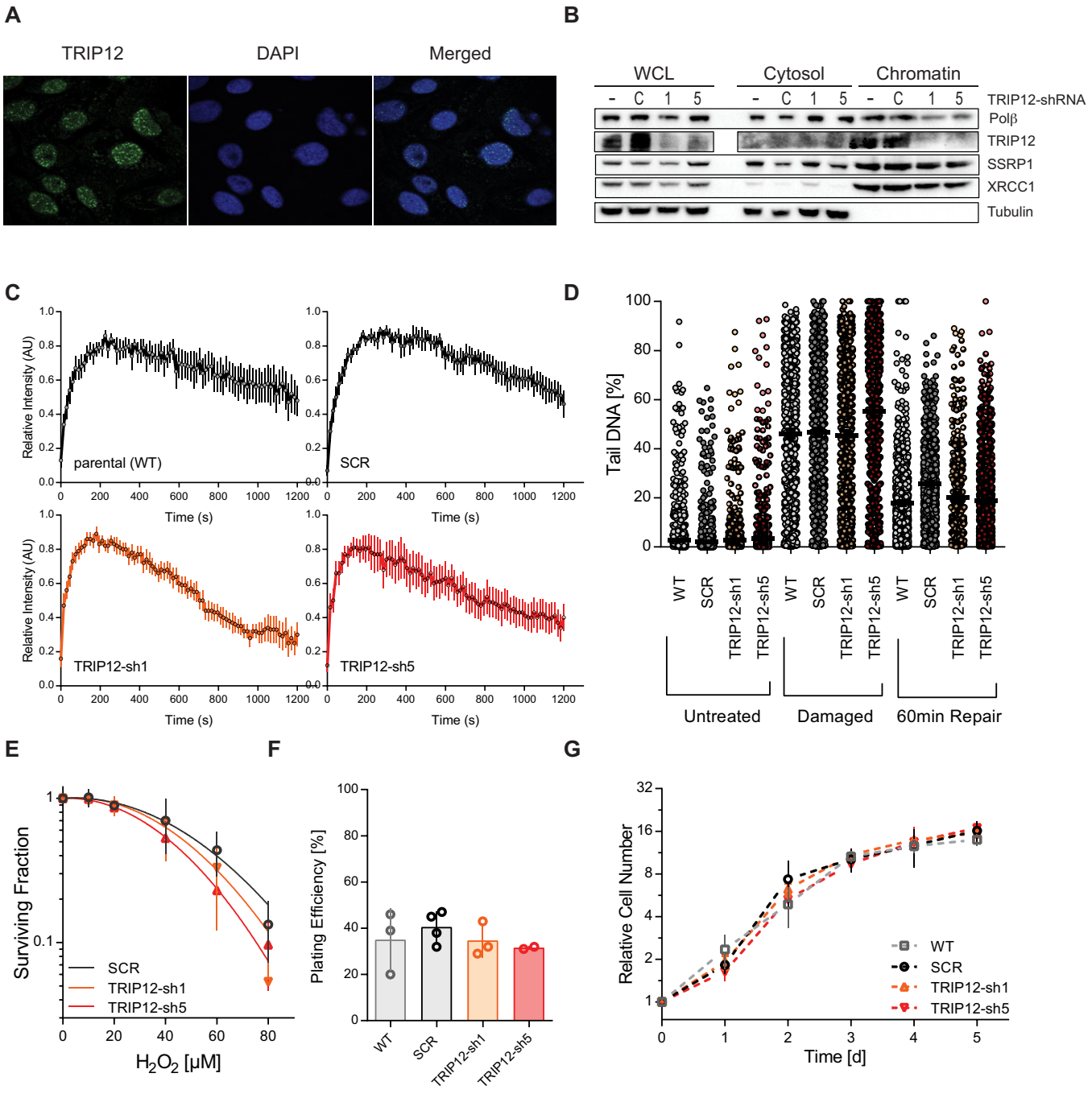


Figure 3. TRIP12-mediated Polβ chromatin retention and BER function (**A**) Prominent nuclear localization of TRIP12. Confocal microscopy images showing nuclear (blue) localization of TRIP12 (green). (**B**) TRIP12-mediated Polβ chromatin retention. Immunoblots show Polβ, XRCC1, tubulin (cytosol fraction loading control), and SSRP1 (chromatin fraction loading control) levels in WCLs, the cytosolic or chromatin fraction in two different TRIP12-KD (knockdown) cell lines (TRIP12-sh1 and -sh5), scrambled controls (C), and the parental LN428 cell line (–) as indicated. (**C**) Laser damage induced focal recruitment of Polβ, independent of TRIP12. Quantification of laser (405 nm)-induced local recruitment and retention of copGFP fused Polβ (copGFP-Polβ) in TRIP12 knockdown (TRIP12-sh1 and -sh5) and control LN428 cell lines. Data are from $n = 2$ independent experiments with each $n = 10$ cells. (**D**) Effective oxidative damage repair in TRIP12-depleted cells. Oxidative damage and repair as determined by alkaline CometChip analyses (tail DNA in %) following a 30-min exposure to 250 μM H_2O_2 (damaged) and at 60 min post-exposure (repair) in TRIP12-KD (TRIP12-sh1 and -sh5), scrambled (SCR), or parental (WT) control cell lines. (**E**) Cellular response to oxidative damage is not affected by the loss of TRIP12. H_2O_2 sensitivity of TRIP12-depleted cell lines (TRIP12-sh1 and -sh5) is not significantly different from scrambled controls (SCR) as determined by clonogenic survival and curve fit comparisons or H_2O_2 IC_{50} determinations (ANOVA). Shown are the mean surviving fractions of three independent experiments ± SD. (**F**) Depletion of TRIP12 does not affect cellular survival. No significant changes (ANOVA) were observed in the clonogenic survival of TRIP12-depleted cell lines (TRIP12-sh1 and -sh5), scrambled controls (SCR), and parental LN428 cells as determined by colony formation assays. The mean of three to four independent experiments, each indicated by dots with ± SD, is shown. (**G**) Depletion of TRIP12 does not affect cell growth. TRIP12-depleted cell lines (TRIP12-sh1 and -sh5), LN428 parental cells (WT), and scrambled control (SCR) growth as determined by the MTT assay (mean and SD of $n = 4$).

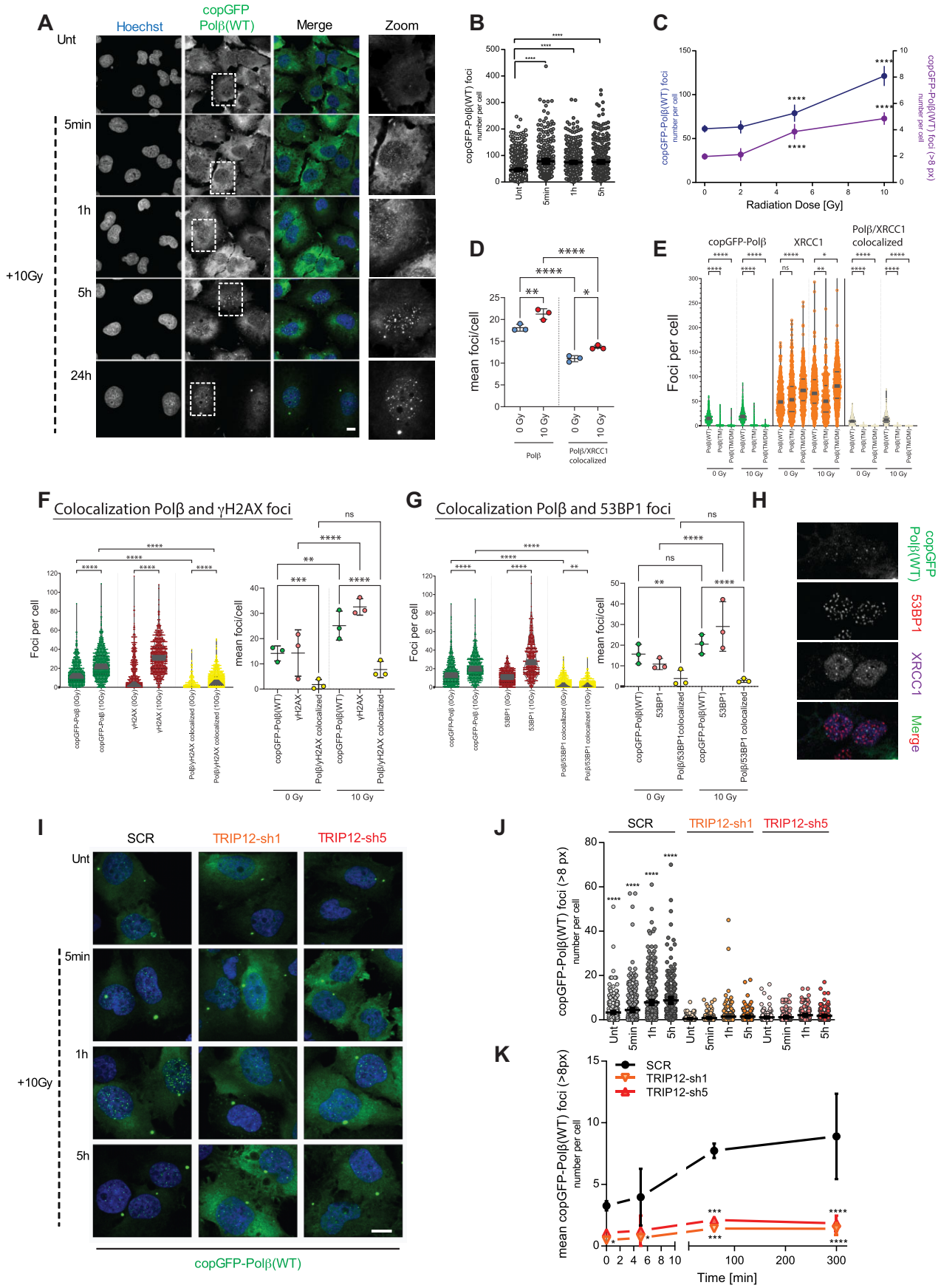


Figure 4. TRIP12 promotes radiation-induced Polβ foci formation. (**A**) Polβ foci formation by radiation. Representative images of copGFP-fused Polβ (copGFP-Polβ) foci in LN428 cells at different time points after radiation (10 Gy). (**B**) Characterization of radiation-induced Polβ foci over time. Dot plots

depletion (Fig. 5A). We therefore argued that, instead, excessive TRIP12-mediated shuttling of Polβ due to Polβ overabundance at supra-physiological levels (overexpression such as seen in some cancer types, developmental stages, and tissues) could expose prioritization of BER and the postulated concomitant suppression of DSB repair signaling. Supporting our proposition, we find that deregulation by Polβ overexpression causes increased residual DSBs after radiation as determined by γH2AX and 53BP1 foci (Fig. 5B). In line with these increased DSB levels, our data further show an increased sensitivity to radiation and reveal that the ultimate impact from Polβ overexpression on the radiation response is as important as the loss of the SSBR/BER protein XRCC1 (Fig. 5C). Together, these data suggest that increased Polβ levels can tip the balance toward persistent BER engagement attempts, thereby disrupting lesion repair leading to secondary and unresolved DSBs.

TRIP12 and its Polβ ubiquitylating activity affect radiation damage repair and response

These findings (Fig. 5) highlight the relevance of a finely tuned balance between BER and DSB repair protein levels in the maintenance of chromosomal integrity. TRIP12's role in Polβ ubiquitylation and chromatin engagement (Figs 2 and 4), together with its reported DSB repair confinement function, points to TRIP12 as a likely candidate for such a regulatory role at the intersection of BER and DSB repair. Confirming TRIP12's governance of Polβ and a ruling in favor of BER, TRIP12 depletion rescued cells from the Polβ overexpression-induced phenotype that caused increased residual DSBs (Fig. 6A) and clonogenic cell death after radiation (Fig. 6B). Abrogating TRIP12 ubiquitylation sites on Polβ [Polβ(DM)] completely suppresses local Polβ accumulation at earlier and later time points after radiation (Fig. 6C). Ubiquitylation of Polβ, by TRIP12, is required for the interference in radiation damage repair, as shown by the reduction of residual DSBs, as determined by γH2AX and 53BP1 foci (Fig. 6D). Finally, abrogation of TRIP12 ubiquitylation also ultimately impedes the survival detriment caused by excess Polβ and further confirms a functional link to TRIP12 (Fig. 6E). Thus, TRIP12 influences radiation response and controls Polβ engagement through Polβ ubiquitylation.

TRIP12 at the intersection of DNA damage repair

Despite the observed decrease and deregulation of Polβ in chromatin, TRIP12 depletion also causes extended RNF168-mediated chromatin signaling that leads to larger 53BP1 foci [21]. This process is supported by the E3 ubiquitin ligase UBR5, which, in conjunction with TRIP12, suppresses RNF168-facilitated 53BP1 chromatin loading [21]. Underscoring TRIP12's unique and active role in Polβ engagement and degradation, we, however, find that, in contrast to TRIP12, UBR5 loss (Supplementary Fig. S5A) does not alter cellular Polβ levels or its chromatin loading (Supplementary Fig. S5B). Since UBR5 also suppresses RNF168, this therefore also excludes indirect effects caused by the excessive spreading of ubiquitylated chromatin.

Our findings suggest that TRIP12 can direct DNA repair activity at complex DSB lesion sites by (i) engaging Polβ for BER at these sites and (ii) temporarily restraining chromatin ubiquitylation, which fosters DSB repair at these sites. In such a scenario, Polβ will be recruited to lesion sites marked by γH2AX, a process accompanied by a TRIP12-mediated delay in chromatin ubiquitylation, thus 53BP1 involvement. Testing this role in local repair segregation further, we conducted a series of colocalization analyses to characterize Polβ foci further. Consistent with a radiation-induced recruitment to complex sites, we observed that a fraction of Polβ foci colocalizes with γH2AX foci (Fig. 4F). We find that recruitment of Polβ to these γH2AX foci is reduced in TRIP12-depleted cells (Fig. 7A–C and Supplementary Fig. S5C–F) and

←

show the distribution of copGFP-Polβ foci in untreated cells (Unt) and in cells at 5 min, 1 h, or 5 h after 10 Gy ($n = 3$ independent experiments with >50 cells each). Mean foci/cell values per experiment and corresponding statistical evaluations are shown in Supplementary Fig. S4A and B. $^{****}P < .001$ in the Kruskal–Wallis test. (**C**) Polβ foci formation is radiation dose dependent. Average copGFP-Polβ foci counts per cell are shown of all foci (left *Y* axis and blue values) and of large foci (>8 px, right *Y* axis with purple values) at 1 h after radiation. Data show mean and SD of pooled foci counts of two to three independent experiments and $^{****}P < .001$ indicate multiple comparisons adjusted to the respective unirradiated controls (ANOVA). (**D**) Polβ foci colocalize with XRCC1. Changes in mean Polβ(WT) foci/cell and Polβ(WT)/XRCC1 colocalized foci of $n = 3$ independent experiments are shown with $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$ as determined by ANOVA. (**E**) Reduced Polβ foci and XRCC1 colocalization of Polβ isoforms devoid of XRCC1 binding [copGFP-Polβ(TM) or copGFP-Polβ(TM/DM)]. Violin plots show copGFP-Polβ isoform foci [copGFP-Polβ(WT), copGFP-Polβ(TM), and copGFP-Polβ(TM/DM)], XRCC1 foci in copGFP-Polβ(WT), copGFP-Polβ(TM), and copGFP-Polβ(TM/DM) expressing cells, and copGFP-Polβ isoform/XRCC1 colocalized foci distributions. Kruskal–Wallis test results are shown and interexperimental variations are shown in Supplementary Fig. S4C and D; representative images are shown in Supplementary Fig. S4E. (**F**) Majority of Polβ foci do not colocalize with γH2AX foci. Numbers of copGFP-Polβ(WT), γH2AX, and Polβ(WT)/gH2AX colocalized foci of individual cells (left) or mean foci/cell values per experiment (right) unirradiated and following 5 h after 10 Gy irradiation are shown; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$ as determined by Kruskal–Wallis (left) or ANOVA comparing the average of the means from three independent experiments (right). (**G**) Polβ foci do not colocalize with 53BP1 foci. Numbers of copGFP-Polβ(WT), 53BP1, and Polβ(WT)/53BP1 colocalized foci of individual cells (left) or mean foci/cell values per experiment (right) unirradiated and following 10 Gy irradiation and 5-h repair are shown; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$ as determined by Kruskal–Wallis (left) or ANOVA comparing the average of the means from three independent experiments (right). (**H**) Representative images illustrating the quantified colocalization pattern of Polβ and XRCC1 foci or lack thereof with 53BP1. (**I**) TRIP12-mediated Polβ foci formation. Representative images of TRIP12-controlled Polβ recruitment after radiation (10 Gy) in scrambled (SCR) control cells and TRIP12-depleted (TRIP12-sh1 and -sh5) cells. (**J**) Late and large Polβ foci and Polβ foci rich cells are particularly affected by TRIP12 depletion. Graph shows quantification of radiation-induced Polβ [copGFP-Polβ)] foci in scrambled (SCR) control and TRIP12-sh1 and -sh5 cells as indicated. Data show the number of large (>8 px) Polβ foci per cell from $n = 3$ independent experiments with >50 analyzed cells each; bars indicate means with SD. Asterisks mark multiple comparison adjusted *P*-values ($^{****}P < .0001$) in the Kruskal–Wallis test comparing the different cell line results at each time point to each other. (**K**) Robust abrogation of radiation-induced Polβ foci by TRIP12 depletion. Quantification of radiation-induced Polβ [copGFP-Polβ)] foci in scrambled (SCR) control and TRIP12-sh1 and -sh5 cells as in panel (J). Graph demonstrates the interexperimental variation not visible in panel (J) and shows the average and SD of the means from $n = 3$ independent experiments over time. Control SCR data used as reference; asterisks indicate significantly different mean foci numbers with $^{*}P < .05$, $^{***}P < .00$, and $^{****}P < .0001$ (ANOVA). ANOVA reports a significant interaction with $P < 0.05$. Radiation-induced foci are significantly different from untreated, only in the SCR for the 1 and 5 h data points with $P < .001$ and $P < .0001$, respectively.

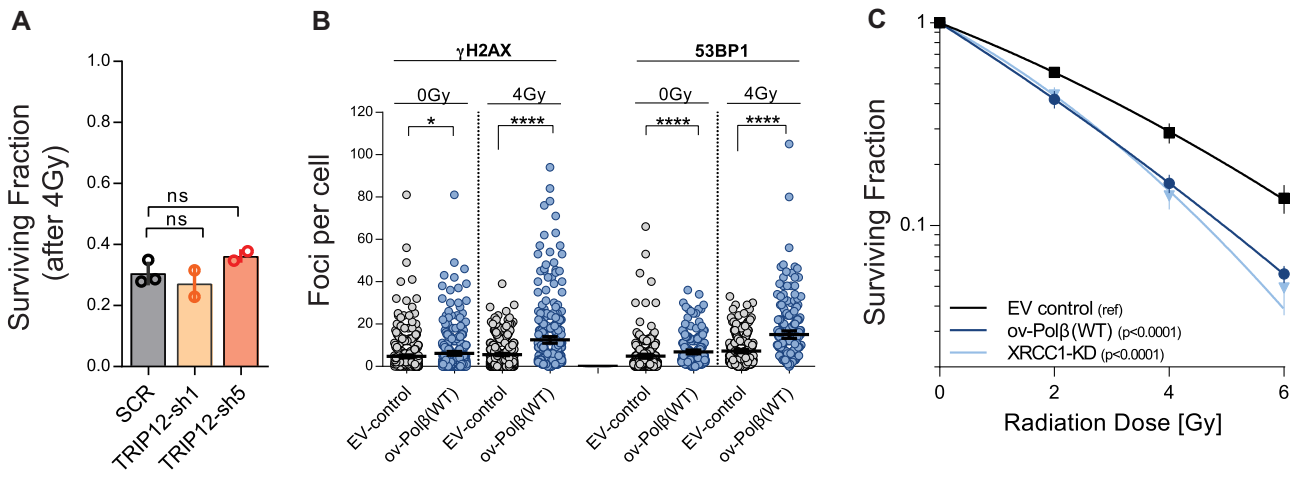


Figure 5. Repair interference by forced Polβ imbalance (**A**) Radiation response is unaltered by TRIP12 depletion. TRIP12-depleted cells (TRIP12-sh1 and -sh5) were compared to scrambled (SCR) LN428 control cells. Clonogenic survival after 4 Gy with $n = 2$–3 independent experiments; errors are SD; and ns = non-significant ANOVA test results. (**B**) Polβ overexpression results in increased residual DSBs. Forced Polβ shuttling by excess Polβ results in increased residual γH2AX and 53BP1 foci per cell 24 h after radiation (4 Gy). Polβ overexpressing LN428 cells [ov-Polβ(WT)] were compared to empty vector controls (EV-control). Dot plots show foci per cell counts of $n = 3$ independent experiments with minimal 50 cells each: $^{*}P < .05$ and $^{****}P < .0001$ (ANOVA). (**C**) Radiosensitization by deregulated Polβ. Cellular survival (clonogenicity as plating efficiency) and survival after radiation drops in Polβ overexpressing cells [ov-Polβ(WT)] to the same extent as in XRCC1-depleted (XRCC1-KD) cells. Bars and values show the mean and SD of the averages from three to seven independent experiments as indicated by the dots. *P*-values indicated in the radiation response curve graphs assess the likelihood of the data curve fits to be similar. Radiation response parameters (D37%) in the ov-Polβ(WT) and XRCC1-KD differ significantly from the reference (ref) EV-control cell line with $P < .01$ and $P < .01$ (ANOVA), respectively.

for the Polβ(TM/DM) variant that cannot be ubiquitylated by TRIP12 (Fig. 7D–F and Supplementary Fig. S5F–H). Consistent with the earlier finding of preferential TRIP12 binding to, and ubiquitylation of, the XRCC1-devoid BER complex binding, abrogation of XRCC1 binding does not affect recruitment to γH2AX foci (Fig. 7D–F and Supplementary Fig. S5F–H). We further observe that TRIP12 restrains nuclear colocalization of Polβ and 53BP1 (Fig. 7G and Supplementary Fig. S5I–K) in unirradiated conditions. Although low in numbers (Fig. 4G), this increased fraction of cells with colocalized Polβ and 53BP1 foci in TRIP12-shRNA expressing cells suggests that pathway segregation has been compromised at these lesions (Fig. 7G and Supplementary Fig. S5L). We also find a prominent, but largely TRIP12-independent, radiation-induced drop in the colocalization of Polβ and 53BP1 foci (Fig. 7G) that can be explained by the reduced engagement of Polβ at DSB sites in TRIP12-depleted cells. Together, these data illustrate how TRIP12 functions as a regulator of repair protein engagement at the intersection of BER and DSB repair pathways by revealing that TRIP12 and TRIP12 ubiquitylation sites support Polβ engagement at a fraction of γH2AX marked DSB sites (Fig. 7A–F) while at the same time reducing 53BP1 involvement as evidenced by reduced Polβ/53BP1 colocalization in its presence (Fig. 7G and Supplementary Fig. S5L).

In conclusion, our data indicate that TRIP12 functions as a DNA damage repair regulator at the intersection of BER and DSB repair processes by ubiquitylating Polβ at K206/K244 in an XRCC1 and BER complex type-dependent manner. TRIP12 facilitates chromatin association of Polβ, promoting radiation-induced engagement to γH2AX marked foci, while also restraining chromatin ubiquitylation. This has the impact of temporally reducing DSB repair pathway interference and ultimately regulating cellular Polβ levels, potentially guided through distinctive (mono- and poly-)ubiquitylation processes (graphically depicted in Fig. 7H).

Discussion

The data presented here grant TRIP12 a central molecular role in DDR and repair pathway orchestration and reveal its important function in Polβ engagement and BER homeostasis. We show that TRIP12 binds to Polβ and through ubiquitylation controls cellular levels of Polβ and regulates Polβ chromatin retention upon DNA damage. The herein discovered role for TRIP12 at the nexus of DDR and repair pathways toward Polβ engagement and Polβ-directed repair results in the segregation of BER from RNF168/53BP1-initiated DSB repair pathways through its previously reported function in RNF168 and 53BP1 containment [21] (see the Graphical abstract). Such a "repair-controller" role is particularly important at complex DNA lesion sites to assure optimal DNA repair pathway choice and sequence to prevent damage aggravation.

Importance of DNA repair pathway orchestration

The vital importance of genome maintenance is underscored by the evolution of multiple genome repair mechanisms, each of which function on a specific type or class of damaged DNA [51]. Of these, the BER pathway plays a critical role in repairing the most abundant type of DNA lesions. DNA SSBs, nicks, and replication-blocking base lesions are the most critical among BER targets. If not repaired, they can give rise to DSBs that ultimately require repair by NHEJ or HR, depending on the context in which they arise. Ionizing radiation induces clustered DNA lesions with base damage, SSBs, and DSBs in close proximity to each other. They are therefore particularly challenging lesions to repair that require a well-coordinated BER-mediated strand incision sequence to prevent DSB formation [50, 52–55]. Since the complexity of this type of damage also hampers DSB repair if located close to or at DSBs, cellular mechanisms that direct the order of repair activities are critical for genome maintenance, suggesting

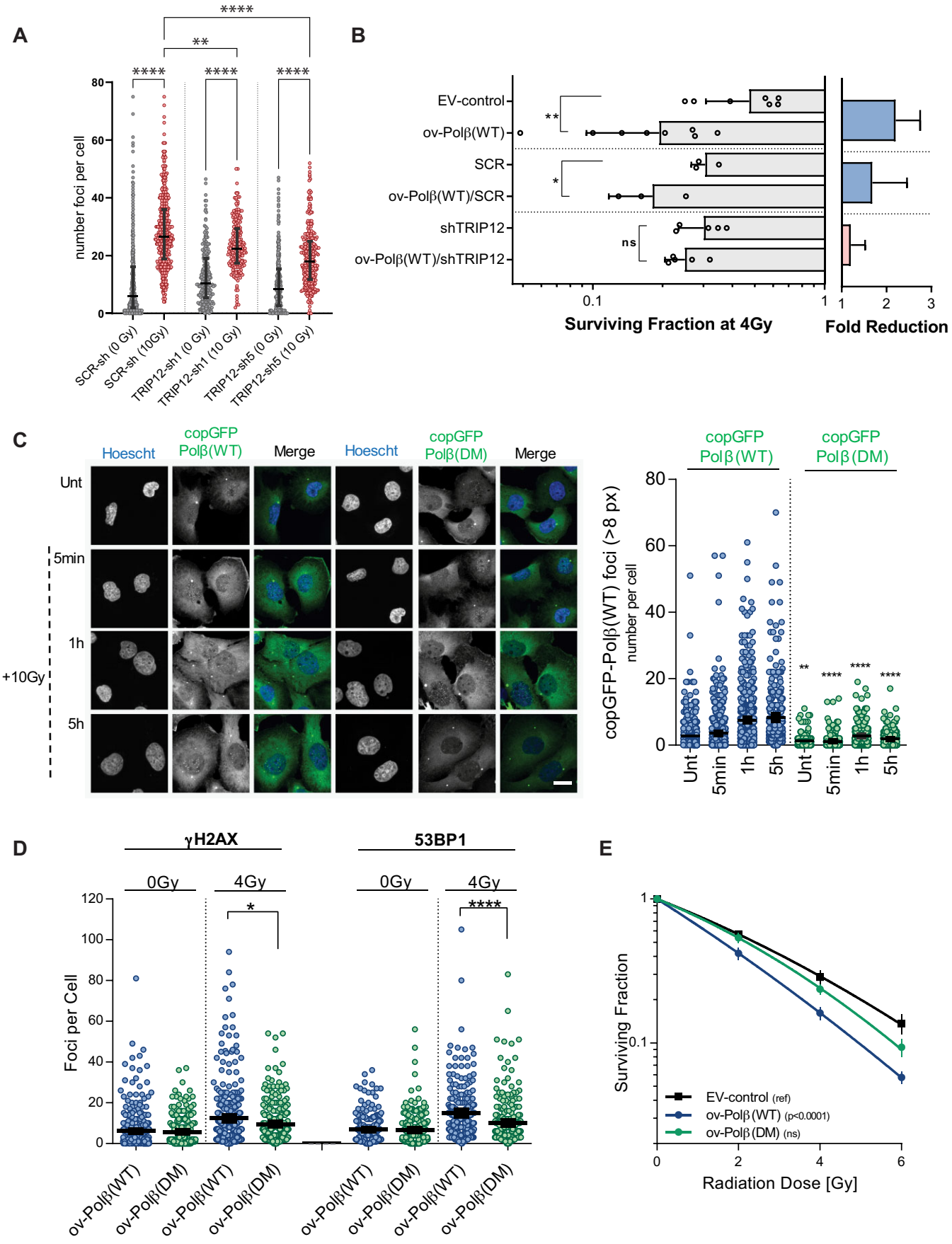


Figure 6. TRIP12 influences radiation response through Polβ ubiquitylation. (**A**) TRIP12 depletion reduces Polβ overexpression-induced DSB formation after radiation. Numbers of γH2AX foci per cell in copGFP-Polβ overexpressing scrambled (SCR) control cells and TRIP12-depleted (TRIP12-sh1 and

that a prioritization of BER and the prevention of, in this case, futile DSB repair may be of vital importance to cells. Indeed, functional or physical crosstalk among DNA repair pathways is emerging as an essential aspect of the overall cellular response to DNA damage [15, 56–58]. This has been extensively described for repair pathways dealing with DSBs or crosslinks. However, the BER/DSB repair pathway orchestration postulated here has not yet been defined and links repair pathways across classes that are intrinsically very different. Our study portrays TRIP12 at the nexus of these main cellular repair activities (Fig. 7H; see the Graphical abstract).

The recently discovered binding of TRIP12 to PARP1, another important BER and SSBR member, and its ability to regulate steady-state levels of PARP1 through (poly-)ubiquitylation, is in line with our model and our proposed repair coordination and control function [22]. Like the notion elucidated by us, the authors suggested that the relevance of this process lies in the prevention of supra-physiological PARP1 accumulation and activity. Indeed, in our study we were able to demonstrate the detrimental effects of supra-physiological Polβ levels in our overexpression models and thus a role for TRIP12 in genomic stability.

TRIP12's role in radiation damage repair

Our study uncovers a new cellular function of TRIP12 and identifies TRIP12 as a key regulator of BER repair activity through Polβ engagement. Here, we show ionizing radiation-induced Polβ foci formation and reveal that TRIP12 is essential for Polβ engagement in the cellular response to radiation. Notably, TRIP12 requirement is also evident for those foci that arise under unchallenged conditions; however, Polβ recruitment at lesions induced by laser irradiation intensities that do not cause DSBs or clustered lesions is not affected (Fig. 3C and Supplementary Fig. S3C), nor is the small induction of Polβ foci by cisplatin, which also does not directly induce DSBs (Supplementary Fig. S4E and F). In contrast, but consistent with the herein proposed role of TRIP12 at the nexus of BER and DSB repair, TRIP12 is involved in foci induction after radiation. This functional link between TRIP12 and Polβ is further strengthened by the inability of Polβ mutants, devoid of TRIP12-mediated ubiquitylation (Fig. 2C and Supplementary Fig. S2B), to form Polβ foci at background levels or after radiation, consistent with the TRIP12 knockdown data (Figs 4I–K and 6C). More so, we were able to confirm TRIP12's involvement in the nuclear activities of Polβ by showing the rescue in DSB induction and the radiation survival detriment from Polβ deregulation in TRIP12 ubiquitylation mutants and TRIP12-depleted cells (Fig. 6).

Initially, and despite the drastic reduction in Polβ engagement, we were not able to identify cellular defects in TRIP12-deficient cells (Fig. 3 and Supplementary Fig. S3C and D) except for the elevated expression of RNF168 (Supplementary Fig. S3B). This lack of an impact on cellular survival or radiation sensitivity is not surprising as Polβ-deficient cells have been reported to survive well in culture and show similar radiation survival as their wild-type counterparts [29, 59–62]. Indeed, a role for Polβ in the cellular radiation response could only be identified after isolating replicating cells or applying dominant-negative transgenes that interfered in BER [59–62]. Overall, this observation is also consistent with earlier reports from Gudjonsson *et al.* [21], who did not report a deficit in cellular survival upon depletion of TRIP12, and from Kajiro *et al.* who describe a minor impact of TRIP12-HECT domain mutations on the proliferation of ES cells [63]. The lack of radiation sensitivity is also in line with the notion that TRIP12 deficiency unlocks DSB repair capacity [21, 64], which may rescue any potential cellular impact from DSBs caused by the loss of Polβ-mediated repair [64–67].

TRIP12's role in Polβ homeostasis

Polβ and XRCC1 are important elements in BER; their equilibrium appears to be critical for BER function [3, 36, 37, 68]. Of these two, Polβ provides both DNA polymerase and 5′dRP lyase activities essential to complete base lesion repair [7, 29, 69]. By performing colocalization experiments after radiation, we found that Polβ resides at most XRCC1 sites (Fig. 4D, E, and H, and Supplementary Fig. S4D and E), underlining the relevance of XRCC1/Polβ complex formation [37]. Our previous findings and reports from others revealed that both Polβ and XRCC1 play common but also separate and independent roles in DNA metabolism and repair [3, 70]. We previously discovered that the composition of BER protein complexes depends on the cell cycle and DNA damage type [3] and observed a shift toward HSP90-bound XRCC1, devoid of Polβ. Since Polβ levels remained constant, it suggested a considerable pool of a Polβ-centric BER complex (herein termed Complex B) and a mechanism to mediate this shift that depended on Polβ as it was absent in Polβ knockout cells [3]. The considerable increase in Polβ degradation, when unable to bind to XRCC1 [Polβ(TM)], further substantiated the suspicion of a complex-dependent and Polβ-specific E3 ubiquitin ligase activity in these previous studies [3, 36]. Herein, we

-sh5) cells are shown, unirradiated and 5 h following 10 Gy irradiation as indicated; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$ as determined by Kruskal–Wallis. (**B**) TRIP12 depletion rescues cells from Polβ overexpression-mediated radiosensitization. Reduced survival after radiation by the overexpression of wild-type Polβ [ov-Polβ(WT)] (Fig. 5C) in empty vector (EV-control) or SCR (scrambled shRNA) controls is rescued by TRIP12 depletion (TRIP12-sh1 and -sh5). The graph shows the average clonogenic survival after 4 Gy and the SD in $n = 3$–7 independent experiments as indicated by the dots. The Polβ overexpression-induced fold reduction in radiation survival is indicated to the right; $^{*}P < .05$ and $^{**}P < .01$ (ANOVA). (**C**) Mutation of the TRIP12 ubiquitylation sites (K206A/K244A) on Polβ abrogates Polβ focal accumulation after radiation. Representative images and quantification of radiation-induced Polβ [copGFP-Polβ(WT)] and ubiquitylation mutant Polβ(DM) [copGFP-Polβ(DM)] foci. The graph (right) shows the number of large (>8 px) mutant or wild-type Polβ foci per cell at different time points after radiation from $n = 3$ independent experiments with means and SD; $^{*}P < .05$ and $^{**}P < .01$ (ANOVA). (**D**) Abrogation of TRIP12 ubiquitylation sites on Polβ reduces DSB induction. Residual γH2AX and 53BP1 foci at 24 h are shown in untreated cells and 4-Gy irradiated cells that overexpress wild-type Polβ [ov-Polβ(WT)] or the Polβ K206A/K244A ubiquitylation mutant [ov-Polβ(DM)]; $^{*}P < .05$ and $^{**}P < .01$ (ANOVA). (**E**) TRIP12 ubiquitylation site mutation in Polβ rescues cells from Polβ overexpression-mediated radiosensitivity. Polβ(WT) overexpressing cells are compared to Polβ(DM) overexpressing and empty vector (EV-control) LN428 control cells. Surviving fractions after 2, 4, and 6 Gy are shown with data points indicating the averages with SEM of the mean values from $n = 3$–8 independent experiments. *P*-values indicated in the legend of the radiation response curve graph assess the likelihood of the data curve fits to be similar to the reference EV-control response curve. Radiation response parameters (D37%) in the ov-Polβ(DM) cell line are not significantly different from the EV-control (ns), compared to the ov-Polβ(WT) that differ significantly from the reference (ref) EV-control cell line with $P < .01$ (ANOVA).

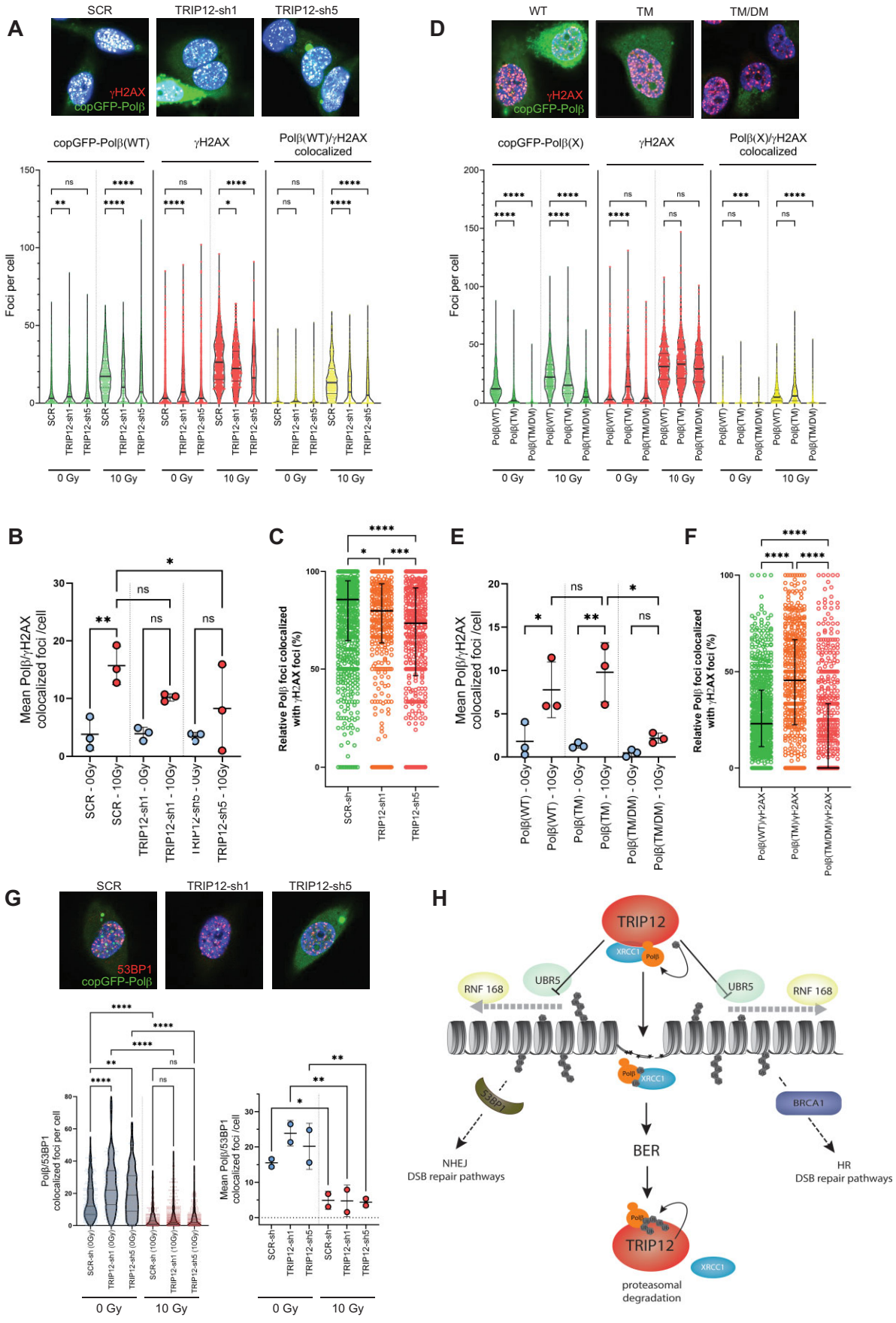


Figure 7. TRIP12's controller function in DNA repair pathway engagement and choice. (**A**) TRIP12's role in Polβ engagement to γH2AX marked chromatin. Representative images show decreased colocalization (white) of Polβ (green) and γH2AX (red) in TRIP12-depleted cells. Violin dot plots

identified, via label-free dMS, TRIP12 as this complex-dependent E3 ubiquitin ligase. Polβ is a substrate for TRIP12 both *in vitro* and in cells, and the interaction is between the SB domain (TRIP12-SB) of TRIP12 and the C-terminal domain of Polβ. At this stage, we are not able to fully disentangle whether TRIP12 has a lessened influence on XRCC1-bound Polβ and can contribute to a shift toward the XRCC1-free complex or whether directed by other mechanisms, this state enables TRIP12 interference, thus ubiquitylation and Polβ foci formation. We did, however, observe a reduction in cytoplasmic Polβ levels after radiation at late time points (Fig. 4A). Together with the expected overall increase in levels (due to the lack of degradation processes), this reduction was absent in TRIP12 knockdown cells (Fig. 4I). TRIP12's contribution to Polβ elimination supports a role in re-establishing baseline values after successful repair, further highlighting the importance of well-balanced Polβ levels. This may address the observed paradoxical role of TRIP12 in promoting Polβ engagement in the nucleus in response to damage on one hand while also facilitating Polβ degradation after the insult on the other [26, 71–76].

Polβ in TRIP12-regulated processes

Little is known about the cellular role of TRIP12, a HECT-type E3 ubiquitin ligase. ARF and App-BP1 have been reported to be among TRIP12 ubiquitylation targets, implicating TRIP12 in ubiquitin fusion degradation, neddylation, or the ARF-regulated p53 response [44, 77–79]. The first indications for its involvement in DNA repair processes came from data revealing a role in 53BP1 foci formation [21] and a link to the DDR through USP7 ubiquitylation [27]. Gudjonsson *et al.* also showed that TRIP12 acts as a suppressor of RNF168, reducing the accumulation of "Lys-63"-linked histones H2A and H2AX at DNA damage sites and revealed its partnership with UBR5 [21]. TRIP12 thereby acts as a guard against the excessive spreading of ubiquitylated chromatin at damaged chromosomes that are thought to facilitate the accumulation of repair proteins [21, 80]. Here, we were able to juxtapose TRIP12's restraining function of DSB repair with a repair-promoting activity by direct engagement of Polβ, an essential BER factor. The lack of colocalization of Polβ with 53BP1 (Figs 4G and 7G, and Supplementary Fig. S5K) supports this link. Interestingly, we find that in contrast to TRIP12, its partner UBR5 does not affect the chromatin association of Polβ (Supplementary Fig. S5A and B).

Indeed, BER may not require extensive chromatin rearrangement as indicated by fast recruitment kinetics in heterochromatic regions [81]. The repair of radiation-induced clustered lesions will benefit from early BER engagement and restricted chromatin rearrangement to prevent DSB formation or DSB repair attempts at lesion sites. In this context, Eccles *et al.* showed the importance of, the then unknown, nuclear factors in the sequence of events at such sites [82]. At this point, we are not able to discern whether the late and large Polβ foci are a consequence of retention due to continued loading attempts by TRIP12 from incomplete repair or the engagement in late, secondary DSB repair processes. The impact of TRIP12 deficiency in the early and small foci, however, confirms a loading role for most of these foci. This is also consistent with a damage-type specific role of TRIP12 as shown by the lack of such a response modulation after H_2O_2 or cisplatin. Since radiation induces a large amount of both Polβ and 53BP1 foci, the drop in Polβ/53BP1 colocalization reveals an active segregation process early after radiation that is, in part, TRIP12-dependent (Fig. 7G and Supplementary Fig. S5K and L). Not all DSBs are highly complex, i.e. coinciding with multiple BER targeted lesions. Indeed, we observed that only few γH2AX marked foci, and substantially less 53BP1 foci, contain Polβ. Different staining conditions and imaging settings impair comparisons across the different colocalization datasets; yet, it appears that, 5 h after irradiation, most Polβ foci can be found colocalized with γH2AX, a process dependent on TRIP12 or TRIP12 ubiquitylation sites (Fig. 7C and F). Since TRIP12 depletion affects radiation-induced engagement with γH2AX chromatin, it is difficult to assess the impact of TRIP12 depletion on pathway segregation (thus test for increased Polβ/53BP1 colocalization). Yet, we observe significantly increased foci colocalization events in unirradiated cells (Fig. 7G) and increased Mander's overlap coefficient values before and after radiation in time course experiments in TRIP12-depleted cells (Supplementary Fig. S5L),

show the distribution of copGFP-Polβ, γH2AX, and Polβ/γH2AX colocalized foci in scrambled (SCR) shRNA-expressing or TRIP12-depleted (TRIP12-sh1 and -sh5) unirradiated control (0 Gy) cells and 5 h after 10 Gy irradiation ($n = 3$ independent experiments with >100 cells each); $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$ as determined by Kruskal–Wallis. (**B**) Mean colocalized foci/cell values per experiment, pooled in panel (A), and corresponding statistical evaluations ($^{*}P < .05$ and $^{**}P < .01$ from ANOVA) are shown. (**C**) The relative numbers of Polβ colocalized with γH2AX (on a cell-by-cell basis) in irradiated cells are shown with medians and interquartile ranges. (**D**) Role of TRIP12 ubiquitylation site in Polβ engagement to γH2AX marked chromatin. Representative images show decreased colocalization (white) of the TRIP12 ubiquitylation-mutated copGFP-Polβ(TM/DM) isoform (green) and γH2AX (red). Violin dot plots show the distribution of the copGFP-Polβ isoforms [copGFP-Polβ(WT), copGFP-Polβ(TM), copGFP-Polβ(TM/DM), as indicated], γH2AX, and Polβ/γH2AX colocalized foci in control (0 Gy) cells and 5 h after 10 Gy irradiation ($n = 3$ independent experiments with >100 cells each); $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$ as determined by the Kruskal–Wallis test. (**E**) Mean foci/cell values per experiment and corresponding statistical evaluations ($^{*}P < .05$ from ANOVA) are shown. (**F**) The relative numbers of Polβ [copGFP-Polβ(WT), copGFP-Polβ(TM), or copGFP-Polβ(TM/DM)] colocalized with γH2AX (on a cell-by-cell basis) are shown with medians and interquartile ranges. (**G**) Influence of TRIP12 on colocalization of Polβ with 53BP1. CopGFP-Polβ and 53BP1 foci colocalization in control (SCR) and TRIP12 knockdown cells (sh1 and sh5) 5 h after 10 Gy irradiation are shown and compared to unirradiated controls (0 Gy) in violin dot plots illustrating changes in median and distribution (left graph) and as mean foci/cell values from two independent experiments with at least $n = 100$ cells each (right graph; see Supplementary Fig. S5 for additional data). Asterisks mark *P*-values (Kruskal–Wallis test, $^{****}P < .0001$, $^{**}P < .01$) assessing the impact of TRIP12 depletion and radiation on these cells. (**H**) TRIP12 in the governance of repair pathway choice. A graphic is shown that illustrates the current working model of TRIP12 involvement in BER. TRIP12-mediated prevention of the RNF168 promoted histone ubiquitylation extension surrounding DNA lesions restrains DSB signaling in favor of Polβ. Enabled by its ubiquitylation activity on Polβ, TRIP12 exerts its repair traffic control function by assisting BER and deviating DSB repair at the same time. Complex damage sites with clustered lesions can cause DSBs if not repaired appropriately and DSB repair attempts at DSBs residing in such regions may fail due to the presence of such BER-targeted lesions, since they prevent synthesis and nuclease activities. A cellular control mechanism that channels repair proteins to yield the right of way to BER may be therefore relevant in these cases. A two-step activity can be proposed in which TRIP12 engages Polβ at nuclear damage sites but also initiates BER complex disassembly, freeing Polβ from XRCC1 for distinct repair activities and thereafter destines Polβ for proteasome degradation upon repair completion.

suggesting that the segregation is compromised. It should be noted that in order to detect any potential Polβ/53BP1 associations in this low colocalization context (Fig. 4G and H), these analyses (Fig. 7G) were conducted at different settings that comprise very small foci or simply protein localization (Supplementary Fig. S5L).

Combined with TRIP12's Polβ-regulatory activity shown here, BER engagement and 53BP1 exclusion further support our hypothesis that assigns TRIP12 a role in the sequence of repair activities. We postulate that the provenance of TRIP12 ubiquitylation activity on Polβ is to enable a timely order of repair by ranking BER activities over those from the DSB repair machinery through an active role in Polβ chromatin association and the concomitant regulation of initial DDR modulators.

Dual function of TRIP12: repair pathway coordination and protein homeostasis

Here, we observed a clear role for TRIP12 in the governance of Polβ chromatin loading as well as cellular Polβ levels. Ubiquitylation plays a role in protein stability for many DNA repair proteins, whereas non-proteolytic functions of ubiquitin (either mono-ubiquitin or poly-ubiquitin) are essential for signaling in DNA replication and DNA repair [83, 84]. As part of our dynamic BER model [3], we deduced that ubiquitylation of Polβ may facilitate repair pathway choice similar to ubiquitylation in DSB repair processes [83, 85]. The poly-ubiquitin mark can play a signaling role as well as facilitate proteolysis [86, 87]. Herein, we suggest that Polβ is regulated by TRIP12-mediated ubiquitylation, possibly assisted by additional E3 ubiquitin ligases. We observe TRIP12-dependent generation of both (mono- and poly-)ubiquitylated species of Polβ in the *in vitro* "on-bead" assay (Fig. 2A and B). This is in line with a role for ubiquitylation in both signaling and protein homeostasis that may depend on ubiquitin chain types. Future analyses will be essential to differentiate what role, if any, is ascribed to (mono- and poly-)ubiquitylation. Consistent with a role for Polβ ubiquitylation in repair and signaling, here we show that TRIP12 and the ubiquitylation of Polβ at K206/K244 are essential for Polβ chromatin association and foci induction and in the maintenance of cellular Polβ levels.

In conclusion, we propose that TRIP12 functions as a "repair-sequence-controller," through ubiquitylation, facilitating pathway crosstalk across significantly different repair processes that require a different extent of chromatin modulation. By coordinating Polβ and DSB repair engagement, TRIP12 may ultimately prevent the conversion of base lesions and single-strand breaks to cytotoxic and genotoxic DSBs.

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Supplementary data

Supplementary data is available at NAR online.

Conflict of interest

None declared.

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Data availability

All data is available upon request. Mass spectrometry proteomics data have been deposited in the MassIVE repository, under accession code MSV000094615.

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