Lysine Residue 185 of Rad1 Is a Topological but Not a Functional Counterpart of Lysine Residue 164 of PCNA

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Abstract

Monoubiquitylation of the homotrimeric DNA sliding clamp PCNA at lysine residue 164 (PCNA^K164) is a highly conserved, DNA damage-inducible process that is mediated by the E2/E3 complex Rad6/Rad18. This ubiquitylation event recruits translesion synthesis (TLS) polymerases capable of replicating across damaged DNA templates. Besides PCNA, the Rad6/Rad18 complex was recently shown to yeast in ubiquitylate also 9-1-1, a heterotrimeric DNA sliding clamp composed of Rad9, Rad1, and Hus1 in a DNA damage-inducible manner. Based on the highly similar crystal structures of PCNA and 9-1-1, Rad18 complex was recently shown in yeast to ubiquitylate also 9-1-1, a heterotrimeric DNA sliding clamp composed of Rad9, Rad1, and Hus1 in a DNA damage-inducible manner. Based on the highly similar crystal structures of PCNA and 9-1-1, K185 of Rad1 (Rad1K185) was identified as the only topological equivalent of PCNA^K164. To investigate a potential role of posttranslational modifications of Rad1K185 in DNA damage management, we here generated a mouse model with a conditional deletable Rad1K185 allele. The Rad1K185 residue was found to be dispensable for Chk1 activation, DNA damage survival, and class switch recombination of immunoglobulin genes as well as recruitment of TLS polymerases during somatic hypermutation of immunoglobulin genes. Our data indicate that Rad1K185 is not a functional counterpart of PCNA^K164.

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Introduction

Maintaining DNA integrity is crucial to the survival and reproduction of all organisms. As a consequence, elaborate mechanisms have evolved to preserve genetic information. Cells rely on a complex protein network capable of sensing specific DNA damage and triggering adequate responses. Distinct DNA damage checkpoints can delay specific phases of the cell cycle and this extra time window allows a cell to repair or transiently tolerate DNA damage. If the damage is too severe, the system can force the cell to go into senescence or apoptosis [1]. Inappropriate DNA damage management has been associated with a variety of diseases, like cancer and premature ageing [2].

DNA sliding clamps and post-translational modification (PTM) thereof play important roles in DNA replication, recombination, and repair, as well as DNA damage responses (DDR), and DNA damage tolerance (DDT) [3]. The homotrimeric DNA sliding clamp Proliferating Cell Nuclear Antigen (PCNA) encircles the DNA and acts as a critical processivity factor for the replicative polymerases δ and ε. In the presence of stalling DNA lesions, for instance caused by DNA alkylation or UV exposure, prolonged exposure of single-stranded DNA may ultimately lead to the formation of DNA double strand breaks. To prevent the formation of such detrimental secondary lesions, DDT enables DNA replication to be continued. This feature renders DDT as an integral component of the overall cellular response in surviving genotoxic stress [3]. In eukaryotes two DDT pathways are distinguished: translesion synthesis (TLS) and template switching [4]. Both pathways, initially identified as the Rad6 epistasis group, strongly depend on DNA damage-inducible, site-specific ubiquitylation of PCNA at lysine (K) 164 [5]. DNA damage-inducible monoubiquitylation at PCNA^K164 (PCNA-Ub) is mediated by the E2 conjugase Rad6 and the E3 ligase Rad18 and recruits TLS polymerases via their ubiquitin binding motifs [6,7,8,9]. These TLS polymerases are capable of replicating directly across damaged DNA templates [3]. TLS polymerases have an extended catalytic domain that can fit non-Watson-Crick base pairs, allowing this class of polymerases to synthesize directly across DNA lesions [10]. Simultaneously, the inherent lack of proofread activity renders TLS polymerases error-prone, even in the presence of an intact template. Further K63-linked polyubiquitylation of PCNA-Ub stimulates template switching, which enables stalled replicative polymerases to bypass the damage by switching transiently to the intact template strand of the sister chromatid [4].

Interestingly, affinity maturation of antibodies takes advantage of error-prone TLS polymerases to introduce point mutations at a high rate into the variable region of immunoglobulin genes of B cells, a process known as somatic hypermutation (SHM) [11]. To initiate SHM, the activation-induced cytidine deaminase AID is induced transiently in activated B cells to create uracil residues in the variable region of Ig genes by deaminating cytidines [11,12]. It is thought that three major pathways can process the U:G mismatch in an error-prone manner. 1) Direct replication of the uracil results in G/C to A/T transitions, as U instructs a template mismatch in an error-prone manner. 2) Excision of the U by the base excision repair protein Ung2, generates a non-instructive abasic site that can be processed by specific TLS polymerases [13,14]. 3) Alternatively, the U can be recognized as a U:G mismatch by the
mismatch recognition complex Msh2-Msh6, resulting in exomerelease 1 (Exo-1) activation, formation of a single-stranded gap, activation of Rad6/Rad18, PCNA-Ub and recruitment of the TLS polymerase η (Pol η) to generate 90% of all A/T mutations around the initial mismatch [16,17,18,19,20]. Interestingly, TLS polymerases involved in G/C transversions, like Rev1, are not controlled by PCNA-Ub. This suggests that G/C transversions are regulated differently [16,17]. For instance, this may involve ubiquitination of the alternative DNA sliding clamp 9-1-1 (see below) [21].

Besides the homotrimeric PCNA DNA sliding clamp, a heterotrimERIC DNA sliding clamp exists, Rad9-Rad1-Hus1 (9-1-1), which is evolutionary and structurally highly related to PCNA [22,23]. While its role as DNA damage sensor in the DDR is well-defined [24], more recent reports revealed a role of 9-1-1 in DDT. The non-catalytic Rev7 subunit of the TLS polymerase ζ, a heterodimer of Rev3 and Rev7, is recruited to DNA in a damage-inducible and Rad9-dependent manner in S. cerevisiae [25]. In addition, in S. pombe polymerase κ physically interacts with 9-1-1, and its recruitment to chromatin is dependent on checkpoint activation [26]. These observations suggest a function of 9-1-1 in controlling TLS and possibly SHM in B cells. Most remarkably, a recent study in S. cerevisiae by Fu et al. indicated that DNA damage activates Rad6/Rad18 to ubiquitylate not only PCNA but also Rad17, the orthologue of mammalian Rad1 at a non-conserved lysine residue, K197 [27]. Furthermore, it was shown that Rad17 ubiquitylation controls phosphorylation of Rad53, the yeast Chk2 orthologue, a downstream component of the DNA damage response [27]. Strikingly, by solving the crystal structure of human 9-1-1, Doré et al. made the observation that the non-conserved Rad17K197 is not a topological equivalent of PCNAK164 [23]. In fact, Doré et al. revealed mammalian Rad1K185 as the only topological equivalent of PCNAK164 [23].

The facts that: 1) a topological equivalent of PCNAK164 exists in mammalian Rad1; 2) PCNA ubiquitylation by Rad6/Rad18 is selective for K164; and 3) that in yeast PCNA and 9-1-1 are both ubiquitylated in a DNA damage-inducible manner by Rad6/Rad18, prompted us to investigate whether the conserved mammalian Rad1K185 is not just a topological equivalent but also a functional counterpart of PCNAK164. To investigate the role of any PTMs of Rad1 in mammals, we introduced a K185R mutation in exon 4 of mouse Rad1. We found that the Rad1K185R mutation does not affect mammalian Chk1 activation, DNA damage survival, TLS function during SHM and class switch recombination (CSR) of Ig genes. These data are consistent with a recent report published by the Ulrich lab, suggesting that DNA damage-inducible ubiquitylation of 9-1-1 as observed by Fu et al. might not exist in yeast [28].

In addition, we simultaneously flanked exon 4 by LoxP-recombination sites. This strategy allows us to determine a putative role of Rad1K185 modification in mammalian DNA damage management and to inactivate Rad1 conditionally in mammalian tissues. Cre-mediated deletion of exon 4 inactivates Rad1, providing an ideal model system to perform structure function analyses of Rad1 in a mammalian system.

Materials and Methods

Cloning of Rad1K185R targeting vector

The 5’ arm of homology (~3 kbp) was amplified with a PmeI site at the 5’ end and an AscI site at the 3’ end (FWD: 5’-TTT TGACGACGCAATTGTTAATGCAAGGTAGATTGAACTTAAGTCAGG-3’ and REV: 5’-TTTTGCGCGCTTCGACGTCGAGGG-3’ (G1 FWD, Figure 1A) and REV1: 5’-GATAAGGGTCTTGACGACGACGGGGCGGGGGG-3’ (G2 FWD, Figure 1A) and REV2: 5’-GTTAGTTGAACTTGACGACGACGGGACGCGGCGG-3’ (Figure 1A)). Germline competent mice were crossed with the Flp deleter strain (provided by S. Dynneck, Harvard Medical School, Boston, MA) to delete the selection cassette in vivo [30]. Genotyping of Flp-deleted Rad1K185R mice: FWD: G1 FWD (Figure 1A) and REV 5’-TGACGATACGTAAC-3’ (G2 REV, Figure 1A).

All experiments were approved by an independent animal ethics committee of the Netherlands Cancer Institute (ID 8065) and executed according to national guidelines.

Derivation of Rad1K185R mouse embryonic fibroblast cell lines

Mouse embryonic fibroblasts (MEFs) were derived from embryos at day 14.5 of gestation. MEFs were maintained in complete medium (IMDM, 8% FCS, 5% N2, 2-mercaptoethanol, penicillin/streptomycin). Immortalization of MEF cell lines was established by lentiviral-mediated shRNAs targeting p53 [31].
DNA damage survival

Naive splenic B cells from three mice per genotype were obtained by CD43 depletion using biotinylated anti CD43 (Clone S7, BD Biosciences), and the IMag system (BD Biosciences), as described by the manufacturer. For UV-C irradiation, 10^5 B cells were irradiated (254 nm, UV Stratalinker H2400, Stratagene) in 0.5 ml complete medium containing 50 μg/ml E. Coli LPS (055:B5, Sigma). For γ-irradiation, a 137Cs source was used. Following irradiation, cells were cultured in 1 ml complete medium and LPS. To determine DNA damage sensitivity, the survival of 10^5 B cells grown in 1 ml complete medium and LPS in the continuous presence of different doses of cisplatin (CisPt) or methyl methanesulfonate (MMS) was determined after four days of culture. The number of viable (propidium iodine negative) B cells was determined by FACS. Data were analyzed using FlowJo 8.8.6 software.

Isolation of germinal center B cells and mutation analysis

Germinal center (CD19+, PNA high, CD95+) B cells were sorted from Peyer’s patches. Genomic DNA was extracted using proteinase K treatment and ethanol precipitation. The JH4 flanking intronic sequence of endogenous rearrangements of VHJ558 family members were amplified during 40 cycles of PCR using PFU Ultra polymerase (Stratagene). PCR products were purified using the QIAquick Gel Extraction kit (Qiagen) and cloned into the pCR-Blunt II TOPO vector (Invitrogen Life Technologies) and sequenced on a 3730 DNA analyzer (Applied Biosystems). Sequence alignment was performed on the first 300 bp starting from the intronic region using Seqman software (DNAStar). Calculations exclude non-mutated sequences, insertions, deletions, and SNPs. Clonally related sequences were counted only once. Statistical analysis was performed as described [17].

Class switch recombination

Naive splenic B cells from three mice per genotype were obtained by CD43 depletion as described above. Purified B cells were cultured in complete medium containing LPS either in the presence or absence of 10% IL-4-containing supernatants generated from X63-m-IL-4 cell cultures [32]. Flow cytometric analysis of surface Ig expression was performed on day 4 of culture using goat anti mouse IgM-APC, IgG1-PE and IgG3-PE (Southern Biotech). Data were analyzed using FlowJo 8.8.6 software.

Chk1 activation Western blotting

One day prior to UV irradiation wild type and Rad1K185R MEFs were seeded at 1.6*10^6 cells per 15 cm dish in 20 ml complete medium. The next day, cells were washed with PBS and irradiated with 100 J/m² UV-C (254 nm, UV Stratalinker 2400, Stratagene) after removal of the PBS. Hereafter complete medium was added. 10, 40 and 70 minutes later cells were harvested by

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Figure 1. Targeting strategy and genotyping Rad1K185R mouse. A) Targeting strategy Rad1K185R mouse. LoxP recombination sites are represented by black triangles. Flpe recombination sites are represented by white triangles. PCR primers are represented by gray arrow heads. Please note that this figure is not drawn to scale. B) Genotyping PCRs for non-flipped (Primers G1 FWD, G1 REV and G2 REV) and flipped Rad1K185R mice (Primers G1 FWD and G3 REV).

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scraping the cells in cold PBS, centrifuged (500 × g). After removal of the supernatant, cells were lysed in 200 µl ELB buffer (150 mM NaCl; 50 mM Heps pH 7.5; 5 mM EDTA; 0.1% NP-40; protease inhibitors [Roche]) and incubated for 30 minutes on ice. Next, samples were centrifuged for 10 minutes at 20,000 × g (4°C). The supernatant was transferred to a new tube and the protein concentration was measured using standard Bradford method. Western blotting was performed using standard protocols. NuPAGE 3-8% Tris-Acetate gels (Invitrogen) were used for protein separation. Antibodies used were: mouse anti-Chk1, 1:1000 (sc-8408, Santa Cruz); rabbit anti-pChk1 S345, 1:1000 (clone 133D3, Cell Signaling); mouse anti-Actin, 1:10,000 (clone C4 (MAB1501R), Milipore).

Results

Generation of Rad1^K185R mutant mice with a floxed exon

To test the possible role of Rad1^K185 modifications in controlling mammalian DDT, we generated a mouse mutant with a site-specific Rad1^K185R mutation in exon 4 of the Rad1 locus (Figure 1A). Simultaneously, we also flanked this exon with LoxP recombination sites, which allows conditional inactivation of the Rad1^K185R allele and functional analysis of Rad1 in higher eukaryotes. To identify homologous recombinants, we established a long range PCR strategy to detect homologous recombinant ES cells (primer sets P1 and P2, Figure 1A). To prevent possible detrimental effects of the selection cassette, Rad1^K185R mice were crossed with the Flpe deleter strain to remove the selection cassette controlling mammalian DDT, we generated a mouse mutant to contribute to the activation of the mammalian DDR. As opposed to Rad1^K185R mice, which allows conditional inactivation of the Rad1^K185R allele and functional analysis of Rad1 in higher eukaryotes. To identify homologous recombinants, we established a long range PCR strategy to detect homologous recombinant ES cells (primer sets P1 and P2, Figure 1A). To prevent possible detrimental effects of the selection cassette, Rad1^K185R mice were crossed with the Flpe deleter strain to remove the selection cassette in vivo [30]. Mice homozygous for Rad1^K185R were obtained by intercrossing heterozygous mice. Heterozygous and homozygous Rad1^K185R mice were born at Mendelian ratios, indicating that the Rad1^K185R mutation has no detrimental effect on mouse development (data not shown).

Rad1^K185 does not control Chk1 activation

Mammalian 9-1-1 has been implicated in the activation of the checkpoint kinase Chk1, a critical activation step for DDR [24]. For example, upon UV irradiation Hus1-deficient MEFs display significantly lower levels of serine (S) 345 phosphorylated Chk1 (pChk1 S345) [33]. Moreover, Fu et al. have shown that PTM of 9-1-1 plays a role in DDR activation as well, as rad17-K197R sg41A yeast cells also have an impaired DDR [27]. These observations led us to postulate that possible PTMs at Rad1^K185 could also contribute to the activation of the mammalian DDR. As opposed to Hus1-deficient MEFs and rad17-K197R sg41A yeast cells, Rad1^K185R MEFs do not display impaired DDR activation after DNA damage as revealed by pChk1 S345 levels (Figure 2).

Rad1^K185R B cells display normal DNA damage sensitivity

Modulation of PCNA plays an important role in the regulation of DDR, as PCNA^K164R cells are extremely sensitive to various DNA damaging agents, primarily DNA damaging agents that cause replication blocking lesions [5]. Besides the importance of PCNA modification in DDR, 9-1-1 modification in yeast seems to cause replication blocking lesions [5]. Besides the importance of PCNA modification in DDR, 9-1-1 was shown to be involved in the repair of DNA double strand breaks (DSBs) by means of homologous recombination [34,35], we also investigated whether Rad1^K185R B cells were more sensitive than WT cells to γ-irradiation. In contrast to rad17-

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Discussion

The DNA sliding clamps PCNA and 9-1-1 are critical docking stations for proteins involved in diverse processes such as replication, recombination, and DNA damage management. Site-specific PTM of these sliding clamps helps to coordinate the activation of specific pathways. Stalled replication forks activate the Ub-conjugase/piggase Rad6/Rad18 complex to mediate PCNA^K164-specific ubiquitylation and subsequent stimulation of DDT. In this regard, the recent finding that in S. cerevisiae the same Rad6/Rad18 complex ubiquitylates Rad17, the yeast Rad1 orthologue, at lysine residue 197 was quite intriguing [27]. However, Rad17^K197 is not conserved and based on structural arguments unlikely to be a substrate of Rad6/Rad18 [23]. Yet, structural comparisons by Doré et al. did reveal a lysine residue (K185) in the Rad1 subunit of 9-1-1 that is indeed a topological equivalent of PCNA^K164 [23].
Figure 3. Rad1K185R B cells do not display sensitivity to various DNA damaging agents. WT (blue) and Rad1K185R (Red) B cells were stimulated with LPS and exposed to increasing amounts of UV-C (A), MMS (B), CisPt (C) and γ-irradiation (D). The percentage of survival is shown on the y-axis after four days of culture. Data represent the mean and SD of individual cultures (n = 3). The results are representatives of two independent experiments.
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Figure 4. Normal SHM in Rad1K185R GC B cells. A) Mutated JH4 regions from WT and Rad1K185R GC B cells. B) Rad1K185R GC B cells display a normal nucleotide exchange pattern in hypermutated Ig genes. In the left panel, values are expressed as the total numbers of mutations. In the right panel, values are expressed as the percentage of total mutations. Chi square testing did not reveal any significant changes in the nucleotide exchange pattern (p>0.01). C) Relative contributions of A/T mutations, G/C transversions and G/C transitions in the different mouse strains. Values are expressed as the percentage of total mutations.
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To investigate whether PCNA\textsuperscript{K164} and Rad1\textsuperscript{K185} are not just topological equivalents, but also functional counterparts, we first tried to identify DNA damage-inducible Rad1 ubiquitylation in different mammalian cell lines. After extensive experimentation we were unable to observe any DNA damage-inducible PTMs, in particular ubiquitin modification of Rad1 (data not shown). As this approach proved unsuccessful for potentially a number of reasons, we simultaneously took a genetic approach by introducing a conditional inactivation of endogenous Rad1. Our data clearly demonstrate that any PTM at Rad1\textsuperscript{K185} does not play a role in DNA damage management. SHM or CSR.

Collectively, our data show that putative PTMs at Rad1\textsuperscript{K185} do not play a role in DNA damage management, which is in line with recent observations made in the Ulrich lab [28]. We conclude that mammalian Rad1\textsuperscript{K185} is a mere topological, but not a functional counterpart of PCNA\textsuperscript{K164}.

Having flanked Rad1 exon 4 with LoxP recombinase sites allows a conditional inactivation of Rad1 in mice and cell lines derived thereof. Upon deletion of exon 4, any alternative splicing gives rise to out-of-frame transcripts downstream of exon 3. As Rad1 null embryos are not viable [38], our and equivalent systems of Rad9 [39] and Has1 [40] will enable a detailed structure/function analysis of the mammalian 9-1-1 DNA sliding clamp in DNA damage management in future studies.

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Author Contributions

Conceived and designed the experiments: NW HJ. Performed the experiments: NW PHLK PCMvdB. Analyzed the data: NW HJ. Wrote the manuscript: NW HJ.

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Figure 5. CSR is not altered in Rad1\textsuperscript{K185R} B cells. WT (gray bars) and Rad1\textsuperscript{K185R} (black bars) B cells were tested for their ability to switch to either IgG3 or IgG1 by stimulation with LPS or LPS and IL-4, respectively. Data represent the mean and SD of individual B cell cultures from three independent mice. The results are representatives of two independent experiments. doi:10.1371/journal.pone.0016669.g005
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