DNA Repair 42 (2016) 82–93

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

RNF138 interacts with RAD51D and is required for DNA interstrand crosslink repair and maintaining chromosome integrity

Brian D. Yard [b], Nicole M. Reilly [a], Michael K. Bedenbaugh [c], Douglas L. Pittman [a,*]

[a] Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, Columbia, SC 29208, USA
[b] Department of Translational Hematology and Oncology Research, Cleveland Clinic Taussig Cancer Institute, Cleveland, OH 44195, USA
[c] Department of Pharmacy Services, Greenville Health System, Greenville, SC 29615, USA

ARTICLE INFO

Article history:
Received 23 February 2016
Received in revised form 8 April 2016
Accepted 15 April 2016
Available online 21 April 2016

Keywords:
Homologous recombination
DNA double-stranded breaks
DNA interstrand crosslinks
RAD51D
RNF138
E3 ligase
Chromosome integrity
DNA repair
Ubiquitination

ABSTRACT

The RAD51 family is integral for homologous recombination (HR) mediated DNA repair and maintaining chromosome integrity. RAD51D, the fourth member of the family, is a known ovarian cancer susceptibility gene and required for the repair of interstrand crosslink DNA damage and preserving chromosomal stability. In this report, we describe the RNF138 E3 ubiquitin ligase that interacts with and ubiquitinates the RAD51D HR protein. RNF138 is a member of an E3 ligase family that contains an amino-terminal RING finger domain and a putative carboxyl-terminal ubiquitin interaction motif. In mammalian cells, depletion of RNF138 increased the stability of the RAD51D protein, suggesting that RNF138 governs ubiquitin-proteasome-mediated degradation of RAD51D. However, RNF138 depletion conferred sensitivity to DNA damaging agents, reduced RAD51 focus formation, and increased chromosomal instability. Site-specific mutagenesis of the RNF138 RING finger domain demonstrated that it was necessary for RAD51D ubiquitination. Presence of RNF138 also enhanced the interaction between RAD51D and a known interacting RAD51 family member XRCC2 in a yeast three-hybrid assay. Therefore, RNF138 is a newly identified regulatory component of the HR mediated DNA repair pathway that has implications toward understanding how ubiquitination modifies the functions of the RAD51 paralog protein complex.

1. Introduction

When DNA damage affects both strands, such as interstrand crosslinks (ICLs) or double-strand breaks (DSBs), cells become more vulnerable to chromosomal deletions and rearrangements. Homologous recombination (HR) is a major DNA repair pathway that resolves these lesions. HR is a dynamic process involving protein complexes that are tightly regulated to guide DNA damage signaling, lesion processing, and invasion of a damaged DNA strand onto a homologous template [1,2]. Decreased HR leads to the accumulation of mutations and genome instability associated with carcinogenesis, whereas increased HR levels may lead to hyper-recombination phenotypes that contribute to radiation treatment and chemotherapy drug resistance [3].

The seven mammalian RAD51 family members are crucial HR components [4]. Even though they are involved in multiple HR steps, the regulatory mechanisms are still being investigated. Ubiquitin-specific post-translational modifications (PTMs) act as key orchestrators of the HR pathway, which include polyubiquitin landscapes and mobilization of histones that surround the damaged chromatin, ubiquitin associated recruitment and signaling, and ubiquitin-mediated protein modifications [5–11]. RAD51 is the only family member known to be ubiquitinated and is subject to proteasome-mediated degradation following exposure to ionizing radiation [12–14]. In the absence of RAD51C, the RAD51 protein is ubiquitinated independently of DSB formation [12]. Therefore, RAD51C appears to be involved in the ubiquitin transition of RAD51 in response to DNA damage and targeting for proteasomal degradation. The E3 ubiquitin ligase RAD18 acts as an adapter between RAD51C and RNF8/UBC13 catalyzed polyubiquitin chains that surround damaged chromatin [5]. In addition, RAP80 has an analogous role by recruiting the BRCA1 complex to RNF8/RNF168 synthesized polyubiquitin chains at DSB [15,16].

RAD51D is the fourth member of the RAD51 family and is a known ovarian cancer and possibly a breast cancer susceptibility gene [17–23]. Mutations in RAD51D confer extensive chromoso-

Abbreviations: HR, homologous recombination; CFA, colony forming assay; NT, N-terminus; ssDNA, single-stranded DNA; ICL, interstrand crosslink; DSB, double-strand break; MEF, mouse embryo fibroblasts; MMC, mitomycin C; MMS, methyl methanesulfonate; PTM, post-translational modification; UIM, ubiquitin interaction motif; Y2H, yeast two-hybrid.

* Corresponding author.
E-mail address: pittman@sccp.sc.edu (D.L. Pittman).

http://dx.doi.org/10.1016/j.dnarep.2016.04.006
1568-7864/

mal instability and sensitivity to DNA damage, primarily crosslinks [24,25]. RAD51D is also necessary for the recruitment of RAD51 to DNA damage sites and facilitates homologous pairing when associated with XRCC2 [25,26]. Identification of post-translational modification mechanisms of RAD51D could make it possible to more efficiently diagnose HR-deficient ovarian cancers and to develop personalized treatment strategies [27]. Therefore, to identify RAD51D interacting proteins, we performed yeast two-hybrid screens against RAD51D and identified the RNF138 E3 ubiquitin ligase as a candidate that may be involved in ubiquitin modification of RAD51D.

RNF138 was initially reported as part of the Wnt signaling pathway involved in secondary axis formation in *Xenopus* embryos [28]. It was later linked to DNA damage response as a phosphorylated ATM target on Serine 124 following ionizing radiation [29]. Given the emerging role of ubiquitin signaling cascades in governing HR, we hypothesized that RNF138 E3 ligase activity regulates RAD51D function. In this manuscript, we demonstrate that RNF138 directly interacts with RAD51D and is required for ubiquitination of the RAD51D protein. Consistent with a role during HR, depletion of RNF138 increased sensitivity to DNA damaging agents, reduced RAD51 foci formation, and increased levels of chromosomal aberrations. The data presented here suggest that RNF138-dependent ubiquitination of RAD51D is an essential step during HR DNA repair and offers a potential explanation regarding the selection for increased RNF138 expression levels during carcinogenesis [30–32].

2. Materials and methods

2.1. Cell culture and transfections

Mouse embryonic fibroblast (MEF) and HeLa cell lines were maintained at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; HyClone) supplemented with 10% newborn calf serum (Atlanta Biologicals), 1% penicillin/streptomycin, and 1% glutamine. The MEF cell lines MEFC20 (*Rad51d*$^{+/+}$*Trp53*$^{-/-}$), MEF258 (*Rad51d*$^{-/-}$*Trp53*$^{-/-}$), M7 (*Rad51d*$^{+/+}$*Trp53*$^{+/+}$), and MEF172AG (*Rad51d*$^{-/-}$ *Trp53*$^{-/-}$ *HARad51d*) were described previously [25]. Plasmid constructs were transfected using Lipofectamine Reagents (Invitrogen) or Mirus TransIT-LT1 according to manufacturer's instructions. Ten micrograms per milliliter cycloheximide (Sigma) was used for protein stability experiments.

2.2. Plasmids

RNF138 (*MmRnf138* NM_207623.1) expression vectors were generated by PCR amplification of mouse liver cDNA with Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) using *MmRnf138*-specific primers RNF138s1 (5′-CTTGGTACCTCCGAGGAACTTTCGGCGG-3′) and RNF138as1 (5′-CTTGGATCCTGTAGGTTGCAAGGAGGCAG-3′) followed by cloning of the amplification products into the *Kpn*I/*Bam*HI sites of pcDNA3.1/Hygro+ (Invitrogen) with either HA or Myc epitope tags. Mutagenesis of the RNF138 RING finger domain (H36A,C39S) was accomplished by subcloning *MmRnf138* cDNA into pUC19 for conventional PCR based site-directed mutagenesis with primers: RNF138mut1 (5′-GGCCTGTCAGGCCGTTTTCTCTAGAAAATGTTT CCTGACTG-3′) and RNF138mut2 (5′ CAGTCAGGAAACATTTTCTAGAGAAAA CGGCCTGACAGGCC 3′). The underlined portion denotes sequence targeted for mutagenesis. All PCR derived expression constructs were confirmed by sequencing both strands. The Myc-ubiquitin expression plasmid was kindly provided by Dr. Xiongbin Lu (Department of Cancer Biology, MD Anderson) and the HA-ubiquitin plasmid purchased from Addgene. The RAD51D Walker A ATPase mutant plasmid constructs were described previously [33], and RAD51D deletion constructs containing residues 4–77 and residues 77–329 were a gift from Dr. Joanna Albala (Lawrence Livermore National Laboratory) [34]. The yeast expression vector pVT100u was a generous gift from Dr. David Schild (Lawrence Berkley National Laboratory, Berkley). *MmRnf138* cDNA was subcloned into the HindIII/BamHI sites of pVT100u for use in yeast three-hybrid experiments. Splice variant constructs, *MmRnf138-Δ7* and *MmRnf138-Δ5*, were cloned into the *Bam*HI and *Eco*RI sites of pGADT7 and pGBKT7 for use in yeast two-hybrid experiments.

2.3. Yeast two- and three-hybrid

For yeast two-hybrid screening, a *Mus musculus* pre-transformed normalized universal cDNA library (Clontech) was screened using mouse full-length RAD51D. A total of 3.3×10^7 clones were assayed (cfu/mL of diploids × resuspension volume). Liquid β-galactosidase assays were performed using *ortho*-nitrophenyl-β-galactopyranoside (ONPG: Sigma) [35]. Yeast two-hybrid expression vectors pGADT7 and pGBKT7 (Clontech) were co-transformed into Y187 haploids using the EZ Transformation Kit (Zymo). Yeast three-hybrid experiments were performed using Y190 haploids transformed with pGADT7, pGBKT7, and pVT100u expression constructs [36].

2.4. Immunoprecipitations

For co-immunoprecipitations, vectors encoding HA-tagged and Myc-tagged proteins were co-transfected into HeLa cells. Whole cell extracts were prepared after 24 h using mammalian protein extraction reagent (M-PER; Thermo-Scientific) or 1X Cell Lysis Buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% TritonX-100) containing a protease inhibitor cocktail (Complete Mini; Roche Life Sciences). Three to five hundred micrograms of whole cell extract was incubated with anti-HA agarose beads (3F10; Roche) or anti-Myc magnetic beads (9E10; Thermo-Scientific) for 16 h at 4 °C with gentle rocking in incubation buffer (20 mM Tris, 100 mM NaCl, 100 mM EDTA) or 1X Cell Lysis Buffer. Precipitated proteins were washed 3 times with 1x PBST or 1X Cell Lysis Buffer, eluted by boiling in Laemmli buffer for 10 min, and resolved on 12% SDS-PAGE or 4–20% SDS-PAGE (Bio-Rad). For *in-vivo* ubiquitination assays, cells were treated with 25 μM MG132 (Sigma) 4 h prior to preparation of whole cell extracts.

2.5. Immunoblotting

Western blot analysis was performed using mouse monoclonal anti-HA (3F10; Roche), mouse monoclonal anti-Myc (9E10; Santa Cruz Biotechnology), rabbit monoclonal anti-Myc (ab9106; Abcam), or rabbit polyclonal anti-RAD51 (H-92; Santa Cruz Biotechnology). Primary antibody incubations were followed by incubation with the appropriate species-specific HRP-conjugated or IRDye 800CW secondary antibody (Licor) secondary antibody (Santa Cruz Biotechnology). Detection was performed by ECL (West-Pico chemiluminescent substrate; Thermo-Scientific) or the Licor Odyssey Sa Imaging System. Quantitative analysis of band intensity was performed using NIH ImageJ.

2.6. RNA interference and quantitative real-time PCR

RNAi-mediated knockdowns were conducted using Mission siRNA oligos (Sigma Proligo, TX, USA). Oligos directed to the mouse *Rnf138* gene corresponded to nucleotides 462–482 corresponding to exon 2 (1_Rnf138_Mm and 1_Rnf138_Mm_as duplex; siRNA1) and 1234–1254 corresponding to the 3′ UTR (2_Rnf138_Mm and