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Distinct functions of human RECQ helicases WRN and BLM in replication fork recovery and progression after hydroxyurea-induced stalling

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ABSTRACT

Human *WRN* and *BLM* genes are members of the conserved RECQ helicase family. Mutations in these genes are associated with Werner and Bloom syndromes. WRN and BLM proteins are implicated in DNA replication, recombination, repair, telomere maintenance, and transcription. Using microfluidics-assisted display of DNA for replication track analysis (ma-RTA), we show that WRN and BLM contribute additively to normal replication fork progression, and non-additively, in a RAD51-dependent pathway, to resumption of replication after arrest by hydroxyurea (HU), a replication-stalling drug. WRN but not BLM is required to support fork progression after HU. Resumption of replication by forks may be necessary but is not sufficient for timely completion of the cell cycle after HU arrest, as depletion of WRN or BLM compromises fork recovery to a similar degree, but only BLM depletion leads to extensive delay of cell division after HU, as well as more pronounced chromatin bridging. Finally, we show that recovery from HU includes apparent removal of some of the DNA that was synthesized immediately after release from HU, a novel phenomenon that we refer to as nascent strand processing, NSP.

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1. Introduction

RECQ helicases are a family of proteins conserved from bacteria to humans. Out of five human RECQ helicase genes, three are associated with heritable disorders. Mutations in the *BLM* [1], and *WRN* [2] genes cause, respectively, Bloom syndrome (BS) and Werner syndrome (WS), and mutations in *RECQL4* [3] are seen in Rothmund–Thomson, RAPADILINO, and Baller-Gerold (BGS) syndromes.

Clinical manifestations of Werner syndrome mimic premature aging, while Bloom syndrome is associated with developmental abnormalities [4]. Bloom and Werner syndromes are cancer-prone diseases, albeit the spectra of cancers they predispose to are different. Cells mutated in *BLM* or *WRN* genes show phenotypes associated with genomic instability and perturbed replication: slower S phase, increased fraction of cells at the G2/M boundary of the cell cycle, and expression of some fragile sites (for review, see [5–8]). *In vitro*, several biochemical features are unique to BLM or WRN, warranting a systematic analysis of the redundancy and cooperation between these two RECQs within a cell. Studies in DT40 cells demonstrated synthetic hypersensitivity of *WRN*/*BLM* knockout cells to a number of genotoxic drugs, including camptothecin [9], as well as unique genetic interactions between these RECQs and other genes [10], pointing toward WRN and BLM's complementary roles within pathways of DNA metabolism, and inviting a more mechanistic inquiry.

The insight into roles of WRN and BLM in DNA replication is complicated by the facts that both RECQs are multifunctional proteins [4,11], and that replication fork metabolism is likely conducted through several interconnected pathways [8,12]. Briefly, when fork progression is interrupted by lesions in the template or by replisome poisoning, extra activities are turned on as part of the S phase checkpoint, and stabilize the replisome-DNA structure against collapse [12,13]. It is thought that collapsed replication forks are susceptible to double strand breaks (DSBs). These DSBs may be an intermediate in an active fork rescue pathway, or merely a breakdown product which necessitates repair (see Refs. above). The exact balance between fork stabilization and fork collapse/rescue may depend on the cell type and the nature of interruption facing a fork.

Early studies have suggested that both WRN and BLM can be involved in elongation of DNA replication (reviewed in [8]). The use of DNA fiber technology allowed further insight into roles of RECQ helicases at a replication fork, demonstrating that WRN [14] and BLM [15] may be required for normal fork progression. In addition, complementing BS patient-derived human fibroblasts with BLM improves resumption of replication fork progression after an arrest with hydroxyurea (HU), a ribonucleotide reductase inhibitor [16]. Defects of fork recovery, albeit variable, were also demonstrated in WRN-depleted HeLa cells, in WS fibroblasts [17,18], and

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in WRN-depleted fibroblasts [19]. Both RECQs are targeted by the checkpoint kinase ATR [18,20–22] and affect checkpoint performance [16,23,24].

In order to delineate redundant *versus* cooperative functions of WRN and BLM, we have established isogenic human fibroblasts depleted of WRN, BLM, or both RECQs [25]. Here, we undertake a detailed analysis of replication fork phenotypes in these cells, and describe both unique and shared functions of WRN and BLM at a replication fork, as well as uncover a novel process of metabolizing nascent strands during recovery from HU.

2. Materials and methods

2.1. Cells and culture

SV40-transformed GM639 fibroblast cell line was obtained from the Coriell Institute Cell Repositories (Camden NJ). GM639cc1 is a pNeoA derivative of GM639 [19,25,26]. Unless stated otherwise, all experiments were performed using this cell line. The large T antigen is at least partially inactivated in this cell line since it does not support replication of SV40 origin-containing plasmids (J.S., unpub.).

The primary human dermal fibroblasts were described [27]. All cell lines were grown in Dulbecco Modified Minimal Essential Medium (DMEM) supplemented with L-glutamine, sodium pyruvate, 10% fetal bovine serum (Hyclone, Ogden, UT) and antibiotics in a humidified 5% CO_2, 37 °C incubator.

2.2. Drugs and dyes

Stock solutions of 5-bromodeoxyuridine (BrdU; 10 mM in water), 5-iododeoxyuridine (IdU, 2 mM in PBS), 5-chlorodeoxyuridine (CldU, 10 mM in water), 5-ethynyldeoxyuridine (EdU, 10 mM in DMSO), hydroxyurea (HU, 1 M in PBS), and cytochalasin-B (600 μg/ml in DMSO) were stored at −20 °C. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) with the exception of EdU (Invitrogen). CldU, BrdU, and IdU were used at concentrations of 50 μM and EdU was used at 10 μM.

2.3. RNAi-mediated depletion of WRN, BLM, and RAD51

Short hairpin (sh) RNA constructs for depletion of WRN and BLM are described [19,25]. pLKO.1-based shRNA constructs against human RAD51 were purchased from Open Biosystems (Cat. No. RHS4533-NM_002875). Depletions were carried out as described [19,25].

2.4. Western blotting

Western blotting of WRN was done as described [19,25] with the rabbit α-WRN (Novus Cat. No. NB100472A) or mouse α-WRN 195 C (provided by Dr. Opresko). Rabbit α-BLM antibody against BLM C-terminal peptide (KPINRPFLKPSYAFS was described [25]. α-RAD51 antibodies were rabbit polyclonal (Cat. No. PC130, Calbiochem, La Jolla, CA), and mouse monoclonal (Cat. No.05-530, Millipore, Temecula, CA). Mouse α-CHK1 antibody was from Santa Cruz (Cat. No. sc-8408). Phosphorylation of CHK1 and CHK2 was analyzed with a Phospho-Chk1/2 Antibody Sampler Kit (Cell Signaling, Cat. No. 9931). All proteins were visualized by ECL (Amersham) and quantified using Storm Phosphorimager and ImageQuant software (Molecular Dynamics). For presentation, images were saved in TIFF format, adjusted for brightness/contrast and cropped using Adobe Photoshop, and assembled into figures in CorelDraw. Brightness/contrast adjustments were made to entire images.

2.5. Staining for BrdU incorporation and FACS

Staining for BrdU was done as described [19]. FACS data analysis and presentation were done with Summit software (Dako, Carpinteria, CA), and cell cycle phase quantitations were done with FACS express software (Phoenix Flow Systems, San Diego, CA).

2.6. Microchannel fabrication, DNA fiber stretching and replication track analysis

These procedures were done as described [19,28]. The mouse antibody against total DNA was from Chemicon (Cat. No. MAB3034). Microscopy of stretched DNAs was performed on the Zeiss Axiovert microscope with a 63× objective. Lengths of tracks were measured in raw merged images (jpegs) using Zeiss AxioVision software. Details of statistical analysis are described in figure legends.

2.7. Nucleoplasmic bridge measurements

Cells were pulse-labeled with 10 μM EdU for 1 h and then arrested with 2 mM HU for 6 h. After release from HU, cytochalasin-B was added at 2 μg/ml. Cells were harvested by trypsinization in 20 h and cytospun onto poly-L-lysin-coated slides (Sigma). Cells were fixed for 10 min in 4% paraformaldehide, 0.2% Triton X-100, 20 mM Pipes pH 6.8, 1 mM $MgCl_2$, 10 mM EGTA, washed with PBS, and stained for EdU incorporation (by Click-It reaction with AlexaFluor 594 azide according to the manufacturer's recommendations (Invitrogen), as well as for total DNA using Hoechst33342. Slides were mounted in Vectashield (Vector labs) and examined under 40× magnification using Zeiss Axiovert microscope and AxioVision software. Images of binucleated cells were collected. Scoring was done according to [29]. 60–200 each of EdU+ and EdU− binucleated cells in each sample were inspected for nucleoplasmic bridges.

2.8. Microscopy image presentation

Visual scoring or measurement of features in microscopy images was done in sets of multicolor jpeg files in AxioVision. For presentation, images were adjusted for brightness/contrast and cropped in Adobe Photoshop, and assembled into figures in CorelDraw. Adjustments were always done to entire images. In some cases, brightness/contrast of individual color channels was adjusted separately.

3. Results

3.1. WRN and BLM contribute additively to fork progression rates during an unperturbed S phase

We depleted WRN and/or BLM with lentiviral shRNAs, as before ([19,25] and Fig. 4A and Figs. S1A, S3C and S4B), achieving at least 80–85% depletion of the target protein(s). Growth rate was lower in WRN-depleted and, more dramatically, in BLM-depleted cell populations, which reflected the size of replicating fraction. To account for it, every assay used in this study discriminated between replicating and non-replicating fractions, or focused exclusively on replicating fraction.

We labeled cells with two nucleotide analogs (CldU and IdU) for 30 min each and used immunofluorescence to visualize tracks of replication in DNA stretched using microfluidics [28]. We measured lengths of 1st and 2nd label segments in two-segment tracks that incorporated both labels in tandem and thus correspond to ongoing