DNA damage response defect in Williams-Beuren syndrome

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Abstract. Williams-Beuren syndrome (WBS, no. OMIM 194050) is a rare multisystem genetic disorder caused by a microdeletion on chromosome 7q11.23 and characterized by cardiovascular malformations, mental retardation, and a specific facial dysmorphism. Recently, we reported that a series of non-Hodgkin's lymphoma occurs in children with WBS and thus hypothesized that a predisposition to cancer may be associated with this genetic disorder. The aim of the present study was to ascertain the role played by three genes hemizygously deleted in WBS (*RFC2*, *GTF2I* and *BAZ1B*) in DNA damage response pathways. Cell proliferation, cell cycle analysis, γ-H2A.X induction, and expression of DNA damage response proteins were investigated upon exposure to genotoxic treatments in WBS patient-derived primary fibroblasts and in the 293T cell line treated with specific siRNAs targeting *RFC2*, *GTF2I* and *BAZ1B*. An impaired hydroxyurea-induced phosphorylation of CHK1 was observed in the WBS cells. However, this defective DNA damage response was not associated with an increased sensitivity to genotoxic agents. In addition, depletion of RFC2, GTF2I and BAZ1B using specific siRNAs did not have a significant impact on the DNA damage response in 293T cells. Our results highlight that the ATR-dependent DNA damage response is impaired in WBS patient cells but is also dispensable for viability when these cells undergo a genotoxic stress. The mechanism by which the ATR pathway is impaired in WBS warrants elucidation through further investigation.

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Key words: Williams-Beuren syndrome, Williams syndrome, DNA damage, BAZ1B, RFC2, GTF2I, non-Hodgkin's lymphoma, 7q11.23, cancer, predisposition

Introduction

Williams-Beuren syndrome (WBS, no. OMIM 194050) is a multisystem genetic disorder resulting from the hemizygous deletion of a region spanning 1.5-1.8 Mb on chromosome 7q11.23 which contains 28 coding genes and 2 miRNAs. This syndrome is characterized by cardiovascular malformations (mostly supravalvular aortic stenosis), mental retardation and a specific facial dysmorphism (1). We recently reported the occurrence of several cases of B-cell non-Hodgkin's lymphoma (B-NHL) in children with WBS as well as the existence of somatic deletion of the WBS critical region (WBSCR) in sporadic B-NHL. We also characterized the WBSCR using CGH-array and high-throughput sequencing in both normal and tumor samples from WBS patients and we found no second hits on the remaining allele. Thus, we suggested that the haploinsufficiency of the WBSCR genes might be associated with a predisposition to cancer, particularly B-NHL (2,3).

Genomic instability is a hallmark of B-NHL. The mechanisms by which B cells somatically engineer their genomes to generate the vast diversity of antibodies induce genomic instability. These mechanisms are highly regulated but, in some rare cases, abnormal B-cells might escape from controls and evolve toward malignancy. A number of congenital disorders associated with a DNA damage response and/or repair defect such as ataxia-telangiectasia, Bloom syndrome or Nijmegen breakage syndrome have been linked to a predisposition to B-NHL (4-7).

In addition, three genes that are hemizygously deleted in WBS encode proteins that are involved in DNA damage response and/or repair signaling pathways (Fig. 1).

First, RFC2 is one of the five subunits of the replication factor C that loads the proliferating cell nuclear antigen (PCNA) onto chromatin during DNA replication thus facilitating DNA polymerase action. RFC2 forms a multiprotein complex with Rad17 that plays a major role in ATR signaling. Notably, it has been shown that mutant cells of *Saccharomyces cerevisiae* having a thermosensitive RFC2 mutation exhibit temperature-sensitive growth, sensitivity to hydroxyurea (HU) and UV light and an increased rate of mitotic recombination

and chromosome loss suggesting an important role of RFC2 in DNA replication and cell cycle checkpoint (8).

Second, GTF2I (also called TFII-I) is a multifunctional transcription factor that activates growth-promoting target genes upon tyrosine phosphorylation in response to mitogenic signaling. In 2005, Desgranges *et al* demonstrated that GTF2I is ubiquitinated and targeted to proteasomal degradation in response to genotoxic stress in a p53- and ATM-dependent manner resulting in cell cycle arrest (9). More recently, Tanikawa *et al* showed that GTF2I plays an important role in regulating BRCA1-mediated homologous recombination (HR) mechanisms. After irradiation, GTF2I forms foci with γ-H2A.X and the depletion of endogenous GTF2I using siRNA knockdown results in the inhibition of HR efficiency (10). GTF2I is also involved in translesion synthesis (TLS) mechanisms. Notably, it has been shown that GTF2I bridges PCNA and Pol ζ to promote TLS (11).

Finally, BAZ1B (also called WSTF) is a component of the WSTF-ISWI chromatin-remodeling complex (WICH) that is involved in maintaining chromatin organization (12,13). Recently, BAZ1B has also been linked to the DNA damage response pathway and was shown to possess tyrosine kinase activity phosphorylating Tyr142 of H2A.X. In response to genotoxic stress, the balance of the phosphorylation state of both Tyr142 and Ser139 of H2A.X appears to determine the cell fate between DNA repair and apoptosis (14,15).

However, it is unclear whether the haploinsufficiency of RFC2, GTF2I and BAZ1B is involved in DNA damage response and/or repair deficiency in WBS patients.

The aim of our study was to investigate the sensitivity to genotoxic stress and the DNA damage response in both primary cell lines derived from WBS patients and in WBS gene-specific siRNA knockdown cells.

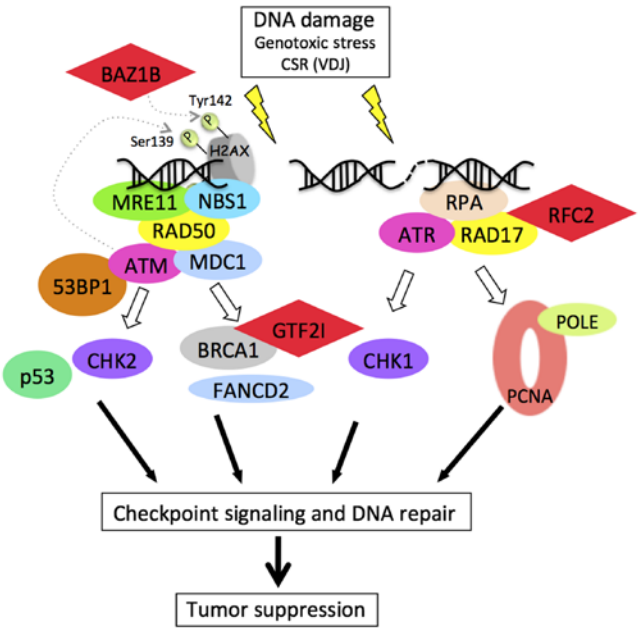

Figure 1. Schematic view of Williams-Beuren syndrome genes involved in DNA damage response pathways.

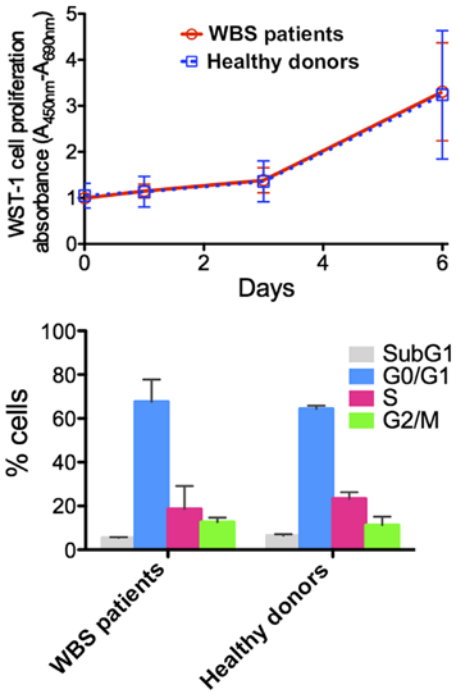

Figure 2. Cell proliferation and cell cycle analysis in primary fibroblasts derived from Williams-Beuren syndrome (WBS) patients and healthy donors.

Materials and methods

Ethical approval. Participation in this study by patients and their relatives along with skin biopsy donations and informed consent procedures were approved by the ethics committees of the Genomic and Genetic Disorder Biobank (Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy) and the University of Franche-Comté (UFC; Besançon, France).

Cell lines, cell culture and transfections. Primary fibroblast cell lines from WBS patients (GDB306FIBRO, GDB863FIBRO, GDB728FIBRO) and from healthy donors (GDB380-2FIBRO, GDB809-1FIBRO, GDB819-1FIBRO) were provided by Dr Giuseppe Merla from the Genomic and Genetic Disorders Biobank (GGDB, Network of Telethon Genetic Biobanks, project no. GTB07001G) in San Giovanni Rotondo, Italy. The 293T cell line (no. CRL-3216) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated endotoxin-free fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA).

293T cells were stably transfected individually with 1 µg of each of the 4 unique gene-specific 29mer shRNA constructs (BAZ1B, no. TG306439; GTF2I, no. TG304176; RFC2, no. TG309864) or no. TR30013 scrambled negative control non-effective shRNA cassette in pGFP-V-RS (OriGene Technologies, Inc., Rockville, MD, USA) using the Effectene transfection kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Transfected cells were selected with 2 µg/m of puromycin (Invitrogen Life Technologies), 48 h following transfection.

Drug treatments and cell proliferation assay. Cells were treated with 0.05, 0.5 and 5 mM of HU (no. H8627, 500 mM

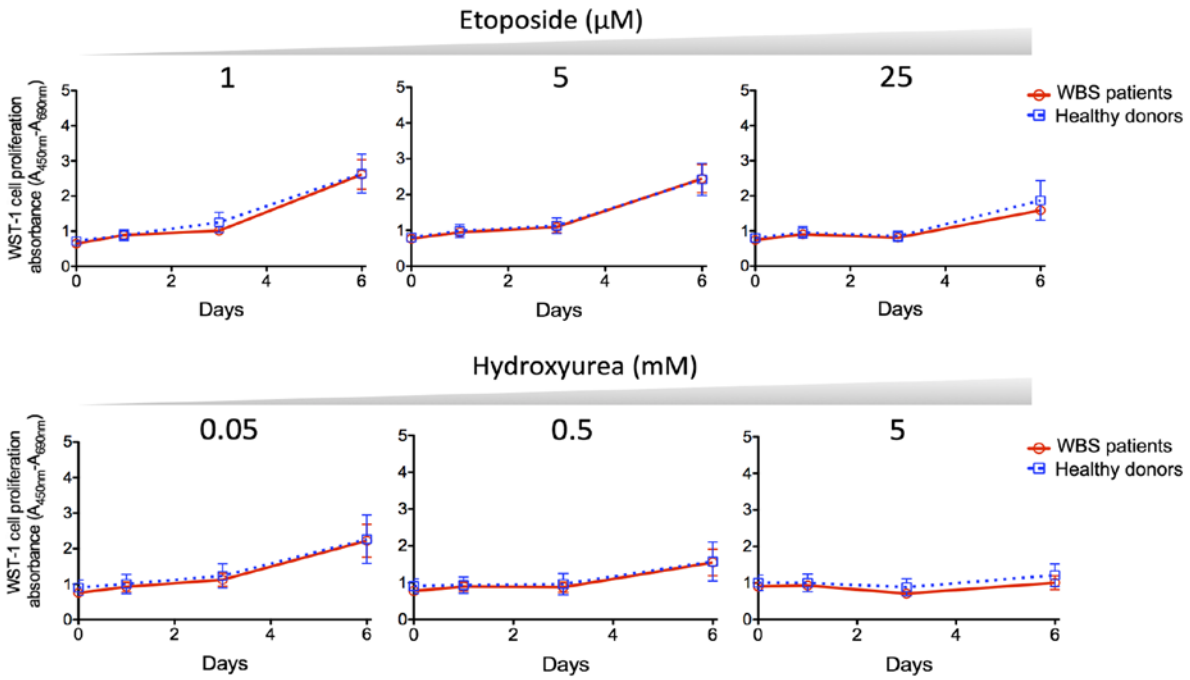

Figure 3. Sensitivity to genotoxic agents in primary fibroblasts from Williams-Beuren syndrome (WBS) patients and healthy donors.

stock solution in ddH_2O; Sigma, St. Louis, MO, USA) or 1, 5 and 25 μM of etoposide (ETP, no. 2200, 50 mM stock solution in DMSO; Cell Signaling Technology, Inc., Danvers, MA, USA) for the indicated times.

For the cell proliferation assay, 10 μl of Premix WST-1 cell proliferation assay reagent (no. MK400; Takara Bio, Dalian, China) was added to the cultured cells in triplicate in a 96-well plate at the indicated times and conditions with 100 μl of culture media and then the cells were incubated for 2 h before measuring the optical density (OD) following the manufacturer's recommendations.

Cell cycle and γ-H2A.X analyses by flow cytometry. For flow cytometry analyses a minimum of 10^5 cells from each condition was washed with PBS and then fixed in pre-cooled 70% absolute ethanol and incubated at -20˚C for 60 min. Fixed cells were washed 3 times with cold PBS and then stained with Alexa Fluor 647 anti-H2A.X-phosphorylated (Ser139) antibody (no. 613408; BioLegend, Inc., San Diego, CA, USA). Finally, the cells were washed once with PBS and resuspended in 50 μl of propidium iodide (PI)/RNase staining solution (no. 4087; Cell Signaling Technology, Inc.) 15 min before fluorescence acquisition with BD FACS Canto II cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting. For western blot analysis, whole-cell lysates were separated by SDS-PAGE and transferred onto PVDF membranes using Criterion TGX gels and Trans-Blot Turbo Midi PVDF Transfer Packs (Bio-Rad, Berkeley, CA, USA). The blots were then incubated with specific primary antibodies as follows: anti-CHK1 (no. 2360), anti-CHK2 (no. 6334), anti-phospho-CHK1 Ser317 (no. 12302), anti-phospho-CHK2 Thr68 (no. 2197), anti-BRCA1 (no. 9010), anti-phospho-BRCA1 Ser1524 (no. 9009), anti-TFII-I (no. 4562) and anti-WSTF (no. 2152) all purchased from Cell Signaling Technology, Inc. The primary anti-RCF2 antibody (no. ab174271) was purchased from Abcam (Cambridge, UK).

Results

A link between WBS and cancer has been suggested by us and other authors. In the absence of epidemiological data on this topic, we carried out a functional study in order to investigate the DNA damage response in cells derived from WBS patients.

Cell proliferation and sensitivity to genotoxic stress. The proliferation of primary skin fibroblasts derived from 3 WBS patients was studied on day 1, 3 and 6 and was compared with the proliferation of fibroblasts derived from 3 healthy controls. The analysis of proliferation curves obtained using the WST-1 proliferation assay showed no difference in cell proliferation between the WBS patient and control fibroblasts. Moreover, cell cycle analysis by flow cytometry revealed a similar distribution of WBS cells in the G0/G1, S and G2/M phases relative to that noted in the control cells (Fig. 2).

DNA damage response and repair defects are usually associated with increased cellular sensitivity to genotoxic agents. We used two different genotoxic agents to investigate the sensitivity of WBS cells. ETP is a topoisomerase II inhibitor commonly used as an antitumor agent that induces DNA double-strand breaks. HU is a replication inhibitor that can induce DNA double-strand breaks by causing replication fork arrest upon nucleotide pool depletion. WST-1 cell proliferation assay of the treated cells showed a dose-dependent effect of ETP and HU but a difference in sensitivity to genotoxic agents between WBS patient and healthy control fibroblasts was not observed (Fig. 3).

In order to further understand the individual functions of WBS genes in DNA damage response and repair, we generated RFC2, GTF2I and BAZ1B knockdown in 293T cells using

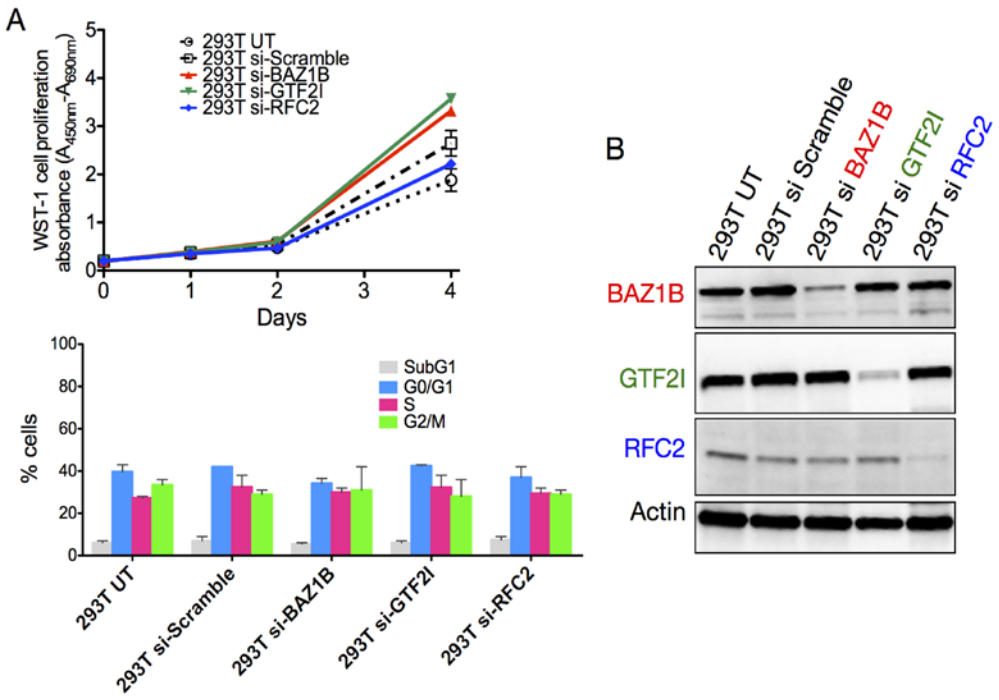

Figure 4. (A) Cell proliferation assay and cell cycle analysis of the 293T cells stably transfected with siRNAs targeting three Williams-Beuren syndrome genes. (B) Validation of the siRNA knockdown effect on target protein expression in the 293T cells. UT, untransfected cells.

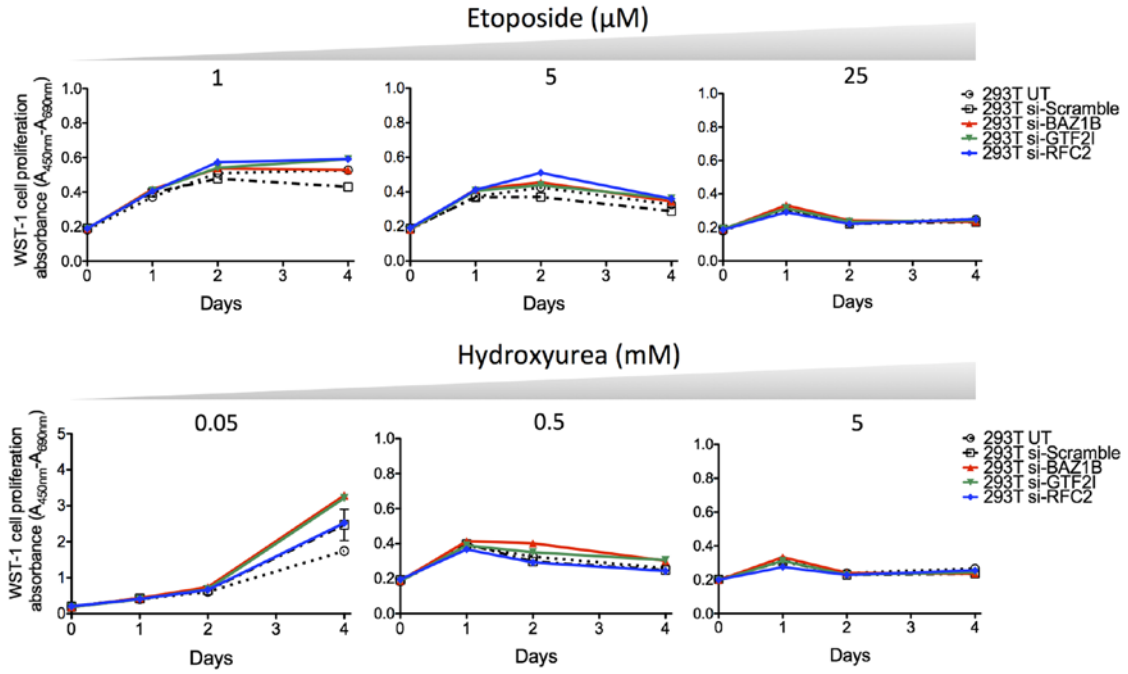

Figure 5. Sensitivity to genotoxic stress of the 293T cells stably transfected with siRNAs targeting three Williams-Beuren syndrome genes. UT, untransfected cells.

specific siRNA expression vectors. The 293T cells were stably transfected and the expression of siRNA targets was validated by western blotting (Fig. 4B).

The 293T cells were subsequently used for proliferation and cell cycle assays (Fig. 4A). In the absence of treatment, 293T cells depleted in BAZ1B and GTF2I showed a slightly increased proliferation rate on day 4 compared with the untransfected cells (UT) and cells transfected with the scramble siRNA. However, these differences were not correlated with the data of the cell cycle analysis that showed almost no differences between the different transfected cells and controls. The sensitivity to HU and ETP was also examined in the siRNA-transfected 293T cells. No difference was observed relative to the UT and scramble controls (Fig. 5).

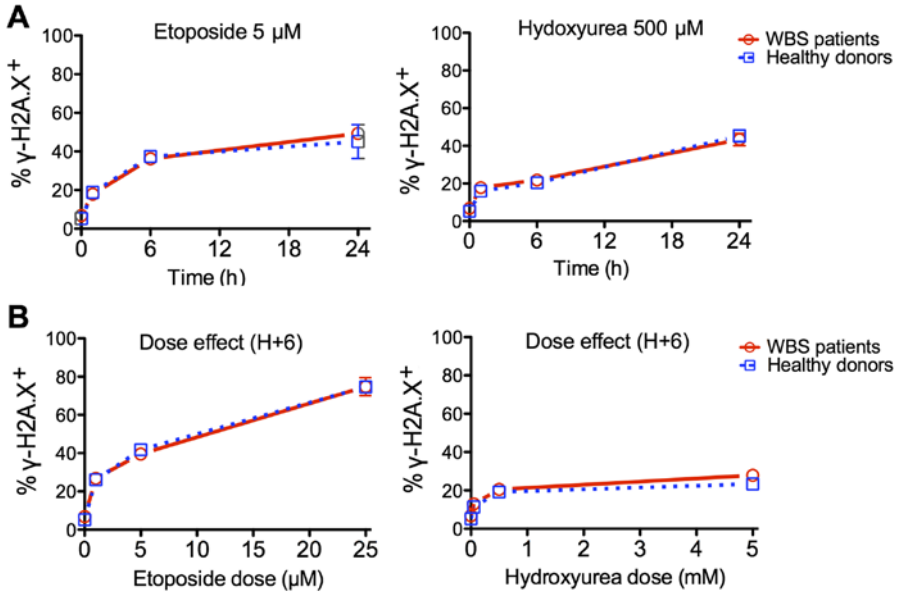

Figure 6. (A) Kinetics of γ-H2A.X induction in primary fibroblasts from Williams-Beuren syndrome (WBS) patients and healthy donors upon exposure to etoposide or hydroxyurea. (B) Dose effect of γ-H2A.X induction after 6 h of treatment with etoposide or hydroxyurea.

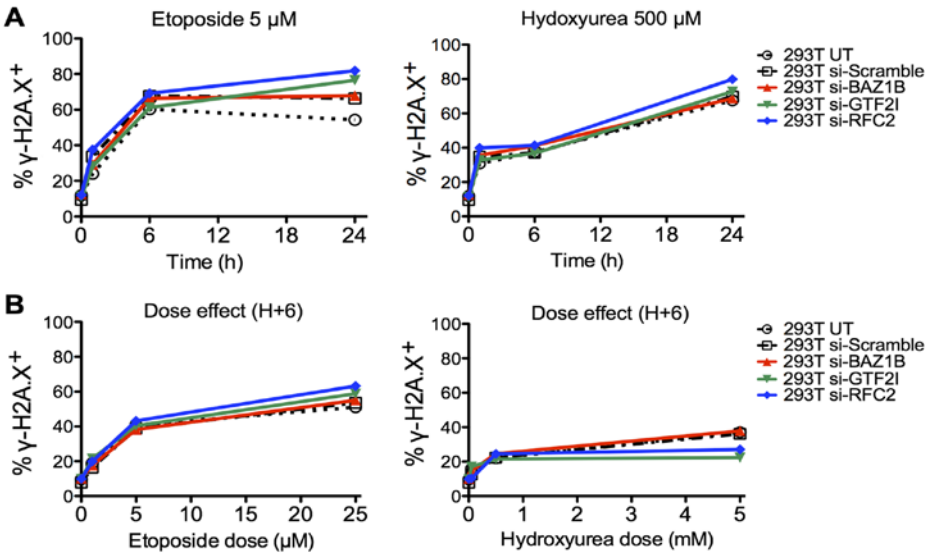

Figure 7. (A) Kinetics of γ-H2A.X induction in the 293T cells stably transfected with siRNAs targeting three Williams-Beuren syndrome genes upon exposure to etoposide or hydroxyurea. (B) Dose effect of γ-H2A.X induction after 6 h of treatment with etoposide or hydroxyurea in the same cells. UT, untransfected cells.

These results suggest that the haploinsufficiency of WBS genes, including BAZ1B, GTF2I, and RFC2, is not associated with a hypersensitivity to genotoxic agents.

γ-H2A.X induction and expression of DNA damage response and repair proteins. During the DNA double-strand break response, chromatin undergoes reorganization marked by H2A.X Ser 139 phosphorylation (γ-H2A.X). In the early phase of DNA damage response, γ-H2A.X forms foci, which are platforms for recruiting molecules involved in DNA damage repair and signaling. It has been demonstrated that γ-H2A.X induction is reduced in cells derived from patients with genetic syndromes associated with impaired DNA damage (16). BAZ1B has a kinase function that is responsible for H2A.X Tyr142 phosphorylation. A crosstalk between Tyr142 and Ser139 of H2A.X has been demonstrated recently and we aimed to ascertain whether the haploinsufficiency of BAZ1B may be associated with abnormal γ-H2A.X induction. We, therefore, analyzed γ-H2A.X induction in WBS fibroblasts, primary cell lines and siRNA-transfected 293T cells.

The dose-effects and kinetics of γ-H2A.X induction following exposure to ETP and HU was investigated using intracellular staining and flow cytometry. WBS patient and healthy control cells displayed a similar proportion of γ-H2A.X-positive cells under each condition (Fig. 6). In the 293T cells, no significant difference was observed between the siRNA-transfected cells and the controls (Fig. 7).

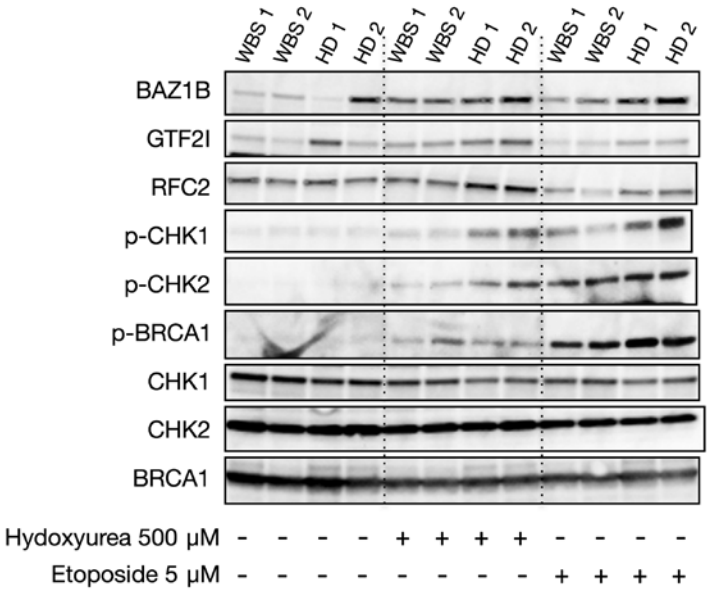

Figure 8. Analysis of the expression of Williams-Beuren syndrome (WBS) proteins and other DNA damage response proteins by western blotting in primary fibroblasts from WBS patients and healthy donors.

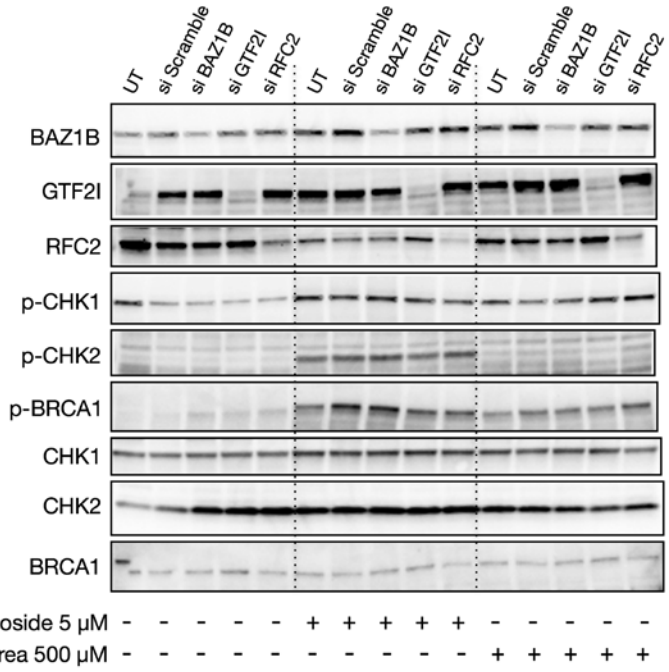

Figure 9. Analysis of the expression of Williams-Beuren syndrome (WBS) proteins and other DNA damage response proteins by western blotting in 293T cells transfected with specific siRNAs targeting WBS genes. UT, untransfected cells.

The expression and phosphorylation of DNA damage response and repair proteins known to interact with RFC2 and GTF2I were then studied by western blotting at the baseline (without any treatment) as well as following genotoxic damage using ETP and HU treatments.

As expected, the baseline expression of BAZ1B and GTF2I in primary fibroblasts from WBS patients appeared to be slightly lower compared with these levels noted in the healthy donors. This difference in expression increased upon treatments. Notably, the expression of RFC2 was similar in the WBS and control cells. Nonetheless, upon treatments with ETP and HU, a lower expression of RFC2 was observed in the WBS cell lines. Interestingly, phosphorylation of CHK1 at Ser317 and CHK2 at Thr68 appeared to be lower in the WBS cells after HU exposure and, to a lesser extent, after ETP exposure. No difference was found in the phosphorylation of BRCA1 in the primary fibroblasts from WBS patients and healthy donors (Fig. 8).

These results are concordant with previous research (16) and demonstrate that an impaired HU-induced phospho-CHK1 and phospho-CHK2 is associated with WBS.

In 293T cells transfected with siRNAs, immunoblot analysis demonstrated effective knockdown of the targets. However, no effect on the expression or phosphorylation of

CHK1, CHK2 and BRCA1 was demonstrated between the transfected 293T cells and the controls (Fig. 9).

Discussion

In the present study, we demonstrated that ATR-dependent CHK1 and CHK2 phosphorylation upon DNA damage is impaired in primary fibroblasts from WBS patients. However, these results were not reproduced following silencing of each of the genes *RFC2*, *GTF2I* and *BAZ1B* that are involved in DNA damage responses. Moreover, exposure to HU or ETP did not impair the cell cycle and proliferation in fibroblasts from WBS patients as compared to normal cells.

Several previous studies on WBS patient-derived cells demonstrated a DNA damage response defect in WBS. In 2011, Savina *et al* demonstrated experimentally a relationship between an abnormal DNA damage response and the 7q11.23 hemizygous microdeletion using a comet assay in lymphocytes isolated from WBS patients (17).

In 2007, O'Driscol *et al* found an impaired ATR-dependent DNA damage response in WBS lymphoblastoid cell lines (LBL) which they linked with the haploinsufficiency of RFC2, a coding gene localized within the WBSCR (16).

Our results are consistent with the observations of O'Driscoll *et al* (16) who demonstrated a link between WBS and impaired ATR-dependent DNA damage response. However, our results did not demonstrate a role of RFC2 in the abnormal DNA damage response observed in WBS patients. This may be due to the cellular model that we used. In the study of O'Driscoll *et al* (16), LBL cell lines were complemented after transfection of RFC2 cDNA and might be more appropriate to show the role of RFC2 since depletion of RFC2 alone in the 293T cells did not reproduce the phenotype of WBS cells.

Additionally, the DNA damage response defect observed in WBS patients was not associated with a hypersensitivity to DNA damaging agents. Thus, our results suggest that the WBS gene plays important roles in DNA damage response but are also dispensable for WBS cell viability when these cells undergo a genotoxic stress.

WBS is a contiguous gene syndrome and the DNA damage response defect in WBS cells is more likely to be associated with the depletion of a combination of genes.

Further studies are needed to elucidate the role of each WBS gene and the combination of these genes in DNA damage response and to understand their links with the susceptibility to LNH-B in WBS patients.

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