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Depletion of RUVBL2 in Human Cells Confers Moderate Sensitivity to Anticancer Agents

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Abstract

Background: Many anticancer agents kill cancer cells by inducing lethal damage in DNA, but the capacity of DNA repair of cells reduce the therapeutic efficacy of anticancer agents. RuvB-like (RUVBL) 2 is part of large protein complexes such as TIP60 and INO80 that are involved in chromatin remodeling and DNA damage responses and repair. Relatively few studies have investigated the role of RUVBL2 in the survival of cells after exposure to anticancer agents.

Methods: We depleted RUVBL2 in human MRC5-SV cells by small interfering (si) RNA and assessed the sensitivity of the cells to chemotherapeutic anticancer agents including cisplatin (cisPt), 2'-deoxy-5-azacytidine (azadC), and mitomycin C (MMC), and to physical DNA-damaging agents including X-rays.

Results: The knockdown efficiency with 10 nM siRUVBL2 was 80% on day 3 post-transfection, and knockdown (>65%) persisted on day 6. The cell growth slowed significantly due to depletion of RUVBL2 when compared to mock- and control siRNA-treated cells, indicating that RUVBL2 is essential for the proliferation of cells. The RUVBL2-depleted cells were moderately sensitive to cisPt, azadC, and X-rays. The increase in the sensitivity to MMC was marginal.

Conclusion: Depletion of RUVBL2 in cells confers moderate sensitivity to anticancer agents and X-rays, presumably through partial impairment of the homologous recombination repair of DNA double-strand break intermediates formed directly or indirectly by anticancer agents or X-rays. Further studies are necessary to clarify the exact role of RUVBL2 in this process.

Keywords: DNA repair; Chromatin remodeling; Cell survival; Anticancer drugs; X-rays

Introduction

RuvB-like 1 (RUVBL1, also known as RVB1, TIP49A and Pontin) and RuvB-like 2 (RUVBL2, also known as RVB2, TIP49B and Reptin) belong to the family of ATPases associated with various cell activities (AAA+ ATPase), and are part of large protein complexes that are involved in chromatin remodeling, transcription regulation, DNA damage responses and repair, and the biogenesis of small nucleolar ribonucleoproteins [1,2]. RUVBL1 and RUVBL2 (RUVBLs) share about 45% identity and 60% similarity in the amino acid sequence and are homologous to the prokaryotic RuvB helicase [3,4]. Remarkably, RUVBL1 depletion leads to down regulation of RUVBL2, and vice versa [5]. RUVBLs also interact with oncogenes such as c-Myc and β-catenin and are implicated in cancer [4,6]. Since RUVBLs participate in many aspects of cellular functions, links between these functions are currently being studied extensively.

In human cells, RUVBLs serve as the essential components of the TIP60 histone acetyltransferase complex. TIP60 is recruited to DNA damage sites [7,8] and plays multiple roles in DNA damage responses [9]. After treatment with various DNA-damaging agents, RUVBL1 is required for the dephosphorylation of phosphorylated histone H2AX, a marker of DNA double-strand breaks (DSBs), through the histone acetyltransferase activity of TIP60 [8]. Likewise, depletion of RUVBL2 increases the persistence of phosphorylated histone H2AX upon treatment with X-rays [10]. Apart from the pathway involving the TIP60 complex, RUVBLs regulate the abundance and activity of phosphatidylinositol 3-kinases such as ataxia telangiectasia mutated (ATM), ATM- and Rad3-related (ATR) kinase, and DNA-dependent protein kinase (DNA-PK) that sense and activate the DNA damage signal [11]. RUVBLs are also components of the INO80 chromatin remodeling complex that is recruited to the DSB site. A recent study has shown that the mammalian INO80 complex mediates DSB repair through its role in DNA end resection [12]. The ATP-dependent helicase activity of RUVBLs demonstrated *in vitro* may be involved in the processing of resected DNA ends [13,14]. Depletion of RUVBLs in human cells impairs the recruitment of the RAD51 recombinase to the DSB site following DNA end resection [15]. Consistent with the finding, it has been shown that the functional inactivation or depletion of TIP60 that contains RUVBLs in mouse and human cells impairs the recruitment of RAD51 to the DSB site [16].

The aforementioned studies have afforded mechanistic insights into the function of human RUVBLs in DNA damage responses and repair. However, few studies have investigated the role of RUVBLs in

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the survival of human cells after exposure to DNA-damaging agents, although decreased cell survival through impaired DNA damage responses and repair is among the major end points of DNA damage. It is also noteworthy that many anticancer agents kill cancer cells by inducing lethal damage in DNA, but the capacity of DNA repair of cells reduce the therapeutic efficacy of anticancer agents [17,18]. To the best of our knowledge, enhancement of sensitivity to mitomycin C (MMC) by the depletion of RUVBL1 [15] and that to ultraviolet light (UV) by the depletion of RUVBL2 [19,20] have only been reported for human cells. We therefore thought it would be useful to analyze the effect of down-regulation of RUVBLs on cell survival upon treatment with chemical DNA-damaging agents such as anticancer drugs and physical DNA-damaging agents such as X-rays. In the present study, we depleted RUVBL2 in human MRC5-SV cells by small interfering (si) RNA and assessed the sensitivity of the cells to anticancer drugs including cisplatin (cisPt), 2'-deoxy-5-azacytidine (azadC), MMC, and to X-rays.

Materials and Methods

Cell culture and overexpression of RUVBL2

DNA repair-proficient MRC5-SV cells, a human fetal lung fibroblast cell line transfected with SV40, were used for depletion of RUVBL2 [21,22], since mutations in DNA repair genes that are frequently found in cancer cells [23] may obscure the direct effect of RUVBL2 depletion. HEK293T cells were used for overexpression of RUVBL2. MRC5-SV and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% inactivated fetal bovine serum (Biological Industries), L-glutamine (584 μg/ml) and kanamycin (20 μg/ml) in a humidified incubator at 37°C with a 5% CO_2 atmosphere. For the overexpression of RUVBL2, HEK293T cells were transfected with pCMV6-XL5-RUVBL2 or pCMV6-AC-RUVBL2 (both from Origene) using Lipofectamine LTX and Plus reagent (Invitrogen) according to the method provided by the manufacturer. After transfection, cells were cultured for 72 h, recovered and assayed for protein expression. Chinese hamster ovary (CHO) cells proficient (AA8) and deficient (51D1) in homologous recombination (HR) were cultured in α-minimum essential medium (Wako) supplemented with 10% inactivated fetal bovine serum [24].

RNA interference

MRC5-SV cells at ca. 50% confluence in a 10 cm dish were transfected with RUVBL2 siRNA (2–30 nM) or control siRNA (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the method provided by the manufacturer. One day after transfection, the cells were divided into four plates, incubated for up to 5 days and assayed for the depletion of RUVBL2. The target sequences of RUVBL2 siRNA were 5'-gctccacgcagtacatgaaggagta-3' (siRUVBL2-1), 5'-gagatccagattgatcgaccagcaa-3' (siRUVBL2-2) and 5'-ccagcttggtgtgccggaaacgcaa-3' (siRUVBL2-3). Two negative-control siRNAs with high (siCONT-H) and medium (siCONT-M) GC contents were also used.

Western blot analysis

MRC5-SV cells were transfected with siRNA as described above, and cells were recovered from the plates after 3 and 6 days. Cells were resuspended in cold RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with 1 mM PMSF for 10 min, sonicated twice for 2 s each and then sedimented at 22,000 ×*g* for 20 min at 4°C. The recovered supernatant was used as whole cell extracts (WCEs). The protein concentration was measured with the BCA protein assay kit (Pierce). WCEs were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel, and proteins were transferred to a PDVF membrane on a Trans-Blot Turbo transfer system (BIO-RAD). The membrane was washed with TBST (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20), blocked with 1% BSA in TBST and then washed again with TBST. RUVBL2 was proved with anti-RUVBL2 antibodies (Abcam EPR4146, 1:5000 dilution). Primary antibodies were detected with HRP-conjugated goat anti-rabbit IgG antibodies (KPL 474-1516, 1:5000 dilution). β-Tubulin was used as a loading control and was detected with anti-β-tubulin antibodies (Sigma T8328, 1:5000 dilution) and HRP-conjugated anti-mouse IgG antibodies (Santa Cruz Biotechnology sc-2005, 1:2000 dilution). Finally, the membrane was incubated with the ECL Western Blotting Substrate (Promega), and chemiluminescence was detected on a ChemiDoc XRS+ system (BIO-RAD). The sizes of bands were estimated relative to the Precision Plus Protein WesternC Standard (BIO-RAD) run in parallel.

Cell proliferation and survival

MRC5-SV cells were transfected with siRNA as described above. One day after transfection, the cells were divided into four plates and incubated for up to 5 days. The cells were stained with trypan blue and the numbers of viable and dead cells were counted. Survival assays with MRC5-SV cells were performed as follows: One day after transfection with siRNA, cells were recovered, seeded in a dish and incubated for 24 h. Cells were treated with cisPt, azadC, or MMC (all from Wako) for 3 h (cisPt and MMC) or 24 h (azadC). Alternatively, cells were irradiated with X-rays generated by an OM-303M X-ray generator (OHMiC) at a dose rate of 1.14 Gy/min. The treated cells were further incubated in fresh medium for 7–8 days, and colonies with more than 50 cells were scored. The surviving fraction was calculated as N/N_0, where N and N_0 are the numbers of colonies for DNA damaging agent-treated and untreated cells, respectively. Survival assays with CHO cells were performed as described for MRC5-SV cells except that siRNA treatment was omitted.

Results

Detection of RUVBL2 by western blot analysis

We optimized the conditions for immunodetection of endogenous RUVBL2 prior to its depletion by siRNA. In the Western blot analysis of RUVBL2, the WCEs from MRC5-SV cells gave rise to two bands at around 50 kDa (expected size = 51.2 kDa, Figure 1A). Comparison with the mobility of the RUVBL2 protein overexpressed from pCMV6-XL5-RUVBL2 or pCMV6-AC-RUVBL2 revealed that the upper band was attributable to endogenous RUVBL2. The cross-reacting protein in the lower band remains to be identified. The band intensity of endogenous RUVBL2 was proportional to the amount of WCEs up to 15 μg (Figure 1B). From the result, we used 10 μg of WCEs for the analysis of the down-regulation of RUVBL2.

Depletion of RUVBL2 by siRNA

Three different siRNAs (siRUVBL2-1, -2 and -3) were designed to target the coding sequence of the RUVBL2 mRNA. The siRNAs were transfected into MRC5-SV cells, and the depletion of RUVBL2 was analysed by Western blotting at 3 and 6 days after transfection. The knockdown efficiency of RUVBL2 increased with increasing the concentrations of siRNA (2–30 nM, data not shown), and near-maximal silencing was achieved with 10 nM siRUVBL2-1, -2 and -3. The knockdown efficiency with 10 nM siRUVBL2s was 80% on day 3

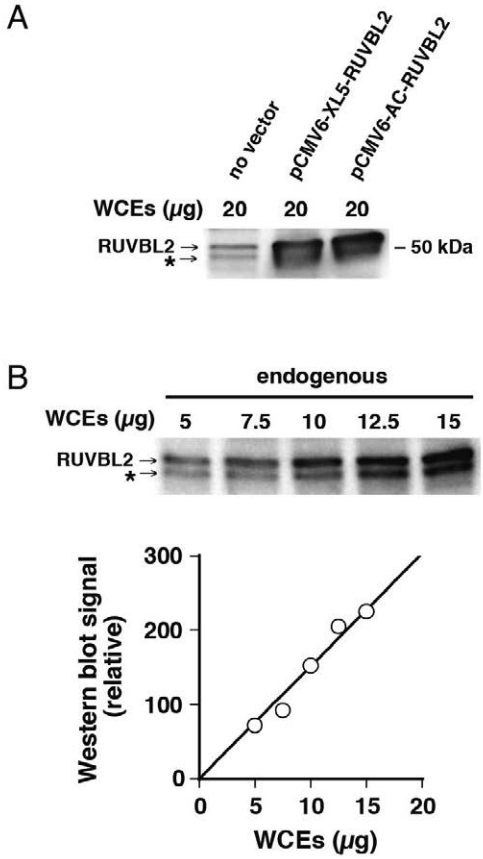

Figure 1: Detection of endogenous RUVBL2. (A) Detection of RUVBL2 by Western blot analysis. WCEs of MRC5-SV cells without transfection and HEK293T cells transfected with a RUVBL2 expression vector (pCMV6-XL5-RUVBL2 or pCMV6-AC-RUVBL2) were separated by SDS-polyacrylamide gel electrophoresis. RUVBL2 protein on a blotted membrane was detected by anti-RUVBL2 antibodies. (B) Correlation between the amount of WCEs of MRC5-SV cells and the Western blot signal of endogenous RUVBL2. Upper panel: Western blot; lower panel: correlation between the amount of WCEs and the Western blot signal (average from two experiments). A cross-reacting protein in the lower band, which remains to be identified, is indicated by the asterisks in (A) and (B).

post-transfection, and knockdown (>65%) persisted on day 6 (Figure 2).

Inhibition of cell proliferation by the depletion of RUVBL2

RUVBLs are essential for the cell proliferation of yeast [3,25], C. elegans [26], drosophila [27] and xenopus [28]. We asked whether this is also true for MRC5-SV cells. Figure 3 shows the growth curves of MRC5-SV cells transfected with RUVBL2 siRNAs (10 nM). The cell growth slowed significantly due to depletion of RUVBL2 when compared to mock- and control siRNA-treated cells, indicating that RUVBL2 is essential for the proliferation of MRC5-SV cells. The result is consistent with the previous observation that silencing of RUVBL2 (and RUVBL1) impedes the proliferation of HeLa cells [29]. More recently it was shown that silencing of RUVBL2 blocks the progression of human hepatocellular carcinoma in xenografts without inducing significant apoptosis [30]. We also compared the fractions of viable and dead cells by using trypan blue dye exclusion at 3 or 4 days after transfection. The fraction of viable cells was 80–90% for mock-, siCONT- and siRNA-treated cells, showing no systematic differences between the treatments. Thus, the slow proliferation of the siRNA-treated cells was due to growth retardation *per se* and not the increase in the fraction of dead cells.

Sensitivity of RUVBL2-depleted cells to anticancer agents and X-rays

In order to obtain insight into the role of human RUVBL2 in protecting the genome, we analyzed the survival of RUVBL2-depleted MRC5-SV cells using colony formation assays after treatment with cisPt, azadC, MMC, and X-rays. Figure 4 shows the typical survival curves of knockdown cells when RUVBL2 was depleted by siRUVBL2-2. The

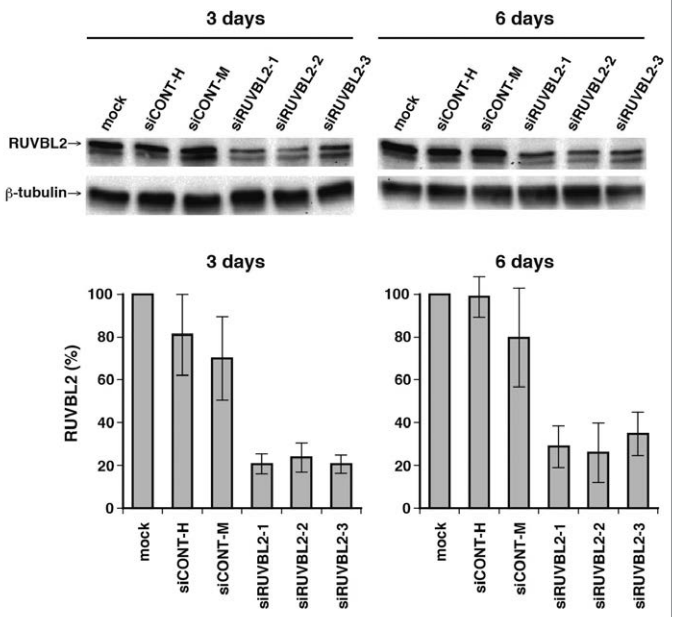

Figure 2: Depletion of RUVBL2 by siRNA. siRNAs for RUVBL2 (10 nM siRUVBL2-1, -2 and -3) or control siRNAs (10 nM siCONT-H and -M) were transfected into MRC5-SV cells, and the depletion of RUVBL2 was analysed by Western blotting at 3 and 6 days after transfection. Upper panels: typical Western blots for RUVBL2 and β-tubulin (loading control); Lower panels: depletion of RUVBL2 in siRNA-treated cells relative to mock-treated cells. Data points are means of 3 independent experiments with standard deviation.

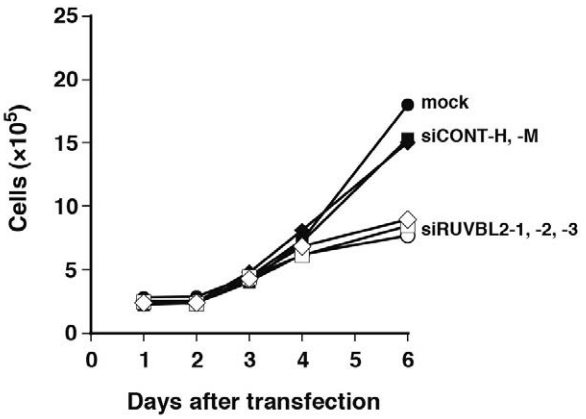

Figure 3: Inhibition of cell proliferation by the depletion of RUVBL2. siRNAs for RUVBL2 (10 nM siRUVBL2-1, -2 and -3, open symbols) or control siRNAs (10 nM siCONT-H and -M, closed square and diamond) were transfected into MRC5-SV cells and the number of cells was counted on the indicated days. The data points are the average from two experiments.

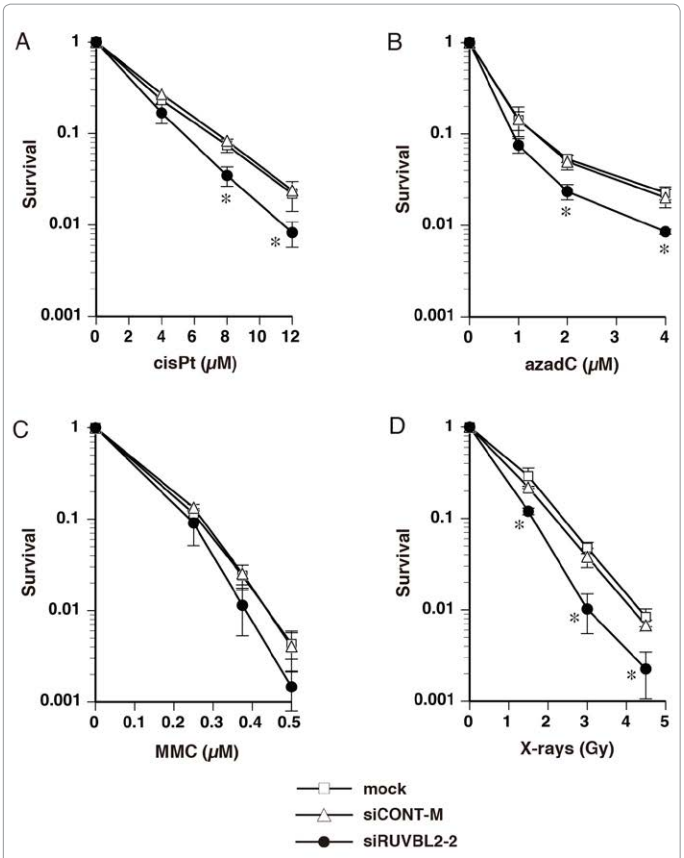

Figure 4: Sensitivity of RUVBL2-depleted cells to anticancer agents and X-rays. siRNA for RUVBL2 (10 nM siRUVBL2-2, closed circle) or control siRNA (10 nM siCONT-M, open triangle) was transfected into MRC5-SV cells and incubated as described in Materials and Methods. Two days after transfection, the cells were treated with cisPt (A), azadC (B), MMC (C), or X-rays (D). The treated cells were further incubated in fresh medium for 7–8 days, and colonies were scored to calculate the surviving fraction. Data points are means of 3 independent experiments with standard deviation. Statistically significant differences in survival (t-test, $p<0.05$) between siRUVBL2-2-treated and siCONT-M-treated cells are indicated by asterisks.

RUVBL2-depleted cells were moderately sensitive to cisPt, azadC, and X-rays (Figure 4A, B, and C). Conversely, the RUVBL2-depleted cells were not sensitive to MMC: the difference in the sensitivity between RUVBL2-depleted and control siRNA (siCONT-M)-treated cells was not statistically significant (Figure 4C). The survival of cells treated with other siRNAs (siRUVBL2-1 and siRUVBL2-3) was also analyzed using the same anticancer agents and X-rays: the results were similar to those obtained with siRUVBL2-2 (data not shown). It should be noted that we scored the colonies with more than 50 cells in the cell survival assays (Figure 4). In a parallel experiment, we examined the colonies with more than 20 cells and found that the cell survival was essentially the same as that obtained with colonies with more than 50 cells, indicating that the cell survival data were not affected by growth retardation by the depletion of RUVBL2.

Sensitivity of HR-deficient cells to anticancer agents and X-rays

RUVBLs play a role in recombination repair of DNA damage as described in introduction. To compare the impacts of depletion of RUVBL2 and HR on the sensitivity to anticancer agents and X-rays, we measured the survival of HR (RAD51D)-deficient CHO cells to cisPt, azadC, MMC, and X-rays. RAD51D-deficient cells exhibited more pronounced sensitivity to the anticancer agents and X-rays than those deficient in RUVBL2 (Figure 5). In contrast to the RUVBL2-depleted cells (Figure 3), the HR-deficient CHO cells showed only mild growth retardation as compared to wild type AA8 cells, which is consistent with the previous observation [24].

Discussion

In the present study we have shown that depletion of RUVBL2 renders human MRC5-SV cells moderately sensitive to chemotherapeutic anticancer agents including cisPt and azadC, and to X-rays (Figure 4). The present data thus expand our knowledge regarding the role of RUVBLs in cell survival after exposure to anticancer agents and X-rays. It has been demonstrated previously that depletion of RUVBL1 and RUVBL2 from human cells confers sensitivity to MMC [15] and UV [19,20], respectively. However, our data showed that depletion of RUVBL2 from MRC5-SV cells conferred no statistically significant sensitivity to MMC (Figure 4C).

It is well established that X-rays induce DSBs as the major lethal lesions. azadC is incorporated into DNA and covalently traps the reaction intermediate of DNA cytosine methyltransferase, giving rise to DNA-protein cross-links (DPCs) [31]. DPCs block DNA replication and transcription [32,33] and hence constitute the major lethal lesions upon treatment with azadC. cisPt is a bifunctional DNA-damaging

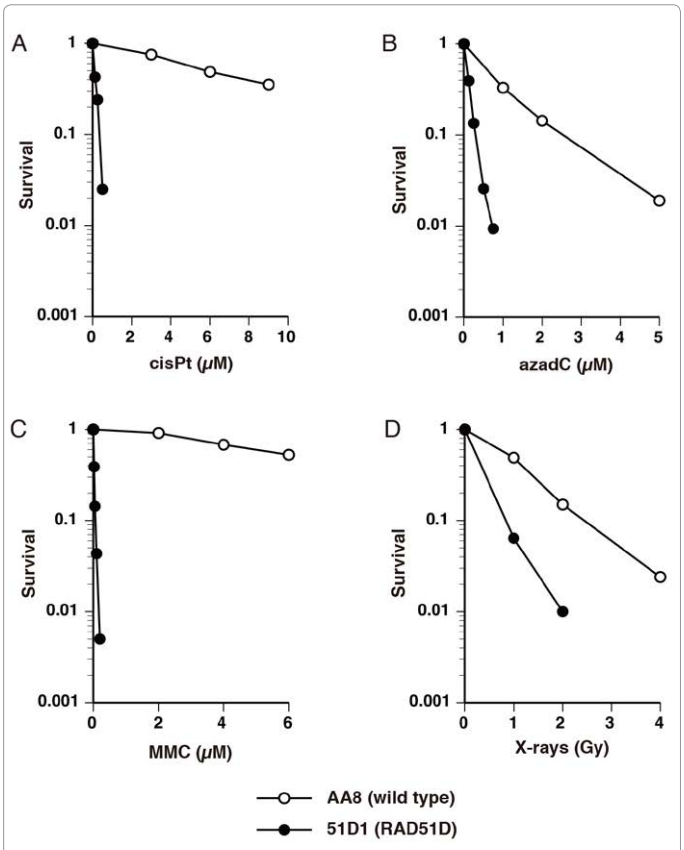

Figure 5: Sensitivity of HR-deficient CHO cells to anticancer agents and X-rays. AA8 (wild type, open circle) and 51D1 (deficient in RAD51D, closed circle) cells were treated with cisPt (A), azadC (B), MMC (C), or X-rays (D). The treated cells were further incubated in fresh medium for 6 days, and colonies were scored to calculate the surviving fraction. The data points are the average from two experiments.

agent and reacts with two guanine residues in the same DNA strand or two opposing DNA strands, yielding intrastrand cross-links and interstrand cross-links (ICLs) [34]. DPCs can also form when cisPt reacts with guanine and a proximal protein. ICLs inhibit the unwinding of duplex DNA upon DNA replication and hence can constitute the lethal lesions upon treatment with cisPt. Although the spectra of lethal lesions associated with anticancer agents used in this study differ considerably, DSBs are commonly involved as intermediates both in the reactivation of the replication fork stalled by DPCs [35] and the repair of ICLs [36], where DSB intermediates resulting from DPCs and ICLs are repaired by HR [35-37]. Thus, the sensitivity to cisPt, azadC, and X-rays could be increased if the depletion of RUVBL2 compromises the repair of DSB intermediates via HR. This mechanism is consistent with the observations made in mammalian cells that depletion of RUVBLs or inactivation of the TIP60 histone acetyltransferase containing RUVBLs impairs the recruitment of RAD51, the key recombinase in HR, to DSBs [15,16]. In addition, depletion of RUVBL2 may compromise the activity of the INO80 chromatin remodeling complex that is involved in the DNA end resection of DSBs [12]. In yeast, INO80 and SWR1 chromatin remodeling complexes contain the orthologs of RUVBLs and are suggested in chromatin remodeling in the vicinity of DSBs [38-40]. Functional inactivation of either yeast INO80 or SWR1 complex renders cells sensitive to DNA-damaging agents including methyl methanesulfonate, UV, ionizing radiation and hydroxyurea to varying extents [41-43]. It is therefore possible that the function of RUVBLs in the survival of cells exposed to DNA-damaging agents is at least partially conserved from yeast to human.

We noticed that RUVBL2-depleted MRC5-SV cells were less sensitive to anticancer agents and X-rays than were CHO cells deficient in HR (Figure 5). The residual RUVBL2 in the knockdown cells (Figure 2) may be sufficient to partially support the repair of DSB intermediates by HR. This could also be true for cell proliferation (Figure 3). Alternatively, RUVBL2 or complexes containing RUVBL2 such as TIP60 and INO80 may act as a promoting but not essential factor in repair. In addition, it has been reported that the human INO80 complex containing RUVBLs positively regulates the expression of RAD54B and XRCC3, which are involved in HR, and may contribute indirectly to DSB repair [44].

In summary, RUVBL2 is involved in the survival of human cells after exposure to a wide range of anticancer agents and X-rays, but further studies are necessary to clarify the exact role of RUVBLs in this process.

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The authors declare that there are no conflicts of interest.

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