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Resveratrol modulates DNA double-strand break repair pathways in an ATM/ATR–p53- and –Nbs1-dependent manner

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Resveratrol (RV) inhibits tumour initiation, promotion and progression which has mainly been explained by its properties in cell cycle control and apoptosis induction. So far, ambiguous observations have been published regarding its influence on genomic stability. To study RV's effects on DNA double-strand break (DSB) repair, we applied the established enhanced green fluorescent protein (EGFP)- and I-*Sce*I-based assay system on RV-treated lymphoblastoid cell lines (LCLs). We show that RV inhibits both, homologous recombination (HR) and non-homologous end joining (NHEJ) independently of its known growth and death regulatory functions. Using (i) the isogenic cell lines TK6 and WTK1, which differ in their p53 status, (ii) LCLs from patients with ataxia telangiectasia, (iii) shRNA-mediated p53 knockdown and (iv) chemical inhibition of ATM/ATR by caffeine, we established an ATM–p53-dependent pathway of HR inhibition by RV. Additional use of LCLs from Nijmegen breakage syndrome patients furthermore provided evidence for an ATM/ATR–Nbs1-dependent inhibition of microhomology-mediated NHEJ after RV treatment. We propose that activation of ATM and/or ATR is a central effect of RV. Repression of error-prone recombination subpathways could at least partially explain the chemopreventive effects of this natural plant constituent in animal cancer models.

Introduction

Over the past years, several natural food constituents have gained major attention worldwide due to their inhibitory activities on the outgrowth of tumour cells in culture and/or *in vivo* (1,2). One of the most promising substances in this regard is resveratrol (RV) (*trans*-3, 4′, 5-trihydroxystilbene), a polyphenol that is synthesized in a variety of plant species after challenges like fungal infection or ultra violet irradiation. The major dietary sources of RV are peanuts, berries, grapes and red wine (2–5). Antioxidant, anti-inflammatory, apoptosis-inducing/-sensitizing and cell cycle regulatory effects of RV were elucidated in diverse cellular systems with involvement of >30 molecular targets in multiple different cellular pathways (2–6). All these responses have been demonstrated to show differences with respect to dose, time of application and cell type. In part, even contradictory results were reported (reviewed in 5). Prominent, not yet completely understood mechanisms involve inhibition of AP-1 and NF-kappaB pathways, influence of intracellular reactive intermediates, downregulation of survivin and activation of p53 (5–7).

Due to its natural origin, RV is considered to be a safe and non-toxic chemopreventive agent (1,4), and it is now even available as an over-the-counter drug. However, seemingly contradictory findings were reported for RV's effects on genomic stability (reviewed in 8). Although RV has been found to act as an effective radical scavenger counteracting oxidative DNA damage and to reduce benzo[a]pyrene-induced carcinogenesis by CYP1A1 inhibition, corresponding to features of a chemopreventive agent (5), it has also been reported to induce micronuclei, mitotic chromosome displacement, chromosomal aberrations, sister-chromatid exchanges and increased homologous recombination (HR) (8,9).

To clarify RV's role in genomic stability—either preserving or possibly destructive—might have a fundamental impact on the further use of RV in chemopreventive or even therapeutic studies. In this work, we investigated for the first time the effect of RV on double-strand break (DSB) repair in various lymphoblastoid cell lines (LCLs) by use of our EGFP-based test system that enables us to study the different repair pathways (10). We provide evidence that RV at 5 and 30 μM directly influences DSB repair independently of its well-known functions in cell cycle regulation and apoptosis induction.

Abbreviations: AT, ataxia telangiectasia; DSB, double-strand break; EGFP, enhanced green fluorescent protein; LCLs, lymphoblastoid cell lines; MM-NHEJ, microhomology-mediated NHEJ; MRN, Mre11-Rad50-Nbs1; NBS, Nijmegen breakage syndrome; NHEJ, non-homologous end joining; PIKK, phosphatidylinositol 3-kinase-related kinase; p53pSer15, p53 phosphorylated on serine 15; RV, resveratrol; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

Materials and methods

Cell lines, cultivation and analysis of DNA content

LCLs were maintained in RPMI 1640 supplemented with 12% foetal calf serum, 3 mM L-glutamine (Biochrom, Berlin, Germany). TK6 and WTK1 were described previously (10) and HA226 was established from a Nijmegen breakage syndrome (NBS) patient who is homozygous for the 657del5 mutation in the *NBS1* gene (11). GM01526E and GM08436A were derived from ataxia telangiectasia (AT) patients and were homozygous for the *ATM* initiation codon mutation M1T or compound heterozygous for truncating mutations 4642delGATA and E1978X, respectively (Coriell Cell Repositories, Camden, NJ). GM02253F is mutated in the XPD gene but otherwise normal (Coriell Cell Repositories) and HA169 was derived from a healthy donor in whom A–T causing mutations were excluded. RV (Sigma, Deisenhofen, Germany), benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk; Bachem, Heidelberg, Germany) and LY294002 (Calbiochem, Darmstadt, Germany) were dissolved in dimethyl sulphoxide (DMSO) and stored at –20°C. Caffeine (Sigma) was freshly dissolved in H_2O prior to use. Cell cycle and apoptosis profiles were obtained by fluorescence activated cell sorting analysis on fixed- and propidium iodide-stained cells.

Recombination assay, p53 knockdown and drug treatment

To analyse different DSB repair pathways in LCLs, we utilized the fluorescence-based test system described in Akyüz *et al.*(10). The DSB repair plasmid substrates used in this study were Δ-EGFP/3′EGFP, Δ-EGFP/5′EGFP, 5′EGFP/HR-EGFP, EJ-EGFP (supplementary Figure 1 is available at *Carcinogenesis* Online). To introduce the repair substrate and the meganuclease expression plasmid (pCMV-I-SceI), LCLs were electroporated with a mixture containing pCMV-I-SceI, the respective recombination substrate, and pBS (control vector) or *wtEGFP* plasmid (for transfection efficiency), giving a total amount of 30–90 μg plasmid DNA. In p53 knockdown experiments, 10–30 μg of pSUPER or pSUPER-p53 were added. After electroporation cells were split into two aliquots and resuspended in fresh culture medium containing DMSO and RV as indicated, and zVAD-fmk (50 μM) in most of the experiments. RV/DMSO were added after a pre-incubation period of 3–6 h in knockdown experiments, and 1 h after caffeine or LY294002 pre-treatment. Bleomycin (50 mU/ml; Sigma) and etoposide (50 μM; Sigma) were added after a pre-incubation period of 4 h. LCLs were cultivated for approximately one generation time, i.e. 12–18 h (TK6), 14–18 h (WTK1) and 20–24 h (GM02253F, GM01526E, GM08436A, HA226), respectively. Subsequently, cells were subjected to flow cytometry by use of a FACS®Calibur FACSscan (Becton and Dickinson, Heidelberg, Germany) as detailed in Akyüz *et al.*(10). Recombination frequencies were calculated through correction for transfection efficiencies. The statistical significance of differences was determined by using Student's unpaired *t*-test.

Extraction of mammalian cells, western blot analysis and antibodies

Total cellular homogenates were prepared as described (10). For lysates, cells were incubated in freshly prepared lysis buffer [50 mM Tris, pH 7.4; 150 mM NaCl; 2 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid; 2 mM ethylenediaminetetraacetic acid; 25 mM NaF; 25 mM β-glycerophosphate; 0.1 mM NaV; proteinase inhibitor, Roche Diagnostics, Mannheim, Germany]. Extracts from 0.25 to 0.5 × 10^6 cells or 40 to 60 μg total protein were separated in a sodium dodecyl sulphate gel (4–15% acrylamide) and transferred to Immobilon-P membranes (Millipore, Bedford, MA) or nitrocellulose (Amersham Corporation, Amersham, UK). For immunodetection, the following primary antibodies were used: anti-p53 (Ab-6, DO-1; Calbiochem), anti-p21 (SX118; BD Pharmingen™, Heidelberg, Germany), anti-Mdm2 (SMP14; Santa Cruz Biotechnology, Santa Cruz, CA), anti-DNA-PKcs (Ab-4, Cocktail; NeoMarkers, Fremont, CA), anti-GAPDH (mAbcam 9484; Abcam, Hiddenhausen, Germany), anti-actin (Ab-1, JLA-20; Calbiochem) and anti-β-actin (AC-15; Sigma) were mouse monoclonal; anti-phospho-p53(Ser15), anti-phospho-chk1(Ser317) and anti-phospho-chk1(Ser345) (Cell Signalling Technology, Beverly, MA), anti-phospho-chk2(Thr68) (Calbiochem), anti-Nbs1 (NB 100-143G1; Novus Biologicals, Littleton, CO), anti-Nbs1 (pSer343) (NB 100-284; Novus Biologicals), anti-ATM (Ab-3; Calbiochem), anti-ATM protein kinase pSer1981 (Rockland Immunochemicals, Gilbertsville, PA), anti-Rad51 (H-92; Santa Cruz) were rabbit polyclonal; anti-actin (I-19, Santa Cruz) was goat polyclonal. Affinity-purified secondary antibodies were peroxidase-conjugated goat anti-mouse and anti-rabbit IgG (Pierce, Rockford, IL), anti-mouse IgM (Calbiochem) and peroxidase-conjugated donkey anti-goat IgG (Biomol, Plymouth Meeting, PA). Bands were visualized by SuperSignal® West Pico Chemiluminescent Substrate and West Dura Extended Duration Substrate (Pierce).

Protein expression levels were assessed by quantification of band intensities after normalizing with actin, or GAPDH signals. Densitometric quantification of band intensities for the different treatment conditions (± RV 30 μM, ± caffeine 2 mM) was performed using a ChemImager 5500 with software (Alpha Imunotech Corporation, San Leonardo, CA).

Results

RV inhibits HR in a manner depending on p53, but independently from cell cycle changes or apoptosis induction

To examine the influence of RV on DSB repair, we used the previously established, fluorescence-based test system (10). This assay relies on a series of plasmids comprising differently mutated *EGFP*genes, which after cleavage by the meganuclease I-*Sce*I will undergo HR or NHEJ. Restoration of functional EGFP is subsequently detected by FACS analysis (supplementary Figure 1 is available at *Carcinogenesis* Online).

Since possible links between RV and p53 had been considered previously (reviewed in 5), we chose TK6 and WTK1 cells from the same donor which harbour wild-type p53 and mutant p53(237I), respectively (10). After cotransfection of these cells with Δ-EGFP/3′EGFP and pCMV-I-SceI, HR in TK6 was reduced to 42% with 30 μM RV, when compared with DMSO-treated cells (Figure 1a, left panel; $P < 0.001$) and to 74% after treatment with 5 μM RV ($P > 0.05$). No significant changes in recombination frequencies, however, were noted in the p53 mutant cell line WTK1 for both RV concentrations used. For comparison, when TK6 cells were electroporated with Δ-EGFP/3′EGFP and pCMV-I-SceI and treated with the DNA-damaging agents bleomycin (50 mU/ml) or etoposide (50 μM), we rather saw a recombination increase than a decrease (data not shown).

RV has been demonstrated to provoke apoptosis and cell cycle changes in p53-dependent and independent ways (reviewed in 5), which might indirectly influence DSB repair. When we analysed the sub-G1 content under the conditions of recombination measurements, no significant difference in the percentage of sub-G1 cells was found after DMSO or RV treatment (not shown). Additionally, we carried out equivalent recombination experiments in the presence of the pan-caspase inhibitor zVAD-fmk (50 μM). Recombination results did not differ from the initial results (Figure 1a, right panel), thus arguing against an involvement of apoptosis in the RV-mediated inhibition of HR. Next, we performed cell cycle analysis under the conditions of repair measurements. Whereas minor differences between TK6 and WTK1 cells were noticed without zVAD-fmk (Figure 1b, upper panels), cell cycle distributions were equivalent upon zVAD-fmk treatment in both cell lines with DMSO and RV (Figure 1b, lower panels). Thus, cell cycle changes are unlikely to explain the observed differences in DSB repair by HR after RV treatment (Figure 1a). Consistently, under the conditions of DSB repair measurements, RV-treated and -untreated TK6 cells had fairly similar p53 levels without p21 induction (Figure 1c).

We wished to confirm these observations in a second experimental system and, therefore, applied our assay system to GM02253F LCLs, another cell line expressing functional p53. As expected from our results with TK6 cells, we saw a downregulation of HR by 30 μM RV ($P = 0.034$) also in GM02253F cells (Figure 1d). To determine, if this RV-mediated effect was p53 dependent, we suppressed endogenous p53 expression by use of vector pSUPER-p53 for the production of specific siRNA (Figure 1e). Densitometric quantification of band intensities after immunoblotting revealed a downregulation to 38%, when corrected for GAPDH signal intensities, in the pSUPER-p53 transfected cells when using DO1 antibody and to undetectable levels when testing for p53 phosphorylated on serine 15 (p53pSer15). When using antibodies directed against the products of the p53 target genes *hdm2* and *p21*, decreased expression was seen after pSUPER-p53 transfection as expected. RV treatment did not induce transcriptional transactivation of these target genes, which was compatible with the absence of a G1 cell cycle arrest induction [G1 content: 67% ± 0.1% (pSUPER/DMSO); 59% ± 1.0% (pSUPER/RV); 63% ± 0.2% (pSUPER-p53/DMSO); 60% ± 0.4% (pSUPER-p53/RV)].

In cells simultaneously transfected with Δ-EGFP/3′EGFP, pCMV-I-SceI and pSUPER-p53, recombination inhibition by RV was lost (Figure 1d). Overall, pSUPER-p53-transfected cells displayed an increase in HR compared with the pSUPER-transfected counterparts, particularly after treatment (Figure 1d; DMSO: $P = 0.062$, RV: $P = 0.001$), which is consistent with the previously established role of p53 in downregulating HR (12–14). Loss of RV-mediated recombination inhibition after p53 downregulation supported our conclusions from the results obtained with the TK6/WTK1 system, suggesting that RV inhibits HR in LCLs in a manner depending on p53. In further support of this interpretation, we measured HR frequency reduction down to 80% ($n = 3$, $P = 0.015$) upon treatment with 30 μM RV even in WTK1, mutant p53 cells, when these cells were transfected with pCMV–wild-type p53 plasmid (10) for wild-type p53 expression.

HR inhibition after RV treatment is ATM dependent

We next sought to determine whether p53 could be the immediate target of RV or whether RV could act via modulation of targets upstream of p53 in the DSB repair cascade. The preceding experiments had indicated no gross changes in p53 levels after RV treatment of GM02253F LCL (Figure 1e, left panel). However, when we specifically analysed the fraction of p53pSer15, cell homogenates from RV-treated cells showed a marked increase in p53pSer15 (Figure 1e, right panel).

p53pSer15 is known to occur after genomic insults by the ATM kinase (15) that is recruited by Nbs1 as part of the Mre11-Rad50-Nbs1 (MRN) complex that represents another phosphorylation target of ATM and modulates its activity as part of a positive feedback loop (16). To understand whether ATM signalling plays a role in HR suppression after RV treatment, we chose the AT-cell lines GM01526E and GM08436A and the NBS-cell line HA226 for further experiments. Deficiency of ATM and Nbs1 proteins, respectively, was verified by western blot analysis of untreated cell lysates, whereas DNA-PK_{cs} and p53 were detected in all LCLs (Figure 2a). In contrast to HR frequency reduction by RV at concentrations of 5 and 30 μM in the control cell line (P not significant with 5 μM RV; $P = 0.044$ for 30 μM RV), ATM-deficient cell lines did not show reduced HR frequencies after RV treatment. Rather, we noticed a recombination increase for both RV concentrations used. The NBS-cell line HA226 displayed an intermediate pattern with only a slight, non-significant decrease after treatment with 30 μM RV (Figure 2b).

To further delineate the mechanisms involved in DSB repair regulation by RV in these cell lines, we analysed phosphorylation events within the ATM signalling cascade. For that purpose, we performed immunoblotting with phosphospecific antibodies using cell

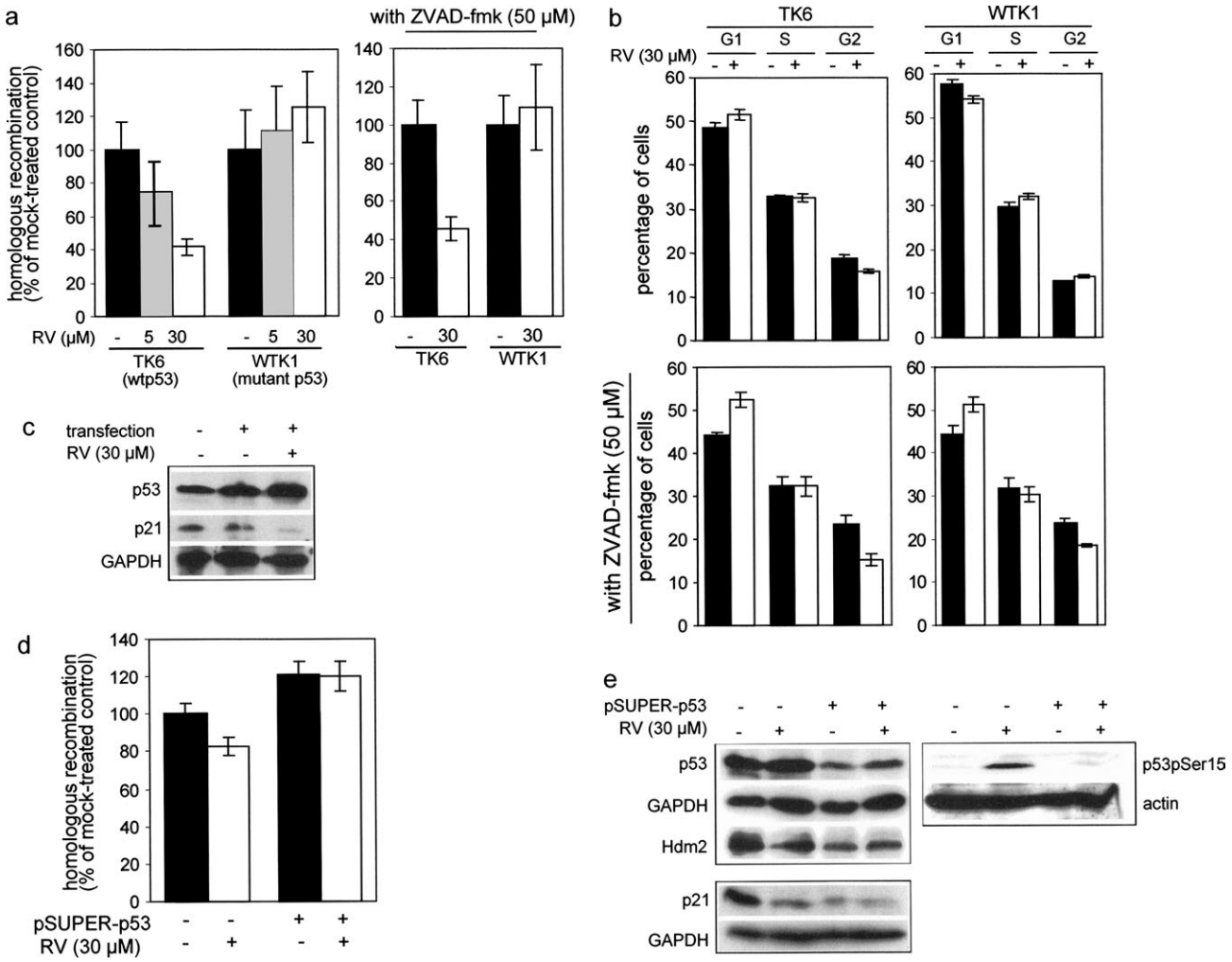

Fig. 1. Effect of RV on HR and cell cycle distribution in LCLs differing in the p53 status. (**a**) HR after RV treatment in TK6 (wild-type p53) and WTK1 cells (mutant p53). Cells were coelectroporated with Δ-EGFP/3′EGFP and pCMV-I-SceI and further cultivated with DMSO (−) or RV for 12–18 h. In a separate set of measurements, 50 μM zVAD-fmk was added (right panel). Recombination frequencies were normalized to 100% for the mean value of DMSO-treated cells (absolute mean values with zVAD-fmk: 0.29% for TK6 and 0.60% for WTK1). Columns, mean values of 4–6 (5 μM RV) and 6–21 (30 μM RV) measurements, respectively; bars, SEM. (**b**) Cell cycle distribution in TK6 and WTK1 cells. Cells were treated as in (a), propidium iodide-stained and subdivided into populations in the cell cycle phases G1, S and G2 (%). Columns, mean values of 3–6 measurements; bars, SD. (**c**) Quantities of p53 and p21. TK6 cells were DMSO-treated without transfection or processed as in (a); total homogenates were prepared and immunoblotted utilizing antibodies against total p53, p21 and GAPDH. (**d**) Recombination inhibition by RV is abolished after p53-specific RNA interference. GM02253F cells were coelectroporated with Δ-EGFP/3′EGFP, pCMV-I-SceI and pSUPER (−) or pSUPER-p53 (+) before DMSO or RV treatment. The mean recombination frequency of DMSO-treated cells transfected with pSUPER was set as 100%. Columns, mean values of 12 measurements each; bars, SEM. (**e**) Downregulation of p53 and influence on target gene products. GM02253F cells were processed as in (d), total homogenates prepared and immunoblotted utilizing antibodies against total p53, p53pSer15, p21 and Hdm2. GAPDH or actin immunostaining served as loading controls. Transfection efficiencies were ∼50%.

homogenates from DMSO- and RV-treated cells, which were harvested concomitantly with the samples for recombination measurements (Figure 2c). After treatment with 30 μM RV, the control line showed an average 5-fold increase of p53pSer15 ($n = 6$), whereas total p53 protein expression did not change markedly. For comparison, p53pSer15 was less prominent in the AT and NBS lines. Nbs1 phosphorylation (Nbs1pSer343) was also diminished in AT cells after RV treatment compared with the control line. Furthermore, ATMpSer1981 autophosphorylation was induced by RV treatment at 30 μM in control and NBS cells. Altogether, these results indicated that an ATM–p53 signalling pathway is induced by RV and is critically involved in the inhibition of HR that was observed in control cells but not in two different AT cell lines.

RV inhibits conservative HR in a manner depending on ATM and/or ATR

To discriminate between different HR pathways with respect to the inhibitory effect of RV, we applied additional plasmid variants of the fluorescence-based assay system, which were designed to measure conservative (Δ-EGFP/5′EGFP) or non-conservative (5′EGFP/HR-EGFP) HR, exclusively (supplementary Figure 1 is available at *Carcinogenesis* Online). In TK6, we found a significant decrease in conservative HR frequencies down to 58% after treatment with 30 μM RV (Figure 3a; $P = 0.032$), whereas no change in non-conservative HR was noticed. These data demonstrate a preferential suppression of conservative HR, i.e. suppression of the strictly Rad51-dependent pathway by RV in these cells. According to a recent report, p53 may exert part of its inhibitory function in HR by transcriptional repression of Rad51 (17). Consequently, we performed western blot analysis with TK6 cell homogenates that were obtained parallel to repair measurements. As shown in Figure 3b, Rad51 protein levels were increased after RV treatment, arguing against transcriptional repression by p53 as the mechanism underlying inhibition of conservative HR.

To test whether RV-mediated inhibition of conservative HR requires ATM or ATR, we next coelectroporated TK6 cells with

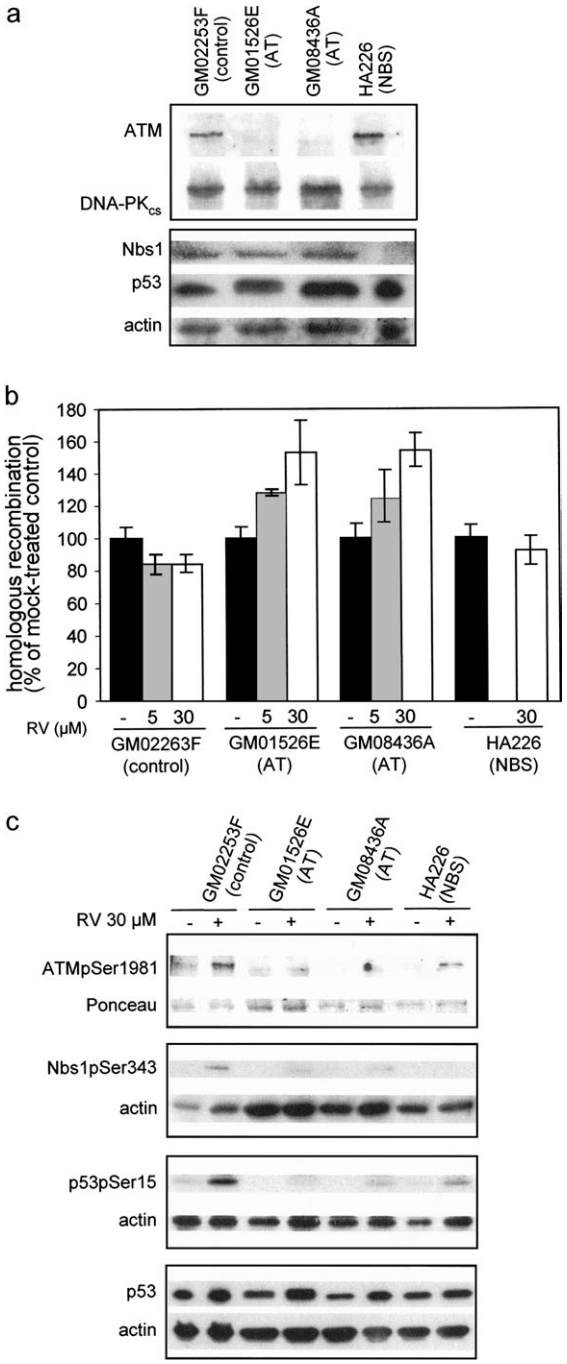

Fig. 2. RV-mediated recombination repression is lost in ATM-deficient cells. (**a**) Western blot analysis. Immunoblotting was performed using protein lysates from LCLs as indicated utilizing antibodies directed against ATM, Nbs1 and p53. DNA-PK_{cs} and actin served as loading controls. (**b**) HR in AT and NBS cells after RV treatment. Cells (AT: GM01526E, GM08436A; NBS: HA226; control: GM02253F) were coelectroporated with Δ-EGFP/3′EGFP and pCMV-I-SceI, followed by treatment with DMSO (−) or RV. The mean recombination frequency for DMSO-treated samples was defined as 100%. Columns, mean values of 3–5 measurements (5 μM) and 6–11 measurements (30 μM); bars, SEM. (**c**) Phosphoprotein-specific immunodetection of ATM, Nbs1 and p53 in AT and NBS cells treated with RV. LCLs were processed as in (b). Total homogenates were subjected to immunoblotting for p53pSer15, Nbs1pSer343 and ATMpSer1981 and total p53. Equal loading was verified by Ponceau or actin immunostaining.

Δ-EGFP/5′EGFP and pCMV-I-SceI and treated the cells with the ATM/ATR inhibitor caffeine (18). H_2O pre-treated samples again showed inhibition of conservative HR after treatment with 30 μM RV. Pre-treatment with caffeine led to an overall reduction of conservative HR as was observed by others (19,20), without further downregulation by RV (Figure 3c). Western blot analysis showed that RV could also induce p53 phosphorylation in TK6 cells with a relative decrease in p53pSer15 levels after caffeine treatment (Figure 3d). These observations provided supportive evidence for an ATM/ATR and p53-dependent mechanism of recombination inhibition by RV. The fact that repression of HR can be mediated through both the ATM/ATR inhibitor caffeine and the activator RV could be explained by caffeine and RV targeting distinct ATM/ATR functions, namely, in the activation of the enzymatic machinery and the fidelity control of HR, respectively (19–23).

RV inhibits NHEJ

Several studies have indicated a negative regulatory role for p53 also in NHEJ, particularly in the mutagenic microhomology-mediated NHEJ (MM-NHEJ) pathway (13,14). Given that RV regulated HR in a p53-dependent manner, we next wished to clarify whether RV also influences MM-NHEJ. To this end, we applied the EJ-EGFP plasmid with microhomologies of 5 bp flanking the I-*Sce*I recognition sequence such that NHEJ reconstitutes the wildtype *EGFP* gene after targeted cleavage (supplemental Figure 1 is available at *Carcinogenesis* Online; 10). Furthermore, to distinguish between p53-dependent and p53-independent NHEJ regulation, we again utilized the isogenic cell pair TK6 and WTK1. Unexpectedly, after electroporation with EJ-EGFP and pCMV-I-SceI, we observed a strong, highly significant inhibition of MM-NHEJ for 30 μM RV in both cell lines (suppression to 37% in TK6 and 41% in WTK1; $P < 0.001$ for both). When using 5 μM of RV, NHEJ was suppressed to 77% in TK6 ($P = 0.035$), whereas WTK1 cells displayed no detectable NHEJ decrease (Figure 4a). In GM02253F cells, RV at 30 μM caused NHEJ inhibition to an extent comparable with that in TK6/WTK1 cells. After downregulation of p53 by pSUPER-p53 NHEJ increased in both, RV- and DMSO-treated samples (DMSO: $P < 0.001$; RV: $P = 0.020$) and, within this system, the relative NHEJ inhibition was similar after treatment with 30 μM RV (36% for pSUPER, $P < 0.001$; 46% for pSUPER-p53, $P < 0.001$) (Figure 4b). These data suggest that p53 might not play a role in RV-induced NHEJ inhibition in LCLs.

Functional ATM/ATR and Nbs1 are required for the inhibition of NHEJ by RV

So far, our results implied an effect of RV on ATM/ATR signalling to p53 in the context of HR, whereas in the context of NHEJ RV appeared to exert a p53-independent regulatory role. To examine whether RV-mediated inhibition of NHEJ depends on ATM, we analysed NHEJ in AT versus control cells after DMSO- versus RV-treatment (Figure 5a). Treatment with 30 μM RV led to a highly significant NHEJ inhibition in all cell lines down to 41% (GM08436A) and 43% (GM01526E) versus 35% (control), respectively ($P < 0.001$). Even with 5 μM of RV, inhibition could be seen to a lesser, though not significant degree. These data indicated that ATM alone might not be the mediator of the observed NHEJ inhibition by RV.

The phosphatidylinositol 3-kinase-related kinase (PIKK) ATR displays a substrate spectrum very similar to ATM and may compensate for loss of ATM function (24,25). Therefore, we decided to pre-treat the cells with the ATM and ATR inhibitor caffeine for further NHEJ measurements. Intriguingly, after the addition of caffeine, only minor differences between RV-treated (30 μM) and DMSO-treated samples could be detected ($P > 0.050$) in GM02253F, GM08436A and GM01526E cells. To further demonstrate that this effect cannot only be seen in cells, in which caffeine treatment alone entails a strong reduction of NHEJ frequencies (down to 35–55%; see Figure 5b, left panel), we also analysed TK6 cells with a less pronounced influence of caffeine on NHEJ (reduction to 74%, Figure 5b, middle panel). However, in caffeine-treated TK6 cells, RV was again much less effective in repressing NHEJ (relative downregulation by RV to 76% with caffeine versus to 42% in the control). To inhibit the activity of DNA-PK_{cs}, another PIKK with well-established role in NHEJ, we utilized LY294002 (26). LY294002 pre-treated GM02253F cells

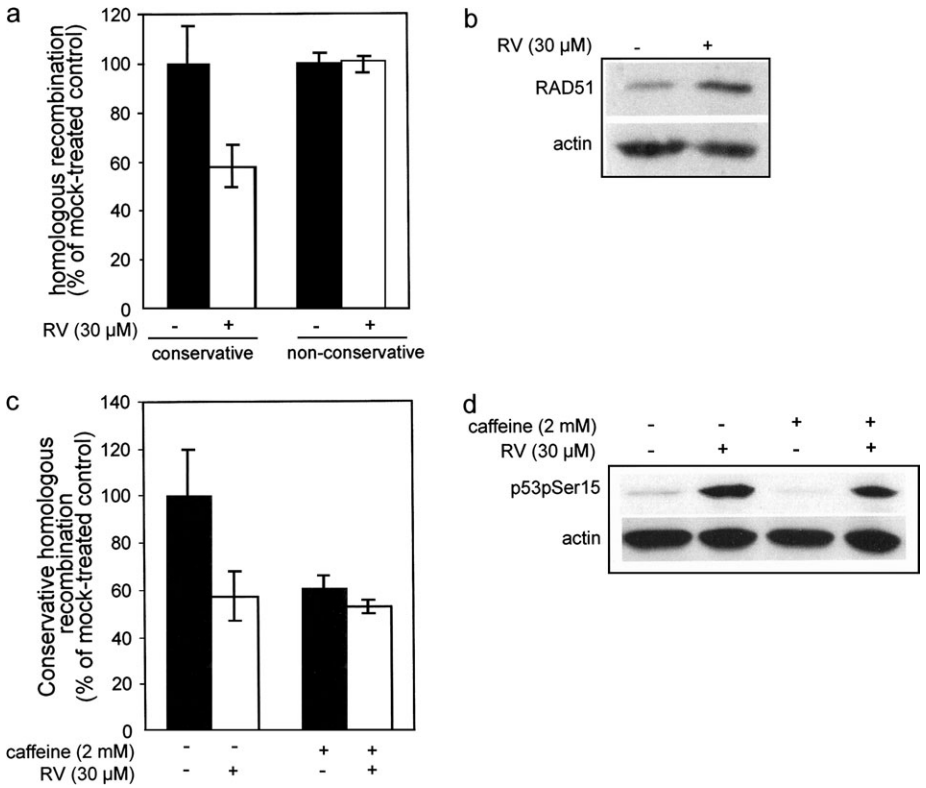

Fig. 3. Effect of RV on conservative versus non-conservative HR. (**a**) Conservative versus non-conservative HR after RV treatment. TK6 cells were coelectroporated with Δ-EGFP/5′EGFP or 5′EGFP/HR-EGFP and pCMV-I-SceI to determine conservative and non-conservative HR, respectively, and cells treated with DMSO (−) or 30 μM RV (+). The mean recombination frequency of DMSO-treated samples was defined as 100%. Columns, mean values of 6–15 measurements each; bars, SEM. (**b**) Immunoblot analysis for Rad51. TK6 cells, treated as in (a), were subjected to western blotting with Rad51 antibody and reprobed with actin antibody. (**c**) Loss of RV-mediated inhibition of conservative HR after treatment with caffeine. After coelectroporation with Δ-EGFP/5′EGFP and pCMV-I-SceI, TK6 cells were treated with 2 mM caffeine (+) or H_20 (−) before DMSO (−) or RV (+) addition. DMSO/H_2O-treated samples (−/−) were set as 100%. Columns, mean values of six measurements; bars, SEM. (**d**) Immunodetection of p53pSer15. Western blot analysis was performed with TK6 cells treated as in (c). Actin: loading control.

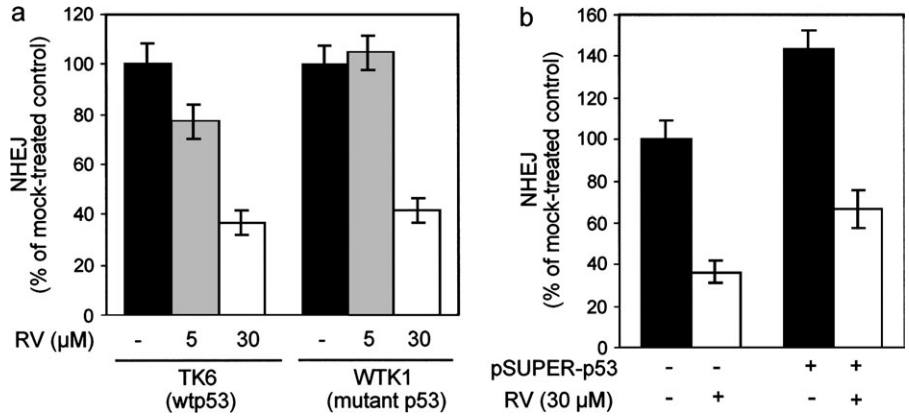

Fig. 4. Effect of RV on MM-NHEJ in cells differing in the p53 status. (**a**) NHEJ in TK6 and WTK1. Cells were transfected with EJ-EGFP and pCMV-I-SceI and treated with DMSO (−) or RV as indicated. The NHEJ frequency for DMSO-treated control samples was set as 100%. Columns, mean of 9–30 measurements; bars, SEM. (**b**) NHEJ after p53 knockdown. GM02253F cells were electroporated with EJ-EGFP, pCMV-I-SceI and pSUPER (−) or pSUPER-p53 (+) followed by incubation with DMSO (−) or 30 μM RV (+). The mean NHEJ frequency of cells transfected with pSUPER without RV treatment (−/−) was set as 100%. Columns, mean values of 9 measurements; bars, SEM.

(50 μM) displayed a marked inhibition of NHEJ, which was comparable with that of caffeine-treated samples. However, in contrast to caffeine pre-treatment, RV-mediated NHEJ suppression was maintained after LY294002 pre-treatment, resulting in an equivalent relative downregulation compared with mock pre-treated samples (42% versus 37%; $P = 0.002$ for both) (Figure 5b, right panel). Interestingly, both effects were additive resulting in the most pronounced reduction of NHEJ in the doubly treated cells. These findings confirmed the role of DNA-PK in the investigated NHEJ assay and excluded that DNA-PK_{cs} is directly implicated in NHEJ inhibition by RV.

To verify kinase inhibition by caffeine, we performed western blots with phosphospecific antibodies (Figure 5c). p53pSer15 in RV-treated

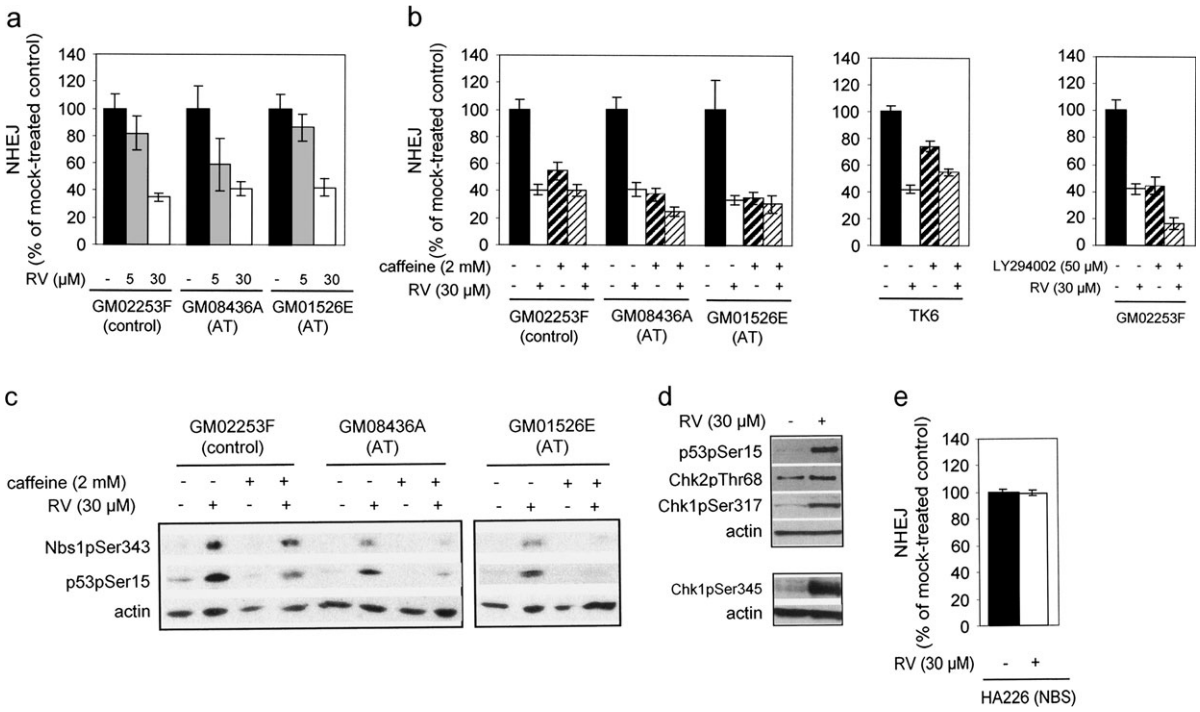

Fig. 5. Role of ATM, ATR, DNA-PK$_{cs}$ and Nbs1 in RV-mediated NHEJ inhibition. (**a**) RV-mediated NHEJ inhibition is maintained in ATM-deficient cells. AT (GM08436A, GM01526E) and control cells (GM02253F) were electroporated with EJ-EGFP and pCMV-I-SceI and treated with DMSO (−) or RV as indicated. The mean value for DMSO-treated samples was set as 100%. Columns, mean values of 6–18 measurements; bars, SEM. (**b**) RV-mediated NHEJ inhibition is lost after caffeine but not LY294002 treatment. GM02253F, GM01526E, GM08436A and TK6 cells were transfected as in (a). Following pre-treatment of the cells with caffeine (2 mM), LY294002 (50 μM) or mock, DMSO (−) or 30 μM RV (+) were added. Mean values from mock-treated cells (−/−) were set as 100%. Columns, mean values of 6–9 measurements; bars, SEM. (**c**) Phosphoprotein-specific immunodetection after caffeine treatment. GM02253F, GM08436A and GM01526E cells were processed as in (b) and immunoblotting performed for p53pSer15 and Nbs1pSer343. (**d**) Phosphoprotein-specific immunodetection of ATM and ATR target sites. HA169 control cells were treated with DMSO (−) or 30 μM RV (+). Lysates were immunoblotted using antibodies against p53pSer15, Chk2pThr68, Chk1pSer317 and Chk1pSer345, followed by reprobing with anti-β-actin antibody. (**e**) RV-mediated NHEJ inhibition requires Nbs1. HA226 cells, defective in Nbs1, were transfected with EJ-EGFP and pCMV-I-SceI and subjected to treatment with DMSO (−) or 30 μM RV (+). The mean value from DMSO-treated samples was set at 100%. Columns, mean values of 6 measurements; bars, SEM.

samples was diminished after caffeine treatment to some extent in control cells (down to 50–70%) and more pronounced in AT cells (down to 20–30%). Although Nbs1pSer343 phosphorylation after RV treatment was almost unaltered by caffeine coincubation in control cells, in AT cells a significant decrease of Nbs1 phosphorylation was also noticed after caffeine pre-treatment. To further delineate the effect of RV on ATM/ATR signalling, we checked phosphorylation of the more specific ATM and ATR target sites Chk2pThr68 and Chk1pSer317/Chk1pS345, respectively (27,28). Confirming the involvement of ATM and ATR, RV induced phosphorylation of Chk2 on threonine 68 and to an even greater extent of Chk1 on serine 317 and serine 345 (Figure 5d).

We finally measured NHEJ in the NBS cell line HA226. In contrast to all previously analysed cell lines, the NBS cells did not show suppression of NHEJ after treatment with 30 μM RV (Figure 5e). This lack of NHEJ suppression by RV was not due to inactive ATM since immunoblotting had revealed that ATM autophosphorylation still occurred in HA226 cells after RV treatment (Figure 2c). These data place Nbs1 at the centre of the RV-mediated NHEJ suppression pathway, possibly as a downstream effector of ATM/ATR.

Discussion

RV modulates DSB repair independently of its activities in growth and death control

In the present work, we analysed whether RV plays a role in DSB repair, an essential process in mammalian cells, which upon deregulation can cause cancerogenic genome mutations. We demonstrate for the first time that RV exerts specific effects on the DSB repair machinery, which are unrelated to previously identified activities in growth and death control. After RV treatment, HR is inhibited in LCLs in a manner depending on ATM and p53, whereas MM-NHEJ turned out to be suppressed in a p53-independent, but ATM/ATR- and Nbs1-dependent manner. Importantly, due to the fast readout of the applied EGFP-based repair assay, measurements could be performed as early as 12 h after the start of RV treatment, i.e. before the manifestation of growth-related changes. Moreover, through the addition of zVAD-fmk, possible influences of RV on apoptosis were minimized. These well-known RV effects on cell cycle and apoptosis could explain some of the contradictory results obtained with regard to genomic stability in previous studies (9).

Because we applied targeted introduction of DSBs, we can exclude that the newly discovered influence on repair was indirectly caused by alternative effects of RV that modulate the formation of potentially recombinogenic DNA lesions within the repair substrate, such as through its anti-oxidative activities (29). RV has also been described to inhibit polymerases α and δ, which might affect recombinative repair through generation of stalled replication forks (30). However, since we intentionally chose to monitor DSB repair in non-replicating plasmid substrates, we can rule out effects related to altered DNA synthesis in the repair substrate. Rather, the mutant cell and chemical inhibition data presented in this work suggest that the inhibitory activities of RV on DSB repair depend on distinct repair factors like p53, ATM/ATR or Nbs1.

The PIKKs ATM and ATR are main players in DSB repair regulation after RV treatment

We propose that the newly established modulatory role of RV in DSB repair is linked to the activation of the PIKKs ATM and ATR by RV followed by the activation of downstream targets like p53 or Nbs1. This model is supported by several lines of evidence: first, our western

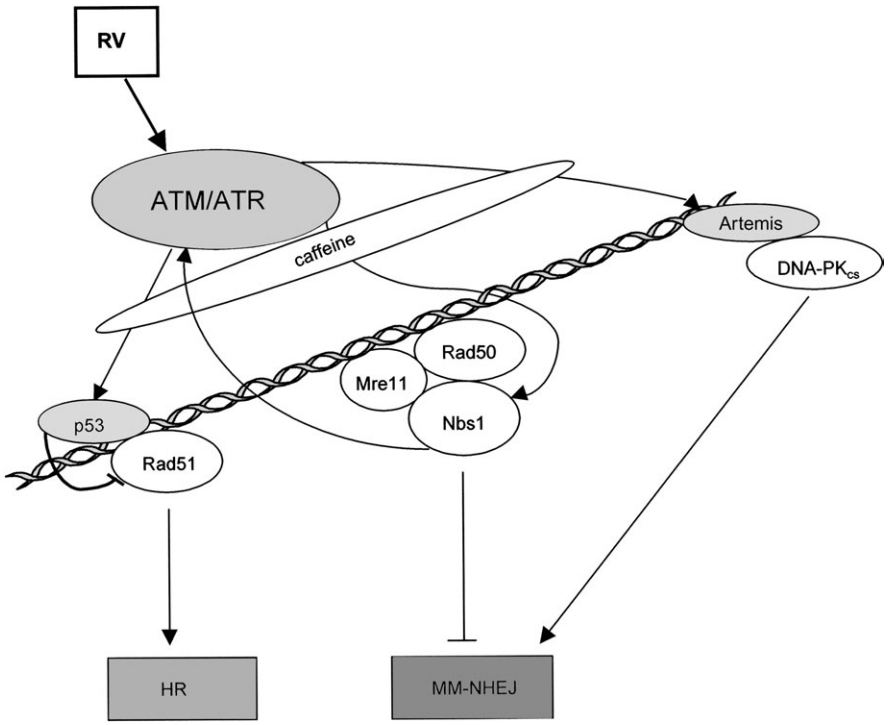

Fig. 6. Model of RV-induced mechanisms leading to inhibition of DSB repair. RV activates ATM and/or ATR via an unknown mechanism. ATM/ATR-mediated phosphorylation of downstream targets including p53 and Nbs1 takes part in DNA repair regulation (including both HR and NHEJ). p53 inhibits HR via inhibition of Rad51 activity (12–14); Nbs1 represses MM-NHEJ possibly via the MRN complex or activation of alternative surveillance proteins that favour HR (40). Activated Artemis stimulates MM-NHEJ activity in concert with DNA-PK_{cs} (31). Caffeine inhibits ATM/ATR downstream activities. Arrow, stimulation; bar, inhibition.

blot analyses clearly demonstrated RV-induced phosphorylation of p53 and Nbs1 at ATM/ATR target sites as well as Chk2 at ATM and Chk1 at ATR target sites. Second, the p53-dependent HR inhibition after RV treatment was not detected in AT cell lines, and the inhibitory effect of RV was masked through treatment with the ATM and ATR inhibitor caffeine. Third, we further discovered that MM-NHEJ suppression after RV treatment is caffeine-sensitive though largely ATM-and p53-independent. These data argue for a compensatory mechanism that involves ATR as was suggested by others (24,25). An employment of ATR as a backup kinase is consistent with the rapid drop of p53pSer15 and Nbs1pSer343 levels after caffeine treatment of AT cells. On the other hand, DNA-PK_{cs}, as one of the players in core NHEJ and of a subpathway requiring end processing by Artemis (31), is not probably to be involved in the NHEJ subpathway suppressed by RV, since treatment of LCLs with LY294002 did not affect the inhibitory effect of RV on NHEJ (Figure 6).

Reminiscent of the model proposed here for RV as an activator of the PIKKs ATM and ATR, several other natural food constituents with postulated chemopreventive potential, among them luteolin, kaempferol, apigenin, quercetin and genistein, have been demonstrated to lead to activation of ATM and/or ATR (32,33). Further evidence for the involvement of ATM/ATR in RV-induced cellular pathways very recently came from studies using different ovarian cancer cell lines. Thus, it could be demonstrated that RV induces S-phase arrest via activation of an ATM/ATR–Chk1/Chk2–Cdc25C pathway that was inhibited after caffeine treatment (34). In line with this work, we here have defined additional pathways triggered by RV and firstly show that activation of ATM/ATR by RV has implications for the fine tuning of DSB repair.

Nbs1, a component of ATM/ATR signalling is essential for MM-NHEJ suppression after RV treatment

The MRN complex consists of the proteins Mre11, Rad50 and Nbs1. Whereas Mre11 and Rad50 have roles in the tethering and trimming of DNA ends, the regulatory component Nbs1 is important for the activation of ATM by DSBs and is itself a substrate of the ATM kinase leading to an amplification loop for ATM activation (16). More recent studies also demonstrated the requirement of a functional MRN complex for ATR kinase activation (35,36). In the study presented here, we have shown that Nbs1 is phosphorylated on Ser343 after RV treatment in ATM proficient and to a lesser extent in deficient cell lines, suggesting that ATM and ATR both function as Nbs1 kinases that are activated by RV.

In budding yeast, *mre11* and *rad50* mutant strains are deficient in HR and MM-NHEJ (37). In vertebrates, an important role of Nbs1 in HR via MRN complex-mediated DNA strand resection is generally agreed upon (38–41); however, data on the relevance of Nbs1 in NHEJ are contradictory. Nbs1 does not appear to be essential for NHEJ in chicken DT40 cells (38). Conversely, Nbs1 was documented to be necessary for fully efficient NHEJ via use of fibroblasts from NBS patients (39) and, after ionizing irradiation, also for a NHEJ subpathway that has been shown to be ATM and DNA-PK_{cs}/Artemis dependent (31). Applying the Cre–loxP technology to mouse embryonic stem cells and fibroblasts, Yang *et al.* (40) demonstrated that Nbs1 represses NHEJ associated with sequence deletion events. Interestingly, we discovered that inhibition of imprecise MM-NHEJ by RV similarly requires functional Nbs1 (Figure 6). It is worth noting, however, that although caffeine pretreatment masked the suppressive effect of RV on MM-NHEJ in all cell lines, phosphorylation of Nbs1 after RV treatment was markedly caffeine sensitive only in AT cells. It should be noted that ATM/ATR inhibition might additionally abolish activation of other signalling molecules in the Nbs1 pathway. If Nbs1 alone or together with effectors like BRCA1 or BLM conveys the RV-mediated NHEJ suppression remains to be established.

The tumoursuppressor p53 is the main factor involved in RV-induced and ATM-mediated suppression of HR

p53 plays a central role in the maintenance of genome stability through transcriptional, cell cycle and pro-apoptotic activities and, independently from these functions, through fidelity control of DSB repair. Previous cell-based recombination and protein interaction

studies provided strong evidence for a role of p53 in the earliest steps of Rad51-dependent HR, thereby targeting inaccurate events involving divergent sequences (12–14). p53 was identified as one of the main players in RV-mediated effects with respect to apoptosis induction and cell cycle regulation in different cellular systems. However, p53-independent settings were established as well (4–6). In this study, we provide evidence for a critical role of p53 in mediating suppression of HR after RV treatment (Figure 6). Our results are reconcilable with reports on the induction of sister-chromatid exchanges and micronuclei formation after RV treatment that at first sight seemed contradictory, because all cell lines used in those studies carry dysfunctional p53 (8,9).

RV treatment led to p53 phosphorylation on serine15 which is of interest with respect to previous reports on colocalization of p53 with ATM and p53pSer15 with Rad51, Rad54, the MRN complex and BLM at DSBs and stalled replication forks, respectively (13,14,42). The observed association with central HR components suggested that p53pSer15 controls regulatory functions of p53 in HR. The fact that p53pSer15 by RV neither led to significant p53 accumulation nor led to transcriptional transactivation of the target genes *p21* or *hdm2*, strengthens the idea that p53pSer15 represents a p53 subpopulation involved in the regulation of recombination events in response to RV. In agreement with this concept, others also failed to see an increase in transcriptional transactivation concomitantly with p53pSer15 accumulation in S-phase synchronized cells or in RV-treated vascular endothelial smooth muscle cells (14,43). Notably, RV treatment in AT lines caused a recombination increase rather than decrease and was accompanied by elevated p53 levels (Figures 2b and c), which is compatible with our most recent findings indicating that inhibition of HR by RV is mediated by p53pSer15, whereas facilitation of HR via topoisomerase I is mediated by p53 independently of serine 15 phosphorylation (44, A.Restle, C.Müller-Tidow, K.H.Scheidtmann, L.Wiesmüller in preparation).

Activation of ATM/ATR with respect to DSB repair surveillance may contribute to RV's chemopreventive and chemotherapeutic properties

Having established that RV mediates DSB repair inhibition via activation of ATM and/or ATR, the main question is how RV achieves this activation in molecular terms. One conceivable mechanism would be that RV primarily introduces DSBs into DNA that are sensed by MRN and ATM and consecutively initiate a DNA damage response. However, this explanation is not compatible with published Comet assay data, which demonstrate that doses up to 100 μM of RV alone do not lead to an increased tail moment and rather indicate a preventive effect of RV (45,46). Moreover, when we treated TK6 cells with the radiomimetic drug bleomycin and the topoisomerase inhibitor etoposide, we did not see DSB repair inhibition as noticed for RV. Therefore, introduction of DSBs within the genome is unlikely to explain the demonstrated effects of RV in repair. It is known that RV binds DNA, as does genistein, and modulates DNA intercalation of substances such as ethidium bromide and acridine orange (47). This feature could bring RV in line with compounds such as chloroquine that was initially used by Bakkenist *et al.* (48) when establishing the central role of ATM in the DNA damage response. By analogy, RV could induce structural changes that lead to ATM and ATR activation in the absence of DNA damage.

The precise role of ATM in DSB repair is not yet clearly defined, mostly due to some contradictory results in different experimental systems (19,21–23). All in all, ATM is believed to minimize error-prone repair events and, therefore, lead to an increment of fidelity control (19,21–23). Assuming that RV is a direct activator of the ATM/ATR pathway, RV is predicted to stimulate this genome stabilizing function of ATM/ATR. This idea is supported by our findings that showed inhibition of the mutagenic MM-NHEJ pathway and activation of the HR surveillance function of p53 (Figure 6). Enhanced fidelity of DSB repair and initiation of DNA damage signalling pathways are basic elements of cancer prevention. As documented recently in studies on different stages of lung, bladder, colon, breast and skin tumours, the DNA damage response is activated in the earliest phases in carcinogenesis (27,49). Given these newly defined qualities, RV-treated cells might be sensitized for damage signalling, and RV treatment might mimic the precancerous stimulus that generates a barrier against carcinogenesis.

Beyond a role in ATM/ATR signalling that may very well also underly the effects in apoptosis induction/sensitization and cell cycle regulation, we, therefore, propose a regulatory influence of RV on DSB repair. This role might help to explain at least partially the promising results in chemoprevention in animal models gained so far (3,4,7). In these and related bioavailability studies, systemic dosages of RV were maximally 3 μM after oral or 20–40 μM after intravenous administration (50), so that the novel effect on DSB repair, which we detected at 5–30 μM, is physiologically relevant. Consistently, ATM/ATR was detectably activated at $\geq$5 μM RV, as was assessed by western blot analysis of p53pSer15 (supplementary Figure 2 is available at *Carcinogenesis* Online). For comparison, RV concentrations of 15–100 μM were required to inhibit proliferation and to induce cell death in cell lines (4,5).

Data on the chemotherapeutic potential of RV in animal models often had an ambiguous outcome (51,52, reviewed in 53). This might in part be explained by the fact that tumours frequently carry alterations in the ATM/ATR-p53 pathway, and, therefore, might not profit from RV treatment as much as early precancerous forms. In contrast, tumours with mutations in this pathway might even show the opposite response, i.e. enhancement of genetic rearrangements as seen here for HR with p53 mutant (WTK1) and AT cells.

Therefore, this paper has an important impact on the discussed chemopreventive and chemotherapeutic role of RV: activation of the ATM/ATR-dependent DNA damage response via RV is in line with the concept of chemoprevention. However, therapeutic regimens will have to be applied with caution, as long as mutations in ATM and p53 have not been excluded.

Supplementary material

Supplementary material can be found at http://carcin.oxfordjournals.org/

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