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Inhibition of *REV3* Expression Induces Persistent DNA Damage and Growth Arrest in Cancer Cells[1,2]

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Abstract

REV3 is the catalytic subunit of DNA translesion synthesis polymerase ζ. Inhibition of *REV3* expression increases the sensitivity of human cells to a variety of DNA-damaging agents and reduces the formation of resistant cells. Surprisingly, we found that short hairpin RNA–mediated depletion of *REV3 per se* suppresses colony formation of lung (A549, Calu-3), breast (MCF-7, MDA-MB-231), mesothelioma (IL45 and ZL55), and colon (HCT116 +/−p53) tumor cell lines, whereas control cell lines (AD293, LP9-hTERT) and the normal mesothelial primary culture (SDM104) are less affected. Inhibition of *REV3* expression in cancer cells leads to an accumulation of persistent DNA damage as indicated by an increase in phospho-ATM, 53BP1, and phospho-H2AX foci formation, subsequently leading to the activation of the ATM-dependent DNA damage response cascade. *REV3* depletion in p53-proficient cancer cell lines results in a G_1 arrest and induction of senescence as indicated by the accumulation of p21 and an increase in senescence-associated β-galactosidase activity. In contrast, inhibition of *REV3* expression in *p53*-deficient cells results in growth inhibition and a G_2/M arrest. A small fraction of the p53-deficient cancer cells can overcome the G_2/M arrest, which results in mitotic slippage and aneuploidy. Our findings reveal that *REV3* depletion *per se* suppresses growth of cancer cell lines from different origin, whereas control cell lines and a mesothelial primary culture were less affected. Thus, our findings indicate that depletion of REV3 not only can amend cisplatin-based cancer therapy but also can be applied for susceptible cancers as a potential monotherapy.

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Introduction

Screening in *Saccharomyces cerevisiae* for mutants defective in UV-induced mutagenesis revealed the so-called reversionless phenotype (REV), which is characterized by a diminished frequency of mutations reverting a specific marker gene deficiency [1]. Two genes that confer this phenotype when absent are *Rev3* and *Rev7*, the catalytic and the structural subunits of the DNA translesion synthesis (TLS) polymerase ζ (Pol ζ), respectively [2,3]. The mammalian *REV3L* gene (hereafter *REV3*) encodes a ~350-kDa protein (REV3) consisting of a large C-terminal DNA polymerase subunit, which misses the characteristic proofreading activity present in other B-family DNA polymerases (reviewed in Waters et al. [4]). REV3 interacts through a specific binding domain with REV7, but no additional protein-protein interaction sites were identified. Deletion of *REV3* is embryonically lethal around midgestation [5–8], whereas overexpression of *REV3* leads to increased spontaneous mutation rates [9], confirming that *REV3* expression has to be tightly regulated to maintain genomic integrity. Conversely, one study found that *REV3* expression was downregulated in colon carcinomas compared with that in adjacent normal tissue [10], whereas another study found that *REV3* expression was elevated in human glioma tissues resected before therapy compared with that in normal brain tissues [11].

Abbreviations: TLS, DNA translesion synthesis; Pol ζ, DNA translesion synthesis polymerase ζ; *REV3*, the mammalian *REV3L* gene; MEF, mouse embryonic fibroblast; DDR, DNA damage response; DSBs, DNA double-strand breaks; ATM, ataxia-telangiectasia mutated; γH2AX, phosphorylated H2AX; P-Chk2, phosphorylated Chk2; AN, aneuploid nondividing; AD, aneuploid dividing

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Pol ζ belongs to the functional group of TLS DNA polymerases, which are characterized by a less-stringent active site and a lower processivity compared with the high-fidelity replicative DNA polymerases (reviewed in Waters et al. [4]). TLS polymerases contribute to the maintenance of the genomic integrity by allowing DNA replication to continue in the presence of DNA adducts, which otherwise could lead to DNA replication fork breakdown and subsequent gross chromosomal instability. Pol ζ is the major extender from mismatches formed when incorrect nucleotides are inserted opposite DNA adducts, thereby contributing to mutation formation on the nucleotide level. Recently, it was shown that REV3 is involved not only in DNA damage tolerance but also in DNA repair mechanisms, for example, interstrand cross-link repair [12–14], homologous recombination [15], and nonhomologous end-joining as indicated by the deficiency of *REV3*-deleted B cells in class switching of immunoglobulin genes [16].

The unique function of REV3 is highlighted by the fact that the *REV3* depletion increases sensitivity and decreases mutagenesis induced by UV light, cisplatin, and other mutagens in human and mouse fibroblasts [15,17,18]. In addition, depletion of *REV3* sensitizes mouse B-cell lymphomas and lung adenocarcinomas to cisplatin [19,20]. Although disruption of mouse *REV3* leads to embryonic lethality, it is possible to generate *REV3*-deleted mouse embryonic fibroblasts (MEFs) in a p53-deficient background [21]. Spontaneous chromosomal instability was observed in *REV3*-deleted MEFs and *REV3*-deleted cell lines [16,22,23].

DNA damage induction results in the activation of an evolutionarily conserved signal cascade known as DNA damage response (DDR) (reviewed in d'Adda di Fagagna [24]). Induction of DNA double-strand breaks (DSBs) results in recruitment and activation of ataxia-telangiectasia mutated (ATM). Activated ATM phosphorylates the histone variant H2AX at serine 139 (γH2AX) near DNA DSBs, subsequently leading to an accumulation of DDR proteins at DSBs, which can be visualized by immunofluorescence microscopy as distinct foci. Once ATM activation reaches a certain threshold, checkpoint kinase Chk2 is phosphorylated, resulting in the accumulation of p53, leading to the accumulation of the cyclin-dependent kinase inhibitor p21. Prolonged activation of p21 after DNA damage is associated with a terminal proliferation arrest, i.e., senescence.

While investigating how inhibition of *REV3* expression affects cisplatin-induced mutagenesis, we observed that depletion of *REV3 per se* reduces cancer cell growth, whereas growth of control cells is less affected. Suppression of *REV3* expression in cancer cells leads to the accumulation of persistent DNA damage independent of the p53 status. In p53-proficient cancer cells, inhibition of *REV3* expression results in the activation of the ATM-dependent DDR cascade, leading to senescence induction. In p53-deficient cancer cells, depletion of *REV3* results in a G_2/M arrest and increases the fraction of aneuploid cells. In contrast, inhibition of REV3 expression in control cell lines and a mesothelial primary culture neither reduces colony formation nor activates the DDR cascade.

Materials and Methods

Cell Lines and Culture

All cell lines used in this study were authenticated by DNA fingerprinting (Microsynth, Balgach, Switzerland). SDM104 was maintained as described previously [25]. All other cell lines were maintained in high-glucose Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich, St Louis, MO) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal calf serum, and 1% (wt/vol) penicillin/streptomycin. All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Additional details can be found in Supplemental Materials and Methods.

Vector Production and Transduction

Replication-deficient lentiviral particles were produced, titrated, and used for transduction as described previously [26,27]. Additional details can be found in Supplemental Materials and Methods.

Plasmid Transfection

Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with pSuperior.puro containing either scrambled control short hairpin RNA (shSCR) or three distinct short hairpin RNA (shRNA) sequences targeting the *REV3* messenger RNA (shREV3). Additional details can be found in Supplemental Materials and Methods.

Colony Formation Assay

Crystal violet staining was performed after colonies were visible by eye and, the number of colonies was determined by eye, applying the same threshold for colony size to all transduced cell lines. The number of colonies obtained by mock treatment was set to 100%.

Quantitative Real-time Polymerase Chain Reaction

RNA from samples was isolated using RNeasy Mini kit (Qiagen, Germantown, MD), and reverse transcription was performed on 300 ng of RNA (QuantiTect Reverse Transcription Protocol; Qiagen). The quantitative expression of *REV3* mRNA was measured by SYBR Green polymerase chain reaction (PCR) assay (PE Applied Biosystems, Foster City, CA) on a Prism 5700 detection system (SDS; PE Applied Biosystems). Additional details can be found in Supplemental Materials and Methods.

Immunofluorescence Microscopy

Immunofluorescence microscopy was essentially performed as described [28]. Details can be found in Supplemental Materials and Methods.

Flow Cytometry

Detection of bromodeoxyuridine (BrdU) incorporation in DNA-synthesizing cells was carried out using the anti-BrdU antibody (no. 555627; BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Additional details can be found in Supplemental Materials and Methods.

Senescence-Associated β-Galactosidase Assay

The expression of senescence-associated (SA) β-galactosidase was determined by SA-β-galactosidase staining as described [29].

Western Analysis

Protein extracts (30 μg) were separated by 4% to 20% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Immunoblot analysis was performed as described [30]. Details can be found in Supplemental Materials and Methods.

Enzyme-Linked Immunosorbent Assay

Cells were washed three times with phosphate-buffered saline (PBS) and serum-free DMEM was added for 24 hours. Conditioned medium was filtered, and cell number was determined in every experiment by