Depletion of Deoxyribonucleotide Pools is an Endogenous Source of DNA Damage in Cells Undergoing Oncogene-Induced Senescence

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Abstract

In normal human cells, oncogene-induced senescence (OIS) depends on induction of DNA damage response (DDR). Oxidative stress and hyper-replication of genomic DNA have been proposed as major causes of DNA damage in OIS cells. Here we report that down-regulation of deoxyribonucleoside pools is another endogenous source of DNA damage in normal human fibroblasts (NHF) undergoing HRAS[G12V]-induced senescence. NHF-HRAS[G12V] cells under-expressed thymidylate synthase (TS) and ribonucleotide reductase (RR), two enzymes required for the entire de novo deoxyribonucleotide biosynthesis, and possessed low dNTP levels. Chromatin at the promoters of the genes encoding TS and RR was enriched with RB tumor suppressor protein and histone H3 tri-methylated at lysine 9. Importantly, ectopic co-expression of TS and RR or addition of deoxyribonucleosides substantially suppressed DNA damage, senescence-associated phenotypes and proliferation arrest in two types of NHF expressing HRAS[G12V]. Reciprocally, shRNA-mediated suppression of TS and RR caused DNA damage and senescence in NHF although less efficiently than HRAS[G12V]. However, overexpression of TS and RR in quiescent NHF did not overcome proliferation arrest, suggesting that unlike quiescence, OIS requires depletion of dNTP pools and activated DNA replication. Our data identify a previously unknown role of deoxyribonucleotides in regulation of oncogene-induced senescence.

Introduction

Oncogene-induced senescence represents an important fail-safe mechanism that suppresses proliferation of pre-malignant cells (1-3). Compelling evidence suggests that the response to DNA damage is one of the intrinsic processes required for the induction of OIS (4-7). It has been shown that aberrant activation of HRAS in human fibroblasts induces hyper-replication of genomic DNA which leads to alterations in progression of DNA replication fork, generation of single and double strand DNA breaks (SSB and DSB, respectively) and activation of DNA damage response (DDR) (6). SSB induce DDR by engaging serine/threonine-protein kinase ATR (ataxia telangiectasia and Rad3-related protein) that transmits signaling to checkpoint kinase 1 (CHK1) (8). CHK1 phosphorylates CDC25 protein, one of the key regulators of cell cycle progression, and targets it for degradation (8). DSB initiate DDR that depends on another serine/threonine protein kinase, ATM (ataxia telangiectasia mutated) (9). Activation of ATM results in phosphorylation of several targets, including the histone H2A variant H2AX (10), p53 tumor suppressor (11, 12) and CHK2 kinase (13). Both ATR and ATM signaling pathways are activated in normal human fibroblasts (NHF) undergoing HRAS[G12V]-induced senescence (4-7), while ATM and CHK2 are required for this senescence since their individual shRNA-mediated inhibition enabled NHF to overcome proliferation arrest and other senescence-associated phenotypes (6,7).

At the same time, studies conducted in yeasts and mammalian cells demonstrate that stalling of DNA replication fork and activation of ATR/CHK1 and ATM/CHK2 pathways can be induced by pharmacological depletion of all or selected nucleotide pools (14, 15). In the present study, we investigated endogenous processes that caused DNA damage in human fibroblasts undergoing oncogene-induced senescence and demonstrated that DNA damage at least partially

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originates from under-expression of key enzymes involved in de novo deoxyribonucleoside biosynthesis and subsequent depletion of endogenous dNTP pools. We propose that nucleotide deficiency caused by aberrant expression of activated HRAS contributes to oncogene-induced senescence.

Materials and Methods

Cell lines and populations

Normal human fibroblasts WI-38 were purchased from ATCC. BJ-ET-RAS^{G12V}-ER^{TAM} fibroblasts were a gift from Dr. Andrei Gudkov (RPCI). Cells were cultured in Dulbecco's modified Eagle's essential minimal medium supplemented with fetal calf serum (10%), 2 mM glutamine, and 100 units/ml penicillin G + 100 µg/ml streptomycin.

Lentiviral constructs and infection

Lentiviral infection protocols and vectors containing cDNAs of HRAS^{G12V} were described previously (16). pLKO1 vector containing shRNA for RR2 was purchased from Sigma Aldrich. cDNA for TS, RR1 and RR2 were amplified by reverse-transcription polymerase chain reaction from total RNA isolated from human melanoma cells and cloned in pLV-SV-puro expression vector (a gift from Dr. Peter Chumakov, Cleveland Clinic).

Assays for cell proliferation and senescene

For the proliferation assay, cells were plated in 96-well plates at ~50% confluence two days prior to the assay. Cells were incubated with a nucleoside analog of thymidine, 5-ethynyl-2´-

deoxyuridine (EdU), for 60 minutes followed by fixation and staining for EdU-incorporated cells using the ClickiT™ EdU Assay kit (Invitrogen).

For the senescence assay, cells were plated in 12-well plates, fixed and incubated at 37°C with staining solution containing the X-Gal substrate (BioVision). The development of blue color was detected visually with a microscope.

Immunoblotting, immunofluorescence and comet assay

For Western blotting, antibodies were used for the following human proteins: ERK1 and phospho-ERK1/2, RR1, RR2 from Santa Cruz Biotechnology (sc-42, sc-93, sc-7383, sc-11733, sc-10844, respectively); TS (ab7398-1) from Abcam, TK1 (34003) from EMD Chemical Group; ATM (2873) and phospho-ATM-Ser1981 (4526) from Cell Signaling. Membranes were developed using alkaline phosphatase-conjugated secondary antibodies and the Alpha-Innotech FluorChem HD2® imaging system. For immunofluorescence-based analysis, formaldehyde-fixed cells were stained with H2AX-γ-specific antibodies (05-636) from Millipore according to the manufacturer's recommendations followed by staining with 4',6-diamidino-2-phenylindole (DAPI). Alkaline comet assay was performed using Trevigen CometAssay® HT kit (4252-040-K) according to the manufacturer's recommendations.

Nucleotide triphosphate quantification

Nucleotide triphosphates were extracted and assayed as described previously (17, 18). All measurements were performed in quadruplicate, and data recorded as standard deviation of the mean. For convenience data are presented in the respective figures in terms of arbitrary units.

However, the actual pool sizes determined, in pmol/cell, were comparable to values previously reported for cultured mammalian cells (18).

Chromatin Immunoprecipitation

Interactions between RB, H3K9^{3Me} and the promoters of TS, RR1 and RR2 were assessed using the EZ-Chip kit from Millipore according to the manufacturer's recommendations with the following antibodies: IgG (Santa Cruz Biotechnology, sc-2027), RB (Santa Cruz Biotechnology, sc-73598) or H3K9^{3Me} (Millipore, 07-442). The following primers were used for the PCR: TS 5'-TTC CCG GGT TTC CTA AGA CT-3' and 5'-TGG ATC TGC CCC AGG TAC T-3'; RR1 5'-GCT GAC AGG GCG GAA G-3' and 5'-GGA AGG GGA TTT GGA TTG TT-3'; RR2 5'-CCT CAC TCC AGC AGC CTT TA-3' and 5'-CAC CAA CCT CGT TGG CTA AG-3'

FACS Analysis

Cells were pelleted, resuspended in phosphate-buffered saline containing 0.5% fetal calf serum (PBS-S) and fixed in 70% ethanol. The cells were washed in PBS-S, resuspended in PBS-S containing propidium iodide (Sigma-Aldrcih), incubated for 30 min at 37°C and analyzed using a FACScan fluorescence activated cells sorter (BD Biosciences).

Results

HRAS^{G12V}-overexpressing senescent WI-38 cells possess low dNTP pools and under-express key enzymes required for deoxyribonucleoside biosynthesis

To determine whether NHF undergoing oncogene-induced senescence possess low levels of endogenous nucleotide pools, we assessed amounts of intracellular ribo- and

deoxyribonucleoside triphosphates in WI-38 cells transduced with control or HRAS[G12V]-expressing lentiviral vector 6 days after infection, i.e. soon after the emergence of senescent phenotypes (Figure 1A). We found that although overexpression of HRAS[G12V] depleted both pools of nucleotide triphosphates, levels of rNTPs were affected less severely than dNTPs levels (20%-50% versus 60%-80% reduction, Figure 1B). Unlike de novo ribonucleotide biosynthesis pathways that include multiple enzymatic activities, the entire de novo biosynthesis of deoxyribonucleosides is largely controlled by only two enzymes. Ribonucleotide reductase (RR) converts ADP, CDP, GDP and UDP respectively to dADP, dCDP, dGDP and dUDP (19). Thymidylate synthase (TS) catalyzes the de novo synthesis of dTMP from dUMP (20). Thus, we measured expression levels of TS and both subunits of RR (RR1 and RR2) in control and HRAS[G12V]-expressing WI-38 cells by western blotting 6 days post-infection. We found that like dNTP pools, the levels of these enzymes were significantly depleted in senescent fibroblasts (Figure 1C). Additionally, the expression of a gene encoding thymidine kinase 1 (TK1), a key enzyme required for the salvage deoxyribonucleoside synthesis (21), was also down-regulated in senescent cells.

Co-expression of TS, RR1 and RR2 or addition of deoxyribonucleosides reduces DNA damage and OIS in NHF

The highest expression of thymidylate synthase and ribonucleotide reductase has been reported in the S-phase of the cell cycle (22, 23). Therefore, depletion of TS, RR, and dNTP pools in HRAS[G12V]-WI-38 cells may be a consequence of G0/G1 or G1/S cell cycle arrest induced by HRAS[G12V] (Figure 1D). Alternatively, this depletion may be a cause of proliferation arrest and senescence in cells overexpressing HRAS[G12V]. To discern between these possibilities, we

attempted to restore TS and RR levels in HRAS^{G12V}-WI-38 fibroblasts following by assessment of senescence-associated phenotypes. To this end, we concurrently transduced WI-38 cells with cDNAs for TS, RR1 and RR2 (hereafter TRR) or in parallel with three empty vectors (hereafter 3V). Two days after infection, 3V and TRR cells were super-infected with viruses containing empty vector or HRAS^{G12V}-expressing vector. Activity of HRAS^{G12V} in 3V and TRR cells was monitored by measuring the levels of phosphorylated ERK1/2 that appeared to be the same in both types of cells (Figure 2A). Expression of TS, RR1 and RR2 proteins was followed by western blotting. As shown in Figure 2A, super-infection of HRAS^{G12V} in TRR-cells did not significantly change total (exogenous and endogenous) amounts of these proteins. Consequently, dNTP levels in HRAS^{G12V}-TRR cells were 2-2.5-fold higher than those in HRAS^{G12V}-3V cells (Figure 2B). Moreover, the number of cells exhibiting DNA damage detected by the comet assay or by staining with H2AX-γ-specific antibodies or the number of SA-β-Gal-positive cells was also 2-3 fold lower in HRAS^{G12V}-TRR versus HRAS^{G12V}-3V populations (Figure 2C). Most importantly, inhibition of proliferation by HRAS^{G12V} in TRR cells was only partial as compared to the complete and stable proliferation arrest of HRAS^{G12V}-3V cells (Figure 2DE, Supplemental Figure 1A). Thus, restoration of thymidylate synthase and ribonucleotide reductasse levels in cells overexpressing HRAS^{G12V} results in the substantial suppression of senescence phenotypes.

To independently verify the importance of maintaining high dNTP pools for suppression of DNA damage and OIS, we attempted to restore intracellular dNTPs in WI-38-HRAS^{G12V} cells by supplementing their culture media with deoxyribonucleosides starting the next day after infection with HRAS^{G12V}-encoding virus, i.e. prior to the emergence of senescence-associated phenotypes. Culture media containing or not deoxyribonucleosides were replaced with the fresh

ones respectively containing or not deoxyribonucleosides every 2 days for the duration of the experiment. Incubation with deoxyribonucleosides did not affect the activity of HRAS^{G12V} as was determined by the absence of changes in the amounts of phosphorylated ERK1/2 between treated and untreated populations of cells (Figure 3A). However, this treatment did increase intracellular amounts of dNTP pools 1.5-2.5 fold (Figure 3B) and decreased the number of H2AX-γ- and comet-positive cells and cells exhibiting SA-β-Gal activity in the treated populations (Figure 3C). Accordingly, HRAS^{G12V} was less effective in suppressing proliferation of WI-38 cells growing in the medium supplemented with deoxyribonucleosides compared to the untreated cells that underwent proliferation arrest (Figure 3DE, Supplemental Figure 1B).

Therefore, even partial restoration of suppressed dNTP pools via either overexpression of thymidylate synthase and ribonucleotide reductase or incubation with exogenous deoxyribonucleosides reduced DNA damage and the full-scale manifestation of oncogene-induced senescence in WI-38 cells.

In order to generalize our findings, we utilized a different type of NHF, primary BJ fibroblasts ectopically expressing the human telomerase reverse transcriptase gene and tamoxifen-inducible RAS^{G12V}-ER^{TAM} chimera gene (BJ-ET-RAS^{G12V}-ER^{TAM}cells). Upon activation of HRAS^{G12V} via addition of 4-hydroxytestosterone (4-OHT) (Figure 4A), proliferation of these cells was reduced gradually until reaching an arrest at approximately day 8 (Figure 4B). The arrest was accompanied by changes in cell morphology, increased SA-β-Gal activity and increased DNA damage visualized by antibodies to H2AX-γ □(Figure 4C). At the same time, continuous presence of deoxyribonucleosides in growth media, while not affecting HRAS^{G12V} activation (Figure 4A), reduced inhibition of cell proliferation and other senescence-associated phenotypes (Figure 4BC) similarly to WI-38 cells.

Depletion of TS and RR2 causes senescence in NHF

To understand whether down-regulation of thymidylate synthase and ribonucleotide reductase is sufficient to cause DNA damage and emergence of senescent phenotypes in NHF with normal, not hyper-stimulated DNA replication, we depleted amounts of thymidylate synthase and ribonucleotide reductase in WI-38 fibroblasts via co-infection with vectors carrying shRNAs for thymidylate synthase and ribonucleotide reductase subunit R2and compared these cells with cells infected with control shRNA vector. Depletion of TS and RR2 (Figure 5A) led to stable proliferation arrest (Figure 5B, Supplemental Figure 1C), albeit with longer latent period compared to HRAS^{G12V}-WI-38 cells (9-11 days versus 4-6 days). Arrested fibroblasts exhibited DNA damage (as was visualized by staining with antibodies to H2AX-γ (Figure 5C) and elevated activity of SA-β-Gal (Figure 5C), however, the proportion of SA-β-Gal- and H2AX-γ-positive cells was also lower compared to WI-38 cells arrested by overexpression of HRAS^{G12V} (compare Figs 2, 3 and 5).

Overexpression or thymidylate synthase and ribonucleotide reductase or addition of exogenous deoxyribonucleosides does not suppress quiescence in normal human fibroblasts

We were interested in addressing the role of thymidylate synthase and ribonucleotide reductase overexpression in suppressing quiescence of NHF, a form of temporary arrest of DNA replication that is not associated with DNA damage and can be achieved by serum deprivation (4, 24).   Incubation of WI-38 cells in medium devoid of fetal bovine serum (FBS) for 48 hours resulted in substantial down-regulation of TS, RR1 and RR2 (Figure 6A), however, no increase was detected in the number of H2AX-γ-positive cells (Figure 6B), suggesting that no DNA

damage was present. Importantly, continuous supplementation with deoxyribonucleosides of FBS-free culture media did not even partially affect proliferation arrest as was determined by EdU incorporation at day 5 after replacing the culture medium (Figure 6C). Accordingly, the same assay demonstrated that proliferation of TRR-expressing WI-38 cells (described above) was suppressed by serum withdrawal as efficiently as the proliferation of control 3V cells (Figure 6C). Thus, we concluded that unlike oncogene-induced senescence, overexpression of thymidylate synthase and ribonucleotide reductase or supplementation with deoxyribonucleosides does not suppress quiescence in normal human fibroblasts.

Promoters of TS, RR1 and RR2 interact with RB and undergo heterochromatization in senescent HRAS^{G12V}-WI38 cells

Transcription levels of RR1, RR2 and TS can be up-regulated by ectopic expression of E2F1 (25). Promoters of some E2F-responsive genes have been shown to interact with RB tumor suppressor proteins in NHF undergoing HRAS^{G12V}-induced senescence (26). Thus, to identify whether silencing of TRR genes in HRAS^{G12V}-WI38 senescent fibroblasts occurs via interactions with RB, we performed chromatin immunoprecipitation in these and vector cells with control antibodies, antibodies to RB or tri-methylated lysine 9 of histone H3 (H3K9^{3Me}), a hallmark of heterochromatin (27) and senescence-associated heterochromatin foci (26). After precipitation, the chromatin was de-crosslinked and the purified DNA was probed in semi-quantitative PCR with primers spanning E2F1-binding sites in the promoters of RR1, RR2 and TS genes. By this method, we demonstrated substantial enrichment of the promoter regions of all three genes in chromatin precipitated with RB- or H3K9^{3Me}-specific antibodies from HRAS^{G12V}-senescent but

not vector cells (Figure 7), suggesting that RB binding and formation of heterochromatin contribute to silencing of TRR genes in senescent HRAS[G12V]-WI38 cells.

Discussion

DNA damage induced by ectopic expression of activated RAS or RAF oncoproteins in normal mammalian cells has been considered as a major cause of senescence, a fail-safe mechanism for suppressing tumor development at a premalignant stage (1,2). In the current study, we tested hypothesis that at least one of the major mediators of HRAS[G12V] DNA damage- and senescence-inducing activity is down-regulation of dNTP pools. Decrease in the amounts of intracellular deoxyribonuclotides in OIS cells may cause DNA damage and affect DNA replication fork progression (which was described in fibroblasts undergoing HRAS[G12V]-induced senescence [6]) via several interrelated mechanisms. The most obvious one is that dNTP deficiency slows processivity or even completely arrests DNA polymerase. However, this is an unlikely scenario since inhibition of DDR proteins ATM or CHK2 abolishes HRAS[G12V]-induced senescence in NHF presumably via dNTP-independent mechanism (4-7). Most likely, stalling or collapse of the replication fork in a senescent cell occurs due to the DNA damage sites pre-existent to the current DNA replication, and these pre-existent lesions may originate from dNTP depletion. For example, it has been shown that inhibition of TS activity results in depletion of dTTP pools and in symmetrical increase in the amounts of deoxyuridine triphosphates which are utilized instead of dTTP for DNA polymerization (28). The incorporated uracil is removed via the uracil base excision repair (BER) system, however, if the number of incorporated uracil bases is high, the repair machinery is overwhelmed and generates abasic sites, as well as double- and/or single-strand breaks (15), which would ultimately cause replication stress and affect the replication fork

progression during the following round of DNA replication. Similarly, inhibition of RR and subsequent depletion of dATP, dGTP and dCTP pools lead to nucleotide misincorporation and activation of the mismatch repair system (MMR) (29) that, if overwhelmed, would also generate double and/or single strand DNA breaks (30). Additionally, both BER and MMR systems utilize de novo DNA synthesis to fill the gaps generated in the course of DNA repair (31). Low levels of dNTPs would affect fidelity of this synthesis and subsequently, DNA repair in general.

The proposed mechanisms of generation of DNA damage in OIS cells are in a good agreement with previously published observations. For instance, as was mentioned above, a large number of HRAS[G12V]-expressing cells were arrested with partly replicated DNA, suggesting stalling or a collapse of replication fork (6). DNA hyper-replication (multiple firing of the same replication origin) was proposed to be a primary cause for this phenotype (6). Stalling or collapse of the replication fork and subsequent induction of the S-phase checkpoint can be induced by pharmacological inhibition of thymidylate synthase or ribonucleotide reductase via mechanisms described above in cells with normal, not hyper-stimulated, DNA replication. Therefore, it is conceivable that under the conditions of low dNTP pools multiple origin firings and subsequently multiple replication forks would generate even higher DNA damage. Accordingly, fragile sites in OIS cells were more prone to undergoing mutations than any other regions of the genome (6). Similarly, pharmacological inhibition of ribonucleotide reductase has been shown to disproportionally increase mutagenesis of genomic regions containing fragile sites (32, 33).

We demonstrated that even partial restoration of depleted intracellular dNTP pools is sufficient for substantial suppression of DNA damage and senescence. Despite significant ectopic overexpression of thymidylate synthase and ribonucleotide reductase or incubation with

excessive amounts of deoxyribonucleosides, we were unable to completely restore dNTP pools in cells undergoing senescence. Complete reconstitution may require involvement of salvage pathway enzymes (especially for phosphorylation of exogenously added nucleosides) that, like thymidine kinase 1 (21), (Figure 1C) may be suppressed in senescent cells, or enzymes involved in biosynthesis of ribonucleotides. Interestingly the genomes of herpes viruses that replicate their DNA in quiescent cells with depleted dNTP pools often encode enzymes for both de novo and salvage pathways, including homologues to cellular RR1 and RR2, thymidylate synthase, thymidine kinase, dihydrofolate reductase and dUTP pyrophosphatase (34). However, we could not recapitulate expression of all of these genes in our experimental settings since overexpression of additional cDNAs in WI-38 cells affected their normal proliferation rates.

It is known that transformed cells utilize aerobic glycolysis as a major pathway for biosynthesis of adenosine triphosphate, a process called the "Warburg effect" (35). Recently, several groups reported that the enhanced rates of glycolysis occur largely due to the increased demand of a transformed cell for macromolecule components including nucleotides that can be synthesized from glycolytic metabolites (36, 37). Bypassing senescence is considered an initial step in oncogenic transformation and our data suggest that elevation of nucleotide levels is an important prerequisite for this step. Thus switching to aerobic glycolysis to increase nucleotide pools may be required already at early stages of tumorigenesis.

Our results suggest that both nucleotide depletion and active DNA replication are required for efficient induction of DNA damage and OIS. Concurrent depletion of TS and RR2 in WI-38 fibroblasts with normal DNA replication induced senescence with longer latent period than overexpression of HRAS[G12V], and senescent populations contained lower fraction of H2AX-γ-positive and SA-β-Gal-positive cells compared to WI-38 cells overexpressing HRAS[G12V] (with

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presumably hyper-replicated DNA) (Figs 2 and 5). Furthermore, serum withdrawal-arrested WI-38 cells, with no DNA replication, do not undergo DNA damage and senescence despite down-regulation of TS, RR1 and RR2 (Figure 5). Accordingly, ectopic overexpression of these enzymes in quiescent WI-38 cells or exogenous addition of deoxyribonucleosides into their media did not overcome this form of proliferation arrest.

We demonstrated that promoters of TS, RR1 and RR2 in HRAS^{G12V}-WI38 senescent cells interacted with RB tumor suppressor proteins and were enriched with heterochromatin marker H3K9^{3Me} (Figure 7). RB has been shown to play an essential role in HRAS^{G12V}-induced senescence by binding to the promoters of several E2F-repsonsive genes and establishing repressive chromatin at the target loci (26). Thus, our findings may offer a novel connection between RB activation and repression of nucleotide metabolism ultimately resulting in the increased DNA damage and senescence.

Taken together, our data provide a previously unidentified mechanism for the induction of DNA damage and oncogene-induced senescence.

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Figure Legends

Figure 1. Senescent HRAS[G12V]-WI38 fibroblasts possess low NTP pools and under-express key enzymes for deoxyribonucleoside metabolism. **(A)** Cells were infected with empty lentiviral vector (V) or lentiviral vector encoding HRAS[G12V] (R). Six days after infection, cells were fixed and processed to visualize SA-β-Gal activity. Numbers indicate a percentage of SA-β-Gal-positive cells. The numbers were derived from counting cells in multiple view fields. **(B)** Cells infected as in (a) were harvested 6 days after infection and intracellular NTPs were extracted and quantified. Amounts of each NTP were normalized by the amounts of a corresponding NTP detected in vector cells. **(C)** Cells infected as in (a) were harvested 6 days after infection, lysed and total protein extracts were probed in western blotting with antibodies shown on the right. **(D)** Cells infected as in (a) were collected, fixed, stained with propidium iodide and analyzed by flow cytometry.

Figure 2. Co-expression of TS, RR1 and RR2 reduce OIS phenotypes in HRAS[G12V]-WI38 fibroblasts. **(A)** Cells were infected with control vectors three times (3V) or with vectors expressing cDNA for TS, RR1 and RR2 cDNAs (TRR). Forty-eight hours later, cells were super-infected with the empty vector (V) or HRAS[G12V] (RAS). Six days after second infection, cells were harvested, lysed and total protein extracts were probed in western blotting with antibodies indicated on the left. **(B)** Cells were infected as in (a). Six days after the second infection, intracellular dNTPs were extracted and quantified. Amounts of each dNTP from HRAS[G12V]-infected cells were normalized by the amounts of corresponding dNTP detected in 3V-Vector cells (left columns) or TRR-Vector cells (right columns). **(C)** Cells were infected as in (a). Six days after infection, cells were fixed and processed to visualize SA-β-Gal activity,

fixed and stained with H2AX-γ-specific antibodies and DAPI or subjected to the comet assay (the type of assay is shown on the left). Numbers indicate percentage of positive cells in each assay. The numbers were derived from counting cells in multiple view fields. **(D-E)** Cells were infected as in (a). Two days after second infection, cells were plated in 12-well plate in triplicate and counted every other day for 6 days. Numbers below the graph correspond to the days post-infection. In parallel, at day 7 post-infection, cells were assayed for proliferation rates (EdU incorporation). In each population, the number of positive cells was divided by the number of total cells.

Figure 3. Exogenous deoxyribonucleosides reduce OIS phenotypes in HRAS^{G12V}-WI38 fibroblasts. **(A)** Cells were infected with empty lentiviral vector (V) or lentiviral vector encoding HRAS^{G12V} (R). Next day after infection, 100 μM of each deoxyribonucleoside was added (+dN) or not to the culture media and media were replaced with the fresh one respectively containing or not deoxyribonucleosides every 2 days. Six days after infection, cells were harvested, lysed and total protein extracts were probed in western blotting with antibodies indicated on the left. **(B)** Cells were infected and treated as in (a) followed by dNTPs extraction and quantification 6 day after infection. All dNTP amounts were normalized by the amounts detected in "vector" cells. **(C)** Cells were infected and treated as in (a). Six days after infection, cells were fixed and processed to visualize SA-β-Gal activity or fixed and stained with H2AX-γ-specific antibodies and DAPI or subjected to the comet assay (the type of assay is shown on the left). Numbers indicate percentage of positive cells. The numbers were derived from counting cells in multiple view fields. **(D-E)** Cells were infected and treated as in (a). Two days after infection, cells were plated in 12-well plate in triplicate and counted every other day for 6 days. Numbers below the

graph correspond to the days post-infection. In parallel, at day 6 post-infection, cells were assayed for proliferation rates (EdU incorporation). In each population, the number of positive cells was divided by the number of total cells.

Figure 4. Exogenous deoxyribonucleosides reduce OIS phenotypes in BJ-ET-RAS^{G12V}-ER^{TAM} fibroblasts. **(A)** Untreated cells (UN), cells treated with 0.1 µM of 4-hydroxytestosterone (4-OHT) or with 4-hydroxytestosterone and 100 µM of each deoxyribonucleoside (4-OHT+dN). Seven days after addition of the chemicals, cells were harvested, lysed and total protein extracts were probed in western blotting with antibodies indicated on the left. **(B)** Cells were plated in 12-well plate in triplicate and treated as in (a) and counted once in two days for 8 days. Numbers below the graph correspond to the days post-infection. **(C)** Cells were treated as in (a) for 7 days. Cells were fixed and processed to visualize SA-β-Gal activity or fixed and stained with H2AX-γ-specific antibodies and DAPI (the type of staining is shown on the left). Numbers indicate percentage of positive cells. The numbers were derived from counting cells in multiple view fields.

Figure 5. Deficiency of TS and RR2 induces senescence in WI-38 fibroblasts. **(A)** Cells were infected with control shRNA lentiviral vector (Cl) or with combination of vectors carrying shRNAs for TS or RR2 shRNAs (T+R2). Two days after infection, cells were harvested, lysed and total protein extracts were probed in western blotting with antibodies indicated on the left. **(B)** Cells were infected as in (a). Two days after infection cells were plated in 12-well plate in duplicate and counted every other day for 9 days. Numbers below the graph correspond to the days post-infection. **(C)** Cells were infected as in (a). Ten days after infection, cells were fixed

and processed to visualize SA-β-Gal activity or fixed and stained with H2AX-γ-specific antibodies and DAPI (type of assay is shown on the left). Numbers indicate percentage of positive cells. The numbers were derived from counting cells in multiple view fields.

Figure 6. Co-expression of TS, RR1 and RR2 or addition of exogenous deoxyribonucleosides does not affect quiescence of WI-38 fibroblasts. **(A)** Cells were incubated in culture medium containing 10% or 0% FBS for 2 days. Cells were collected, lysed and total protein extracts were probed in western blotting with antibodies indicated on the left. **(B)** Cells treated as in (a) were fixed and stained with antibodies for H2AX-γ☐☐The numbers were derived from counting cells in multiple view fields. **(C)** Uninfected WI-38 cells, 3V WI-38 cells and TRR WI-38 cells were grown in the media containing 10% or 0% FBS supplemented or not with 100 μM of deoxyribonucleosides (+dN) for a period of 5 days. Cell proliferation was assessed by EdU incorporation assay. The numbers were derived from counting cells in multiple view fields.

Figure 7. Promoter regions of TS, RR1 and RR2 are enriched with RB and tri-methylated lysine 9 of histone H3. **(A)** Cells were infected with empty lentiviral vector (V) or lentiviral vector encoding HRAS^{G12V} (R). Six days after infection, cells were harvested, lysed and total protein extracts were probed in western blotting with antibodies shown on the left. **(B)** Cells were infected as in (a). Six days after infection, cells were cross-linked, lysed, and chromatin was immunoprecipitated with the antibodies shown on the top followed by the reversal of the cross-linking and DNA isolation. DNA obtained from chromatin immunoprecipitated with the antibodies designated on the top or 0.1% of the input DNA (Inp.) was used in PCR with the primers specific to the promoter regions of the genes shown on the left.