



# O<sup>6</sup>-methylguanine–induced transcriptional mutagenesis reduces p53 tumor-suppressor function

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**Altered protein function due to mutagenesis plays an important role in disease development. This is perhaps most evident in tumorigenesis and the associated loss or gain of function of tumor-suppressor genes and oncogenes. The extent to which lesion-induced transcriptional mutagenesis (TM) influences protein function and its contribution to the development of disease is not well understood. In this study, the impact of O<sup>6</sup>-methylguanine on the transcription fidelity of p53 and the subsequent effects on the protein's function as a regulator of cell death and cell-cycle arrest were examined in human cells. Levels of TM were determined by RNA-sequencing. In cells with active DNA repair, misincorporation of uridine opposite the lesion occurred in 0.14% of the transcripts and increased to 14.7% when repair by alkylguanine–DNA alkyltransferase was compromised. Expression of the dominant-negative p53 R248W mutant due to TM significantly reduced the transactivation of several established p53 target genes that mediate the tumor-suppressor function, including *CDKN1A* (p21) and *BBC3* (PUMA). This resulted in deregulated signaling through the retinoblastoma protein and loss of G1/S cell-cycle checkpoint function. In addition, we observed impaired activation of apoptosis coupled to the reduction of the tumor-suppressor functions of p53. Taking these findings together, this work provides evidence that TM can induce phenotypic changes in mammalian cells that have important implications for the role of TM in tumorigenesis.**

transcription fidelity | transcriptional mutagenesis | O<sup>6</sup>-methylguanine | p53 tumor suppressor

The impact of DNA lesions on replication processivity and fidelity is well understood, and their roles in mutagenesis and tumorigenesis are equally well established (1). However, DNA lesions also influence RNA synthesis, not only by causing transcriptional arrest, which in turn activates transcription-coupled DNA repair (2, 3), but also by inducing transcriptional misincorporation into nascent RNA, an event referred to as “transcriptional mutagenesis” (TM) (4). TM, which has been observed *in vitro* (5–8) and *in vivo* (9–12), results most often from transcriptional bypass of small lesions formed by oxidation or alkylation of the bases in DNA. Although TM has been shown to induce phenotypic changes in mammalian cells by inducing altered protein function (9–11), its role in disease development remains unclear. It has been suggested that expression of mutant proteins via TM could play a significant role in tumor development by stimulating cell proliferation or enhancing the evasion of growth-inhibitory signals, thus allowing expansion of precancerous cells (13).

Tumor development is kept in check in part by a set of genes collectively known as “tumor-suppressor genes.” *TP53* is an important example of a tumor-suppressor gene in humans. It encodes the p53 protein, which represses tumor formation due in part to its role as a regulator of both cell-cycle progression and apoptosis (14). The p53 protein primarily functions as a transcription factor, and a considerable number of target genes are under its control (15). Mutations in the *TP53* gene are commonly found in human tumors, and they frequently result in compromised p53 activity. Typically, the mutations confer a dominant-negative phenotype to

the p53 protein by altering its DNA-binding domain, which in turn reduces its ability to transactivate target genes (16).

The *CDKN1A* gene is among those that the p53 protein regulates and provides an important example of the role of p53 protein in regulating cell growth (17, 18). The protein product of the *CDKN1A* gene is p21, which is a cyclin-dependent kinase (CDK) inhibitor that interacts with CDK2, preventing progression through the G1 phase of the cell cycle (19). Among the well-established effects of mutated p53 protein is its reduced ability to transactivate the *CDKN1A* gene, resulting in limited production of p21 protein and ensuing failed G1 arrest (20, 21). This is particularly problematic when DNA damage occurs in the genome, since lack of G1 arrest in such cases allows the cell to progress to S phase, resulting in an increased risk of mutagenesis and subsequent tumor development. Similarly, inactivation of p53 severely limits the transactivation of proapoptotic genes of the Bcl-2 family, including *BBC3*, *PMAIP1*, and *BAX* (22). This results in the loss of programmed cell death due to an inability of the cell to regulate the intrinsic pathway of apoptosis.

O<sup>6</sup>-methylguanine (O<sup>6</sup>-meG) is among the highly mutagenic lesions found in DNA following exposure to endogenous and exogenous methylating agents, including *S*-adenosylmethionine and *N*-nitroso compounds (23, 24). Indeed, the formation of alkyl lesions at the O<sup>6</sup> position of guanine is also an important event in the cytotoxic mechanisms of some chemotherapeutic drugs, including carmustine and temozolomide (25, 26). O<sup>6</sup>-meG is repaired by the

## Significance

The impact of DNA lesions on replication and mutagenesis is of high relevance for human health; however, the role of lesion-induced transcriptional mutagenesis (TM) in disease development is unknown. Here, the impact of O<sup>6</sup>-methylguanine–induced TM on p53 function as a tumor suppressor was investigated in human cells. Results showed that TM in 15% of the transcripts resulted in a reduced ability of p53 protein to transactivate genes that regulate cell-cycle arrest and induction of apoptosis. This resulted in the loss of functional cell-cycle checkpoints and in impaired activation of apoptosis, both canonical p53 tumor-suppressor functions. This work provides evidence that TM can induce phenotypic changes in mammalian cells that have important implications for its role in tumorigenesis.

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The authors declare no conflict of interest.

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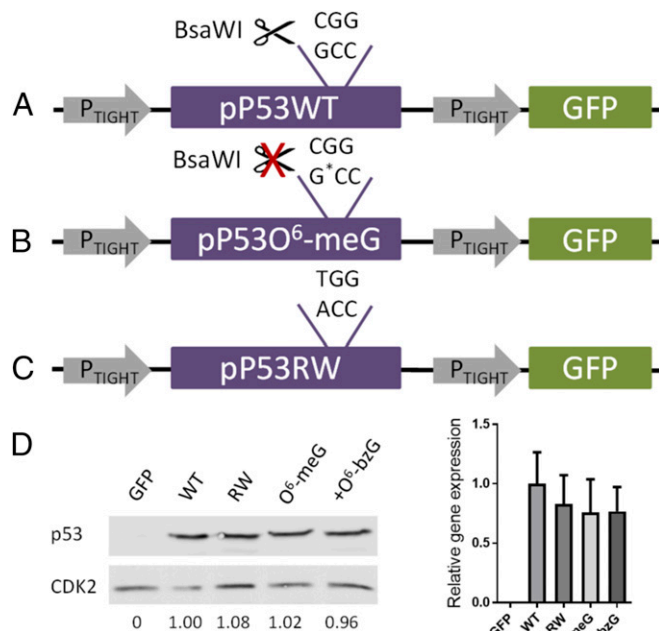
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protein alkylguanine–DNA alkyltransferase (AGT) that transfers the methyl group from the damaged base to a cysteine residue within the protein, resulting in restoration of guanine in DNA. However,  $O^6$ -meG that escapes repair is readily bypassed by RNA polymerases, which can lead to significant levels of TM when uridine rather than cytidine is misincorporated into RNA (7, 11, 12). In fact, TM induced by  $O^6$ -meG can result in changes in protein function in vivo (11) and can attenuate the splicing fidelity of G-rich exon–intron junctions in mammalian cells (12).

To increase the understanding of how TM can contribute to tumorigenesis, the impact of  $O^6$ -meG-induced TM on p53 protein function was investigated by examining the activation of cell-cycle checkpoints and the induction of apoptosis. The results show that TM of p53 reduces its tumor-suppressor function, further strengthening the link between TM and cancer etiology.

## Results

**Synthesis of a *TP53* Plasmid Containing a Site-Specific  $O^6$ -MeG.** To investigate the impact of TM on p53 function in human cells, an expression system consisting of three vectors was developed. Each vector contained one variant of the *TP53* gene: pP53<sup>WT</sup>, pP53<sup>RW</sup>, or pP53 <sup>$O^6$ -meG</sup> (Fig. 1 *A–C*). pP53<sup>WT</sup> encodes wild-type p53 protein, whereas pP53<sup>RW</sup> contains a C-to-T transition at codon 248 that results in the conversion of an arginine (R) to tryptophan (W) at that position. In pP53 <sup>$O^6$ -meG</sup>, a single  $O^6$ -meG was positioned on the template strand within codon 248 (Fig. S1). If this lesion were repaired by AGT, mRNA would be produced that encode wild-type p53 protein. However, transcriptional bypass of the unrepaired lesion would result in the misincorporation of uridine into nascent mRNA, yielding the tryptophan codon *UGG* and the expression of



**Fig. 1.** Overview of the p53 TM vectors. The vectors used in this study contained two genes, *TP53* and *GFP*, controlled by two independent  $P_{TIGHT}$  promoters. (A) The pP53<sup>WT</sup> vector contained the *TP53* wild-type sequence. (B) The pP53 <sup>$O^6$ -meG</sup> vector contained a site-specific  $O^6$ -meG (G\*) opposite the 5' C in codon 248. (C) pP53<sup>RW</sup> contained a single C>T base change from pP53<sup>WT</sup> in codon 248, resulting in the expression of the R248W p53 mutation. *GFP* was used as a reporter gene. Restriction sites of BsaWI, which was used to confirm presence of  $O^6$ -meG (Fig. S2), are also indicated. (D, Left) Representative blot of p53 protein. Mean relative p53 protein expression levels are shown below the blot ( $n = 4$ ). CDK2 was used as a loading control. (Right) Real-time qPCR analysis of p53 mRNA (mean  $\pm$  SE,  $n = 3$ ) from sorted GFP-positive cells 24 h post transfection.

**Table 1.** Analysis of uridine incorporation at codon 248 and the position corresponding to the site-specific  $O^6$ -meG (c.742)

| Plasmid   | No. transcripts analyzed | U incorporation at codon 248, % |
|---|--------------------------|---------------------------------|
| pP53 <sup>WT</sup>                                | 7,410                    | 0.05 $\pm$ 0.03 <sup>†</sup>    |
| pP53 <sup>RW</sup>                                | 7,639                    | 99.9 $\pm$ 0.02*                |
| pP53 <sup><math>O^6</math>-meG</sup>              | 2,333                    | 0.14 $\pm$ 0.14                 |
| pP53 <sup><math>O^6</math>-meG</sup> + $O^6$ -bzG | 1,513                    | 14.7 $\pm$ 1.0* <sup>†</sup>    |

\* $P < 0.0001$  compared with pP53<sup>WT</sup>.

<sup>†</sup> $P < 0.0001$  compared with pP53 <sup>$O^6$ -meG</sup> (one-way ANOVA).

<sup>‡</sup>Mean  $\pm$  SE,  $n = 3$ .

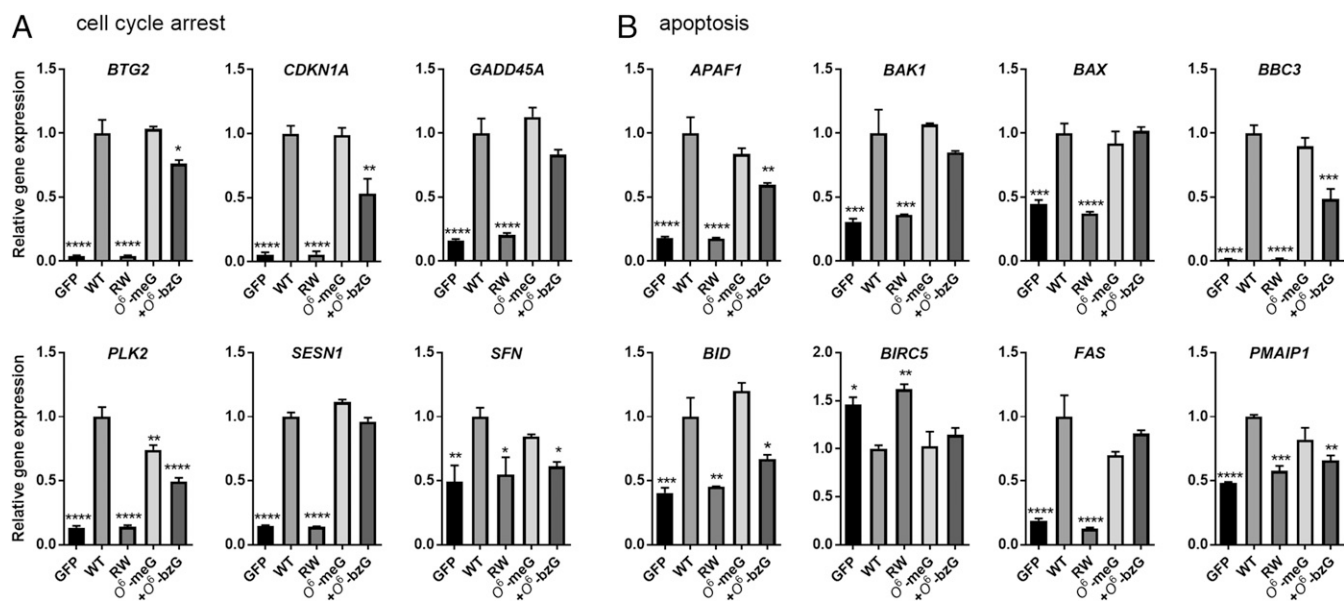
mutant p53 RW protein. Note that the mutation at codon 248 in exon 7 (c.742C>T, p.R248W) is indeed a hotspot mutation with dominant-negative effects over wild-type p53 and is associated with tumor formation (27, 28).

The presence of the  $O^6$ -meG in the pP53 <sup>$O^6$ -meG</sup> vector was verified by its essentially complete resistance to digestion by BsaWI (Fig. 1 and Fig. S2). Importantly, the plasmids used in these studies contain no known mammalian origin of replication, thereby limiting the effects of plasmid replication that would potentially mask TM (11).

**$O^6$ -MeG Induces TM.** For transcripts resulting from human RNA polymerase II (RNAPII) transcription past  $O^6$ -meG in vitro, 21.3  $\pm$  5.4% (mean  $\pm$  SE,  $n = 3$ ) contained a base that blocked digestion by BsaWI (Fig. S3). To determine the extent of TM induced by  $O^6$ -meG in vivo, *TP53* transcripts from H1299 cells transfected with one of the three p53 vectors were analyzed by RNA-sequencing (RNA-seq). These cells, which are p53-null for both alleles and are a common model for studying the effects of p53 mutagenesis (20), were used to avoid any interfering effects from endogenous p53. Cells expressing wild-type p53 protein displayed nominal uridine incorporation at codon 248 (c.742) in 0.05% of the transcripts, while 99.9% of the *TP53* transcripts from cells expressing the mutant p53 RW protein contained the expected uridine at this position (Table 1). In cells transfected with the vector containing  $O^6$ -meG, uridine incorporation opposite the lesion was 0.14%, a value that increased 100-fold to 14.7% ( $P < 0.0001$ ) when cells were treated with  $O^6$ -benzylguanine ( $O^6$ -bzG; 10  $\mu$ M), which inactivated cellular AGT activity >95% (Table S1). A low number of transcripts (0.02–0.06%) contained an adenosine or guanosine at this site (Table S2).

**TM Reduces p53-Dependent Transactivation.** To determine if TM of p53 mRNA resulted in altered protein function, the transactivation of 15 established target genes that play a central role in the tumor-suppressor function of p53 through their regulation of cell-cycle arrest and apoptosis was tested (15). Importantly, analysis of p53 in transfected cells showed similar mRNA and protein expression levels in all conditions (Fig. 1D), and pretreatment of cells with  $O^6$ -bzG did not affect these expression levels or induce cytotoxicity (Fig. S4).

The results showed that TM at codon 248 reduced the expression of genes involved in the regulation of cell-cycle arrest and apoptosis (Fig. 2). Reduced expression levels of *BTG2*, *CDKN1A*, *PLK2*, and *SFN* were obtained in cells when AGT was inhibited compared with wild-type p53-expressing cells ( $P < 0.05$ – $0.0001$ ) (Fig. 2A). The expression of *PLK2* was even reduced in cells with active AGT ( $P < 0.01$ ). *GADD45A* and *SESNI* only showed moderate reductions in expression levels, and no effects of *MDM2* on gene-expression levels were observed in response to either vector (Fig. S4). *BTG2*, *PLK2*, and *SESNI* are involved in the G1/S checkpoint, *GADD45A* and *SFN* are involved in the G2/M checkpoint, and *CDKN1A* is involved in both (15). The strong impact on the transactivation of *BTG2*, *CDKN1A*, and *PLK2* suggests a TM-mediated loss of the G1/S cell-cycle checkpoint.



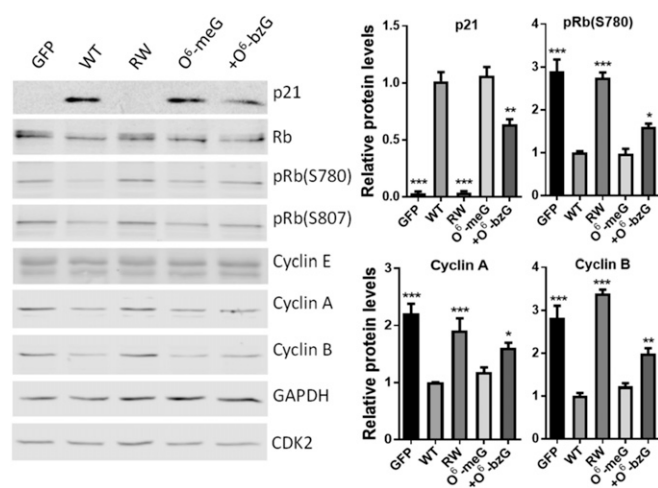
**Fig. 2.** Transcriptional mutagenesis reduces p53-dependent transactivation. Quantitative analysis of gene-expression levels of p53 target genes regulating cell-cycle arrest (A) and apoptosis (B) 24 h after transfection. Data show the mean  $\pm$  SE ( $n = 3$ ) normalized to the expression level in response to wild-type p53 (WT). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  by one-way ANOVA.

For genes with a function in regulating apoptosis, TM at codon 248 resulted in reduced transactivation of *APAF1*, *BBC3*, *BID*, and *PMAIP1* ( $P < 0.05$ – $0.001$ ) (Fig. 2B) but had no significant effect on expression levels of *BAK1*, *BAX*, *BIRC5*, or *FAS*. Most of the affected genes are involved in the intrinsic pathway of apoptosis (29), implying that TM of p53 may well reduce the activation of this route of programmed cell death. As expected, the expression levels of all genes studied were strongly affected in response to expression of the p53 RW mutant compared with wild-type p53 ( $P < 0.05$ – $0.0001$ ) (Fig. 2). Together, these results demonstrate that expression of mutant p53 protein due to TM can impair the transactivation of genes that are important for effective p53 tumor-suppressor activity.

**TM of p53 Impairs Its Control of Cell-Cycle Arrest.** Given the central role of p21 (encoded by *CDKN1A*), in the p53-dependent regulation of cell-cycle arrest, the effect of p53 TM on p21 protein level was analyzed. The reduced transactivation described above resulted in reduced p21 protein levels ( $P < 0.01$ ) (Fig. 3). Gene-expression data indicated that TM of p53 resulted in the loss of a functional G1/S cell-cycle checkpoint. The G1/S checkpoint is controlled by p53/p21 through a negative regulation of phosphorylation of retinoblastoma (Rb) (30). Phosphorylation of Rb (pRb) at certain serine sites releases and activates transcription factor E2F, resulting in the induction of cyclins that further drive the G1/S transition (31). In agreement with the reduced levels of p21, TM of p53 resulted in increased levels of pRb and cyclin A compared with cells expressing wild-type p53 ( $P < 0.05$ ) (Fig. 3). The same effects were observed in cells expressing only GFP or the p53 RW mutant ( $P < 0.001$ ). No effect on protein levels of cyclin E was found. TM of p53 also resulted in increased protein levels of mitotic cyclin B, indicating a loss of the G2/M checkpoint ( $P < 0.01$ ) (Fig. 3).

Loss of checkpoint control was further confirmed by cell-cycle analysis. Detection of BrdU incorporation showed an approximately twofold reduction of cells in S phase in response to wild-type p53 compared with cells expressing only GFP ( $P < 0.0001$ ) (Table 2 and Fig. S5). This was also associated with an increased number of cells in G1 and G2/M ( $P < 0.0001$ ), which is in agreement with the role of p53 in checkpoint activation and cell-cycle arrest. In cells with inactivated AGT,  $O^6$ -meG-induced TM reduced the ability of p53 to arrest the cell cycle, resulting in a higher number

of cells that transitioned into S phase compared with wild-type p53 and p53  $O^6$ -meG in cells with active repair ( $P < 0.0001$ ). An equivalent reduction of cells in G1 and G2/M was also seen ( $P < 0.0001$ ). Notably, the  $\chi^2$  test showed that control of the G1/S checkpoint also was diminished in response to  $O^6$ -meG in cells with active AGT, resulting in a small increase in cells that transitioned from G1 to S compared with wild-type p53 ( $P < 0.0001$ ) (Table 2). As expected, expression of the p53 RW mutant had no effect on the cell-cycle distribution compared with cells expressing only GFP. Together, these results demonstrate that p53 TM impairs the control of cell-cycle arrest via deregulated p21/Rb signaling, resulting in a reduced function of p53 as a tumor suppressor.



**Fig. 3.** Transcriptional mutagenesis of p53 impairs its control of cell-cycle arrest. Representative blots (Left) and densitometry analysis (Right) of protein levels 24 h post transfection. CDK2 was used as a loading control for total Rb, pRb (S807), and cyclin B and E. GAPDH was used for p21, pRb (S780), and cyclin A. Densitometry data show the mean  $\pm$  SE ( $n = 3$ – $4$ ) normalized to protein levels in response to wild-type p53 (WT). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  in comparison with p53 WT by one-way ANOVA.

**Table 2. Cell-cycle distribution of H1299 cells in response to p53 status**

| Plasmid  | Cell-cycle phase, %       |                           |                          |
|--|---------------------------|---------------------------|--------------------------|
|  | G <sub>1</sub>            | S                         | G <sub>2</sub> /M        |
| pGFP   | 68.9 ± 1.5 <sup>§</sup>   | 17.2 ± 2.1                | 8.3 ± 1.3                |
| pP53 <sup>WT</sup>   | 74.6 ± 3.2*               | 9.2 ± 1.3*                | 9.7 ± 2.3*               |
| pP53 <sup>RW</sup>   | 70.0 ± 1.9                | 17.3 ± 2.5                | 7.9 ± 1.8                |
| pP53 <sup>O<sup>6</sup>-meG</sup>                          | 74.3 ± 2.5 <sup>†</sup>   | 10.0 ± 1.0 <sup>†</sup>   | 9.9 ± 1.6                |
| pP53 <sup>O<sup>6</sup>-meG</sup> +<br>O <sup>6</sup> -bzG | 73.5 ± 1.2 <sup>†,*</sup> | 14.2 ± 1.4 <sup>†,*</sup> | 7.4 ± 0.5 <sup>†,*</sup> |

\* $P < 0.0001$  in comparison with pGFP.

<sup>†</sup> $P < 0.0001$  in comparison with pP53<sup>WT</sup>.

<sup>‡</sup> $P < 0.0001$  in comparison with pP53<sup>O<sup>6</sup>-meG</sup> by  $\chi^2$  test.

<sup>§</sup>Mean ± SE,  $n = 4$ .

**TM of p53 Impairs Induction of Apoptosis.** Further studies of the effects of p53 TM on the induction of apoptosis showed that protein levels of Apat1, PUMA (*BBC3*), and Bid, whose transactivations were reduced (Fig. 2), were not affected by p53 status (Fig. S5). However, the impact of p53 on the induction of cell death could be discerned by differences in sub-G<sub>1</sub> populations. Expression of wild-type p53 increased the percentage of dead cells from  $2.4 \pm 0.3\%$  (mean ± SE,  $n = 4$ ) to  $6.1 \pm 1.4\%$  compared with expression of GFP ( $P < 0.05$ ). In comparison, expression of the p53 RW mutant or the presence of O<sup>6</sup>-meG in cells with inactive AGT had no significant impact on cell viability ( $2.4 \pm 0.2\%$  and  $3.5 \pm 0.5\%$ , respectively).

To examine the effect of p53 TM on the induction of apoptosis, the impact on two hallmarks of apoptosis, loss of mitochondria membrane potential (MMP) and caspase activation, were measured (Fig. 4). Expression of wild-type p53 (from either pP53<sup>WT</sup> or pP53<sup>O<sup>6</sup>-meG</sup> with active AGT) resulted in a 1.5- to twofold reduction of MMP ( $P < 0.001$ ), while the p53 RW mutant had no effect compared with cells transfected with the GFP vector (Fig. 4A and B). Importantly, inactivation of AGT and induction of TM reduced this effect, resulting in increased MMP compared with wild-type p53 ( $P < 0.05$ ). The reduced ability of p53 to activate apoptosis due to TM was further supported by the observation that the expression of wild-type p53 increased cellular caspase activity nearly 2.5-fold ( $P < 0.001$ ) (Fig. 4C) compared with the expression of GFP. Similar to the MMP, TM of p53 due to inactivation of AGT strongly reduced the caspase activity compared with wild-type p53 ( $P < 0.001$ ). The results from these two endpoints demonstrate that TM reduces the ability of p53 to activate apoptosis and consequently affects the function of p53 as a tumor suppressor.

## Discussion

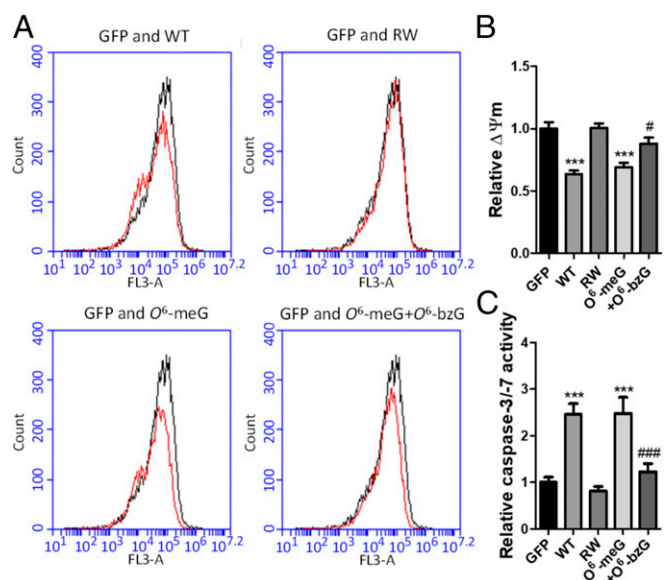
The data presented here show that the presence of DNA lesions on the transcribed strand in mammalian cells can induce TM and subsequent production of a protein that exhibits altered function, especially when DNA repair is absent. This, in turn, can result in downstream effects on the cellular phenotype that could lead to pathology. Specifically, O<sup>6</sup>-meG can induce TM of p53 when AGT is lacking, resulting in the expression of mutant p53 protein that attenuates its tumor-suppressor function by deregulating cellular signaling that is crucial in controlling cell-cycle checkpoints and apoptosis (Fig. 5).

In agreement with earlier studies performed in vitro and in vivo (7, 11, 12, 32, 33), this work demonstrates that O<sup>6</sup>-meG is mutagenic during transcription. Furthermore, DNA repair yields a significant impact on the frequency of TM induced by O<sup>6</sup>-meG, an observation that has been made for other DNA lesions (9, 12, 33, 34). As reported here, RNA-seq analysis indicated that 14.7% of p53 transcripts from cells lacking AGT activity contained a uridine misincorporation at codon 248, a number that was 100-fold lower in cells exhibiting AGT activity. These numbers are lower than

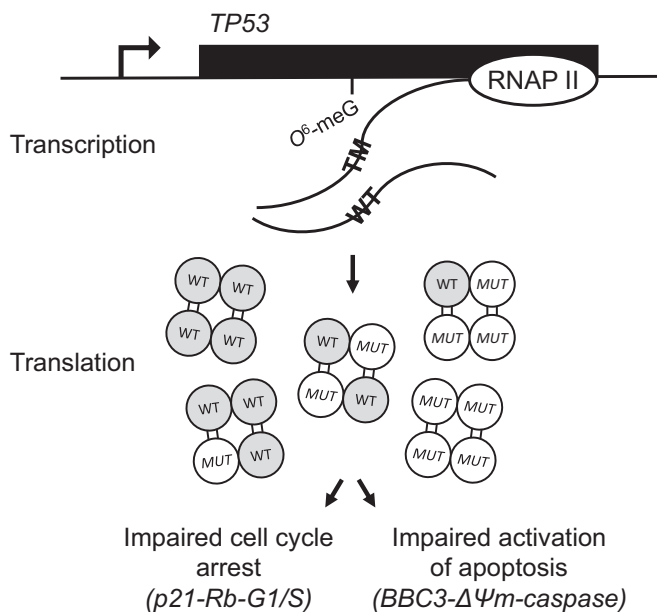
previously reported levels in vivo (11, 12), which could be explained by the different cell systems used and/or the DNA sequence context of the lesion (35). The low levels of misincorporation (<1%) observed when AGT is active could reflect errors occurring during library preparation and sequencing (36). To confirm that the low levels of uridine misincorporation are indeed true TM events, a more accurate RNA-seq protocol would be required (37, 38).

Expression of AGT, which is encoded by the *MGMT* gene, varies considerably among human organs, with the highest expression in liver and the lowest in brain and lung tissue (39), suggesting that some organs will be more sensitive to alkylating agents than others. The AGT activity in H1299 cells following pretreatment with O<sup>6</sup>-bzG was one order of magnitude lower than the lowest reported activity in normal tissues (39). Indeed, loss or reduced expression of *MGMT* is associated with increased susceptibility to alkylating chemicals and with the appearance of *TP53* and *KRAS* mutations in human tumors, including colorectal and non-small-cell lung carcinomas (25, 40). Lack of AGT activity has been found in human tumors (e.g., from brain and lung) and has been suggested to be an early driving event during tumorigenesis (41, 42), and TM might well be one aspect of such events. In addition, AGT plays an important role in the treatment of cancer, since the loss of *MGMT* in malignant tissue is associated with increased sensitivity to alkylating chemotherapeutic agents such as carmustine and temozolomide (25, 40). Expression levels and activity of AGT are thus important determinants for replicative mutagenesis in DNA and transcriptional mutagenesis in RNA and for the resulting detrimental biological effects.

Although transcriptional errors are deemed as transient, available data show that they can instigate splicing defects, heritable reprogramming of transcriptional networks, and shortening of cellular lifespan in bacteria and yeast (43–45). Moreover, chronic



**Fig. 4. Transcriptional mutagenesis of p53 impairs its induction of apoptosis.** (A) Measurement of MMP ( $\Delta\Psi_m$ ) by flow cytometry using tetramethylrhodamine ethyl ester (TMRE) staining 24 h after transfection. Representative histograms show each vector (red) compared with cells expressing only GFP (black). (B) Quantitative analysis of TMRE fluorescence intensity. Data show the mean ± SE ( $n = 3-4$ ) of median intensities normalized to that of cells expressing only GFP. Results using the positive control carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) are shown in Fig. S5. (C) Quantitative analysis of caspase-3/-7 activity 24 h after transfection. Data show the mean ± SE ( $n = 4$ ) of fluorescence intensities normalized to the total protein concentration and the fluorescence signal of cells expressing only GFP. \*\*\* $P < 0.001$  in comparison with GFP; # $P < 0.05$  and ### $P < 0.001$  in comparison with wild-type p53 (WT) by one-way ANOVA.



**Fig. 5.** *O*<sup>6</sup>-meG-induced transcriptional mutagenesis can reduce p53 tumor-suppressor function by impairing control of cell-cycle arrest and apoptosis.

transcription errors, such as transcriptional slippage, can both ameliorate and contribute to disease development in mammalian systems (46, 47). The present study and previous reports clearly show the additional impact DNA lesions can have on transcription elongation and its fidelity, with consequences such as abrogated mRNA expression and a significantly increased error rate (reviewed in refs. 48 and 49). Therefore, lesion-induced transcriptional errors could lead to significantly higher expression levels of transcripts that encode mutated proteins with gain or loss of function, thereby contributing to disease development. The same mutated proteins could also function as tumor neoantigens, increasing cellular immunity; such events might be exploited for improved immunotherapy (50).

Unlike many tumor-suppressor genes, which are inactivated through deletions during tumorigenesis, *TP53* is often found to undergo single-base missense mutations, many of which are dominant negative, thus making it an especially susceptible target for TM (16). Mutations in the DNA-binding domain of p53, to which the RW mutation at codon 248 belongs, strongly reduce the DNA-binding affinity and thus the ability to transactivate target genes (51, 52). The data reported here show that expression of the RW p53 mutant due to TM in 15% of the transcripts resulted in reduced transactivation of *CDKN1A/p21* with a concomitant loss of a functional G1/S cell-cycle checkpoint in H1299 cells. This is in accordance with a previous study expressing similar levels of wild-type and RW-mutant p53 from a dual inducible expression system in H1299 cells (20). In contrast, the same mutation only exerted dominant-negative effects on *BAX* transactivation and downstream activation of apoptosis in CaLu 6 and Saos-2 cells using a similar dual expression system (53). Thus, the impact of a dominant-negative p53 mutation on the regulation of cell-cycle arrest and apoptosis clearly depends on which biological system is studied and the level of p53 expressed (14, 22).

A mutant allele in the DNA-binding domain of p53 is often adequate to exert a dominant-negative effect associated with an increased susceptibility to develop cancer due to the dimer-of-dimers organization of p53 (54, 55). The results reported here demonstrate that a mutation at codon 248 in 15% of the transcripts is sufficient to significantly reduce the tumor-suppressor function of p53. Accordingly, with the dimer-of-dimers organization, this would

correspond to 28% of the expressed p53 protein being inactivated due to TM. However, assuming that the presence of one mutant monomer within the dimer-of-dimers structure is enough to inactivate the function of p53, which to the best of our knowledge has not been assessed, 48% of p53 activity would be highly compromised. This latter number is in agreement with the effects of p53 TM on the transactivation of *CDKN1A*.

There is a prior study showing that TM can lead to phenotypic changes in mammalian cells related to tumorigenesis (9). TM caused by the presence of 8-oxoguanine (8-oxoG) in codon 61 of the oncogene *HRAS* in mouse embryonic fibroblasts that lack 8-oxoG glycosylase, an enzyme that catalyzes the removal of 8-oxoG from the genome, increased the expression of mutated, constitutively active Ras protein (9). This gave rise to sustained downstream oncogenic signaling through the mitogen-activated protein kinase pathway. This is in accordance with the data presented here showing that TM induced by *O*<sup>6</sup>-meG can initiate the inactivation of a tumor suppressor. Importantly, this work extends previous studies by showing again that TM induces changes in protein function and deregulation of downstream signaling events and adds evidence that the expression of mutant protein due to TM affects cellular processes that are important for the maintenance of tissue homeostasis. That significant biological effects are observed when TM occurs in only 15% of the p53 transcripts demonstrates that low frequencies of TM can induce significant phenotypic changes in mammalian cells. This has important implications for the potential role of TM in disease development. Hence, TM may well play an important role in allowing initiated cells to escape growth-inhibitory or apoptotic signals, especially when DNA repair is missing, thus leading to additional tumor growth and aggressiveness. Indeed, the loss of p53 plays an important role in the progression of colorectal and prostate cancers (56, 57).

## Materials and Methods

**Construction of Vectors.** The p53 vectors were constructed on the basis of our previous work (11) and were adapted to the present study to obtain the final plasmids pP53<sup>WT</sup> and pP53<sup>RW</sup>. Generation of a vector containing a site-specific base modification, pP53<sup>O<sup>6</sup>-meG</sup>, involved the production of ssDNA, annealing of a site-specific modified 11-mer oligo to a gapped duplex construct, ligation, and purification of closed circular dsDNA, as described previously (11, 34). For a more detailed description of this procedure and the *in vitro* transcription, see [Supporting Information](#). The sequences of all oligonucleotide primers employed are presented in [Table S3](#).

**Cell Culture and Transfection.** The human p53-null lung carcinoma H1299 cell line (CLR-5803; ATCC) was cultured in DMEM (Gibco by Life Technologies) supplemented with 10% FBS, 1 mM L-glutamine, and penicillin/streptomycin in a humidified 5% CO<sub>2</sub> incubator at 37 °C and was used between passages 6 and 16. Cells were transiently transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. For all assays, cells were harvested with trypsin 24 h after transfection. Transfection efficiencies of the different vectors were 30–50%. *O*<sup>6</sup>-meG repair by AGT was inhibited by 1 h of preincubation with 10 μM *O*<sup>6</sup>-bzG (in DMSO; Santa Cruz Biotechnology) as previously described (11, 12). Measurement of cellular AGT activity was performed as described in [Supporting Information](#).

**RNA-Seq.** RNA was isolated as described above, and samples with an RNA integrity number >9 (Agilent 2200 TapeStation) were used for preparation of Illumina sequencing libraries using the TruSeq Stranded mRNA kit (Illumina) and subsequently were sequenced on a HiSeq 2500 system (Illumina) with at least 14 × 10<sup>6</sup> sequenced read pairs per sample. Following download of FASTQ files, reads were mapped using RNA-Seq v.2.4.0i (58) to the reference genome hg38, downloaded from Ensembl. Alignments mapped to the genomic region corresponding to the coding strand of p53 were selected and analyzed using custom Perl scripts. The script counted bases present at the specified position in each library, resulting in a table with counts and frequencies of the different bases. Only bases with a quality score >20 were considered.

**Sorting and Analyses of GFP-Positive Cells.** *GFP* was used as a reporter gene for transfected cells, and positive cells were sorted using a Beckman Coulter MoFlo XDP or a BD FACS Vantage Diva system. The sorted cells were used for

RT-qPCR, Western blotting, and cell-cycle analysis [propidium iodide (PI)/BrdU] as described in [Supporting Information](#).

**Assessment of Apoptosis.** Measurements of MMP ( $\Delta\Psi_m$ ) and caspase activity were performed according to protocols described in [Supporting Information](#).

**Statistics.** All data presented are means  $\pm$  SE from three or four independent experiments. Student's *t* test, one-way ANOVA with Dunnett's correction, or a  $\chi^2$  test was used to determine statistical significance ( $P < 0.05$ ).

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