8-Oxoguanine-mediated transcriptional mutagenesis causes Ras activation in mammalian cells

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8-Oxoguanine (8OG) is efficiently bypassed by RNA polymerases *in vitro* and in bacterial cells *in vivo*, leading to mutant transcripts by directing incorporation of an incorrect nucleotide during transcription. Such transcriptional mutagenesis (TM) may produce a pool of mutant proteins. In contrast, transcription-coupled repair safeguards against DNA damage, contingent upon the ability of lesions to arrest elongating RNA polymerase. In mammalian cells, the Cockayne syndrome B protein (Csb) mediates transcription-coupled repair, and its involvement in the repair of 8OG is controversial. The DNA glycosylase Ogg1 initiates base excision repair of 8OG, but its influence on TM is unknown. We have developed a mammalian system for TM in congenic mouse embryonic fibroblasts (MEFs), either WT or deficient in Ogg1 (ogg$^{-/-}$), Csb (csb$^{-/-}$), or both. This system uses expression of the Ras oncogene in which an 8OG replaces guanine in codon 61. Repair of 8OG restores the WT sequence; however, bypass and misinsertion opposite this lesion during transcription leads to a constitutively active mutant Ras protein and activation of downstream signaling events, including increased phosphorylation of ERK kinase. Upon transfection of MEFs with replication-incompetent 8OG constructs, we observed a marked increase in phospho-ERK in ogg$^{-/-}$ and csb$^{-/-}$ogg$^{-/-}$ cells at 6 h, indicating persistence of the lesion and the occurrence of TM. This effect is absent in WT and csb$^{-/-}$ cells, suggesting rapid repair. These studies provide evidence that 8OG causes TM in mammalian cells, leading to a phenotypic change with important implications for the role of TM in tumorigenesis.

DNA repair | MAP kinase | Csb | transcription-coupled repair | tumorigenesis

Cells use numerous mechanisms to neutralize various reactive species before they can damage DNA, as well as overlapping pathways to repair the damage, thus protecting genomic integrity. DNA damage can result in a number of potential outcomes for a mammalian cell (1), including permanent fixation of the damage during replication, leading to a heritable mutation. Replication-centric models of mutagenesis have provided valuable information regarding routes of mutagenesis under conditions of continuous cell growth and replication. These models, however, largely ignore transcription, the other major nucleic acid transaction essential to the cell. In slowly growing or nondividing cells, in which DNA replication is greatly diminished or altogether absent (e.g., terminally differentiated mammalian cells [2]), transcription must continue to provide the cell with the proteins necessary for normal physiologic processes. As such, the interaction of DNA damage with the transcription machinery occurs more frequently than with the replication apparatus.

Many types of DNA damage are repaired with greater efficiency if they occur in the template strand of a transcribed gene rather than the nontemplate strand or a nontranscribed region of the genome (3, 4). Bulky lesions that distort the DNA helix will arrest RNA polymerase (RNAP) at the site of damage, promoting transcription-coupled repair (TCR) and allowing the generation of full-length, normal transcripts (5, 6). However, frequently occurring nonbulky lesions, such as 8-oxoguanine (8OG), uracil, and O^6-methylguanine, are efficiently bypassed by RNAPs *in vitro* (7, 8). In these instances, incorporation of incorrect bases opposite the damage would lead to mutant transcripts that could direct the synthesis of mutant proteins, a process termed *transcriptional mutagenesis* (TM) (6).

Experiments addressing TM have largely used *in vitro* assays with purified components, and most have focused on phage or bacterial transcription systems (ref. 6 and references therein). In the few studies conducted using *in vitro* mammalian systems (9–11), in which efficient bypass of DNA damage by RNAP was established, the mutagenic potential was not explored. One exception is a study using mammalian RNAP II suggesting that 8OG does indeed have mutagenic potential *in vitro* (12). The small number of *in vivo* studies conducted to date have relied on luciferase reporter assays in bacterial cells to gauge TM caused by uracil or 8OG (13, 14). Interestingly, a recent study of transcriptional encounters with 8,5′-cyclo-2′-deoxyadenosine and cyclobutane pyrimidine dimers in mammalian cells indicated that even bulky lesions that normally pose a significant block to transcription can be occasionally bypassed with mutagenic effects (15).

We have developed an *in vivo* mammalian system for studying a TM-driven biologic change with a relevant cellular outcome by using the Ras oncogene. Ras proteins comprise a family of small GTPases that serve as molecular switches, bridging the gap between extracellular signals transduced through receptor tyrosine kinases and downstream signaling pathways that ultimately influence cell proliferation or death (reviewed in ref. 16). There are three classic isoforms of Ras (H-, K-, and N-Ras), and nearly one third of all human cancers have a mutation in one of these Ras genes (17). One hotspot for transforming mutations is the codon for glutamine 61. Mutations at this site compromise the Ras GTPase activity, maintaining the protein in the active, GTP-bound state (18). This increased activation leads to measurable downstream events, including increased phosphorylation and activation of components of the MAPK pathway. Such mutant Ras proteins are dominant, exerting their effects on cellular outcomes even in the presence of WT Ras. That a single point mutation in the Ras gene is sufficient for its transforming potential makes it particularly amenable to TM studies.

8OG, a frequently occurring oxidative lesion, is mutagenic for DNA polymerases, and cells unable to efficiently repair this lesion accrue numerous GC>TA mutations (19, 20). The major cellular defense against 8OG is the base excision repair (BER) pathway, initiated by 8-oxoguanine glycosylase (Ogg1). Mice lacking the gene for this protein, however, demonstrate slow repair of the lesion, suggesting that an alternate repair pathway

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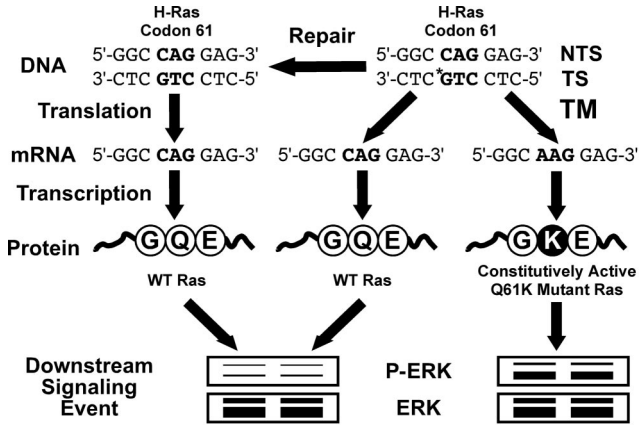

Fig. 1. Mammalian TM system. WT, codon 61 transcription results in a glutamine (Q) upon translation (*Left*). However, if an 8OG residue (*) replaces G at the first base position, it can direct the incorporation of either C or A into the nascent mRNA (*Right*). If a C is incorporated, the resulting translation product will be a WT Ras with Q at position 61. However, if an A is incorporated, the Ras generated will contain a lysine (K) at this position and be constitutively active, resulting in an elevation in cellular levels of phosphorylated ERK (P-ERK). Codons and corresponding amino acids flanking position 61 are also indicated. NTS, nontranscribed strand; TS, transcribed strand.

exists (19). The identity of this pathway has been the subject of some controversy, although TCR has been implicated given that mice lacking the gene for the TCR Cockayne syndrome B (Csb) protein in an Ogg1 knockout background accumulate more genomic mutations and oxidative damage with age than mice lacking Ogg1 alone (21, 22). With the mammalian TM system reported here the respective contributions of Ogg1-mediated BER vs. Csb-mediated TCR in the repair of 8OG lesions can be directly compared in congenic mouse embryonic fibroblasts (MEFs). Our results have important implications for the role of DNA damage-driven TM in tumorigenesis, as well as the pathways involved in the *in vivo* repair of 8OG.

Results

Development of a Mammalian Model System for Assessing TM. 8OG was used in place of G in the first base position (template strand) of Ras codon 61, a hotspot for cancer-associated mutations (17). If the lesion is repaired, the normal coding sequence will be restored, resulting in a WT Ras protein (Fig. 1). If the lesion is bypassed during transcription, however, at least two potential populations of transcripts are expected on the basis of the coding properties of 8OG: either C insertion, which will also generate a normal transcript and WT protein, or mutagenic A insertion, resulting in generation of the constitutively active Q61K mutant protein capable of activating the MAPK cascade and elevating the phosphorylation of ERK1/2.

8OG Causes TM in Mammalian Cells Deficient in Ogg1. To assess the ability of 8OG to cause TM, cell lines either WT in their repair capacities or deficient for Ogg1 or Csb or both were transfected with an 8OG-containing Ras construct (Fig. 1) that is replication incompetent (23). Transfections lacking DNA were done to assess the background level of phospho-ERK (P-ERK) in the absence of Ras overexpression; normal and mutant constructs were also used as controls to provide the lower and upper limits of the P-ERK signal. Cells were plated and incubated for 6 h, the final 1.5 h in media lacking serum, which greatly reduced the background level of P-ERK resulting from growth factors in serum. Protein lysates were resolved by SDS-PAGE and subjected to Western analysis (Fig. 2*A*). Signals for P-ERK, total ERK, and Ras expression level were quantified and normalized

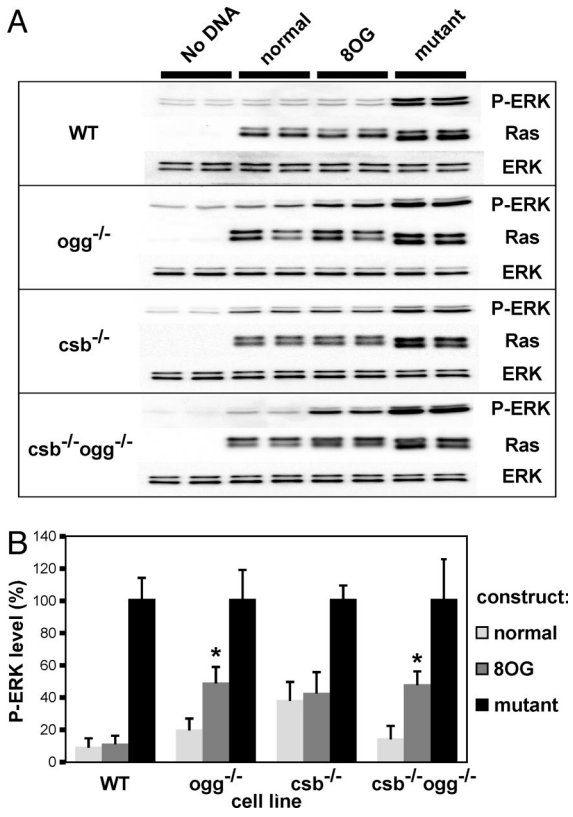

Fig. 2. 8OG causes TM in $ogg^{-/-}$ and $csb^{-/-}ogg^{-/-}$ cells. (*A*) Representative Western analysis of lysates from transfected cells. Transfections were done in duplicate. Specific antibodies were used to detect P-ERK, Ras, and total ERK (*Materials and Methods*). (*B*) Bands from Western blots were quantified and normalized as described in the text. P-ERK levels relative to those obtained with the mutant construct (set to 100% for each cell line) are plotted. Error bars represent standard deviations from six to eight independent transfection experiments. *Signal obtained with 8OG construct was statistically significantly different from that observed with the normal construct ($P < 0.05$), as assessed by Student's *t* test. Differences between the mutant construct and normal construct were significant in all cell lines.

as described in *Materials and Methods* (Fig. 2*B*). In $ogg^{-/-}$ and $csb^{-/-}ogg^{-/-}$ cells, the normalized P-ERK signal increases significantly when cells are transfected with the 8OG-containing construct compared with the normal construct, reflecting the presence of mutant, constitutively active Ras. In contrast, WT and $csb^{-/-}$ cells showed no increase in P-ERK for 8OG-containing construct transfections over normal, indicating that the vast majority of Ras being produced in these cells is WT, most likely owing to repair of the lesion. These results indicate that 8OG causes TM in mammalian cells, resulting in activation of the MAPK cascade and an increase in ERK phosphorylation. It is unclear why the background level of P-ERK is higher in $csb^{-/-}$ cells when WT Ras is overexpressed, but we observed no significant difference in ERK phosphorylation upon transfection with the normal or 8OG-containing constructs.

Mutant Transcript Levels are Elevated in $ogg^{-/-}$ and $csb^{-/-}ogg^{-/-}$ Cells. Because the cellular half-life of Ras protein is ≈24 h (24), our Western analysis measurements of P-ERK at 6 h after transfection could result from early transcription past the lesion followed by repair. Thus, RNA was isolated at earlier times (2 h after transfection) to observe RNAP bypass events at 8OG before substantial repair could occur. cDNA was generated, subcloned into pUC18, and subjected to a PCR screen to identify transcripts with mutant sequence (Table 1). Despite the lack of

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Table 1. Mutant transcript analysis

Cell line	No. of clones analyzed	No. of mutant transcripts (%)*	Mutant class	No. of clones (% of mutant)
WT	229	6 (2.6 ± 1.8)	C to A	2 (33)
			Δ 1 nt	4 (67)
ogg$^{-/-}$	232	33 (13.9 ± 2.2)†	C to A	28 (85)
			Δ 1 nt	5 (15)
csb$^{-/-}$	237	6 (2.6 ± 2.1)	C to A	2 (33)
			Δ 1 nt	4 (67)
csb$^{-/-}$ogg$^{-/-}$	239	26 (10.8 ± 3.1)†	C to A	20 (77)
			Δ 1 nt	6 (23)

*Average of three experiments ± standard deviation.
†$P < 0.05$ vs. WT (Student's *t* test).

P-ERK elevation by Western analysis, a small percentage of mutant transcripts was detectable in the WT and csb$^{-/-}$ cells (≈3%), indicating that at 2 h some of the 8OG remained, even in cells with a normal DNA repair background. We observe an approximately fourfold increase in the proportion of mutant transcripts identified in the ogg$^{-/-}$ and csb$^{-/-}$ogg$^{-/-}$ cells, consistent with the elevation of P-ERK observed by Western analysis (Fig. 2). A PCR screen of cDNAs from all cell lines after transfection with the normal construct identified 2 clones that did not amplify with the WT primer (out of 368 analyzed). Sequencing indicated that both contained a single-nucleotide deletion in the previous codon (codon 60) and no change at the nucleotide of interest.

Previous TM experiments carried out in *Escherichia coli* revealed that 8OG not only directed the incorporation of C or A into the nascent mRNA but also resulted in transcripts containing a one-nucleotide deletion (Δ 1 nt) at this site (13). Because the nucleotide downstream of that which would be incorporated opposite the 8OG is also an A, the PCR screen using the mutant-specific primer would not distinguish between an A insertion or this single-nucleotide deletion. Thus, the mutant clones identified in the screen were sequenced. Intriguingly, in cells with a functional Ogg1 protein (WT and csb$^{-/-}$), although the number of mutant transcripts identified was small, the majority (≈67%) was of the Δ 1 nt class. In cells in which the Ogg1 is absent (ogg$^{-/-}$ and csb$^{-/-}$ogg$^{-/-}$), however, the majority of mutant transcripts (≈80%) contain the C-to-A mutation expected for the miscoding properties of 8OG, indicating that repair of this lesion is compromised.

ogg$^{-/-}$ and csb$^{-/-}$ogg$^{-/-}$ Cells Are Compromised for Repair of 8OG. Unlike in *E. coli*, in which the percentages of transcripts containing C and A insertions or the Δ 1 nt deletion were approximately equivalent (13), we observed a bias toward transcripts with the correctly inserted C in the MEFs used here. One possible reason for this observation is that 8OG is rapidly being repaired to restore the normal coding sequence, despite the reported severe repair defect in ogg$^{-/-}$ and csb$^{-/-}$ogg$^{-/-}$ cells, indicating an additional route for handling 8OG in these cells. To address this possibility, the 8OG-containing construct was reisolated from all cell lines at various times after transfection and analyzed for repair of 8OG. Reisolated plasmid DNA was digested with Fpg, an *E. coli* enzyme with DNA glycosylase/AP (apurinic/apyrimidinic) lyase activity specific for 8OG, nicking at the site of the lesion. Digested samples were resolved on agarose gels containing ethidium bromide, allowing for separation of covalently closed circular constructs that were refractory to digestion (and thus repaired) and relaxed species that had been nicked by Fpg. DNA was subjected to Southern blot analysis to detect the construct (Fig. 3*A*). Repair was assessed up to 24 h after transfection and expressed (fraction repaired) as the fraction of signal representing the covalently closed species divided by the sum of the signals for covalently closed and relaxed species (Fig. 3*B*).

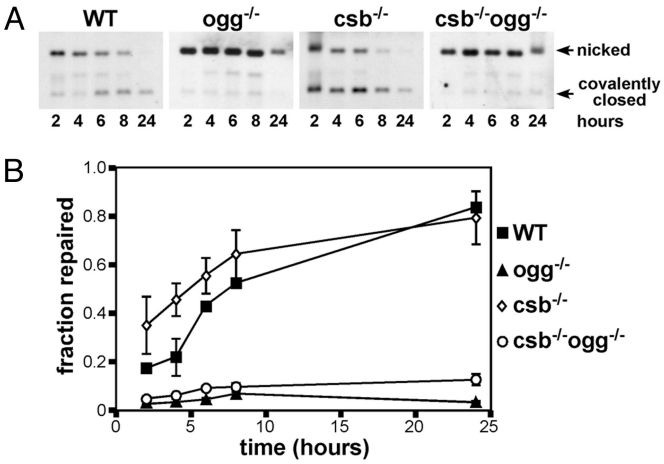

Fig. 3. ogg$^{-/-}$ and csb$^{-/-}$ogg$^{-/-}$ cells are impaired in their repair of 8OG. (*A*) Representative Southern blot of reisolated construct digested with Fpg to assess the residual levels of unrepaired 8OG at various times after transfection. (*B*) Quantification of the data (two independent experiments). Error bars represent standard deviations.

In WT and csb$^{-/-}$ cells, we observed a time-dependent increase in the proportion of repaired species occurring with approximately the same kinetics, and repair is nearly complete by 24 h. In contrast, most of the construct isolated from ogg$^{-/-}$ and csb$^{-/-}$ogg$^{-/-}$ cells is sensitive to Fpg, even after 24 h, indicating that repair of 8OG is severely impaired. These results also indicate that the bias observed for C insertion over A insertion opposite the lesion is not due to transcription past a repaired sequence, but is most likely an innate property of the RNAP. Unexpectedly, we observed a relatively large percentage of unrepaired molecules in WT and csb$^{-/-}$ cells at 2 h. This could indicate that only a portion of the DNA is in the nucleus and subject to repair or transcription at any given time (25).

Discussion

We have developed an *in vivo* mammalian model system for studying the potential of DNA lesions to cause TM with a biologically relevant outcome, the activation of the Ras oncogene. Additionally, our system provides a novel tool for assessing the roles of various proteins in the repair of the lesions investigated. In this work, we have examined the effects of deficiency in Ogg1 and Csb on the repair of 8OG and the mutagenicity of this lesion with respect to transcription.

We demonstrate that 8OG can cause TM, even in WT cells fully competent for DNA repair. However, mutant transcripts are detected at a very low level in these cells, indicating that repair of the lesion is relatively rapid, and no detectable change in cellular phenotype is observed. In contrast, in cells lacking Ogg1 (ogg$^{-/-}$ and csb$^{-/-}$ogg$^{-/-}$), the number of mutant transcripts is elevated four- to fivefold, and there is a significant elevation in P-ERK as a result of such mutant transcripts encoding a constitutively active, mutant Ras protein. These results indicate that repair of 8OG by Ogg1 is paramount for preventing TM. This finding is in contrast to previously published data indicating that Ogg1 plays little or no role in the repair of 8OG from the transcribed strand of a plasmid-borne gene (26). Additionally, in our system any role mediated by the Csb protein in the repair of 8OG is negligible, both alone and in combination with the knockout of Ogg1. Again, these data

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contradict previously published work indicating that whereas Ogg1 is important for repair of the nontranscribed strand (26), Csb is required for the repair of the lesion in the transcribed strand (21).

The participation of TCR in the repair of oxidative lesions, including 8OG, has been controversial. Traditional assays to assess bias toward repair in the transcribed strand of an endogenous nuclear gene over the nontranscribed strand demonstrated no such preference (27). Assays examining the repair of a single 8OG lesion in a shuttle vector with or without a promoter to direct transcription did indicate a strand bias, albeit very small (a difference of ≈2 h in the rate of repair) (26). It is difficult to imagine how 8OG might initiate TCR, because one requirement for this process is thought to be arrest of RNAP at the lesion site, and multiple laboratories using various systems have demonstrated that transcription past 8OG leads to a small population of truncated transcripts in the context of abundant full-length transcripts (11, 12, 28, 29). The level of RNAP pausing can be influenced by various factors, including the sequence context and promoter strength (30), distance of the lesion from the transcription start site (30, 31), the relative abundance of CTP and ATP in the reaction (12, 29), and the presence of various elongation factors (31, 32), but ultimately full-length transcripts are produced. Nevertheless, there is evidence for involvement of Csb in the global repair of 8OG, particularly when combined with the absence of Ogg1 (21, 22), and there is some suggestion that this role is independent of TCR (21). The data presented here do not support a significant role for Csb and/or TCR in the repair of 8OG. However, a role for Csb may only become apparent in the context of a large cellular burden of oxidative DNA damage. Cells from Cockayne syndrome patients harboring mutations in Csb are hypersensitive to oxidative damage (33, 34) and impaired in their ability to repair multiple oxidative lesions in a host–cell reactivation assay (34). The negligible role of TCR for the repair of 8OG in mammalian cells contrasts to *E. coli*, in which both BER and TCR seem to contribute significantly to its removal (13).

mRNA sequence analysis revealed two distinct classes of mutant transcripts, one with an A insertion opposite the template 8OG, and one in which the 8OG had been skipped, leading to a single-nucleotide deletion. This is similar to the transcript population observed in *E. coli* with an 8OG template (13), although the abundance of such mutant transcripts is greatly reduced in the mammalian system (≈10% rather than ≈60%). Thus, it seems that RNAP preferentially incorporates the correct C opposite the 8OG, effectively limiting the mutagenic potential of this lesion with respect to transcription. Previous reports using cell-free systems have indicated that ≈8% of transcripts generated by elongation past 8OG are mutant (31), with the majority containing a C opposite the template lesion (12), in agreement with the data presented here. Interestingly, sequence analysis of truncated transcripts arising from RNAP pausing at the site of damage also indicates that the insertion of A occurs only approximately 8% of the time (32), suggesting that these transcripts are likely to be eventually elongated to full-length, in agreement with data provided by single initiation transcription reactions (29). This bias toward nonmutagenic C insertion, however, could be influenced by the sequence context flanking the lesion.

Intriguingly, the relative abundance of mutant transcripts harboring the Δ 1 nt depended on the presence of Ogg1. When Ogg1 was absent, the majority of mutant transcripts contained the C>A transversion expected from the base-pairing properties of 8OG. In contrast, when Ogg1 was present, most of the mutant transcripts observed were of the Δ 1 nt class. This finding might suggest that in the presence of Ogg1 protein, removal of the 8OG is relatively fast, and the Δ 1 nt mutant mRNA is the result of transcription past some downstream intermediate of 8OG repair. Indeed, prokaryotic polymerases have the capacity to bypass small gaps in DNA during transcription, generally leading to a small deletion in the transcript corresponding to the size of the gap in the DNA (35).

Although the data presented here do not provide a direct link between TM and cancer, they suggest that 8OG-mediated TM could lead to activation of an oncogenic pathway. In nonproliferating cells, the contribution of TM to the mutant protein pool, and thus the cellular phenotype, could confer a growth advantage or an opportunity to escape growth arrest. Under these circumstances, the cell is more likely to re-enter the cell cycle, increasing the chance that the DNA damage leading to the mutant protein will now be encountered by the DNA replication machinery. If studies from phage and bacterial systems are applicable to higher organisms, the bases preferentially inserted opposite the DNA damage by RNAP will, in many cases, be the same as those inserted by the DNA polymerase during replication (36). This ensures permanent retention of the "advantageous" mutation in the genome, resulting in a heritable change in a process termed *retromutagenesis* (reviewed in ref. 37). A transition from a "nongrowth state" to a "growth state" induced by the presence of a mutant protein generated by TM could then potentially explain the development of certain tumors in mammalian systems (Fig. 4). Normally quiescent cells in tissues acquire DNA damage-driven mutations (via TM) in proteins essential for maintenance of cell cycle arrest or for initiation of apoptosis. Such mutant proteins allow escape from growth constraints and acquisition of other characteristics of tumor cells (38). Because occurrence of this event is influenced by the repair capacity of the cell, variation in intrinsic Ogg1 within the normal human population (39) could be of great importance. Additionally, mutations in Ogg1 and other BER proteins have been associated with human cancers (40).

Materials and Methods

Cell Lines and Cell Culture. Congenic WT, ogg$^{-/-}$, csb$^{-/-}$, and csb$^{-/-}$ogg$^{-/-}$ MEF cells were generated as previously described (19, 21, 41). All cells were cultured in DMEM supplemented with 15% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin and grown at 37 °C and 10% CO_2.

Generation of Q61K Mutant Ras Expression Plasmid. The WT human H-Ras coding sequence cloned into the pcDNA3.1(+) expression vector (Invitrogen) was obtained from the Guthrie cDNA Resource Center. To generate the Q61K mutant, a PCR-based mutagenesis protocol was used. Briefly, the complementary primers QK1(F) (5′-ACATCCTGGATACCGCCGGCAAGGAGGAGTAC) and QK2(R) (5′-GTACTCCTCCTTGCCGGCGGTATCCAGGATGT) were used to introduce the mutation using the WT Ras vector as template. In the first round of PCR, separate reactions were established using the following primer pairs: pcDNA(F) (5′-ATACGACTCACTATAGGGAGACCC) with QK2(R), and pcDNA(R) (5′-GGCAACTAGAAGGCACAGTCGAGG) with QK1(F), whereby pcDNA(F) and pcDNA(R) are specific to the vector outside of the multiple cloning site. PCR products were purified with a Gel Purification Kit (Qiagen) and then mixed for a second round of PCR. Six cycles of annealing and polymerization were conducted before the pcDNA(F) and pcDNA(R) primers were introduced to the reaction. The resulting PCR product was gel purified, restricted with BamHI and XhoI, and ligated into the pcDNA3.1(+) vector. The ligation mixture was used to transform XLI Blue *E. coli*, and plasmid preparations obtained from individual colonies were sequenced to verify the presence of the mutation.

Generation of 8OG-Containing Constructs. Constructs were generated as previously described (13), with the exception that primers were annealed to ssDNA by incubating at 75 °C for 10 min and then allowing the mixtures to slowly come to room temperature. The damage-containing construct was generated using WT Ras ssDNA and the 8OG primer (5′-P-GTACTCCTCCT(8OG)GCCGGCGGTATCCAGGATGT). Additionally, constructs bearing the normal and Q61K mutant Ras coding sequences were generated by the same protocol.

Transfection. Cells were grown to 50%–70% confluence in T75 flasks. For each transfection, 1.5×10^6 cells were spun down and resuspended in 100 μl of MEF1 solution (Amaxa) containing 1 μg of DNA construct. This mixture was

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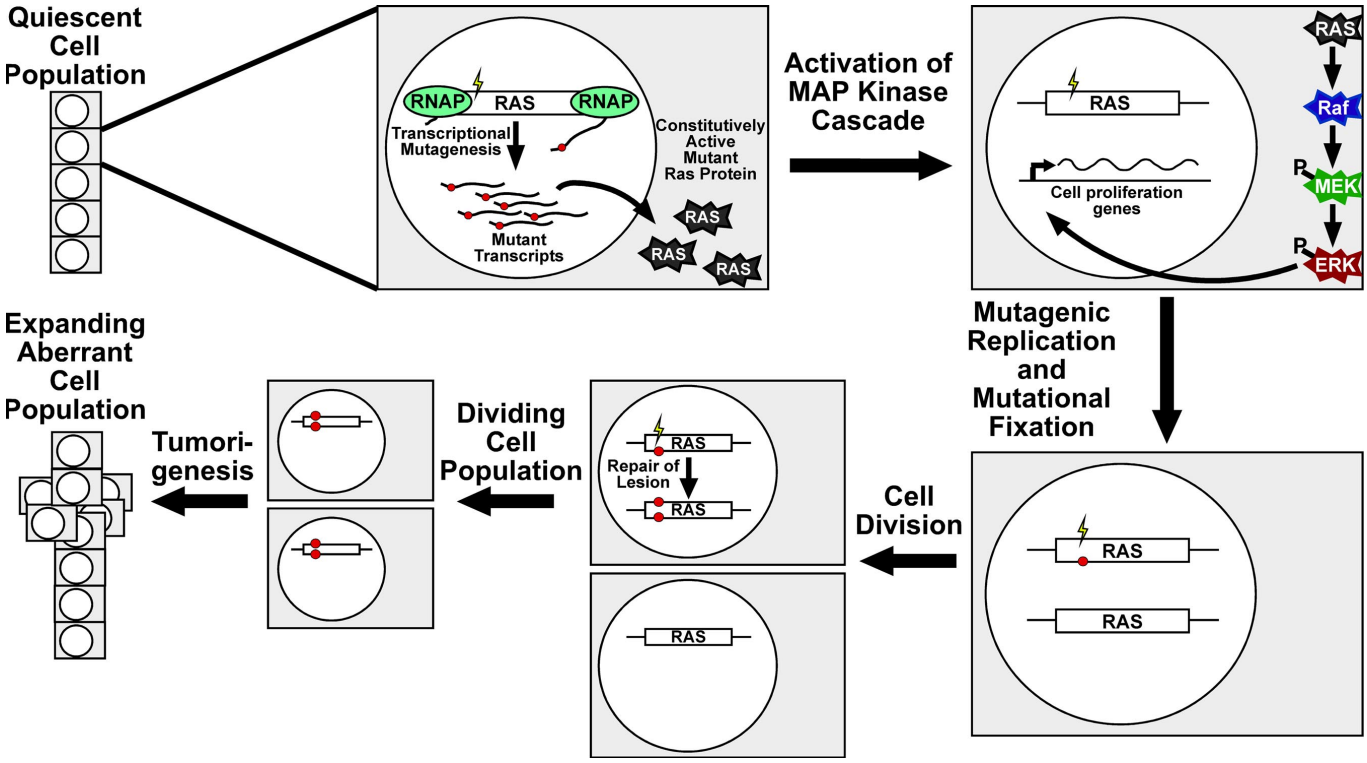

Fig. 4. Proposed role for TM in tumorigenesis. DNA damage (jagged symbol), either spontaneous or induced, occurs within the coding region of an oncogene (e.g., Ras) on the transcribed strand. Transcriptional bypass of this lesion leads to a population of mutant mRNA that, upon translation, gives rise to mutant protein. The resulting mutant protein is capable of signaling in the absence of appropriate stimuli, activating the MAPK cascade and downstream genes that mediate cell proliferation. This signaling promotes a round of DNA replication over the same, unrepaired DNA lesion, allowing for the fixation of a permanently heritable mutation (red dot) at the site of the damage. Upon cell division, one daughter cell will inherit this mutation and will be able to pass it on to progeny regardless of whether the damage is subsequently repaired, giving rise to an aberrant, dividing cell population that, with appropriate additional genetic changes, ultimately develops into a tumor.

electroporated using a Nucleofector II (Amaxa) on the T-20 setting. Immediately after electroporation, cells were resuspended in 500 μl of prewarmed media, plated in six-well plates, and incubated at 37 °C/10% CO_2 for the length of time indicated for each experiment.

Cell Lysis and Western Blot Analysis. Cells were incubated for 4.5 h after transfection, washed once with PBS, and incubated an additional 1.5 h in DMEM lacking serum. Cells were washed twice with cold PBS and lysed with 150–200 μl Hepes lysis buffer (50 mM Hepes [pH 7.0], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 50 mM NaF, and 1 mM Na_3VO_4) supplemented with protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 100 μM PMSF). Lysates were cleared at maximum speed in a microcentrifuge at 4 °C, and the protein concentration of each sample was determined using the BioRad Protein Reagent.

For Western blots, proteins were resolved on 12% SDS-PAGE gels and transferred to PVDF membrane. For P-ERK blots, 30 μg of protein was loaded per lane, whereas 10 μg of protein per lane was loaded to probe for Ras expression and total ERK levels. The following antibodies were used: P-ERK and total ERK (Cell Signaling Technology), H-Ras (Santa Cruz Biotechnology; C20 antibody), and goat anti-mouse and anti-rabbit HRP conjugates (Promega). Blots were developed using SuperSignal West Pico chemiluminescence reagent (Pierce) and either exposed to film or quantified with a FluorS Max imaging system (BioRad). For each cell line, P-ERK signal was normalized to ERK as a loading control, background levels from No DNA controls were subtracted, and the values were normalized to Ras expression level. The values obtained with the mutant construct were set to 100%, and all other numbers were normalized accordingly.

mRNA Analysis. RNA was isolated 2 h after transfection with the 8OG-containing construct. Cultures were treated with DNase I (New England Biolabs; 0.04 U) for 30 min before collection of cells to remove any plasmid DNA that had not been taken up. RNA was isolated with a MasterPure RNA isolation kit (Epicentre) according to the manufacturer's instructions, with the exception that RNA was treated multiple times with RNase-free DNase I until PCR using the RNA as template no longer yielded a product, indicating the removal of construct DNA. cDNA was generated with the OneStep RT-PCR Kit (Qiagen) using pcDNA(F) and pcDNA(R) primers. Twenty to twenty-five percent of the isolated RNA was used as a template, and 40 cycles of amplification were performed at an annealing temperature of 55 °C. Excess primers and dNTPs were removed with a PCR Purification Kit (Qiagen).

Amplified cDNA and pUC18 cloning vector were digested with XbaI and Hind III overnight at 37 °C, resolved on a 1% agarose gel, and gel purified. cDNA was ligated into pUC18, and the ligation reactions were used to transform DH5α *E. coli*. For the PCR screen, isolated colonies were picked into 10 μl of water in a 96-well PCR plate and then streaked onto LB plates containing ampicillin. Five microliters of each cell suspension was transferred to a new well so that parallel PCR reactions using either a primer specific for the WT sequence (5′- ACATCCTGGATACCGCCGGCC) or for the Q61K mutant sequence (5′-ACATCCTGGATACCGCCGGCA) could be performed. PCR was done for 25 cycles at an annealing temperature of 72 °C using Taq DNA polymerase (Qiagen). PCR products were resolved on a 1% agarose gel. Plasmids were isolated from colonies that amplified with the mutant-specific primer and sequenced (Macrogen).

Time Course of Repair Assays Cells were transfected with the 8OG-containing construct, and the plasmid was reisolated at various times after transfection with a modified alkaline lysis protocol, as previously described (26, 42). Plasmid was digested with 1U Fpg (Trevigen) for 2 h at 37 °C and resolved on a 0.6% agarose gel containing 5 μg/ml ethidium bromide. DNA was transferred to nylon membrane, and blots were processed with an AlkPhos Direct Labeling and Detection Kit (GE Healthcare) using a probe against the f1 origin. Blots were exposed to film, and films were scanned and quantified using ImageQuant software (GE Healthcare).

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