NOVEL ROLES FOR TEN-ELEVEN TRANSLOCATION 1 (TET1) 5-METHYLCYTOSINE DIOXYGENASE IN THE CELLULAR RESPONSE TO STRESS

by

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Abstract

Cells maintain exquisite control over gene expression in the struggle to preserve homeostasis. While some genes remain constitutively active to function in energy production or provide mechanical support, other batteries of genes respond only in the context of specific stresses, and must be silenced when homeostasis is achieved. Numerous transcription factors have been identified as responders to stress, whose gene targets aid in maintaining homeostasis. An additional layer of regulation over the relatively static genetic code involves chemical modifications of histones and DNA, particularly in or near gene promoters. Recent advances in the understanding of such epigenetic changes show that methylation at the 5-carbon position of cytosines neighboring guanosines (CpG) is an important player in the regulation of mammalian genes and chromatin architecture. Moreover, methyl groups can be further modified by a reaction catalyzed by Ten-Eleven Translocation (TET) 5-methylcytosine dioxygenases, whose products were shown to be intermediates in DNA demethylation as well as stable, final products that may have functional relevance. DNA methylation has therefore become increasingly viewed as a dynamic process, yet the proteins regulating epigenetic marks and gene expression remain poorly-understood. Because changes in CpG methylation underlie numerous threats to health, including cancer and neurodegenerative diseases, these proteins are potential targets in developing biomarkers and in drug development.

This dissertation explores the role of TET1 in response to cellular stress. Toward this end, an *in vitro* approach was taken in order to manipulate TET1 levels and measure the response to toxicant-induced stress. Two major roles for TET1 were elucidated. First, reactive oxygen species generated by exposure to the benzene metabolite hydroquinone (HQ) led to genome-wide and gene-specific CpG demethylation in a TET1-dependent manner. Moreover, cytoprotective genes induced by HQ were dependent on TET1, suggesting the protein is involved in transcriptional responses to stress. Secondly, a role for TET1 in the response to DNA damage was uncovered and found to be unrelated to catalytic activity, indicating a novel, non-enzymatic role for TET1. Taken together, these data suggest that TET1 represents a heretofore unappreciated interface between the environment and the cellular response to stress.

The findings presented are used to support the underlying hypothesis that TET1 is involved in mediating responses to stresses which would be harmful to the genome if left unconstrained. The idea that TET1 plays a protective role linked to disease is strengthened by reports of nearly uniformly low levels of TET proteins and their products in cancers. Highlighting the role of TET1 as a critical protector of the genome may serve as a foundation upon which a better understanding of the role of DNA methylation in disease may be built. Ultimately, unraveling the protein's functions – both enzymatic and non-enzymatic - will lead to improved prevention and treatment strategies in diseases involving inappropriate DNA methylation.

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Dedication

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I. INTRODUCTION

1.1 The role of DNA methylation in the cell

1.1.1 DNA methylation and its machinery

Mammalian cells have the capability of regulating genes by chemically modifying DNA in a way that is stable and heritable, yet without changing the sequence of bases. Greater than 50% of human genes contain regions of the genome that have a high density of cytosines neighboring guanines (CpG). Cytosines are frequently methylated at the 5-position of the carbon ring, and are susceptible to deamination. This vulnerability over the course of mammalian evolution led to CpG clusters within gene promoters, most of which are unmethylated (38). These CpG Islands are located in gene promoters and are capable of being methylated as a means of controlling gene expression. DNA methylation is a covalent modification catalyzed by DNA methyltransferases (DNMTs).

DNMT3A and DNMT3B, known as the "*de novo* methyltransferases" establish new methylation marks on cytosines, which are thought to be critical in silencing the expression of retrotransposons and satellite repeat sequences, as well as controlling the dosage of genes via imprinting of one allele. DNMT1, known as the "maintenance methyltransferase," recognizes hemimethylated DNA and ensures that methylation patterns are copied during DNA replication. Interestingly, DNMT1 interacts with proliferating cell nuclear antigen (PCNA), allowing it to target proliferating foci during DNA synthesis. DNMT3L is homologous to DNMT3A and DNMT3B, but does not have catalytic activity (39). The gene is highly expressed in germ cells, and is involved in regulating DNMT3A and DNMT3B activity. The importance of DNA methylation was highlighted through the use of knockout mice. *Dnmt3a* knockout mice developed to term,

but were not viable after 3-4 weeks. *Dnmt3b* knockout attempts were not successful, as it was embryonic lethal at E14.5-18.5 (39). These models indicated that *de novo* DNA methylation is essential in mammalian development and growth. *Dnmt1* knockout mice die early in embryogenesis. However embryonic stem cells derived from the embryos have been cultured, but lack a significant amount of DNA methylation and die during induced differentiation (38, 39). *Dnmt3L* –deficient mice have profound genome-wide demethylation and display developmental arrest at E8.5 (39). The profound effects of losing these enzymes are indicative of their importance in development.

1.1.2 Roles of DNA methylation in mammalian cells

CpG methylation, particularly in gene promoters, is generally associated with gene silencing. The methyl groups on the 5-position of the cytosine ring project into the major groove of DNA. While it is thought that this bulky projection may be sufficient to inhibit transcription, it has been shown that methyl groups prevent transcription factors from binding (2, 40). In fact, methylation patterns may act as a code exploiting the differential sensitivity of some transcription factors to bind methylated DNA. Specific methyl-DNA binding proteins are also known to interact with histone deacetylases (HDACs), which participate in changing the chromatin structure from permissive euchromatin to restrictive heterochromatin by removing transcription-promoting acetylation marks from histones (38). The interaction between methyl-DNA binding proteins and histones illustrates the close mechanistic relationship between DNA methylation and chromatin marks. Lysines and arginines in histones are methylated and acetylated. Depending on the location and makeup of the local marks, the modifications

can greatly influence the histone structure, making the chromatin more or less accessible to transcriptional machinery (40).

Epigenetic modifications are a major determinant of tissue identity and differentiation, as the genetic code remains the same, yet differentiated cells must silence genes involved in pluripotency and genes of other tissue types to maintain their identity. DNA methylation participates in mammalian development, and the properly established patterning is crucial for proper functioning of the adult (2,41). An extreme example of DNA methylation occurs when the parental genome of a fertilized egg undergoes rapid global demethylation. This process is an active demethylation, known to be due to TET3 (discussed below). The maternal genome is demethylated in a passive manner, by a decreases in expression and activity of DNMTs (40). DNA methylation patterns, quickly reestablished by DNMT enzymes, are essential for the development of the organism (41). As embryonic programs are set toward a specific lineage, *de novo* methylation is set by DNMT3A and DNMT3B. Embryonic stem cells lacking DNMTs are incapable of differentiation (39). Pluripotency-associated promoters such as *Pou5f1* (*Oct4*) and *Nanog* are silenced by methylation upon differentiation, resulting in the inability to dedifferentiate (42). A well-known example of the involvement of DNA methylation is neural progenitor cell (NPC) differentiation. NPCs, capable of differentiating into neurons, astrocytes, and oligodendrocytes, restrict their ability to become astrocytes as they differentiate toward a neuronal fate. A CpG site in the STAT-binding element in the promoter of numerous astrocytic genes is methylated, and prevents the transcription of astrocytic genes (43). This progressive restriction of gene expression is thought to control, in part, differentiation of stem cells into more differentiated lineages.

An additional function of DNA methylation is the silencing of retrotransposable elements, some of which make up approximately 19% of the genome (26). For example, Long Interspersed Nuclear Element 1 (LINE-1), a retrotransposon encoding an RNA binding protein and an endonuclease with reverse transcriptase activity, makes copies of the DNA via an RNA intermediate, which is then inserted into the host genome at a new site. Left unchecked, LINE-1 would quickly overtake the genome and directed, orderly transcription would be inhibited, if possible at all. LINE-1 is heavily methylated to prevent such an attack on the genome. Methyl-CpG binding protein 2 (MeCP2) is known to help repress LINE-1 activity (23). Interestingly, recent evidence shows that new experiences and exercise cause increases in LINE-1 retrotransposition in rat neurons. It is hypothesized that this "jumping gene" effect contributes to genetic variability in neurons. (44).

1.1.3 DNA methylation and disease

Cancer

Altered DNA methylation has long been recognized as a distinguishing feature in disease (2). In cancers, DNA is typically globally hypomethylated, yet hypermethylated in CpG islands. Non-cancer cells typically have very few methylated CpG islands , though CpG island methylation occurs in X-chromosome inactivation and imprinting and increases with age. Tumors suppressor genes are often methylated in human cancers, silencing their ability to halt the cell cycle, repair, or initiate programmed cell death (39).

A causative role for methylation in carcinogenesis is supported by the fact that reducing DNA methylation in $Apc^{Min/+}$ mice led to fewer intestinal tumors. In addition, methylation of tumor suppressor genes often occurs early in tumorigenesis (39), and has been shown to act as the "second hit" in Knudson's Two-Hit Hypothesis, where one tumor suppressor allele is lost by mutation and the other is methylated (40). *Rb, APC,* and *BRCA1* are examples of commonly methylated tumor suppressor genes involved in cell-cycle regulation, Wnt signaling, and DNA repair, respectively. Cytosine analogue drugs inhibiting DNA methylation, including Decitabine and Zebularine, are currently in use as a form of chemotherapy (45,46) . The idea behind the therapy involves relieving the repression of tumor suppressor genes that will halt cell division or initiate apoptosis. A potential benefit over targeting specific oncogenes could be that tumors are heterogeneous and evolve to evade inhibition of oncogenic drivers. By combining targeted therapies along with demethylating agents, it is hoped that tumor cells will become sensitive to activated tumor suppressor genes they have evolved to silence (45).

Neurologic Diseases and Syndromes

Fragile X Syndrome

Fragile X Syndrome is among the most common forms of inherited syndromes involving cognitive impairement and learning disabilities. The syndrome is caused mainly by a massive expansion of C-G-G triplet repeats found in the 5' UTR of the *FMR1* gene (47). Patients with Fragile X Syndrome have hypermethylation of the C-G-G

repeats, leading to silencing of the *FMR1* gene (47). The gene product is an RNA-binding protein that translocates between the cytoplasm and nucleus. It is thought that the absence of the FMRP protein inhibits protein synthesis in dendrites, leading to massive cognitive deficits (48).

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neuropsychiatric illness characterized by brain atrophy, cognitive impairment, inflammation, and emotional disturbance. The disease commonly also causes memory loss, apathy, depression, and irritability. Both hyper- and hypomethylation of genes have been observed in AD. For example, hypomethylation of COX-2 promoter has been observed in a study involving tissues from 10 diseased and 10 non-diseased frontal cortex samples (49). Hypermethylation of BDNF was observed in the same study, which was accompanied by a significant decrease in mRNA. Perhaps most significantly, CpG methylation of the NF-k B promoter was found to be hypomethylated in AD brain. The investigators noted an increase in p50 and p65 expression as well, suggesting that the neuroinflammation observed in AD patients could result from hypomethylation of the NF-• B promoter (49). Conflicting reports of global DNA methylation have yet to be resolved; both global hypermethylation and hypomethylation have been reported in AD samples. Using an ELISA-based approach, Rao *et al.* observed an increase in global DNA methylation along with global histone H3 acetylation (49). However, Chouliaras *et al.* observed a global decrease in both methylation and hydroxymethylation in the hippocampus of Alzheimer's disease (50). Both groups measured DNA methylation using antibody-based approaches, but it is not

clear which cell types were represented in each sample. Because the brain contains numerous cell types in close proximity to one other, it can be difficult to tell whether changes in methylation are due to changes in methylation of neurons, or whether the death of neurons and the proliferation of astrocytes contributed to the changing methylation values.

1.2 Fe^{2+} - and a-ketoglutarate-dependent dioxygenases

1.2.1 Background and mechanisms

Members of the family of Fe^{2+} - and a-ketoglutarate (aKG) -dependent dioxygenases catalyze numerous oxidation reactions involving a wide range of substrates, including proteins, fatty acids, amino acids, and nucleic acids. The large number of substrates corresponds to the wide-range of cellular processes to which these dioxygenases contribute. They are distinguished from the well-known family of cytochrome P450 monooxygenases by their incorporation of both oxygen atoms in the dioxygen molecule into organic products (61). While P450 enzymes rely on reductants such as $NADPH_2$ to reduce an oxygen atom to water, aKG dioxygenases use a-ketoglutarate as a substrate for oxidation in addition to the principal substrate. Additionally, cytochrome P450 monooxygenases contain heme-bound iron at their catalytic sites, while aKG-dependent dioxygenases directly bind an Fe^{2+} via histidine, aspartate/glutamate, and histidine coordination, referred to as a HXD/E…H motif (62) .

aKG dioxygenases contain a double stranded B-helix (DSHB) fold made up of eight B-strands known to contain cofactor -coordinating pockets. a-helices located n-terminal to the B-strands are capable of coordinating a zinc ion, which may play a role in DNA binding (61).

aKG dioxygenase active sites are located in the DSBH core, where Fe^{2+}, aKG, and substrate bind, followed by dioxygen. It is thought that the ordered binding minimizes the time substrate is kept in close proximity to potentially reactive oxidizing species, thus protecting substrate from damage (61). An Fe^{2+}-derived electron generates a superoxide radical that attacks the carbon-2 position of aKG producing a covalent bond between the resulting Fe^{4+} center and aKG. The aKG intermediate is decarboxylated, resulting in succinate and CO_2, while leaving an Fe^{4+}-oxo intermediate in the active site. This remaining intermediate becomes reduced via substrate-derived hydrogen atom, leaving a substrate-radical. The carbon of the substrate is oxidized by the addition of an oxygen atom, yielding a hydroxyl group on substrate (61). This final oxidation step, in the presence of ascorbate, regenerates the Fe^{2+} in the active site. It is thought that all aKG dioxygenases follow this general mechanism, and most identified members of this family of enzymes add a hydroxyl group to the non-aKG substrate. Interestingly, aKG dioxygenases are emerging as a family of proteins capable of sensing and responding to cellular microenvironmental conditions, suggesting they may play a critical role in diseases such as cancer and in maintaining homeostasis in the face of various stresses.

1.2.2 Prolyl hydroxylases as environmental sensors

The prolyl hydroxylases are by far the most well-known Fe^{2+} and aKG-dependent dioxygenases whose activities respond dramatically to changes in the cellular microenvironment and therefore serve as the prototypical interface between the microenvironment and programmed response to stress. It was recognized long ago that the 4-hydroxyproline necessary for collagen function in tissues was formed by collagen prolyl-4-hydroxylases, and deficiency in these proteins or ascorbate, leads to scurvy. Other members of the same family of Fe^{2+} and aKG-dependent dioxygenases were later found to be involved in the sensing of oxygen availability (63). Transcriptional regulation of genes involved in the cellular response to hypoxia was determined to be controlled by the transcription factor Hypoxia-inducible factor (HIF). Proteomic analysis revealed that two proline residues of HIF-a became hydroxylated under normoxia, and it was known that HIF-a underwent ubiquitin-mediated proteasomal degradation. Under conditions of hypoxia, the HIF-a subunit (but not HIF-B) is liberated from proteasomal degradation pathways and translocates to the nucleus to activate a battery of more than 60 genes involved in overcoming hypoxia, including vascular endothelial growth factor (VEGF), erythropoietin, and glucose transporters (64). The discovery that two proline residues were critical to the responsiveness of HIF-a and the later finding that HIF-a hydroxylation was dependent on Fe and aKG implicated Fe^{2+} and aKG-dependent dioxygenases in the hydroxylation of HIF-a and the overall cellular response to hypoxia. The genes responsible for hydroxylating HIF-a were found to be the prolyl hydroxylases PHD1-3 and a HIF asparaginyl hydroxylase, FIH-1 (63).

Numerous cancers have shown the ability to circumvent hypoxia-mediated toxicity and, not surprisingly, these cancer often display high levels of HIF proteins,

which confer resistance to overgrowth of the tumor blood supply by signaling growth of new vasculature. HIF proteins also support the invasion of cancer cells into adjacent tissues by increasing the expression of matrix metalloproteinase 2 (64). HIF proteins have therefore become targets of anticancer therapeutic agents, and have become an important player in our understanding of cancer biology. Interestingly, Isocitrate dehydrogenase -1 and -2 (IDH1,2) mutations (R132H) result in the formation of 2-hydroxyglutarate (2HG) rather than a-ketoglutarate, which reportedly inhibit numerous aKG-dependent dioxygenases. However, enantiomer-specific changes in collagen prolyl 4-hydroxylase activity have been observed in cells with mutant IDH1 or IDH2. R-2HG was reported to increase EglN1 and EglN2 activity, thereby decreasing HIF levels, while S-2HG had no effect (65). This raises the possibility that IDH status may confer differential sensitivity to specific therapies via the production of cofactors that alter aKG-dioxygenase activity.

1.3 Ten Eleven Translocation 5-methylcytosine dioxygenases

1.3.1 The discovery of TET proteins

Ten Eleven Translocation (TET) proteins are named for the ten-eleven translocation (t(10;11)(q22;q23)) observed in rare acute myeloid and lymphocytic leukemia (7). The product of the translocation is a fusion of the mixed-lineage leukemia (*MLL1*) gene, on chromosome 10, with the TET1 gene on chromosome 11. Following the discovery of the *TET1* gene, three proteins have been described: TET1, TET2, and TET3. It was predicted that the proteins may affect DNA by the known DNA modifying activity of the J-binding protein 1 (JBP1) protein from *Trypanosoma brucei*, the trypanosome causing human African Sleeping Sickness (51). These organisms contain a modified thymine molecule known as “base J,” a glucosylated hydroxymethyluracil. JBP1 and

JBP2 are capable of oxidizing the methyl group of thymine to form hydroxymethyluracil and, based on the amino acid sequence, the protein was identified as a homologue to the metazoan TET protein (51). A gene resembling the TET-JBP family fused with a gene containing a CXXC DNA-binding domain and led to the three members found in metazoans. Both TET proteins and the JBP proteins are divalent iron- and a-ketoglutarate-dependent dioxygenases, though the target of the TET proteins is 5mC rather than thymidine (7).

1.3.2 Functional domains of TET proteins

TET1 and *TET3* contain an amino terminal CXXC domain, and all three *TET* genes contain a carboxy terminal catalytic domain. The TET proteins contain a cysteine-rich domain toward the carboxy terminus, which the JBP members lack. Interestingly, the ancestral *TET* gene underwent a triplication event, leading to three similar *TET* genes. *TET2*, however, lost its CXXC domain in an apparent inversion (51). The exon is now a separate gene transcribed in the opposite direction, known as IDAX. IDAX has been shown to recruit TET2 to the nucleus, targeting it for caspase-mediated destruction following enzymatic activity. The remaining CXXC domains are not necessary for enzymatic activity of *TET1* or *TET3*, and the domains may not even bind DNA with the same affinity as the similar CXXC domains found in DNMT proteins (52). It is still unclear how TET1 and TET3 are regulated, and whether specific domains – other than the catalytic core – have any function.

The catalytic domains of TET proteins are typical divalent iron- and α-ketoglutarate-dependent dioxygenases, which involves a His-Xaa-Asp/Glu motif. A His residue, C-terminal to the catalytic domain, coordinates divalent iron, and an Arg residue coordinates the a-ketoglutarate (51).

1.3.3 Demethylation of 5mC via oxidation

Maintenance of DNA methylation involves DNMT1, which recognizes hemimethylated CpGs (39). 5mC is hydroxylated by TET proteins to 5-hydroxymethylcytosine (5hmC) and, because DNMT1 has greatly decreased affinity for 5hmC, methylation of the newly synthesized CpG does not occur. This possibility of passive demethylation is similar to the chemical inhibition of DNA methylation by 5-aza-2'-deoxycytidine (52).

1.3.4 Active demethylation of 5mC via oxidation

TET enzymes have been shown to yield 5-formylcytosine (5fC) and 5-carboxycytosine (5caC). In this proposed mechanism, 5mC is oxidized to become 5caC through iterative TET activity. 5caC could be excised by thymidine DNA glycosylase (TDG) and replaced by cytosine. While a plausible mechanism, the extremely low abundance of 5caC found in cells known to undergo active demethylation makes the mechanism less likely or perhaps a minor one.

An alternative mechanism involves the deamination of TET-derived 5hmC, forming 5-hydroxymethyluracil (5hmU). Base excision repair (BER) enzymes could then replace 5hmU with unmethylated cytosine (7). One caveat in this proposed pathway is the finding that deaminases (e.g. AID, APOBEC1-2) have greater affinity for single-stranded DNA than double stranded DNA. It is reported, however, that AID can alter the sub-cellular localization of TET proteins from the cytoplasm to the nucleus (53), though the role of AID in the demethylation pathway would then be an indirect one.

Evidence suggests that the oxidation-deamination pathway may be a significant contributor to active DNA demethylation. Guo *et al.* demonstrated that both overexpression and knockdown of deaminases led to decreases and increases in 5hmC levels, respectively, in TET1-overexpressing cells, supporting deaminase activity in the deamination of 5hmC (7). Additionally, small molecule inhibitors of BER led to a decrease in demethylated 5mC, further supporting the pathway (7).

II. The benzene metabolite hydroquinone leads to active cytosine demethylation through increased Tet1 activity

Adapted from:

Coulter, *et al.* Hydroquinone Increases 5-Hydroxymethylcytosine Formation through Ten Eleven Translocation 1 (TET1) 5-Methylcytosine Dioxygenase. J Biol Chem. 2013 Oct 4;288(40):28792-800.

2.1 ABSTRACT

DNA methylation regulates gene expression throughout development and in a wide range of pathologies such as cancer and neurological disorders. Pathways controlling the dynamic levels and targets of methylation are known to be disrupted by environmental and pharmaceutical chemicals and are therefore of great interest in both prevention and clinical contexts. Benzene and its metabolite hydroquinone have been shown to lead to decreased levels of DNA methylation, although the mechanism is not known. This study employs a cell culture model to investigate the mechanism of hydroquinone-mediated changes in DNA methylation. Exposures that do not affect HEK293 cell viability led to genomic and methylated reporter DNA demethylation. Hydroquinone caused reactivation of a methylated reporter plasmid that was prevented by the addition of N-acetylcysteine. Hydroquinone also caused an increase in Ten Eleven Translocation 1 activity and global levels of 5-hydroxymethylcytosine. 5-Hydroxymethylcytosine was found enriched at LINE-1 prior to a decrease in both 5-hydroxymethylcytosine and 5-methylcytosine. Ten Eleven Translocation-1 knockdown decreased 5-hydroxymethylcytosine formation following hydroquinone exposure as well as the induction of glutamate-cysteine ligase catalytic subunit and 14-3-3σ. Finally, Ten Eleven Translocation 1 knockdown decreased the percentage of cells accumulating in G_2+M following hydroquinone exposure, indicating that it may have a role in cell cycle changes in response to toxicants. This work demonstrates that hydroquinone exposure leads to active and functional DNA demethylation in HEK293 cells in a mechanism involving reactive oxygen species and Ten Eleven Translocation 1 5-methylcytosine dioxygenase.

2.2 INTRODUCTION

DNA methylation is dynamic, and the mechanisms involved in methylation and demethylation represent an interface between the environment and gene expression (1). Changes in DNA methylation have been implicated in a wide range of pathologies including cancer (2), neurodevelopmental disorders (3), and chronic inflammation (4). Efforts to understand mechanisms involved in DNA methylation and demethylation therefore have enormous translational potential. DNA methylation is established and maintained by the family of DNA methyltransferases (DNMTs),3 although mechanisms underlying demethylation have been more difficult to elucidate. Whereas Arabidopsis DNA glycosylases DME and ROS1 participate in exchanging methylcytosines with cytosines, no analogous pathway has been discovered in mammalian cells (5). A number of chemicals, such as 5-aza-2-deoxycytidine, decrease DNA methylation passively by inhibiting maintenance methylation as DNA is synthesized (6). However, this mechanism depends on cell division and may not explain active demethylation in post-mitotic cells such as neurons (7) and in the early stages of embryogenesis.

Pharmaceutical, industrial, and environmental chemicals have also been shown to decrease DNA methylation, but the mechanisms are unclear (8, 9). Several chemicals have been shown to have a passive effect on methylation by modifying DNMTactivity (10, 11).An interesting target that could explain active demethylation involves the Ten Eleven Translocation (TET) family of Fe^{2+} and α -ketoglutarate-dependent 5mC dioxygenases (12, 13). In a mechanism proposed by Guo et al., TET proteins catalyze the

oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), followed by deamination to 5-hydroxymethyluracil by cytidine deaminases. Subsequent base excision repair machinery would then replace 5-hydroxymethyluracil with unmethylated cytosine (14).

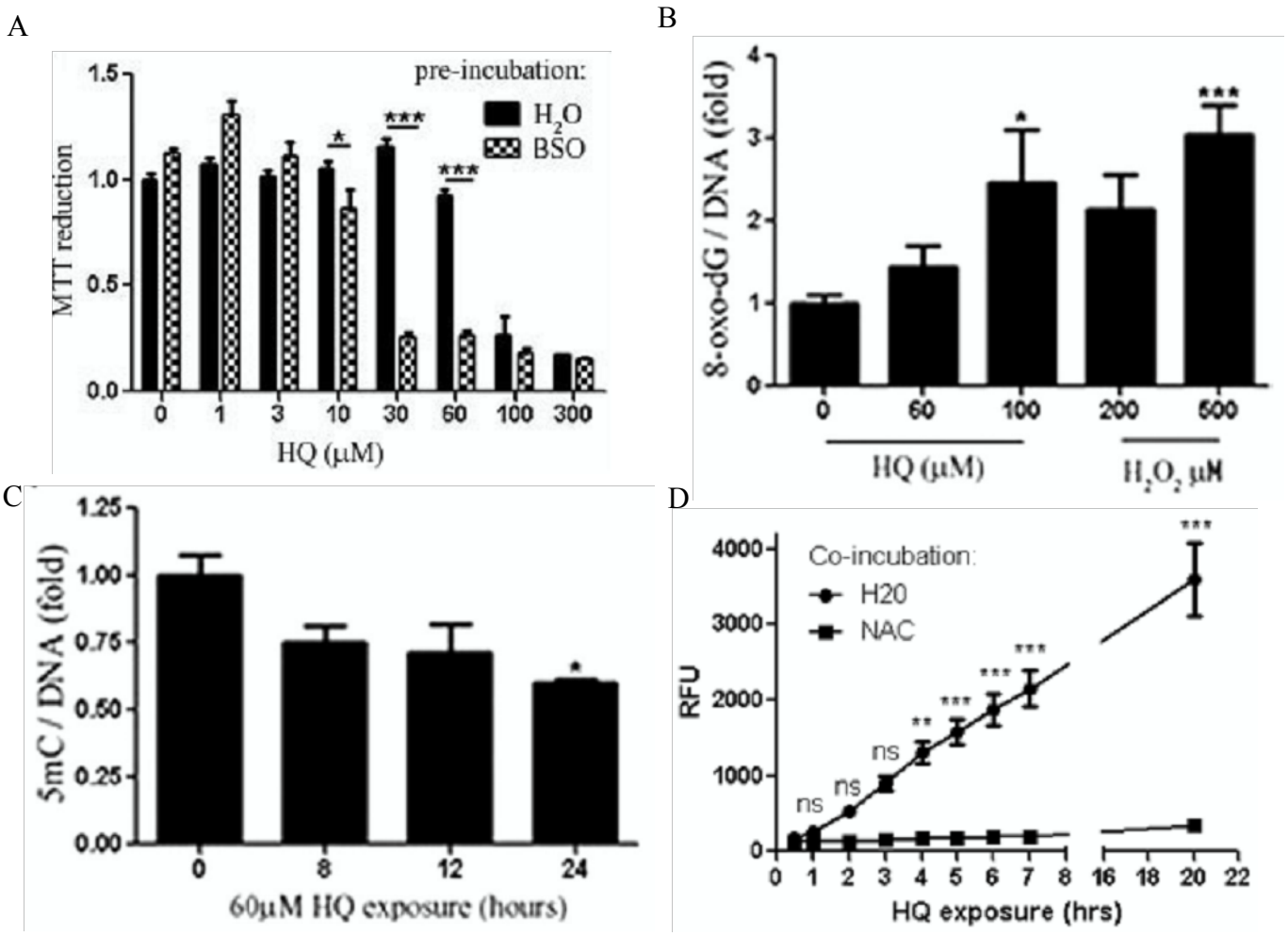
In this study, we focused on changes in DNA methylation by the benzene metabolite hydroquinone (HQ). Benzene is a ubiquitous environmental toxicant found in petroleum products and cigarette smoke (15) that has been associated with aplastic anemia and acute myelogenous leukemia (16).HQis one of the most abundant metabolites of benzene (17, 18) and has been shown to increase levels of reactive oxygen species (ROS) (19, 20), induce mitotic arrest (21), and promote apoptosis (20). Although benzene and benzene metabolite exposures have also been shown to be associated with loss of genomic methylation (9, 22), no mechanisms have been described to explain the observed decreases. The current study investigates a mechanism of DNA demethylation induced by exposure to HQ in a cell culture model, demonstrating that HQ mediates DNA demethylation in a mechanism involving ROS and the TET1 pathway.

2.3 RESULTS

Low Concentrations of Hydroquinone Decrease DNA Methylation

The MTT assay was used to determine concentrations of HQ which did not affect viability of HEK293 cells. Significant changes in MTT reduction were observed following exposure for 24 h to concentrations of 100μM and higher (Fig. 1*A*). Preincubation of cells with buthionine sulfoximine, an inhibitor of glutathione synthesis, increased the sensitivity of cells to HQ, indicating the involvement of glutathione and that toxicity is mediated in part by ROS. 8-oxo-dG was also measured by immunodotblotting genomic DNA. Significant increases in 8-oxo-dG staining were observed at 100μM HQ and 500μM H_2O_2 (Fig. 1*B*) but not at 60μM HQ, although a 40% decrease in genomic 5mC was observed (Fig. 1*C*). Increases in ROS were observed as early at 4 hours following exposure to 60μM HQ and levels of ROS were lower in cells incubated with the anti-oxidant (NAC) (Fig. 1*D*).

Figure 1: Effects of hydroquinone on viability and DNA methylation in HEK293 cells. ***A***, MTT assay results following HQ exposure for 24 hours are shown. Cells were incubated for 14 hours with 0.5 mM buthionine sulfoximine or an equivalent volume of vehicle prior to HQ exposure. ***B***, cells were exposed to indicated concentrations of HQ for 20 hours or H_2O_2 for 0.5 hours . ***C***, 250 ng of genomic DNA was then denatured, vacuum-baked to a nitrocellulose membrane, and probed with antibody against 8-oxo-dG or 5mC and normalized to methylene blue staining. ***D***, ROS was measured by DCF-DA oxidation following 60 μM HQ exposure with and without NAC. Values are mean ± S.E. (*error bars*).

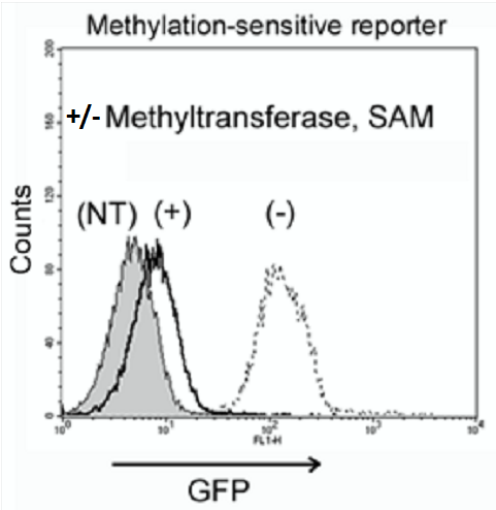


Hydroquinone-Mediated Decreases in Methylation are Active and Functional and Involve Reactive Oxygen Species

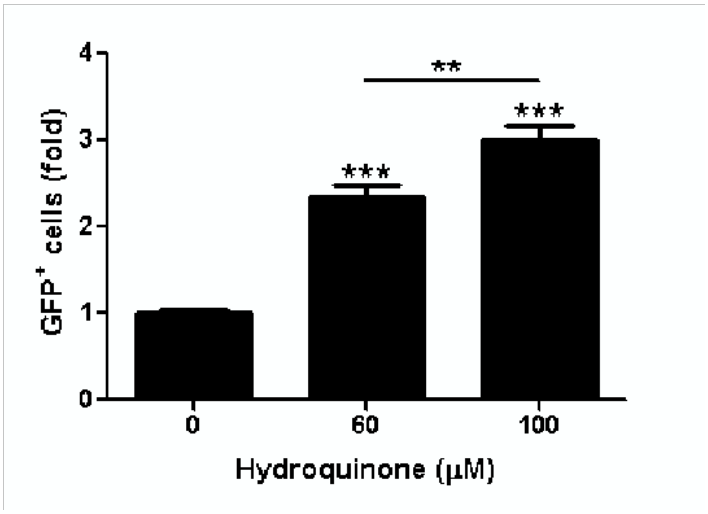
A possible mechanism involved in decreased methylation in the absence of oxidative DNA damage could involve inhibition of DNMT1, as many of the cells would have passed through S phase within 24 hours. A reporter assay was established to examine this possibility. The Nit-GFP plasmid is replication-incompetent and would therefore be less likely to undergo demethylation passively (16). Moreover, the functionality of CpG methylation can be ascertained by measuring GFP expression. Nearly complete repression of GFP was observed in cells transfected with the reporter plasmid that was methylated with CpG methyltransferase and *S*-adenosylmethionine (Fig. 2*A*). A 24 hour exposure to HQ was sufficient to reactivate the methylated reporter. A 2- and 3-fold increase in GFP+ cells was observed in cells transfected with the reporter and exposed to 60- and 100μM HQ, respectively (Fig. 2*B*). Interestingly, the increase in GFP+ cells was prevented by co-incubating 60μM HQ-exposed cells with NAC, suggesting that ROS was involved in reactivation of the methylated plasmid by HQ (Fig. 2*C*). Bisulfite sequencing of the promoter confirmed both pre-transfection methylation of the plasmid promoter and an increase in unmethylated CpGs following 24 hour exposure to HQ (Fig. 2*D*).

Figure 2: HQ causes ROS-dependent active and functional DNA methylation. ***A,*** Histogram of GFP intensity in unmethylated (dotted curve) and methylated reporter plasmid (solid curve) relative to untransfected cells (filled curve). ***B,*** Dose-response of plasmid reactivation in cells transfected with methylated reporter plasmid and exposed to HQ for 24 hours. ***C,*** *R*eactivation of methylated reporter plasmid by 60 μM HQ alone or in combination with NAC is shown. GFP+ cells were quantified by flow cytometry. *n* = 4, Values are mean ± S.E. (*error bars*). ***D***, Bisulfite sequencing of methylated methylation-sensitive reporter plasmid promoter in control and HQ-exposed HEK293 cells. Six CpGs were measured in 23 clones.

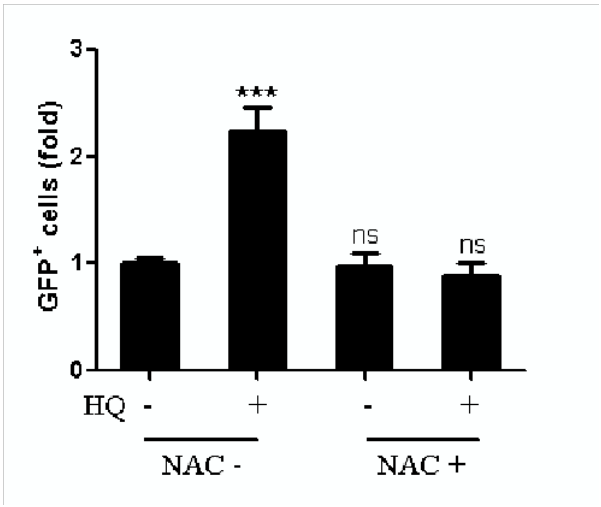
A



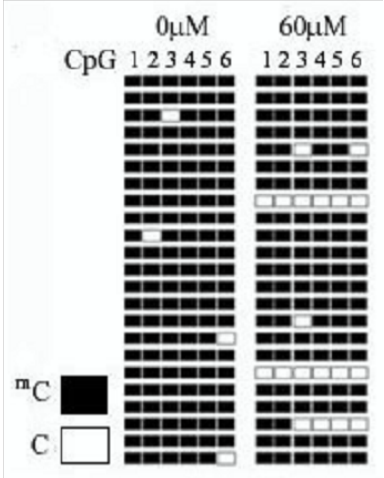
B



C



D

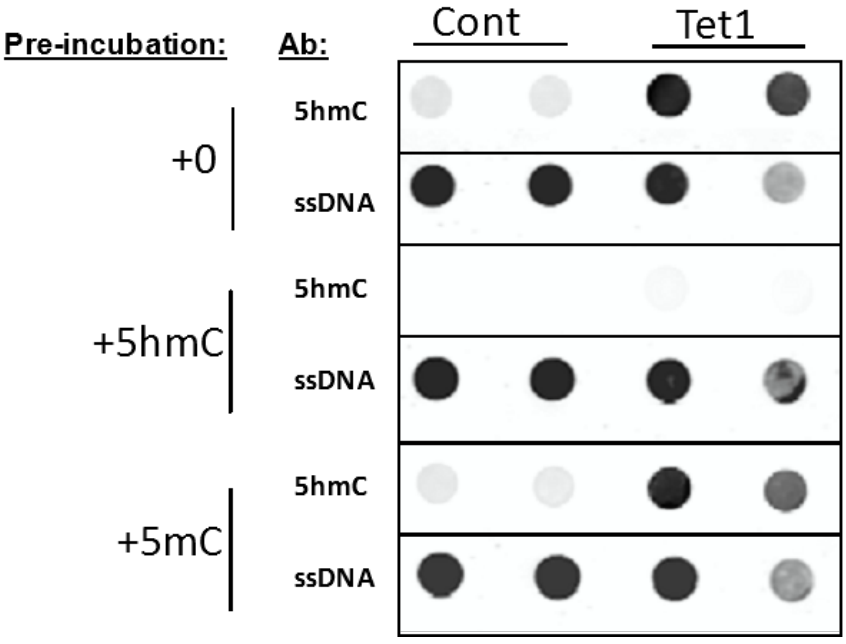


Hydroquinone Increases Genomic 5-Hydroxymethylcytosine Levels

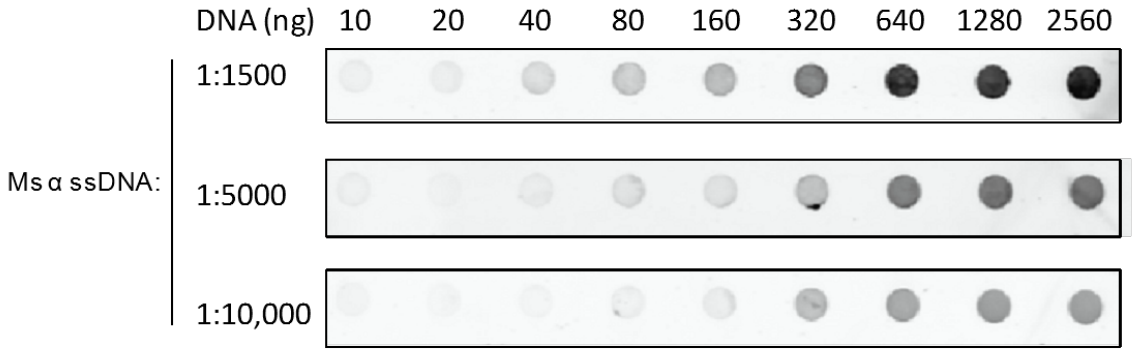
Due to the recent interest in active demethylation pathways involving TET proteins, immunodotblotting was used to measure 5hmC in genomic HEK293 DNA following HQ exposure. In order to establish a method to measure genomic 5hmC with specificity, genomic DNA from untransfected or TET1 catalytic domain (TET1cd) overexpressing HEK293 cells was immobilized onto a nitrocellulose membrane and probed using an antibody against 5hmC that was pre-incubated with 2µg/mL 5hmC or 5mC. Pre-incubation of the antibody with 5hmC, but not 5mC, diminished the signal (Fig. 3*A*), indicating specificity for 5hmC. To normalize 5hmC staining, an antibody against single-stranded DNA (ssDNA) was used. To avoid overloading membranes, increasing amounts of genomic DNA from HEK293 cells was immunoblotted using three concentrations of antibody (Fig. 3*B*). Because more than 640ng of DNA was out of the linear range of detection, immunodotblotting was accomplished using less than 500ng of genomic DNA. Noting a decrease in 5mC following 24 hours of HQ exposure, a time course of 5hmC was conducted to examine the potential for 5hmC as a demethylation intermediate. 5hmC levels increased at 8 and 12 hours following HQ exposure, but were not significantly higher at 24 hours (Fig. 3*D*). A dose response was also observed for 5hmC levels 18 hours following exposure to HQ and menadione (Fig. 3*E*), which is chemically similar to HQ and other ROS-inducing quinones.

Figure 3: Hydroquinone increases genomic 5hmC levels. **A**, Antibody specificity was confirmed by immunodotblot of 500ng HEK293 genomic DNA with and without TET1cd overexpression. Anti-5hmC antibody was incubated with 2µg/mL 5hmC or 5mC for 2 hours at room temperature and used to probe DNA. Anti-ssDNA antibody was used to normalize to total DNA. IR dye-conjugated IgG antibodies (mouse: 600, rabbit: 800) were used for simultaneous visualization by Licor Odyssey Imager. **B**, Varying amounts of HEK293 DNA were loaded onto nitrocellulose membrane and probed with 3 concentrations of ms anti-ssDNA antibody to confirm DNA amounts used in immunodotblotting were within a linear range for 5hmC normalization. Antibody signal was quantified and plotted in **C.** **D**, 5hmC in HEK293 cells following 8-, 12-, and 24-hour exposures to HQ measured using immunodotblotting. **E**, Immunodotblot showing dose-response for 5hmC following various concentrations of hydroquinone or menadione after 18 hours. Results were quantified and are represented in **F**. Values are mean_S.E. (*error bars*), *n*=3. *, significance compared with control one-way ANOVA with Tukey post-test.

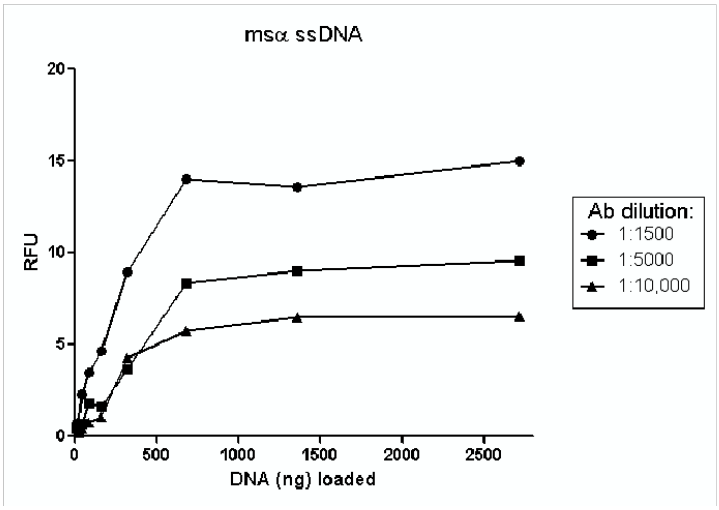
A



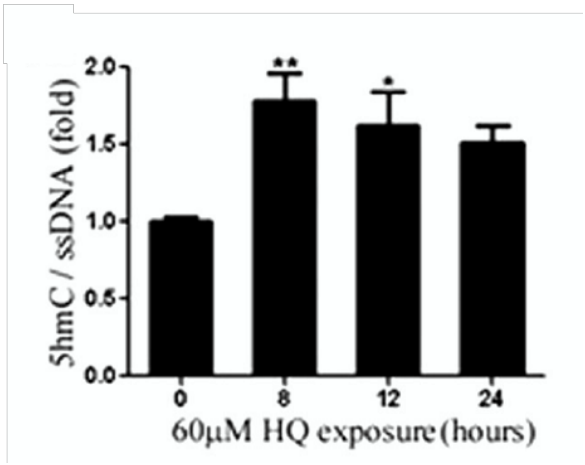
B


DNA (ng) 10 20 40 80 160 320 640 1280 2560
Ms α ssDNA:
1:1500
1:5000
1:10,000

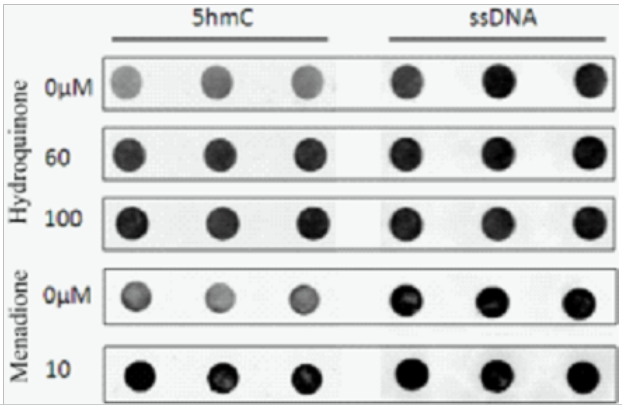
C


msα ssDNA
RFU
DNA (ng) loaded
Ab dilution:
1:1500
1:5000
1:10,000

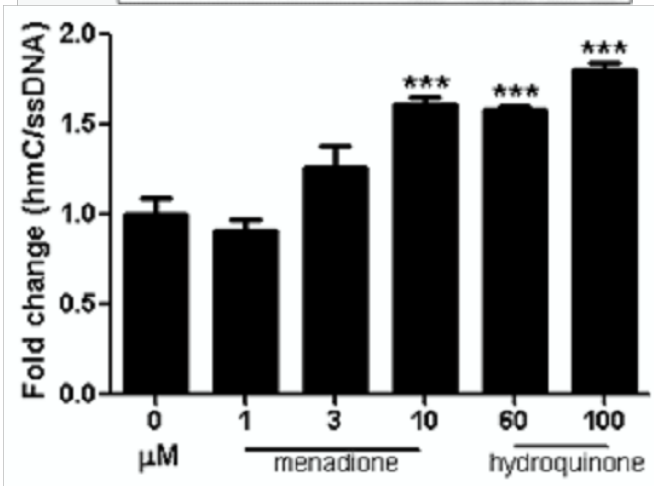
D


5hmC / ssDNA (fold)
60μM HQ exposure (hours)

E


5hmC
ssDNA
Hydroquinone
0μM
60
100
Menadione
0μM
10

F

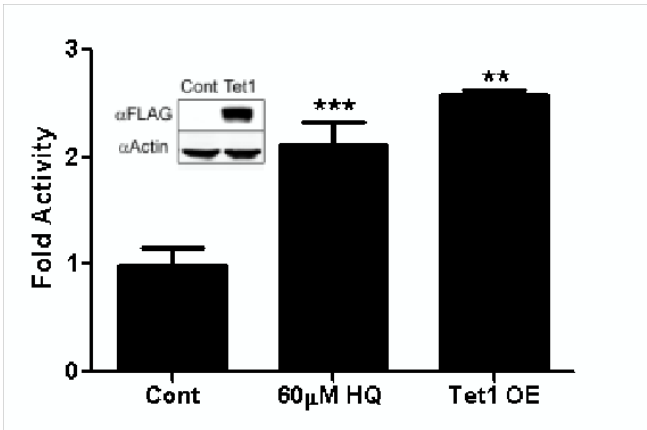

Fold change (hmC/ssDNA)
μM
menadione
hydroquinone

Hydroquinone-Induced 5-Hydroxymethylcytosine Formation is Dependent on TET1 5mC Dioxygenase

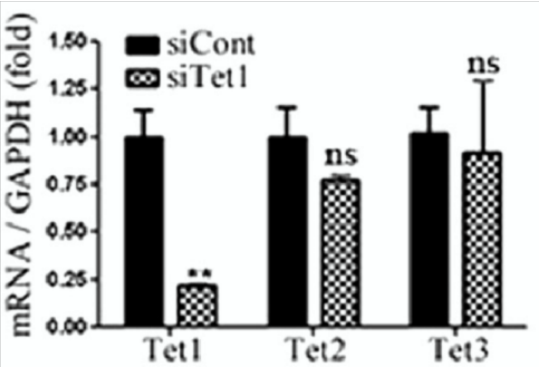
TET activity was higher in HQ-exposed HEK293 cells to levels similar in cells overexpressing TET1 catalytic domain (Fig. 4*A*). Although there are three distinct family members of TET proteins, we found that siRNA-mediated knockdown of TET1 led to an approximate 75% decrease in both *Tet*1 mRNA (Fig. 4*B*) and total TET activity (Fig. 4*C*), suggesting that TET1 is the major TET protein contributing to 5hmC in HEK293 cells. siRNA against *Tet*1 did not affect *Tet*2 or *Tet*3 mRNA levels (Fig. 4*B*), but completely abrogated HQ-induced increases in 5hmC (Fig. 4*D*), indicating that the increase in 5hmC in HEK293 cells exposed to HQ requires TET1.

Figure 4: Hydroquinone increases 5hmC through TET1.**A**, 5hmC formed from nuclear extracts of HEK293 cells exposed to 0 or 60 μM HQ or overexpressing (*OE*) FLAG-tagged Tet1 catalytic domain. **B**, *Tet1, 2, and 3* mRNA measured by qRT-PCR following transfection with Tet1-targeted or control siRNA. Tet expression was normalized to *GAPDH*. **C**, 5hmC formed from HEK293 nuclear extracts following control or Tet1 knockdown. **D**, immunodotblotting of genomic DNA from cells exposed to 60μM HQ following control or Tet1-targeted knockdown. Values are mean±S.E. (*error bars*), *n*=3. * significance compared with control by *t* test (*A*, *B,* and *C*) and one-way ANOVA and Tukey post-test (*D*). #, significance compared with siCont+HQ.

A



B



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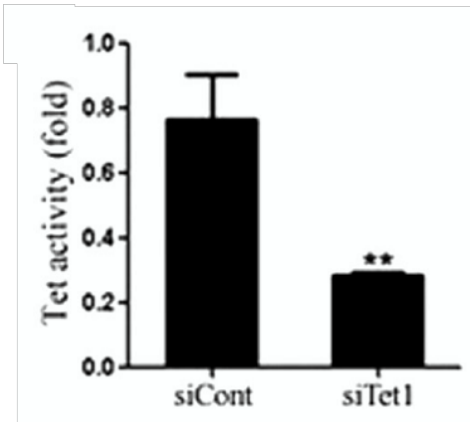
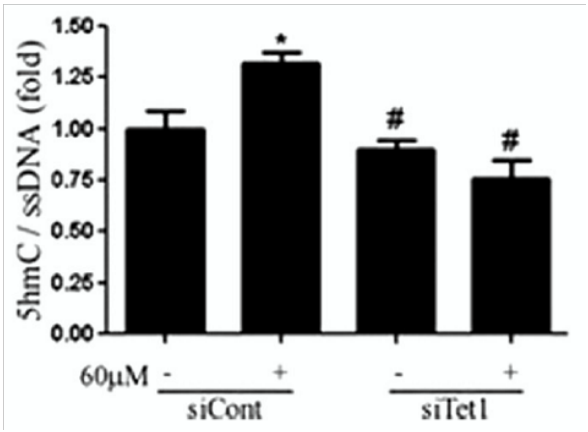


D



Hydroquinone Exposure Leads to Increased Nuclear TET1Levels in HEK293 Cells

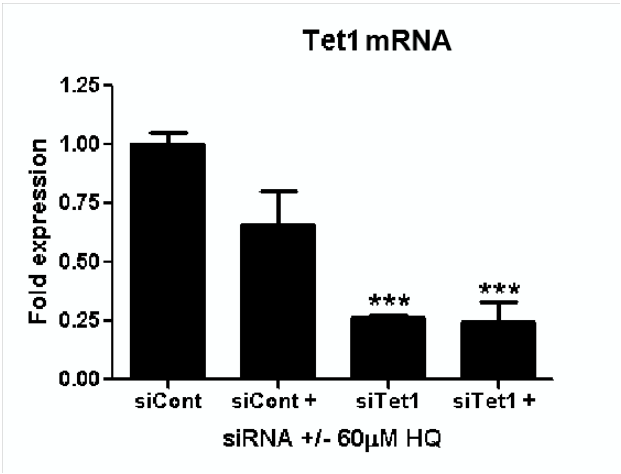
Because TET1 loss-of-function abrogated HQ-induced increases in 5hmC, efforts were directed toward understanding how HQ alters TET1 activity. No increase in TET1 mRNA was observed following exposure to HQ (Fig. 5*A*), suggesting HQ may alter TET1 post-transcriptionally. To determine the relationship between TET1 protein and activity, levels of TET1 protein in nuclei were measured by Western blotting. A 75% increase was observed in TET1 protein following exposure to 60μM HQ (Fig. 5*B* and *C*). A 60% decrease in the band was observed in cells transfected with TET1 siRNA, thereby confirming the identity of the band as TET1 (Fig. 5, *D* and *E*).

To further test the possibility that HQ affects TET1 post-transcriptionally, flag-tagged TET1 driven by CMV promoter was overexpressed and measured by Western blot following HQ exposure. An increase in both cytoplasmic and nuclear TET1 protein (Fig. 5*F* and *G*) was observed in cells exposed to HQ. The increase in protein driven by an alternate promoter supports the idea that HQ affects TET1 levels in a post-transcriptional manner.

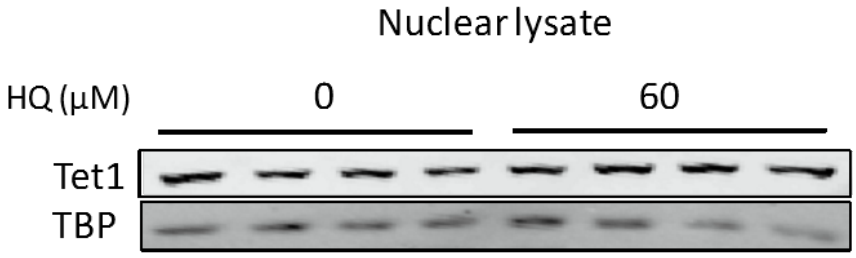
Because HQ exposure led to functional demethylation, 5hmC formation, and an increase in total Tet activity and nuclear TET1 levels, it was reasoned that overexpression of deaminase proteins should enhance reactivation of the methylated GFP reporter. Indeed, overexpression of V5-tagged human Apobec 2 (AP2) increased the GFP+ population approximately 2-fold following 60 μM HQ exposure (Fig. 5*H*), suggesting that HQ-mediated DNA demethylation occurs through recently proposed oxidation-deamination mechanism.

Figure 5: Effects of hydroquinone on nuclear TET1 levels. **A,** *Tet1* mRNA from HEK293 cells transfected with control or *Tet1*-targeted siRNA was measured by qRT-PCR with and without 60μM HQ exposure for 24 hours. **B**, Western blot of nuclear lysates from HEK293 cells exposed to 0 or 60μM HQ for 24 hours. Tata-binding protein (TBP) was used as a loading control. Quantification of TET1/TBP is shown in **C**. **D**, Western blot of TET1 levels in whole cell lysates from HEK293 cells transfected with control or *Tet1*-targeted siRNA. Quantification of TET1 levels is shown in **E**. **F**, reactivation of methylation-sensitive reporter by HQ alone or with overexpression of human V5-tagged cytidine deaminase AP2. GFP+ cells were quantified by flow cytometry. Western blot of cytoplasmic (**G**) and nuclear (**H**) lysates from HEK293 cells transfected with FLAG-tagged TET1cd driven by CMV promoter and exposed to 0 or 60μM HQ. GAPDH and TBP were used as loading controls for cytoplasmic and nuclear lysates, respectively. Values are mean_S.E. (*error bars*), $n_{\equiv}3$. *, significance compared with control by *t* test (*A* ,*C*, and *E*) and one-way ANOVA and Tukey post-test (*F*).

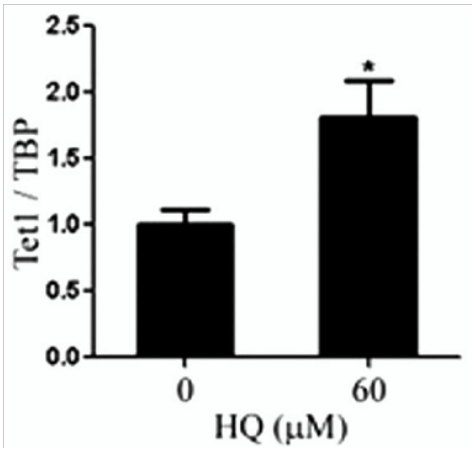
A



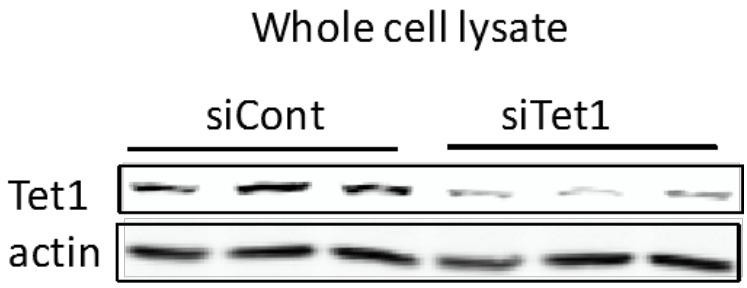
B


Nuclear lysate
HQ (μM)
0
60
Tet1
TBP

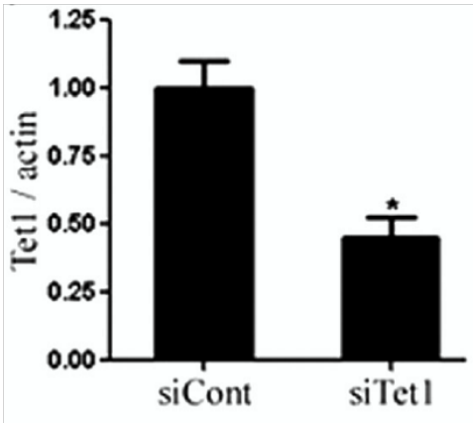
C


Tet1 / TBP
2.5
2.0
1.5
1.0
0.5
0.0
*
0
60
HQ (μM)

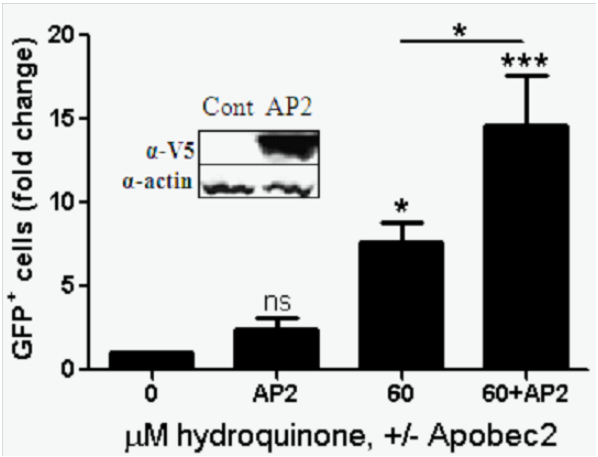
D


Whole cell lysate
siCont
siTet1
Tet1
actin

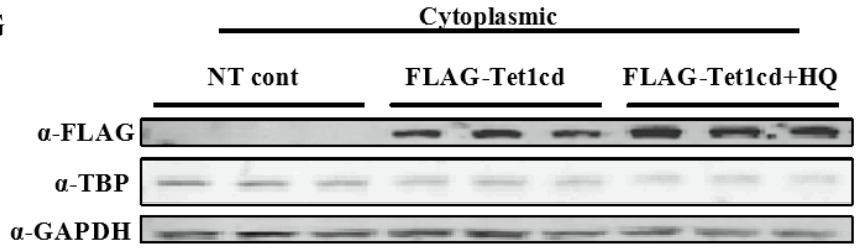
E


Tet1 / actin
1.25
1.00
0.75
0.50
0.25
0.00
*
siCont
siTet1

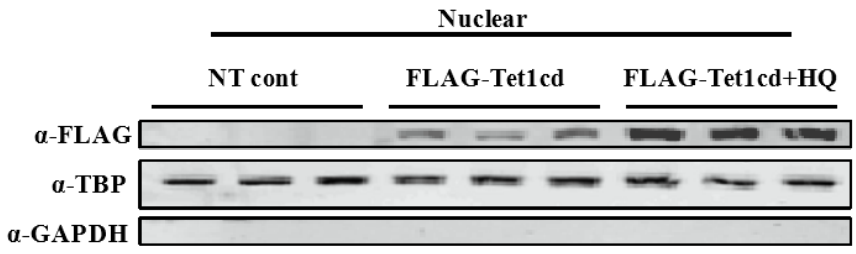
F


GFP+ cells (fold change)
20
15
10
5
0
Cont AP2
α-V5
α-actin
*
***
*
ns
0
AP2
60
60+AP2
μM hydroquinone, +/- Apobec2

G


Cytoplasmic
NT cont
FLAG-Tet1cd
FLAG-Tet1cd+HQ
α-FLAG
α-TBP
α-GAPDH

H


Nuclear
NT cont
FLAG-Tet1cd
FLAG-Tet1cd+HQ
α-FLAG
α-TBP
α-GAPDH

TET1 Mediates Gene Expression Induced by Hydroquinone

The results from the experiments involving the reporter plasmid suggest that HQ increases 5hmC and the involvement of TET1 in the mechanism. Genomic regions enriched for 5hmC and 5mC were isolated by immunoprecipitation and probed using qPCR. A 3-fold enrichment of the open reading frame 2 (ORF2) of the retrotransposon LINE-1 was observed in cells exposed to 60 μM HQ for 12 hours without a significant change in 5mC (Fig. 6*A*). At 24 hours, the enrichment of 5mC and 5hmC was decreased by 10- and 2-fold, respectively. The data support a role for HQ-induced 5hmC formation *prior* to demethylation, consistent with Tet-mediated oxidation-deamination demethylation. In addition, the effects of HQ on DNA methylation are widespread, considering the frequency of LINE-1 in the human genome (26).

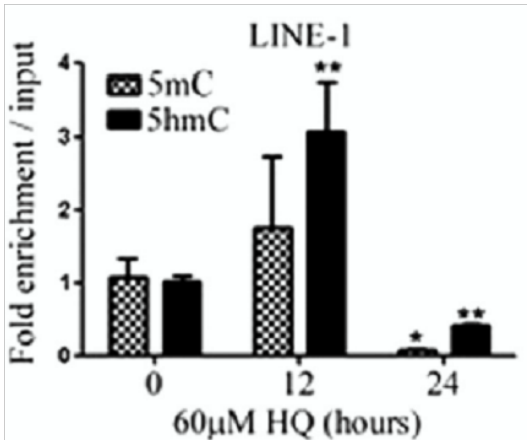
Attention was then focused on 5hmC formation at promoters of genes that are known to be induced by HQ. GCLC is a component of the glutamate-cysteine ligase protein, the rate-limiting enzyme in glutathione synthesis. ROS disrupts Keap1-dependent sequestration of the transcription factor Nrf2, resulting in the transcription of numerous antioxidant response element-containing genes, including GCLC. 5hmC was enriched approximately 12-fold at the promoter of GCLC following a 12 hour exposure to HQ, whereas 5mC levels were significantly lower at 12 and 24 hours (Fig. 6*B*). While *GCLC* mRNA was induced by 2.5-fold in response to HQ in HEK293 cells, induction was attenuated in cells following siRNA-mediated TET1 knockdown (Fig. 6*C*). No changes in oxidized glutathione were observed following exposure to HQ with or without TET1 knockdown (data not shown).

Because previous studies reported accumulation of cells in G_2+M phases of the cell cycle after exposure to HQ, the effect of HQ on methylation of the 14-3-3σ promoter was studied. 14-3-3σ is a p53-regulated inhibitor of cdc2-cyclinB1 nuclear translocation and is known to be regulated by promoter methylation (27–30). An enrichment of 5hmC was observed (Fig. 6*D*) at the 14-3-3σ promoter following a 12 hour exposure to HQ and decreased 5mC at 12 and 24 hours. Induction of 14-3-3σ expression by HQ was also blocked by TET1 knockdown (Fig. 6*E*). To confirm the role of TET1 in 5hmC formation at these promoters, TET1cd was overexpressed and cells were exposed to HQ for 24 hours. ChIP confirmed an increase in binding at GCLC and 14-3-3σ promoters following exposure to HQ compared to control (Fig. 6*F* and *G*).

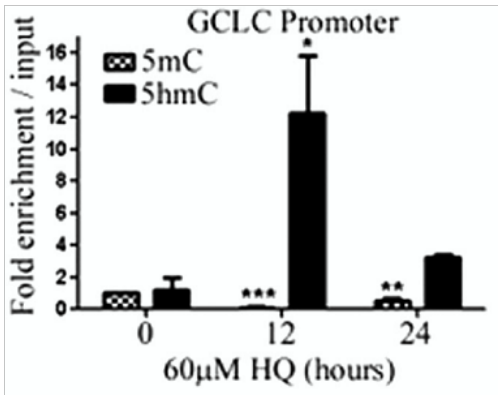
Interestingly, 14-3-3σ promoter hypermethylation has been reported in numerous cancers and correlates strongly with a decrease in expression (31). In addition, 14-3-3σ expression can be increased by exposure to 5-aza-2'-deoxycytidine (32), and its overexpression leads to a mitotic arrest in different cell types (29). Similar to studies shown to link HQ to cell cycle arrest, HQ caused an increase in cells in G_2+M (Fig. 6*H*). Further experiments were directed to examine whether TET1 plays a role in the changes to cell cycle that result from exposure to HQ.

Figure 6: Cytoprotective genes induced by hydroquinone are TET1-dependent. **A**, Genomic DNA was sonicated and immunoprecipitated using antibodies against 5hmC or 5mC and used as templates for qPCR to measure enrichment of LINE-1 (***A***), GCLCpromoter (***B***), and 14-3-3σ promoter (***D***) in HEK293 cells following 0-, 12-, and 24-hour exposure to 60μM HQ. ddCt values were normalized to input values. mRNAwas isolated from cells exposed to 60μM HQ for 24 hours following knockdown with control siRNA or siRNA targeting Tet1, and qRT-PCR was used to measure levels of GCLC (***C***) and 14-3-3σ(***E***). GAPDH was used as a control in all qRT-PCRs. Values are mean ± S.E. (*error bars*), *n* = 3. *, significance compared with control by one-way ANOVA with Dunnett's post-test. #, indicate significance compared with siCont + 60μM HQ (***C*** and ***E***). **F** and **G**, ChIP analysis of mouse IgG isotype control or FLAG-tagged TET1cd bound to GCLC (F) and 14-3-3σ (**G**) promoters following 24 hour exposure to 0 or 60μM HQ. **H**, Cell cycle profiles of HEK293 cells exposed to 0, 60, 100, or 300μM HQ for 24 hours was determined by flow cytometric measurements of DNA content. Values are mean ± S.E. (*error bars*), *n* = 3. *, significance compared with control by one-way ANOVA with Dunnett's post-test. #, indicate significance compared with siCont + 60 μM HQ (*C* and *E*).

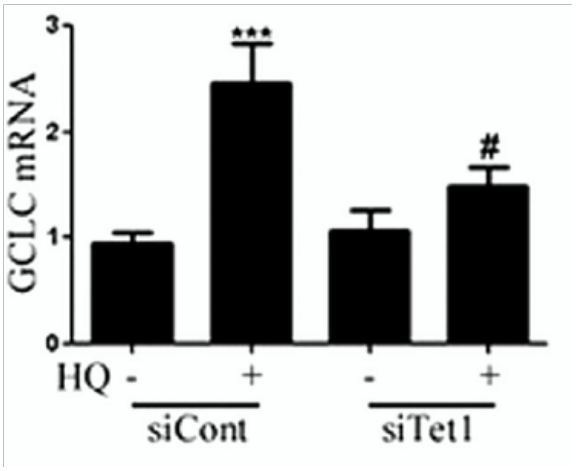
A



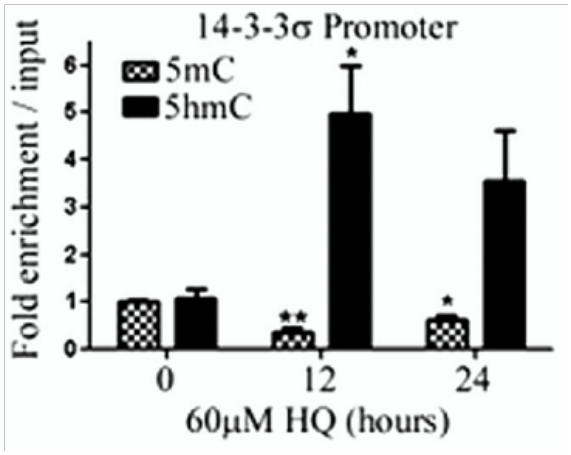
B


GCLC Promoter
5mC
5hmC
Fold enrichment / input
16
14
12
10
8
6
4
2
0
*
***
**
0
12
24
60μM HQ (hours)

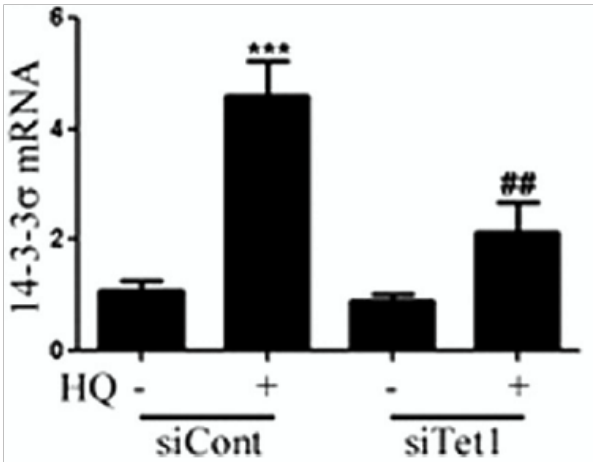
C


GCLC mRNA
3
2
1
0
***
#
HQ - + - +
siCont
siTet1

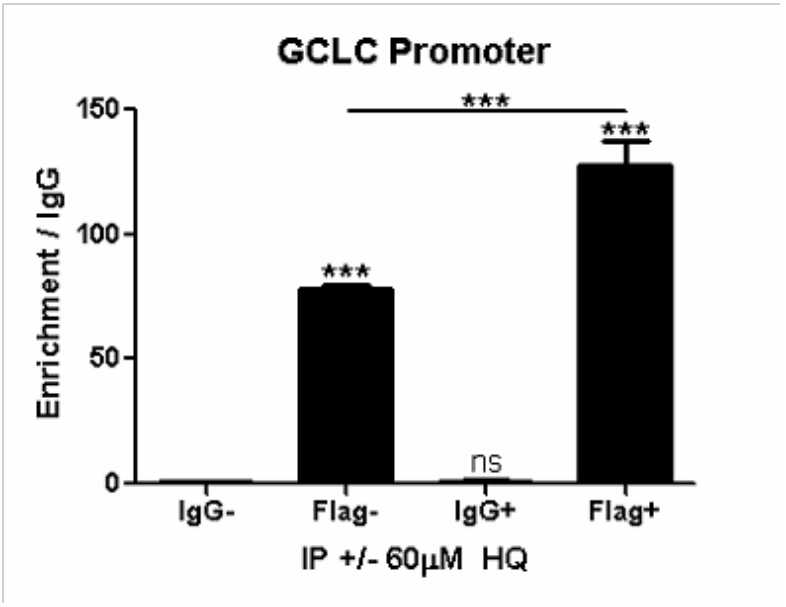
D


14-3-3σ Promoter
5mC
5hmC
Fold enrichment / input
6
5
4
3
2
1
0
**
*
*
0
12
24
60μM HQ (hours)

E


14-3-3σ mRNA
6
4
2
0
***
##
HQ - + - +
siCont
siTet1

F


GCLC Promoter
Enrichment / IgG
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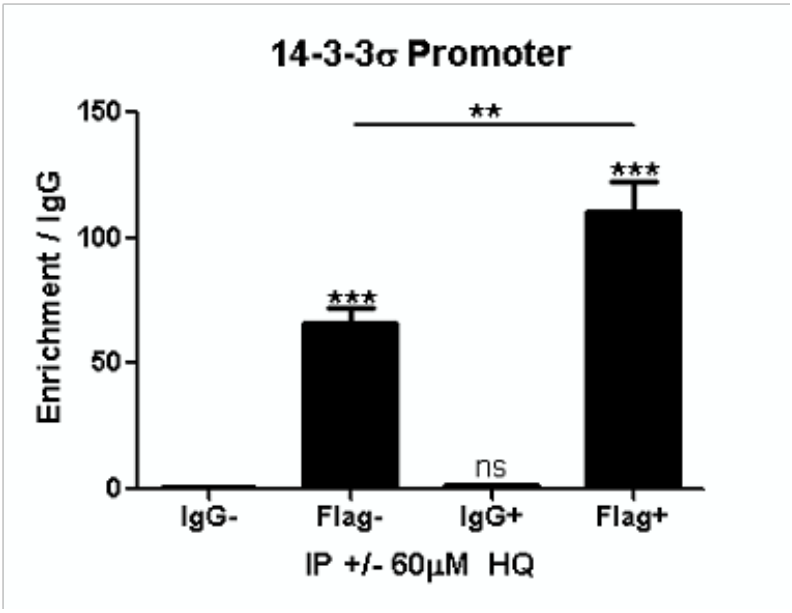
100
50
0
***
***
***
ns
IgG-
Flag-
IgG+
Flag+
IP +/- 60μM HQ

G


14-3-3σ Promoter
Enrichment / IgG
150
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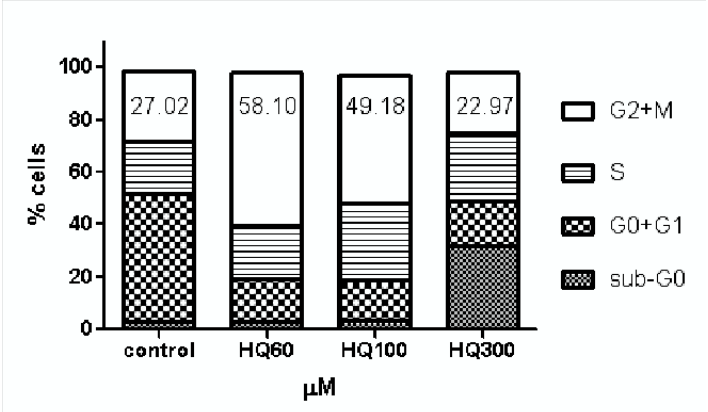
IgG+
Flag+
IP +/- 60μM HQ

H

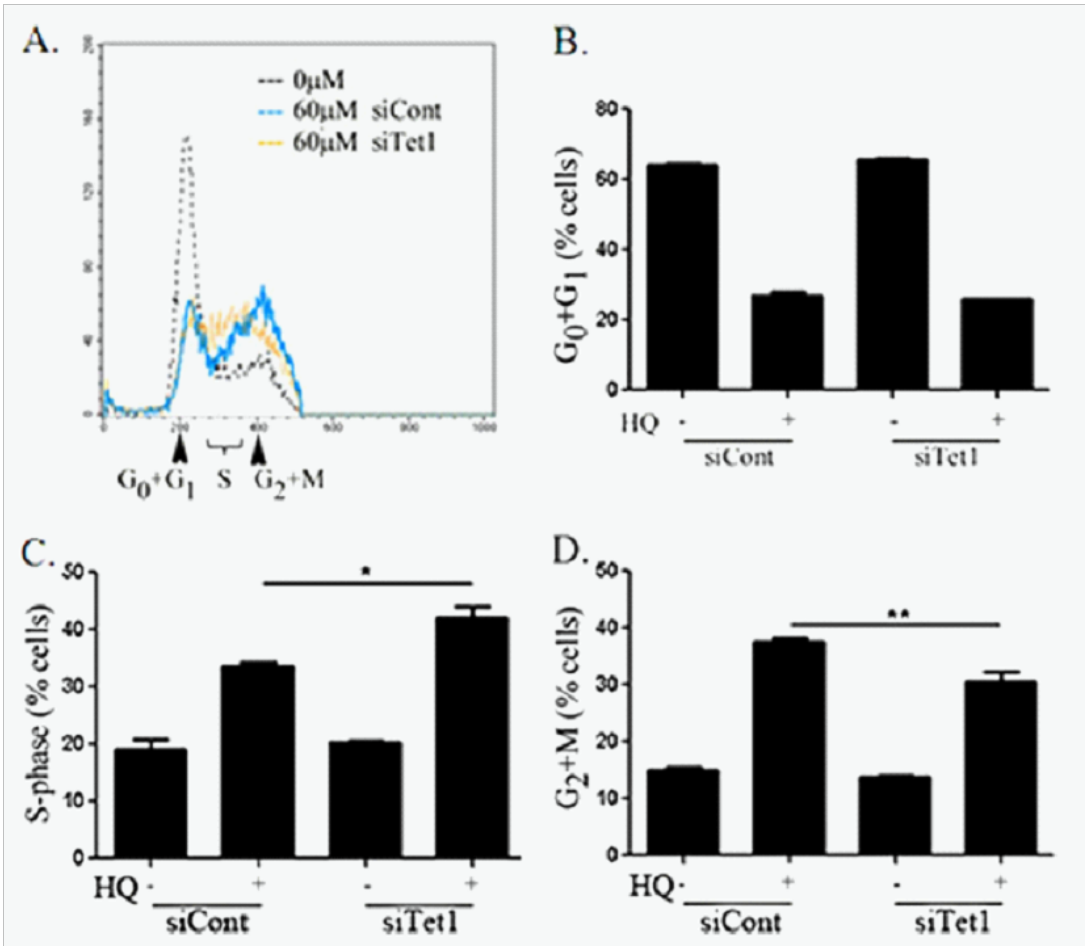

% cells
100
80
60
40
20
0
27.02
58.10
49.18
22.97
G2+M
S
G0+G1
sub-G0
control
HQ60
HQ100
HQ300
μM

TET1 Mediates Cell Cycle Changes following Hydroquinone Exposure

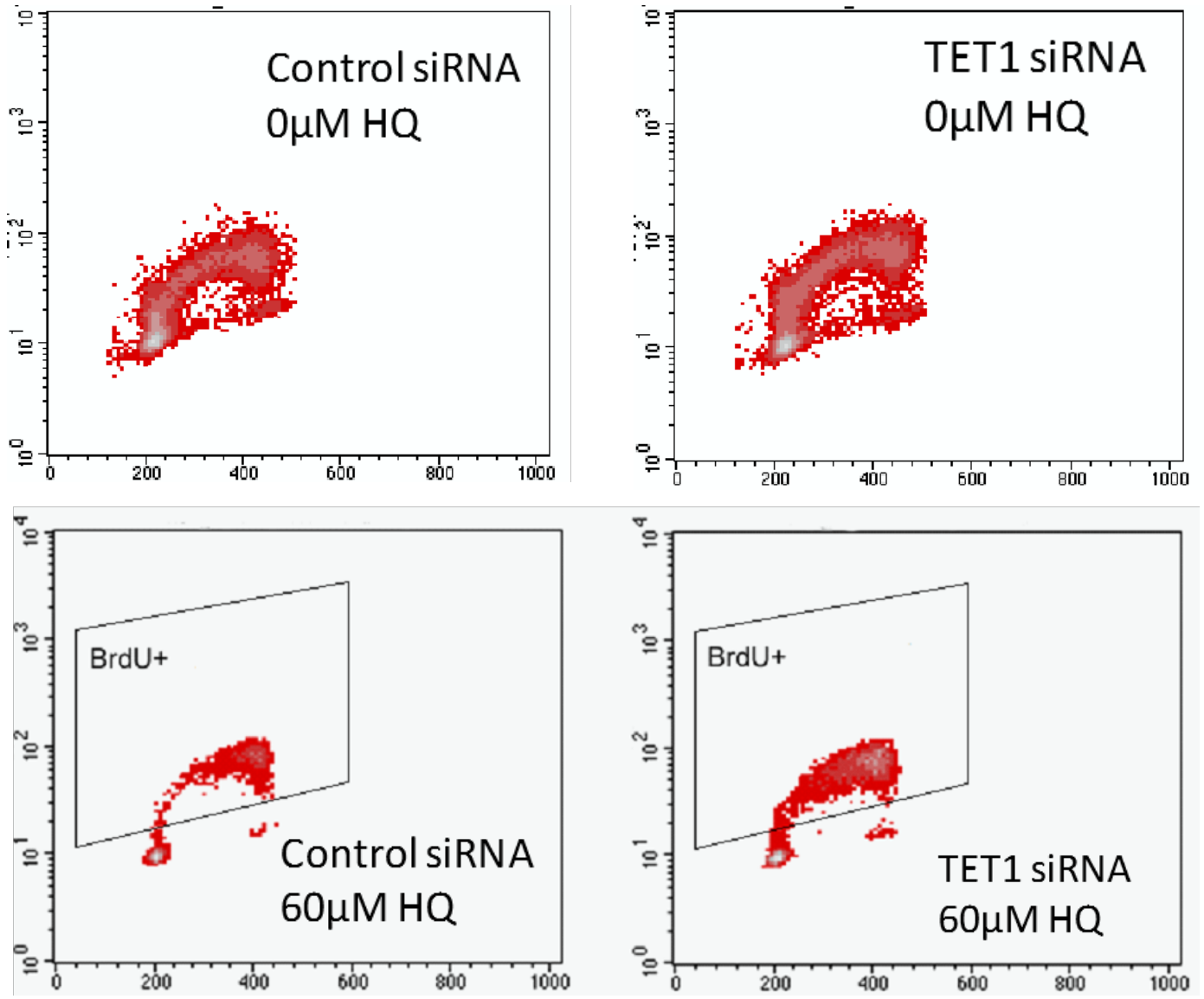
The effects of HQ on the cell cycle were undertaken by measuring DNA content with and without HQ exposure in cells transfected with control- or TET1-targeted siRNA (Fig 7A-D). HQ led to an increase in the percentage of cells in G_2+M from 15% in unexposed cells to 37.5% (Fig. 7*D*). There was no change in the sub-G0 population by HQ exposure. The percentage of cells in G_0+G1 phases decreased following HQ exposure, but this was likely due to the accumulation of cells in G_2+M; TET1 knockdown did not affect this change in cells in G_0+G1 (Fig. 7*B*). The percentage of cells in S phase increased following TET1 knockdown in HQ-exposed cells, from 33 to 42% (Fig. 7*C*). TET1 knockdown decreased the number of cells in G_2+M following HQ exposure to approximately 30% (Fig. 7*D*). Neither TET1 nor control knockdown had any effect on unexposed cells, nor did control knockdown affect the cell cycle compared with untransfected cells.

The observed increase in TET1-deficient cells in S-phase following HQ exposure is measured, by definition, as cells with DNA content between 2n and 4n. A more sensitive measure of S-phase quantification, involving labeling DNA-synthesizing cells with BrdU, was used to confirm the increase. An increase in BrdU+ cells was observed in HQ-exposed cells following TET1 knockdown (Fig. 7E), thereby supporting the conclusions made from DNA-content analysis. The data suggest that TET1 may play a role in promoting the cellular response to ROS-inducing agents, such as promoting arrest of the cell cycle to allow for efficient DNA repair.

Figure 7: The effect of TET1 on hydroquinone-mediated changes in the cell cycle. ***A***, representative cell cycle profiles of HEK293 cells exposed to HQ for 24 h following control or Tet1 knockdown are shown. Cells were fixed using methanol:acetone (1:1) and stained with propidium iodide. ***B–D***, 10,000 events were collected in each sample following control or Tet1 knockdown and exposure to 0 (-) or 60μM HQ (+) and percentage of cells in G_0+G_1 (***B***), S (***C***), and G_2+M (***D***) phases were quantified. **E**, BrdU labeled cells following control or TET1 knockdown and exposure to 0 or 60μM HQ. Cells were pulsed with BrdU for 6 hours at the end of a 24 hour HQ exposure and BrdU+ cells were labeled with anti-BrdU antibody and anti-mouse secondary prior to quantification by flow cytometry. Propidium iodide was used as a counterstain to indicate DNA content. *n*=3, * p<0.05 by one-way ANOVAand Tukey post-test. *Error bars*, S.E.



E


Control siRNA
0μM HQ
TET1 siRNA
0μM HQ
BrdU+
Control siRNA
60μM HQ
BrdU+
TET1 siRNA
60μM HQ

2.4 DISCUSSION

Epidemiologic and laboratory data suggest a negative correlation between DNA methylation and benzene or hydroquinone exposures (9, 22). We investigated the mechanism to account for the decrease by examining the effects of the major benzene metabolite, HQ, in HEK293 cells. While the use of an *in vitro* system as a model for changes in tissues is limited by its simplicity, the use of a single cell type avoids the often underestimated contribution of the selection of cell types or sub-populations of cells to observed changes in DNA methylation. Here, we demonstrate that concentrations of HQ that did not affect viability led to decreased DNA methylation in HEK293 cells in a mechanism involving ROS. Using an immunodotblotting technique, we show that genome-wide DNA methylation levels are decreased following a 24 hour exposure to HQ. The functionality of these decreases in DNA methylation was tested using a GFP reporter plasmid, which revealed that HQ-mediated demethylation results in an increase in gene expression. Because the plasmid is replication-incompetent (14), it is likely that the demethylation is active in nature and does not involve inhibiting the activity of DNMTs. HQ is a well known inducer of ROS and oxidative stress through the depletion of glutathione (19, 20). The addition of NAC abrogated the reactivation of methylated reporter plasmid, suggesting that HQ caused demethylation in a mechanism involving ROS. ROS and oxidative stress have been shown to lead to both increases (31) and decreases (32, 33) in DNA methylation. Interestingly, the low concentrations of HQ used did not significantly increase oxidized glutathione, lipid peroxidation (data not shown), or 8-oxo-dG formation, indicating that DNA demethylation was induced by relatively low levels of ROS that do not result in oxidative stress. This may be particularly relevant in

cells and tissues that are sensitive to changes in redox status or in cases of signaling events that may be mediated by ROS. For example, endogenously produced ROS have been shown to modulate phosphatase activity at physiologic levels, suggesting that these enzymes evolved to be responsive to ROS.

Whereas mechanisms explaining DNA demethylation have been largely focused on inhibition of the DNMT family of DNA methyltransferases, recent evidence suggests a role for the TET family of 5mC dioxygenases in active DNA demethylation. The proposed mechanism entails the conversion of 5mC to 5hmC catalyzed by TET1, followed by deamination to 5hmU and subsequent base excision repair, resulting in an unmethylated cytosine (12–14). Our studies provide support that this mechanism is responsible for DNA demethylation in cells exposed to HQ. Immunodotblots of genomic DNA from HEK293 cells exposed to 60 or 100 μM HQ reveal significantly higher levels of 5hmC than unexposed controls. 5hmC was also enriched at ORF2 of the retrotransposon LINE-1 12 hours following HQ exposure, followed by a decrease in both 5mC and 5hmC at 24 h. As LINE-1 is estimated to comprise 15–19% of human genomic DNA (26), these results support the data from immunoblots of genome-wide increases in 5hmC and also show that the increase in 5hmC is followed by a decrease in genomic methylation, which suggests that 5hmC is an intermediate in DNA demethylation in HQ-exposed cells. The increase and decrease in 5hmC and 5mC, respectively, are also associated with gene expression. Enrichment of 5hmC at the promoters of GCLC and 14-3-3σ was associated with increased expression of their mRNAs after exposure to HQ. Because 5mC and 5hmC have different binding proteins, suggesting very different biological roles in epigenetic regulation, it is not clear whether demethylation is

necessary for gene expression or if 5hmC formation alone is sufficient. A possible explanation for the rapid decrease in 5mC in GCLC and 14-3-3σ but not LINE-1, could be that LINE-1 is heavily methylated to approximately 75-90%, and changes in 5mC may not be detectable in our immunodotblotting technique until 24 hours post-HQ exposure. However, increases in 5hmC which are smaller in magnitude could be detectable in LINE-1, as the mark is reportedly relatively low at LINE-1.

Previous studies involving chemical- and radiation-induced changes to cytosine suggest that direct oxidation of 5mC can lead to 5hmC (34, 35). The lack of 8-oxo-dG formation suggests that ROS resulting from 60 μM HQ does not directly oxidize 5mC. Instead, exposure to HQ resulted in an increase in nuclear TET activity. Moreover, siRNA-mediated knockdown of TET1 significantly decreased total TET activity and prevented HQ-mediated 5hmC formation, highlighting the role of the protein in the epigenetic changes following HQ exposure. We also detected higher levels of nuclear TET1 by Western blotting with no change in mRNA levels. Although it is unclear how protein levels are affected by HQ, TET1 has been shown to form multimers via oxidation (12), raising the possibility that its nuclear transport was altered. It is also possible that the increase in activity was due to post-translational modifications that were caused by ROS. Importantly, the increase in activity is likely due to changes in protein levels or post-translational modifications and not in cofactors, because an excess of cofactors is included in the TET activity assay, and cofactors such as a-ketoglutarate are required for numerous other enzymes required for cell survival. Finally, the involvement of a TET1-mediated DNA demethylation mechanism was supported by the increase in

GFP expression in cells transfected with the cytidine deaminase AP2, further supporting an oxidation-deamination mechanism in DNA demethylation.

HQ exposure has long been known to increase ROS and affect the cell cycle, an effect attributed to disruption of microtubule assembly through sulfhydryl reactivity (18, 21). Here we demonstrate that TET1 mediates the expression of *14-3-3σ*, a p53 target gene, in response to HQ exposure. Moreover, TET1 partially mediates the HQ-induced accumulation of cells in G_2+M phases of the cell cycle, which could be due to its influence over 14-3-3σ . It is possible that TET1 is involved in preventing cells from progressing through the cell cycle when ROS levels are elevated to protect the genome against damage and the accumulation of mutations that could promote uncontrolled cell growth. It is not clear whether TET proteins play a role in carcinogenesis or cell stress responses, although the role of TET1 in HQ-mediated expression of genes involved in cell cycle regulation and antioxidant defense warrants further study. Tumor tissues are reportedly low in 5hmC levels, and loss of TET expression has been demonstrated in malignant glioblastoma (36) and hepatocellular carcinoma samples (37), suggesting that loss of TET activity may contribute to tumorigenesis. To our knowledge, this is the first study to demonstrate that responses to ROS-mediated perturbations in the cell cycle are modulated by TET1.

In summary, we have shown that a ROS-inducing chemical increases genome-wide and gene-specific 5hmC formation through TET1 methylcytosine dioxygenase. Future studies should examine whether the observed effects are unique to HQ or shared by other xenobiotics that increase ROS. We suggest that TET1 represents a link between cellular redox status and maintenance of the epigenome.

2.5 METHODS

Chemicals and Reagents

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich. Nit-GFP, human APOBEC2 (AP2), and TET1 catalytic domain plasmids were obtained from Dr. Hongjun Song's laboratory (JHU) and used according the published methods (14). Silencer Select Predesigned siRNA against *Tet1*(4392420; ID s37194) and control (4390846) were obtained from Ambion and used at 10 nM.

Cell Culture

HEK293 cells were obtained from ATCC and grown in DMEM supplemented with 10% FBS. Hydroquinone, menadione, buthionine sulfoximine, and *N*-acetylcysteine (NAC) stocks were freshly prepared in H2Oand sterile-filtered.

Cell Viability MTT Assay

Cytotoxicity was measured using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) protocol. In brief, 1.5 x 10^4 cells were seeded in each well of a 96-well plate. Cells were exposed to the indicated concentrations of HQ for 24 h, washed with PBS, and incubated with 10 mg/ml MTT reagent in PBS at 37 °C for 1 h. Cells were lysed, and formazan crystals were dissolved in 100 µl of dimethyl

sulfoxide. Absorbance was read at 570 nm using a microplate reader, and data were expressed as a percentage of non-exposed cell MTT reduction.

Western Blotting

Whole cell lysates were prepared using radioimmunoprecipitation assay buffer and included addition of protease inhibitors. Protein concentrations were measured using the Bradford assay, and 30μg of whole cell lysates was separated on a 4–20% Tris-glycine gradient gel (Invitrogen). Nuclear lysates were prepared using Panomics Nuclear Extract kit (Fremont, CA) and measured using the Bradford assay. 15 μg of nuclear lysates was separated on a 4–12% Tris-glycine gradient gel (Invitrogen). Protein was transferred to a nitrocellulose membrane, blocked with 0.5% casein, and incubated overnight at 4 °C with antibodies against V5© (Invitrogen; 1:5000), FLAG-M2© (Sigma; 1:2000), actin (Sigma; 1:5000), TET1 (Abnova; 1:1000), or TATA-binding protein (Millipore; 1:1000). Blots were incubated with secondary antibody (1:10,000) for 1 h before visualization on the Licor Odyssey Imager.

Quantitative RT-PCR

RNA was isolated using TRIzol and quantified using NanoDrop. 1μg was used to generatecDNA (SuperscriptIII; Invitrogen). qPCR was performed using a Bio-Rad iCycler and iQ SYBR Green supermix according to the manufacturer's instructions. Primers used were as follows:

14-3-3σ forward, GGCCATGGACATCAGCAAGAA

14-3-3σ reverse, CGAAAGTGGTCTTGGCCAGAG;

GAPDH forward, ACATCGCTCAGACACCATG

GAPDH reverse, TGTAGTTGAGGTCAATGAAGGG;

GCLCforward, AAAAGTCCGGTTGGTCCTG

GCLC reverse, CCTGGTGTCCCTTCAATCATG;

TET1 forward, TTCGTCACTGCCAACCTTAG

TET1 reverse, ATGCCTCTTTCACTGGGTG;

TET2 forward, CACTGCATGTTTGGACTTCTG

TET2 reverse, TGCTCATCCTCAGGTTTTCC;

TET3 forward, TCCGGATTGAGAAGGTCATC

TET3 reverse, CCAGGCCAGGATCAAGATAA.

Immunodotblotting of Genomic DNA

Genomic DNA was isolated using GenElute Mammalian Genomic DNA kit (Sigma) and measured using a NanoDrop (Thermo Scientific). 250 ng was denatured with 0.4 M NaOH at 95 °C for 10 min. DNA was immobilized on nitrocellulose membrane using a 96-well vacuum apparatus and then dried and fixed by vacuum baking at 80 °C. The membrane was then rehydrated in room temperature PBS and blocked with 0.5% casein for 1 hour prior to probing with antibodies against 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) (Trevigen; 1:2500), 5-mC (Active Motif; 1:1000), or 5-hmC (Active Motif; 1:8000). Mouse anti-single-stranded DNA (Abcam; 1:1500) antibody was used simultaneously for normalizing 5hmC. Antibody binding was measured using the Licor Odyssey. Methylene blue staining of DNA (0.04% with 0.3 M sodium acetate) was used for normalization of

8-oxo-dG and 5mC antibodies. Intensity of methylene blue was measured using ImageJ software (National Institutes of Health). Linear range of detection of DNA was determined by loading increasing amounts of single-stranded DNA and probing with increasing dilutions of anti-single-stranded DNA antibody. 5hmC Ab specificity was confirmed by blocking antibody with 2µg/ml 5hmC or 5mC at room temperature for 2 hours and blotting DNA from mock-transfected and TET1-overexpressing HEK293 cells.

Methylation-Sensitive Reporter Assay

Nit-GFP plasmid was methylated using 4 units of CpG methyltransferase and 0.6mM *S*-adenosylmethionine (New England Biolabs) at 30 °C for 12 hours and purified using Qiagen gel extraction columns. 2.5 x10^5 HEK293 cells were transfected with 0.5 µg of methylated or unmethylated Nit-GFP plasmid, and co-transfections were used at 1:4 GFP:TET1 (positive control), AP2, or Bgal for 48 hours using Lipofectamine. Cells were exposed to 60 µM HQ for 24 hours, beginning 24 hours after transfection. Cells were harvested using 0.25% trypsin-EDTA, fixed using ice-cold methanol:acetone (1:1), and stored at -20 °C. Cells were washed three times and resuspended in 4 °C PBS. GFP fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using CellQuest software. Dead cell and doublet discrimination were accomplished using forward scatter *versus* side scatter gating, and at least 10,000 events were analyzed in each sample for all experiments

Bisulfite Sequencing

Bisulfite sequencing was performed as reported by Guo *et al.* (14). At the end of the 24 hour exposure to 60μM HQ, DNA from cells transfected with methylated Nit-GFP plasmid was isolated and bisulfite modified using the EpiTect bisulfite kit (Qiagen). The Nit-GFP promoter was PCR-amplified using primers recognizing the bisulfite-modified sequence and gel-purified. Bands of the predicted size were cloned into TOPO TA vector (Invitrogen) for sequencing (Johns Hopkins University DNA Analysis Facility). 6 CpGs within the promoter were read in 23 clones. Primers used for bisulfite-sequencing of Nit-GFP promoter were forward, TTTTTTATTAGTGATAGAGAAAAGTGAAA and reverse,

CAAATAAACTTCAAAATCAACTTACC (14).

Measurement of Reactive Oxygen Species

Cells were grown in opaque, black 96-well plates and incubated with 50μM (final concentration) 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) in DMEM lacking phenol red for 30 min. DCF-DA was removed by washing twice with PBS, and cells were exposed to HQ, NAC (5 mM), or in combination. Fluorescence was measured at 485em 522ex beginning at 30 min and followed through indicated time points.

Immunoprecipitation of Hydroxymethylated/Methylated DNA

Genomic DNA was isolated from cells exposed to hydroquinone or control conditions. 8μg of DNA was sheared using intermittent sonication and validated to be 100–500 bp by gel electrophoresis. 2μg of DNA was heat-denatured for 10 min and placed immediately

in an ice bath. DNA was incubated with antibody against 5mC, 5hmC, or matched isotype control at 4 °C overnight. Protein G magnetic beads (Invitrogen) were added for 1 hour at 4 °C with agitation, and bead-antibody-DNA complexes were magnetically separated, followed by three washes in immunoprecipitation buffer (4 °C PBS with 0.05%Triton X-100). Protein was digested using proteinase K digestion buffer (50 mM Tris,pH8.0, 10mMEDTA) overnight at 50 °C. Proteinase K was inactivated by incubation at 80 °C for 30 min. DNA was purified using nucleic acid columns (Sigma). Immunoprecipitated DNA was used as a template for qPCR, and -fold enrichment was calculated using input DNA. Primers used for qPCR following hydroxymethylated/methylated DNA immunoprecipitation were as follows:

LINE1 forward, TGCGGAGAAATAGGAACACTTTT

LINE1 reverse, TGAGGAATCGCCACACTGACT (23);

14-3-3σ forward, CATTTAGGCAGTCTGATTCC

14-3-3σ reverse, GCTCACGCCTGTCATCTC (24);

GCLC forward, CGTCCCAAGTCTCACAGTCA

GCLC reverse, CTTTACGCAAACGCGACATA (25).

Total Ten Eleven Translocation Activity Assay

Total TET activity was measured using the Epigentek Epigenase5mChydroxylase kit (Farmingdale, NY) according to the supplier's protocol. Nuclear lysates were prepared using Panomics Nuclear Extraction kit (Fremont, CA) and measured using the Bradford assay. Hydroxymethylated product formed was measured within a standard curve (0–2

ng), and activity was calculated as ng/min/mg. Data are expressed as -fold change over unexposed nuclear lysates. Overexpression of the TET1 catalytic domain was used as a positive control.

DNA Content and Cell Cycle Analysis

Cells were harvested using 0.25% trypsin-EDTA, followed by inactivation in DMEM containing 10% FBS. Cells were washed in cold PBS and then resuspended in 300 µl of PBS at 4 °C, and fixed overnight at -20 °C in 1:1 methanol:acetone, added dropwise with intermittent vortexing. Cells were washed with cold PBS and incubated with 0.25 ml of 5 µg/ml RNase, 15 min at 37 °C. 0.25 ml of 100 µg/ml propidium iodide was added for 1 hour at room temperature. DNA content was measured using a FACSCalibur flow cytometer and analyzed using Cell Quest software (BD Biosciences). Doublet discrimination was accomplished by gating on forward scatter-H v. FL3A and FL3A v. FL3-H. At least 10,000 events were analyzed in each sample, and each condition was replicated in triplicate.

BrdU Labeling

shEV and shTET1 HEK293 cells were pulsed with 10µM freshly prepared BrdU for 6 hours at the end of a 24 hour exposure to 0- or 60µM HQ. Cells were harvested using 0.25% trypsin-EDTA, followed by inactivation by 10% FBS-containing DMEM. Cells were washed twice with PBS and resuspended in 300µL PBS. Cells were fixed by adding ice-cold 70% ethanol drop wise with intermittent vortexing and stored overnight at -20˚C. Cells were washed with cold PBS, and resuspended in a 2mL solution of 0.4mg/mL

pepsin in 0.1M HCl, made fresh, for 30 minutes. Cells were then washed in PBS, and resuspended in 300µL PBS. 1mL of 2M HCl was added drop wise with intermittent vortexing, followed by an additional 1mL of 2M HCl, and incubation at 37˚C. pH was neutralized by adding 12mL of 0.1M borax buffer, made fresh, for 10 minutes. Cells were pelleted and resusbpended in PBS with 0.05% Tween for permeablization. Approximately $1x10^6$ cells were aliquoted into 100µL and stained with 20µL anti-BrdU antibody for 1 hour at room temperature, followed by 1 hour incubation with secondary antibody in the dark. Cells were washed twice with PBS and resuspended in 1mL of PBS containing 10µg/mL propidium iodide. Cells were analyzed using a FACSCalibur flow cytometer and analyzed using Cell Quest software (BD Biosciences).

Chromatin Immunoprecipitation (ChIP)

Protein-DNA complexes were crosslinked using 37% formaldehyde, diluted to 1% final concentration in DMEM for 20 minutes at room temperature. Crosslinking was quenched by adding 1M glycine to 125mM final concentration for 10 minutes at room temperature. Cells were washed with PBS and pelleted. Cells were lysed using RIPA buffer with protease inhibitor cocktail (Sigma) and incubated on ice for 10 minutes. Lysates were sonicated for 10 cycles of 10 seconds on/10 seconds off on ice to produce chromatin approximately 500-1000bp. Chromatin size was validated using gel electrophoresis. Cell debris was removed by refrigerated centrifugation and supernatants were transferred to new tubes. DNA concentration was measured using nanodrop in input fraction saved from supernatants. Approximately 25µg DNA was used per immunoprecipitation, and 1µg anti-FLAG or non-immune mouse IgG antibody was added overnight at 4˚C with

rocking. After incubation with antibody, 25µL magnetic protein G beads (Invitrogen) was added for 2 hours at 4˚C with rocking. Bead-antibody complexes were washed three times with RIPA containing protease inhibitors, and DNA was eluted by adding 100µL elution buffer containing 1% SDS and 100mM $NaHCO_3$. DNA was purified using PCR purification kit (Sigma) and DNA was used as template for qPCR. Primers were as follows:

14-3-3σ forward, CATTTAGGCAGTCTGATTCC

14-3-3σ reverse, GCTCACGCCTGTCATCTC (24);

GCLC forward, CGTCCCAAGTCTCACAGTCA

GCLC reverse, CTTTACGCAAACGCGACATA (25).

Statistical Evaluation

GraphPad Prism version 5 was used for statistical analyses. All data are summarized as mean ± S.E. Student's *t* test was performed for all single analyses. Multiple comparisons were made using one-way analysis of variance (ANOVA) and Tukey's, Dunnett's, or Newman-Keul post hoc tests. p values < 0.05 were considered significant.

III. TET1 supports γH2AX formation and protection against DNA breaks in response to topoisomerase inhibition.

3.1 ABSTRACT

Altered DNA methylation is a common trait observed in cancers, neurological diseases, and inflammatory syndromes. The frequent observation of altered epigenetic marks early in disease progression suggests they may play a causal role in the initiation of events leading to disease. Much effort has therefore been directed toward understanding regulation of DNA methylation in hopes of identifying targets for biomarkers and treatments. In general, cancer cells display genome-wide hypomethylation and promoter hypermethylation. It has been proposed that tumor cell DNA methyl transferases (DNMTs) have altered activities and binding sites, leading to inappropriate silencing of tumor suppressors. An additional regulator of gene expression may be Ten-Eleven Translocation (TET) 5-methylcytosine dioxygenases, which have been shown to lead to active DNA demethylation in mammalian cells. The role of TET1 in changing the DNA methylation landscape and supporting the expression of cytoprotective genes such as GCLC and 14-3-3σ raises the possibility that TET1 may be involved in preventing DNA damage that could result in tumor-promoting mutations. Consistent with this idea are the observations that hepatocellular carcinomas and malignant glioblastomas, among other cancer types, often display downregulated TET expression and 5hmC levels. It is unclear whether the altered TET system provides an advantage to tumor cells

In this study, TET1 was knocked down using lentiviral vectors cells in HEK293 to test its potential role in the response to the DNA double-strand break-inducing topoisomerase I inhibitor camptothecin (CPT). As expected, CPT exposure caused an increase in phosphorylated serine-139 of histone variant H2AX (γH2AX), G_0+G_1 arrest, and cell death. However, TET1-defiecient cells exposed to CPT accumulated significantly less H2AX and fewer cells stained positive for annexin V, suggesting that TET1-deficient decreases sensitivity to CPT. γH2AX is induced by agents that cause DNA strand breaks and is often used to monitor DNA damage. γH2AX is an early step in the pathway in which DNA strand breaks are first recognized and then repaired. To determine whether differential levels of γH2AX accumulation may indicate a role for TET1 in sensitivity to DNA damage or DNA repair, TUNEL labeling was used to measure exposed 3'OH ends in DNA. Interestingly, TET1-deficient cells exhibited significantly more DNA breaks in the presence or absence of CPT. These data suggest TET1 plays a role in the formation of γH2AX and promotes cell death in response to DNA damaging agents. Confirming the role of TET1 in the formation of γH2AX, overexpression of the catalytic domain of TET1 was able to rescue its induction in TET1-deficient cells. Unexpectedly, catalytically-inactive mutant TET1 also rescued γH2AX induction in response to CPT, indicating a non-enzymatic role for TET1 in response to DNA damage.

While TET enzymes are primarily known for their role in gene expression by hydroxylating CpG methyl groups, this work demonstrates a novel non-enzymatic role for TET1. Moreover, a role for TET1 as a modifier of the response to DNA damage was

uncovered, which may form a foundation for future studies into the contribution of the pathway in disease.

3.2 INTRODUCTION

Preservation of the genetic code is an essential cellular function, ensuring proliferation occurs in a regulated manner. Genetic material is persistently threatened by both endogenous and exogenous mutagens, such as reactive oxygen (ROS) and nitrogen (RNS) species and alkylating agents, which have great potential to alter the genetic code (1,2). Mutations in tumor suppressor genes have been shown to contribute to tumorigenesis and a multitude of neurological (3) and immunological diseases (4). For example, mutations in *TP53*, which have been observed in most tumor types, interfere with cell cycle regulation, attenuate DNA repair, and impair cells from undergoing programmed cell death following genotoxic insults. Experimental models of lung tumorigenesis in mice show that tobacco-derived carcinogens result in G→A transitions at the second base of codon 12 and AT-GC transition or AT-TA transversions in codon 61 in *KRAS,* an oncogene known to contribute to lung cancers in humans (54). Such mutations, if unchecked, may lead to uncontrolled cellular proliferation– an imbalance in mitosis *and* apoptosis – and malignancy.

While pre-cancerous cells accumulate mutations throughout the genome, gene promoters are more often methylated early in tumorigenesis. The tumor suppressors 14-3-3σ , p16 (INK4a), and p15 (INK4b) are often hypermethylated in their respective promoters in human cancers, and are thought to contribute to tumor formation by preventing cell cycle checkpoint regulation (55). GSTP1, a major phase II metabolism

enzyme known to facilitate clearance of reactive electrophiles, has been shown to be downregulated by promoter methylation in over 90% of prostate cancers (55). The MGMT gene, which repairs 0^6-alkyl-guanine, contributes to resistance to the alkylating agents Carmustine and Temozolomide in the treatment of malignant glioma. Interestingly, however, a significant survival benefit was observed in patients with methylated MGMT promoter and treated with alkylating therapeutic agents (56). Though still emerging, the roles of methylation-mediated gene silencing in disease are well-accepted and discoveries are providing prognostic benefit. However, mechanisms of regulation over the complex DNA methylation dynamic are far from understood and present new challenges.

The DNA methylation landscape is established by DNA methyltransferases (DNMTs 1, 3a, 3b), which transfer a methyl group from S-adenosylmethionine to the 5-carbon of cytosine. DNMT1, known as the maintenance methyltransferase, is thought to recognize hemi-methylated DNA during synthesis and transfer a methyl group to the daughter strand CpG. DNMT3a and DNMT3b are known as *de novo* methyltransferases, transferring methyl groups to both hemi- and unmethylated sites (39). Though the mechanisms involved in promoter-specific hypermethylation in cancer are poorly understood, it has been shown that oncogenic transcription factors can facilitate promoter methylation through their association with DNMTs. The PML-RAR fusion protein in acute promyelocytic leukemia is one of the first discoveries to illustrate this mechanism. Differential DNMT expression in various stages of the cell cycle in normal and cancer cells has also been suggested to contribute to the genomic hypomethylation observed in cancer cells (56).

An additional exciting area of investigation is the epigenetic regulation of genes in stem-like tumor propagating cells that likely contribute to recurrence following therapeutic intervention. Evidence has been published linking promoter methylation of genes involved in differentiation of stem and progenitor cells and activating histone modifications associated with promoters of transcription factors promoting stemness (57). These modifications in methylation will establish an epigenetic landscape promoting tumorigenic potential and therapeutic resistance in cell culture and xenografts studies of glioblastoma (57). Suva *et al.* described a network of four transcription factors capable of reprogramming more differentiated cells to stem-like cells, each of which are known to associate with the activating histone-3 acetylation (H3K27ac) mark. DNA methylation and histone modifications are functionally linked, and efforts to target enzymes involved in either pathway may have far reaching effects throughout the genome. For example, the methyl-DNA binding protein MeCP2 is known to bind histone deacetylase (HDAC), coupling CpG methylation to the removal of activating histone marks. Targeting DNA methylation, therefore, has the ability to affect larger scale chromatin architecture and the expression of numerous genes located far from the methylation site. Clinical trials of drugs shown to cause demethylation, such as Decitabine and Zebularine, are currently being investigated and have shown clinical benefit (45). A significant goal in designing novel therapies is defining the epigenetic circuitry allowing for reprogramming in hopes of targeting the pools of stem-like cells in the bulk tumor (57).

Recent work has shown that TET 5-methylcytosine dioxygenases, a-ketoglutarate- and divalent iron-dependent enzymes that hydroxylate methyl groups added by DNMTs, lead to stable 5hmC or initiate a multistep process of active

demethylation (7). In light of the significant role of TET proteins' contribution to active demethylation, the DNA methylome is being appreciated as more dynamic than previously thought. The idea that TET-mediated 5hmC is a stable base is an intriguing one. High levels of 5hmC in human and mouse brain and embryonic stem cells support this notion, though there is also evidence to suggest neuronal activity cooperates with TET1 to demethylate CpGs and activate transcription of activity-regulated genes (7). TET activity is therefore thought to influence gene expression in multiple ways. First, if 5hmC is indeed a stable "sixth base," then the base is predicted to change the binding affinity of methyl-binding domain-containing proteins, such as MBD2 and MeCP2, which are known to participate in blocking transcription factor binding as well as orchestrating chromatin architecture changes which repress gene expression (39). Secondly, the presence of 5hmC in a promoter may affect the ability of DNMT1 to recognize the position as methylated, thus leading to a demethylated daughter strand (51). Third, TET activity that results in demethylation of cytosine would essentially erase the restrictive methyl mark, relieving the repression brought about by the mark itself or its numerous binding proteins. It has also been proposed that some transcription factors may bind to 5hmC-containing promoters with increased affinity (51).

It has been demonstrated that numerous cancers have decreased TET expression and activity, and the global content of 5hmC is nearly uniformly low compared to non-tumor tissue (36,37). Increased methylation of CpGs by DNMTs along with the lack of TET activity in tumor cells – either by loss of TET expression or through oncometabolite-induced inhibition of activity - is thought to contribute to the so-called CpG Island Methylator Phenotype (CIMP) of cancers (39). It is tempting to think that

downregulation of TET enzymes provides advantages to tumor cells by shifting the methylation dynamic toward a hypermethylation phenotype. For example, could the lack of TET-promoted cytoprotective genes lead to increased genomic instability or resistance to DNA damaging agents? Could low TET enzyme levels or activity lead to silencing of pro-apoptotic genes that would otherwise be active in a non-tumor cell? Are low levels and activities of TET enzymes merely supportive of tumorigenesis or required? The consequences of low TET expression and 5hmC levels are not well understood, and provide a potentially rich area for exploration into mechanisms of tumor initiation and therapeutic resistance.

In this study, evidence suggesting that TET1 plays a role in the response to DNA damage is presented. TET1 was stably knocked down in HEK293 cells using lentiviral vectors to evaluate the effects of the topoisomerase I inhibitor camptothecin (CPT). The inhibitor is a prototypical inducer of DNA double strand breaks resulting in activation of the p53 pathway and apoptosis. Control cells displayed phosphorylation of histone variant H2A.X at serine-139 (γH2AX), accumulation of cells in G_0+G_1, and decreased viability following exposure to CPT. TET1-deficient cells treated with CPT accumulated significantly less γH2AX and fewer cells stained positive with annexin V, indicating lower sensitivity to the drug. In order to determine whether differential levels of γH2AX represented resistance to CPT-induced damage or a deficient response to damage, TUNEL labeling was conducted to quantify cells with damaged DNA. Interestingly, TET-1 knockdown led to a significant increase in damaged DNA both with and without CPT exposure compared to control cells. While TET1 is known to support the expression of cytoprotective genes in the context of stress, both active and inactive TET1 catalytic

domain overexpression surprisingly rescued γH2AX formation in TET1 knockdown cells exposed to CPT, indicating a non-enzymatic role for the protein in response to DNA damage.

These data are the first to indicate a non-enzymatic role for TET1 in the response to a DNA damaging agent, and suggest that TET1 plays a significant function in supporting genomic stability in the context of both endogenous and exogenous stressors.

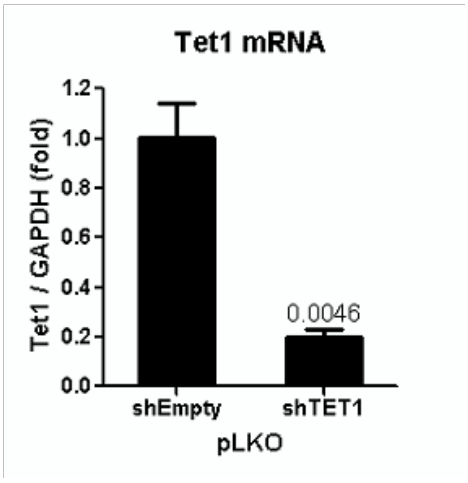
3.3 RESULTS

TET1 Supports γH2AX Formation Following Exposure to Camptothecin in HEK293 Cells

As expected, a 14 hour treatment of 1μM camptothecin (CPT) led to an increase in formation of serine-139 phosphorylation of histone variant H2A.X on (γH2AX) in HEK293 cells (Fig. 1*A*). However, the amount of γH2AX was lower in TET1-deficient cells treated with CPT (Fig. 1*B* and *C*). The effects of CPT were dose-dependent though the differences between cell lines were observed at each dose (Fig. 1*D*). Immunofluorescent staining was used to confirm that the differential levels of γH2AX formed as characteristic foci in cells exposed to CPT and not a diffuse increase throughout nuclei (Fig 1*E*).

Figure 1: TET1 knockdown decreases formation of γH2AX following camptothecin exposure. ***A***, *Tet1* mRNA levels measured by qRT-PCR in HEK293 cells following infection with lentivirus encoding shRNA against *Tet1*(shTET1) or empty pLKO vector (shEV). *GAPDH* levels are used as control. ***B***, Western blot of γH2AX in shEV and shTET1 cells following 1μM CPT exposure for 14 hours. GAPDH is used as loading control, and ratios are represented in ***C***. ***D***, Western blot of γH2AX in shEV and shTET1 cells following 0-, 1-, 3-, or 10μM CPT for 14 hours. GAPDH levels are used as control. ***E***, Representative immunofluorescent images of γH2AX (*red*) in shEV and shTET1 cells following 14 hours of 0-, 1, or 3μM CPT. DAPI (*blue*) represents heterochromatin. Arrows indicate γH2AX foci.

A



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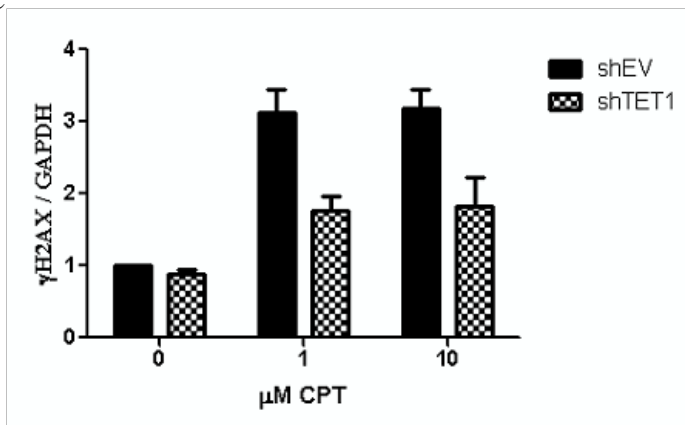

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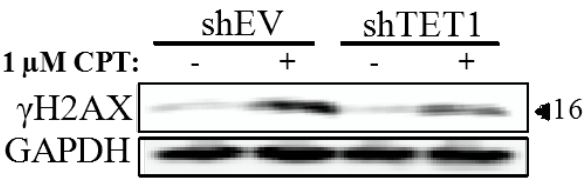
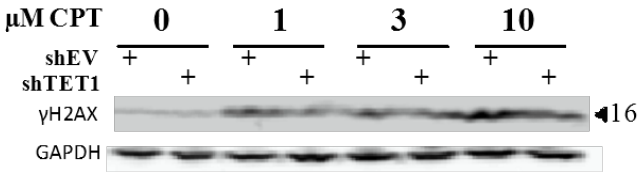
B.





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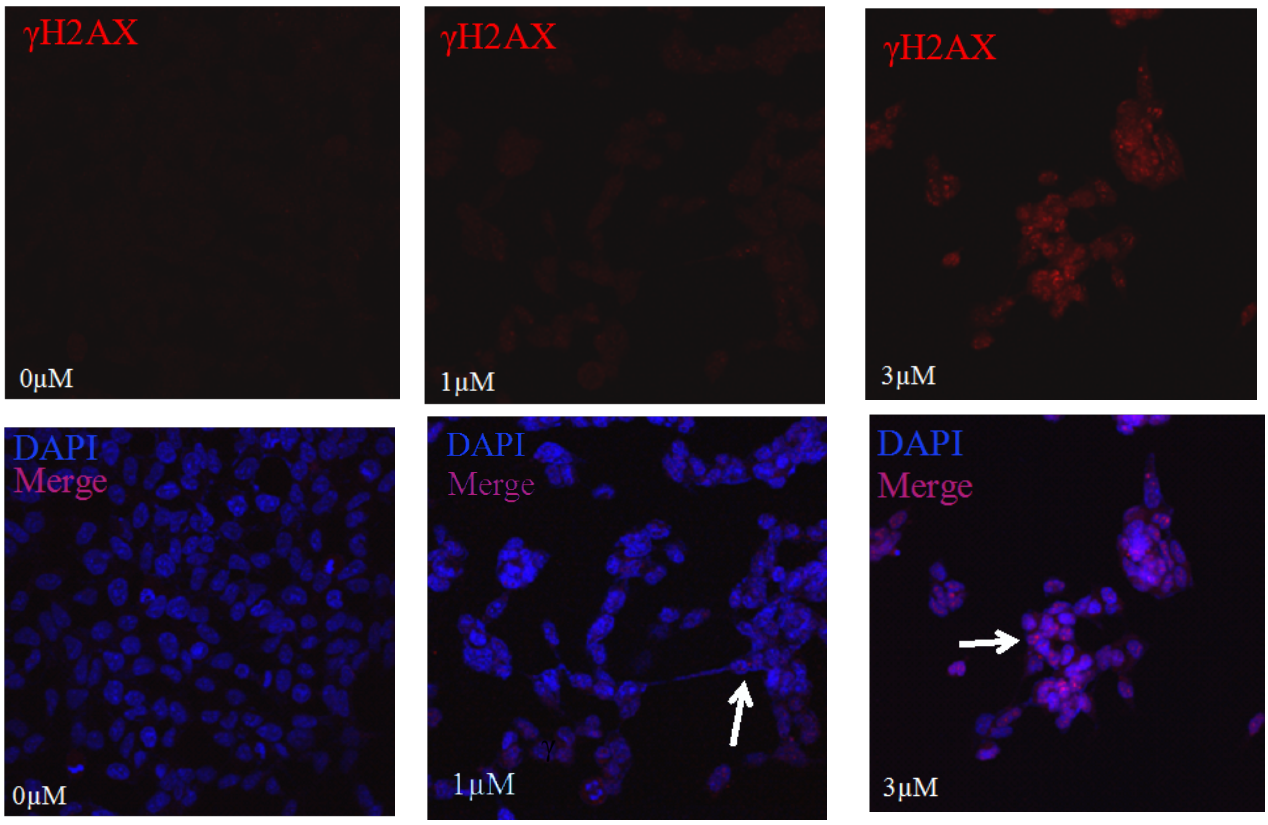
shEV
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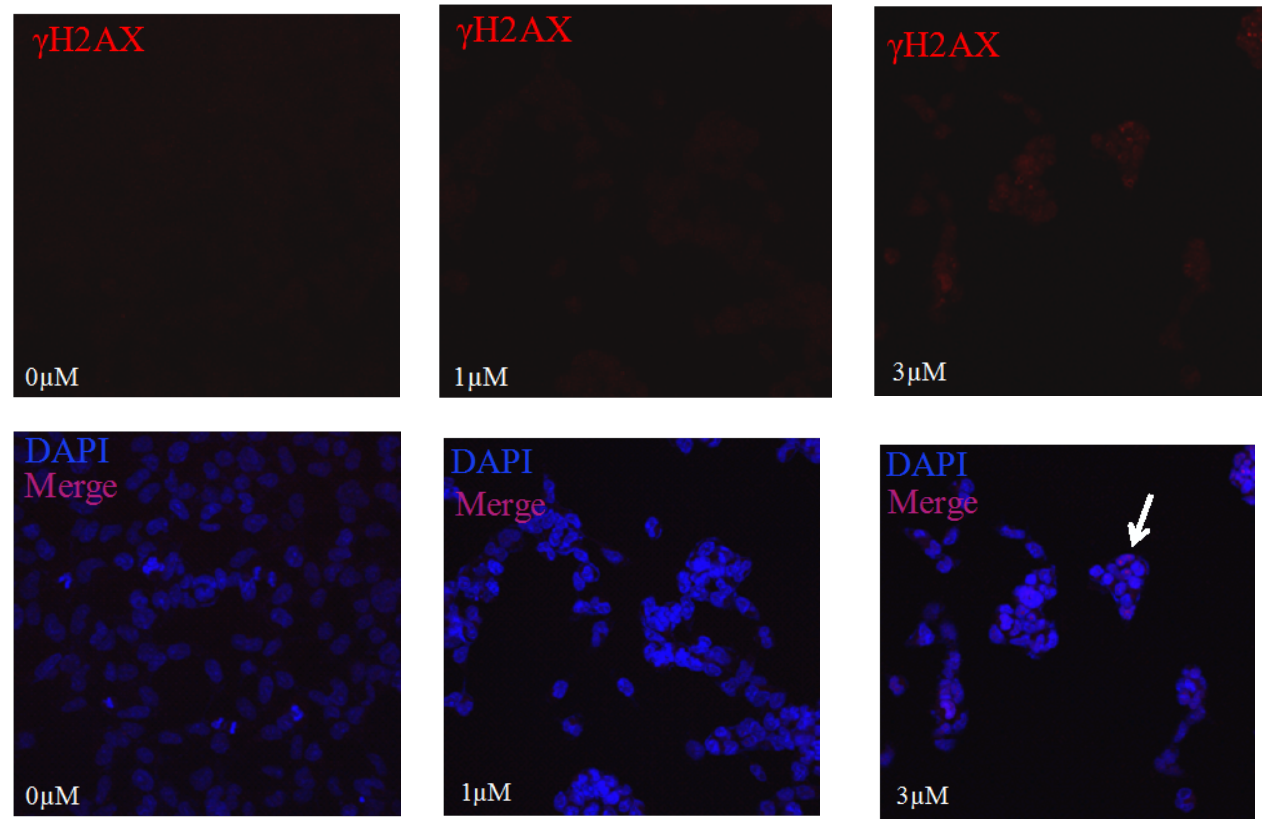
shTET1
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γH2AX
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DAPI
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0μM
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Merge
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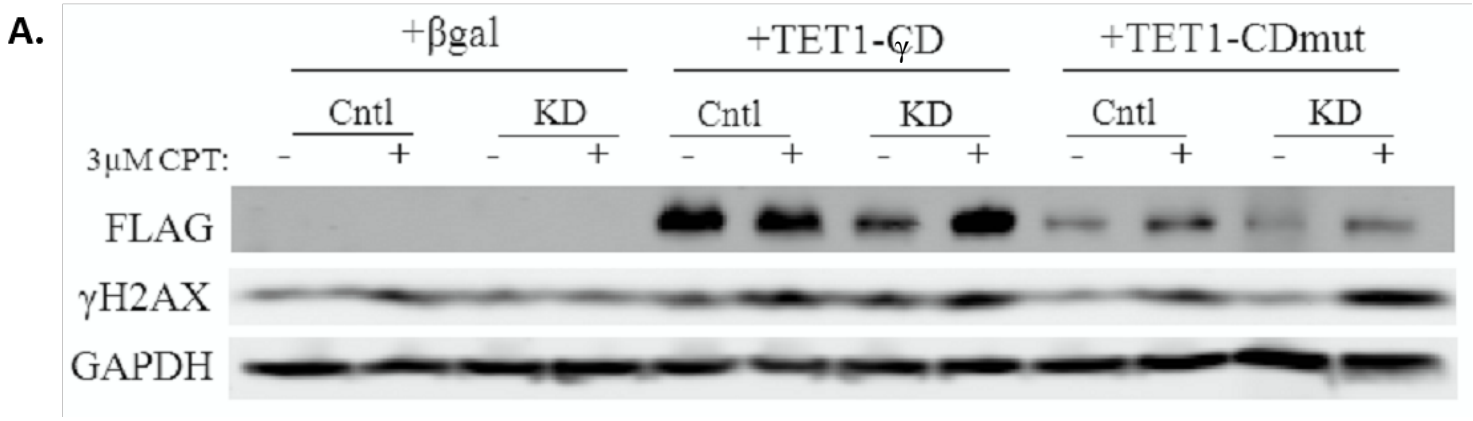
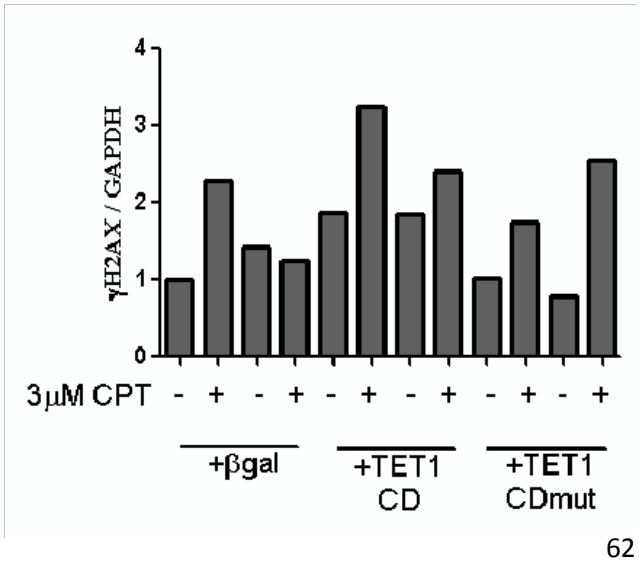
DAPI
Merge
3μM

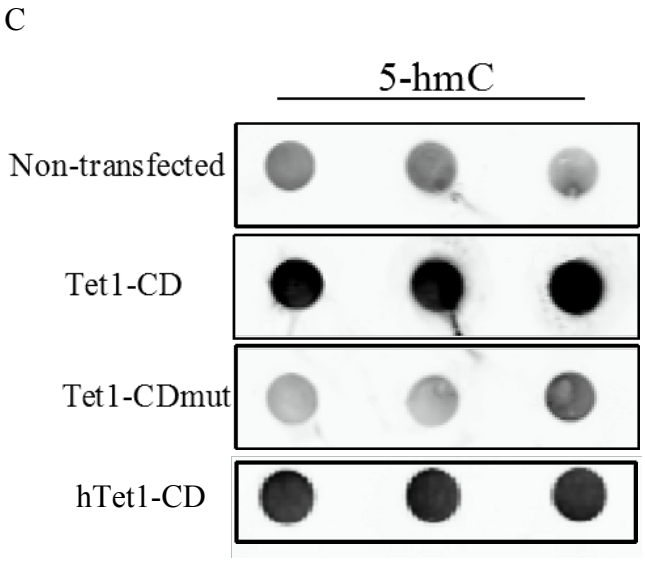
Camptothecin-Induced γH2AX Formation is Dependent on TET1 Catalytic Domain, but Independent of Catalytic Activity

To confirm the dependence of γH2AX on TET1 and test the requirement for TET1 activity on γH2AX formation in response to CPT exposure, shEV and shTET1 cells were exposed to 3μM CPT for 14 hours 24 hours following transfection of βgal, catalytically active mouse FLAG-tagged TET1 (Tet1-CD), or mouse mutant catalytically inactive FLAG-tagged TET1cd (Tet1-CDmut). Both Tet1-CD and Tet1-CDmut overexpression were able to rescue formation of γH2AX following exposure to CPT, suggesting that CPT-induced γH2AX is not associated with hydroxylation of 5mC by TET1 (Fig 2*A* and *B*). Immunodotblotting confirmed that the highly conserved mouse TET1 catalytic domain increased 5hmC similarly to human TET1 catalytic domain cloned from HEK293 cells. An increase of 5hmC in HEK293 cells transfected with catalytically active mouse Tet1-CD, but not catalytically inactive mouse Tet1-CDmut (Fig 2*B*).

Figure 2: Activity-independent rescue of camptothecin-induced γH2AX by TET1. ***A***, Western blot of γH2AX in shEV and shTET1 cells following 0- or 3μM CPT exposure for 14 hours following transfection with βgal, FLAG-tagged Tet1-CD, or FLAG-tagged Tet1-CDmut. GAPDH was used as loading control, and ratios of γH2AX / GAPDH are shown in ***B***. ***C***, Immunodotblot of 5hmC in untransfected HEK293 cells or overexpressing human TET1-CD, mouse Tet1-CD, or mouse Tet1-CDmut. (KD, knockdown)



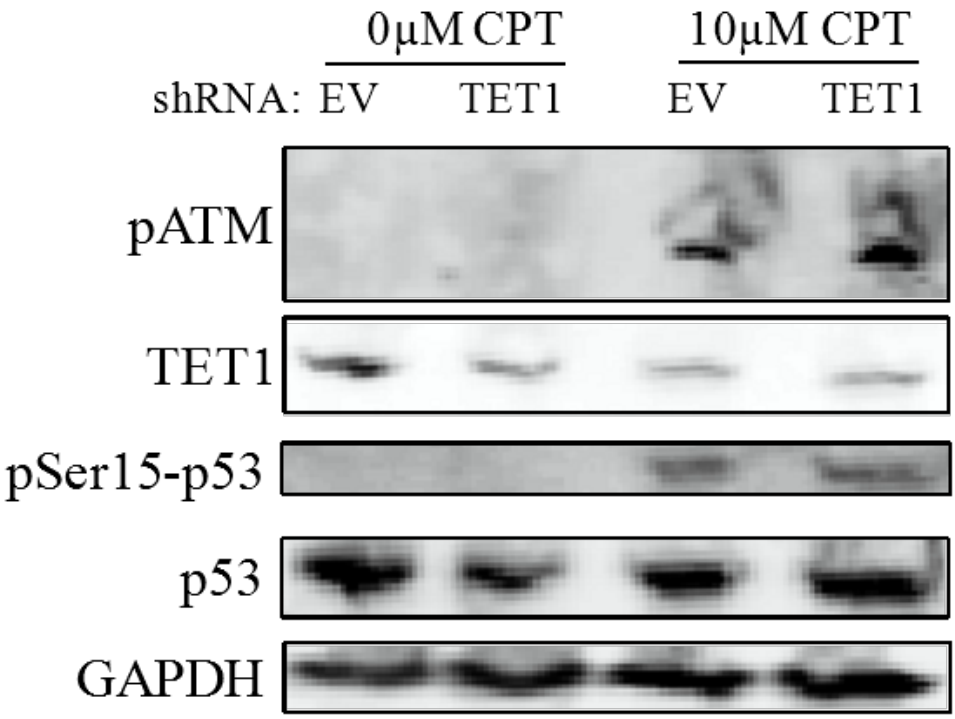




TET1-deficiency Leads to Dampened γH2AX Levels Independent of ATM or p53 Activation

The ability of TET1 overexpression to rescue γH2AX levels following CPT treatment raises the possibility that TET1-deficient cells are less vulnerable to damage or have altered activation of ATM, a kinase known to phosphorylate H2AX in response to DNA damage. Western blotting of phospho-ATM (pATM), and phospho-p53 (pSer15-p53) show that control and TET1-deficient cells had similar levels of activated ATM and p53 following CPT treatment (fig. 3). These data suggest CPT leads to DNA damage and activation of sensors of damage in TET1-deficient cells, and that differential γH2AX formation is independent of the levels and activation of ATM or p53 effectors.

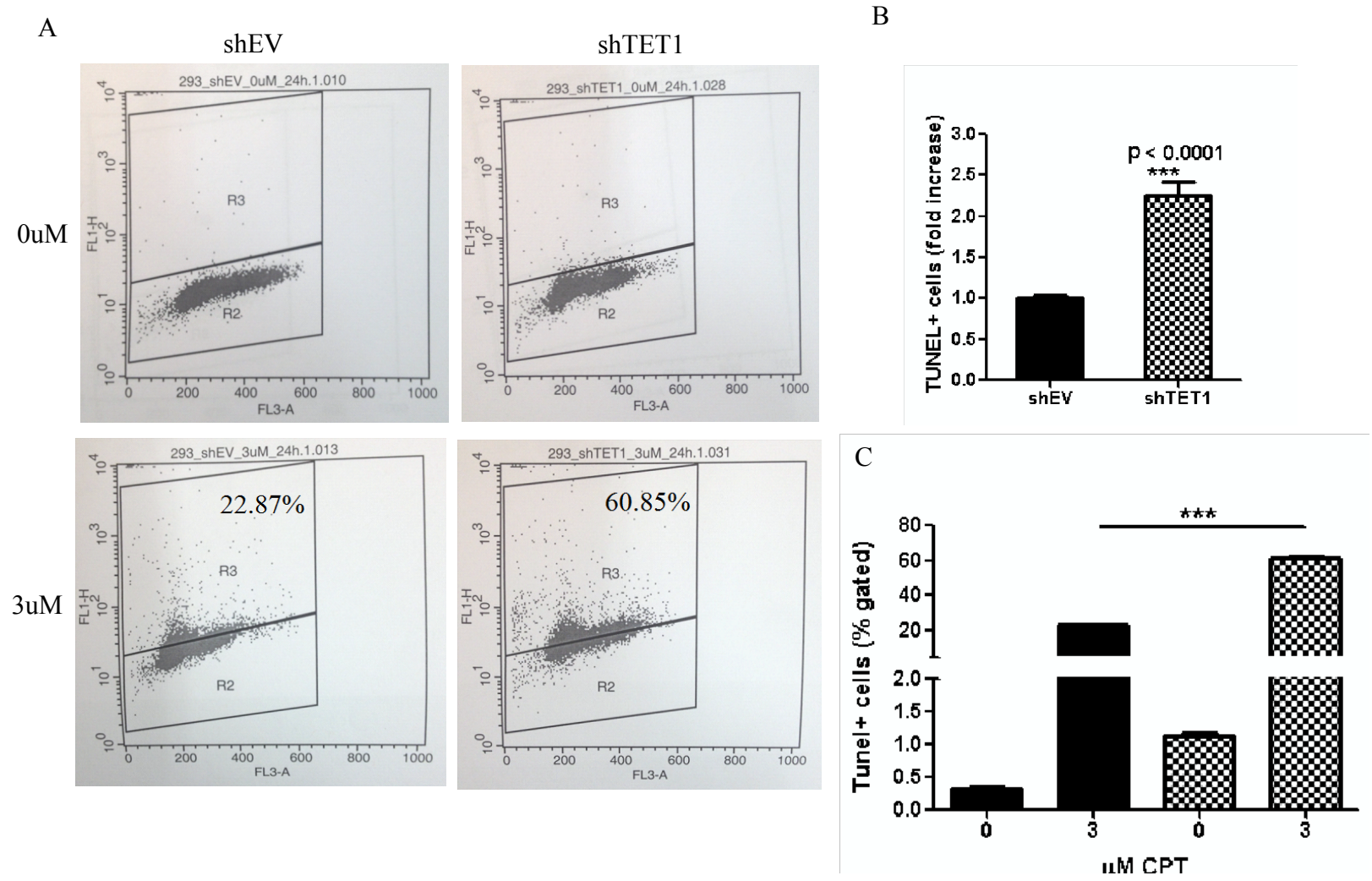
Figure 3: TET1 influence over γH2AX is independent of ATM or p53 activation. Representative western blots of whole cell lysates from shEV and shTET1 cells showing phosphorylated ATM (pATM), TET1, p53 serine-15 phosphorylation (pSer15-p53), and total p53 levels. GAPDH was used as loading control.



TET1 Is protective Against DNA Breaks in HEK293 Cells

While γH2AX formation is often interpreted as an indicator of DNA damage, the phosphorylated histone also plays an integral role in the recognition of DNA damage and the coordination of complexes of proteins involved in repair. To determine whether TET1-supported γH2AX formation leads to protection against DNA breaks, Terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL) labeling of free 3'OH ends was carried out in shEV and shTET1 cells following CPT treatment. Interestingly, an increase in the number of TUNEL+ cells was observed in TET1-deficient cells with and without CPT exposure (Fig. 4*A*-*C*). While a greater number of shTET1 cells displayed (average = 60.85%) breaks compared to shEV cells (average = 22.8%) at 24 hours after treatment with CPT, a greater number of shTET1 cells displayed breaks (greater than 2-fold compared to shEV) in the absence of CPT. While the TUNEL+ population in untreated shTET1 cells was less than 1% of the gated cells, the observation may represent a role for TET1 in protection against endogenous stresses on DNA.

Figure 4: TET1 is protective against DNA breaks *in vitro*. ***A***, Representative dot plots of DNA breaks measured by flow cytometry in shEV and shTET1 cells exposed to 0- or 3μM CPT for 24 hours. Exposed 3'OH ends were labeled with brominated-dUTP, stained with FITC-conjugated anti-BrdU antibody, and counterstained with propidium iodide to visualize DNA content. The x-axis represents relative DNA content and the y-axis represents log-scale relative BrdU labeling. TUNEL+ cells are quantified and represented in ***B*** and ***C***. At least 10,000 events were collected in technical triplicates and the experiment was conducted 3 times.

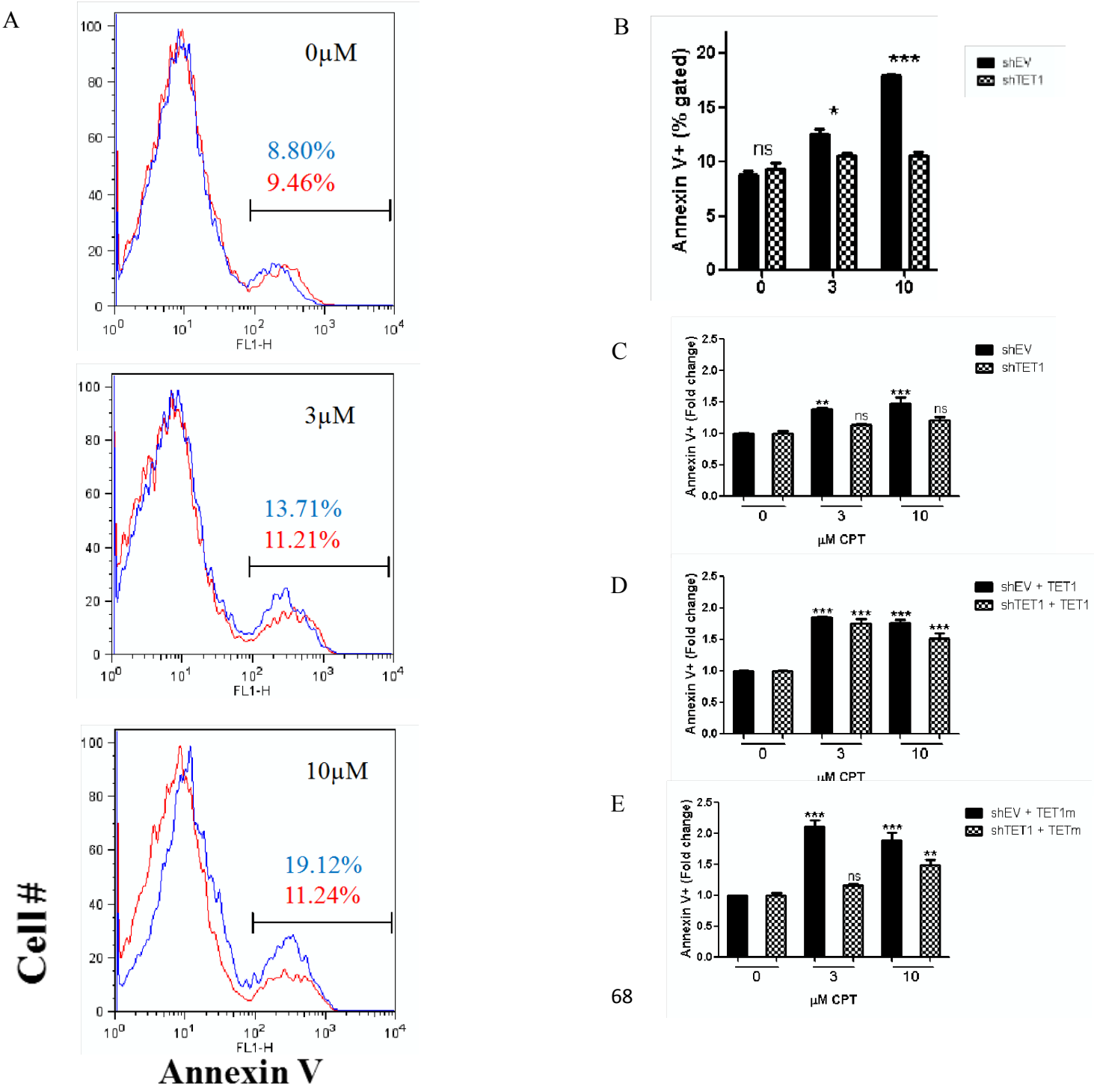


TET1 Supports Cell Death Following Exposure to Camptothecin in HEK293 Cells

TET1 knockdown led to an increase in the number of cells labeled by TUNEL staining. However, the TUNEL assay does not necessarily correlate with apoptosis. Nor does it distinguish apoptotic cells from necrotic cells, or cells with breaks as a result of chemical or physical insult. To determine the potential contribution of TET1 to cell death, shEV and shTET1 cells were exposed to 0-, 3-, or 10μM CPT for 24 hours and stained with annexin V, which binds to phosphatidyl serine either extracellularly, indicating apoptosis, or intracellularly, suggesting loss of membrane integrity (Fig. 5*A*-*B*). An increase in annexin V staining of 1.6 and 2-fold was observed in shEV cells exposed to 3- and 10μM CPT, respectively. Differences were not observed in apoptosis between shTET1 cells and control cells without treatment, suggesting that TET1 promotes or permits cell death to occur in response to CPT.

While the catalytic domain of TET1, but not TET1 activity, was involved in γ H2AX formation and protection against DNA damage, the requirement for TET1 protein and activity in CPT-mediated cell death was tested by overexpressing TET1-CD and TET1-CDmut and staining for annexin V. Catalytically active TET1-CD was able to enhance annexin V labeling in shEV cells and rescue sensitivity to CPT at both 3- and 10μM in shTET1 cells (Fig. 5*D*). Catalytically inactive TET1-CDmut was able to rescue sensitivity to CPT at 10μM (Fig. 5*E*), suggesting that TET1 catalytic domain, but not activity, is sufficient to support cell death following CPT exposure.

Figure 5: Non-enzymatic promotion of cell death by TET1 catalytic domain. ***A***, Representative histograms of annexin V fluorescence in shEV (*blue*) and shTET1 (*red*) cells exposed to 0-, 3-, or 10μM CPT for 24 hours. At least 10,000 events were collected in each sample, and results from 3 experiments are shown in ***B***. Annexin V staining of shEV and shTET1 cells exposed to 0-, 3-, or 10μM CPT for 24 hours following transfection with Bgal (***C***), TET1-CD (***D***), or TET1-CDmut (***E***).



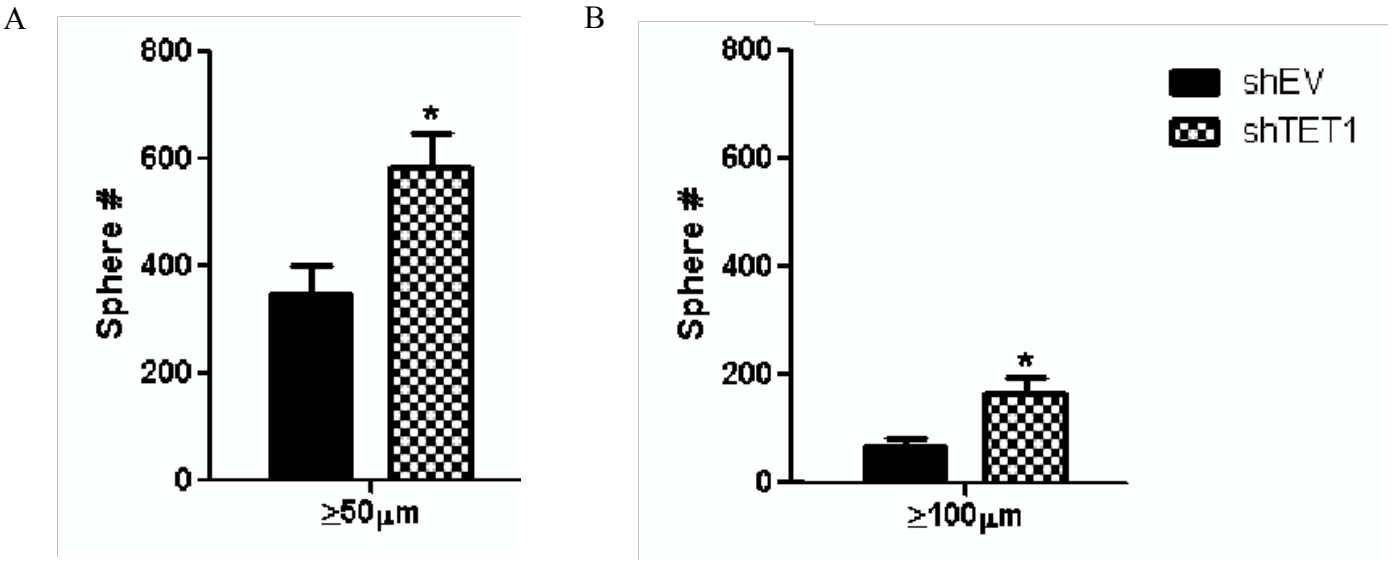
TET1 Knock Down Increases Sphere-forming Capacity of Glioblastoma Tumor Initiating Cells

Because TET1 knockdown led to characteristic features of cancer cells - an increase in DNA damage and decreased sensitivity to the DNA damaging agent camptothecin – the ability of TET1 knockdown to affect the growth of glioblastoma (GBM) sphere-forming cells was tested. 20913 GBM cells were maintained as non-adherent spheres in culture with defined medium containing EGF and FGF growth factors and passaged by breaking into single cells. Lentiviral-mediated shRNA against TET1 was used to generate a stable TET1 knockdown in GBM cells (Fig. 6*A*) and the ability of the cells to form spheres was assayed after 5 days. shTET1-20913 GBM cells generated more spheres compared to shEV-20913 cells (>50 μM or $>100\mu M$) , and the spheres were larger (>100μM) (Fig 6*B* and *C*). mRNA of KLF4, Sox2, Nanog, and Pax6, genes involved in maintaining stemness of GBM cells were found to be higher in shTET1-20913 cells, though the differences were slight and not likely biologically significant (data not shown). However, POU5f1 (Oct4) mRNA was found to be upregulated > 2-fold following TET1 knockdown (Fig. 6*D*). Oct4 has been shown to be a critical factor involved in reprogramming of both induced-pluripotent (iPS) cells and glioblastoma cells to a stem-like state. Additionally, NeuroD1, a transcription factor promoting neuronal differentiation was found to be downregulated following TET1 knockdown.

Noting the increase in sphere-forming capacity of GBM cells following TET1 knockdown, the Rembrandt database containing gene expression and survival data from human brain tumors was used to probe for a potential association between TET1 expression and survival in glioma (Fig 6*E*). A positive association between TET1

expression and survival was observed in patients with tumors containing up-regulated TET1 (n=29) versus intermediate expressed TET1 (n=302) (Log-rank p-value: 0.0099). The association was also observed in patients with tumors containing up-regulated TET1 versus down-regulated TET1 (n=12) (Log-rank p-value: 3.53×10^{-6}), and intermediate expressed TET1 versus down-regulated TET1 (Log-rank p-value: 0.006).

Figure 6: Knockdown of TET1 in human glioblastoma tumor-initiating cells enhances sphere forming capacity. ***A***, Sphere-formation assay showing numbers of shEV or shTET1 GBM-20913 spheres >50 μM (***A***) or >100μM (***B***) formed 5 days after dissociation into single cells. ***C***, Representative image of spheres in agar following 5 days growth in suspension. ***D***, *Oct4* and *NeuroD1* mRNA in shEV and shTET1 GBM -20913 cells measured by qRT-PCR. ***E***, Kaplan-Meier curves indicating glioma patient survival following stratifying tumors into up-regulated (n=29), intermediate (n=302), or down-regulated (n=12) TET1 expression.



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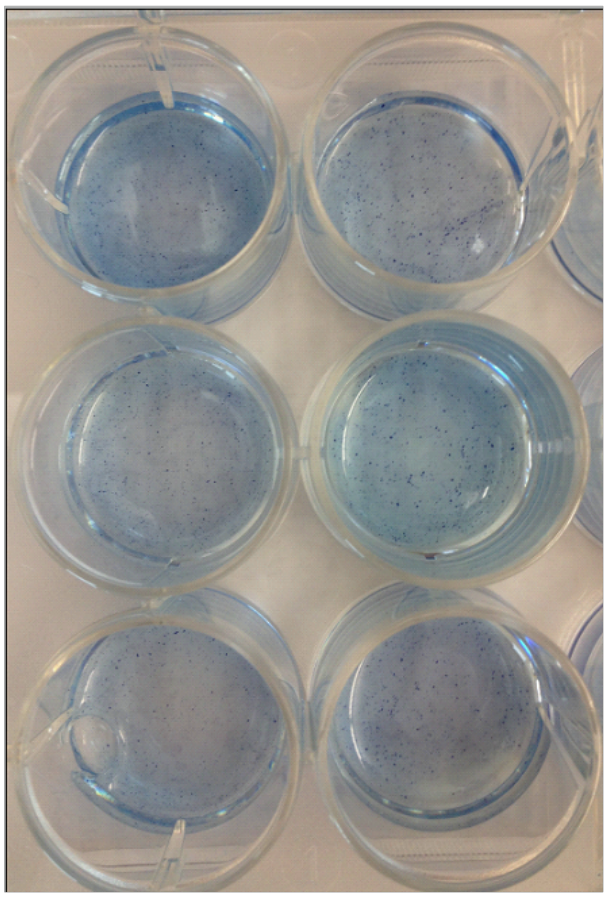

spheres formed from $1x10^5$ cells over 5 days

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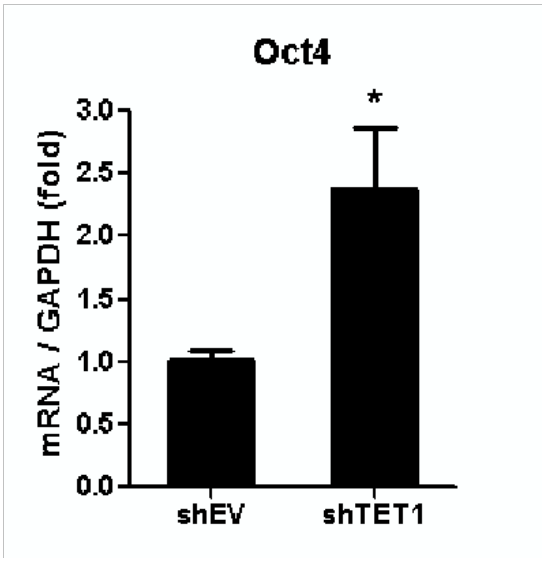
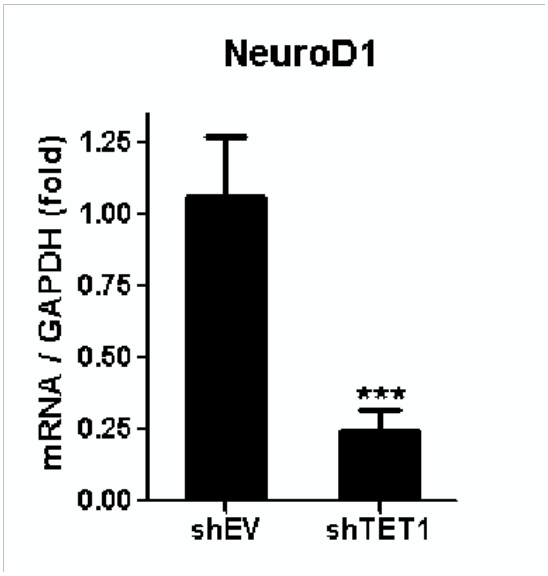
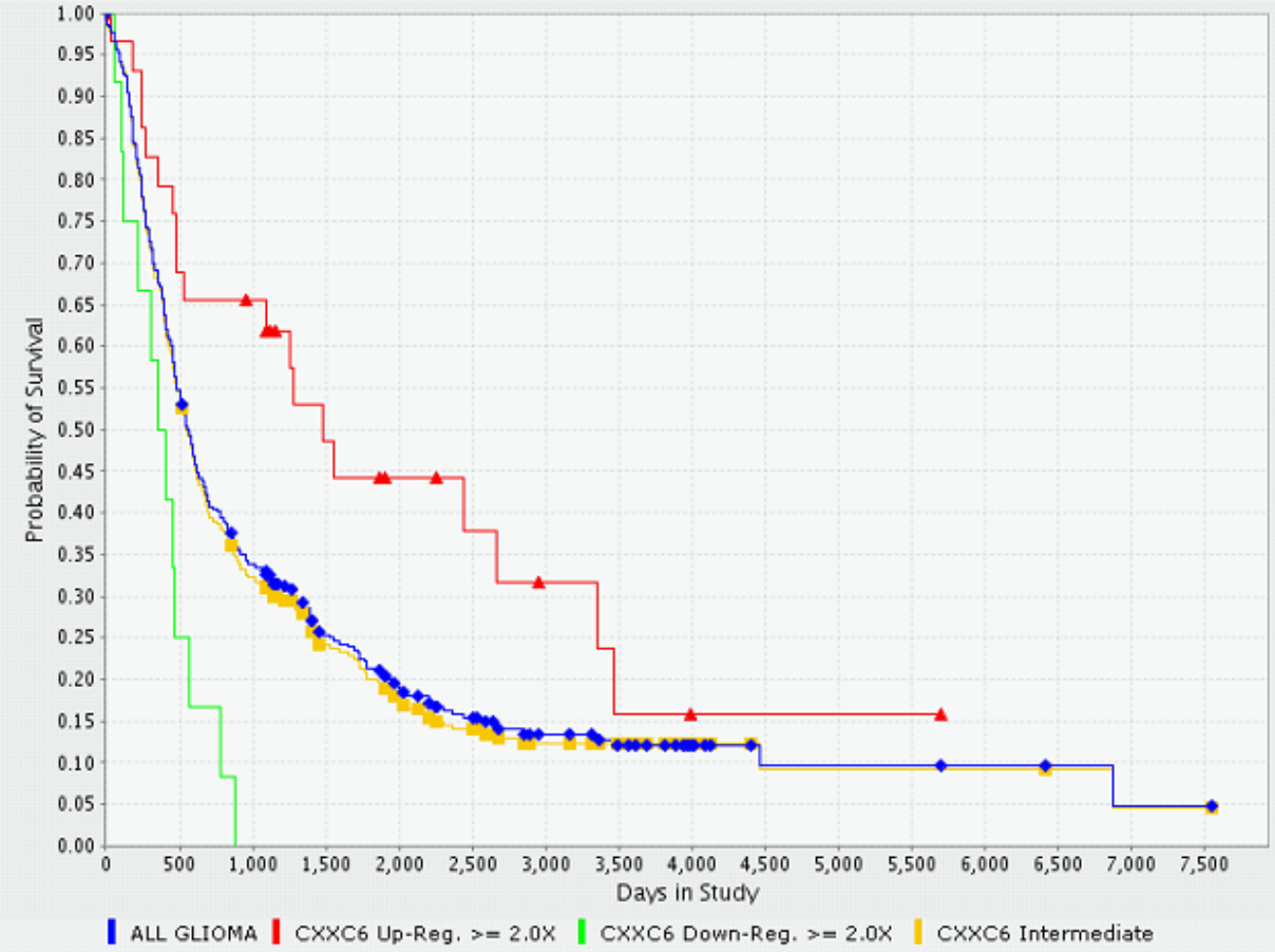




E

Kaplan-Meier Survival Plot for Samples with Differential CXXC6 Gene Expression



View Clinical Reports

Upregulating Samples | Downregulating samples | Intermediate Samples

Statistical Report:

CXXC6

Number of samples in group:

Up-Regulated:	29
Down-Regulated:	12
Intermediate:	302

Log-rank p-value(for significance of difference of survival between group of samples)

Up-Regulated vs. Intermediate:	0.0099147812
Up-Regulated vs. Down-Regulated:	3.53203E-5
Down-Regulated vs. Intermediate:	0.0055880904

3.4 DISCUSSION

Here we present evidence that TET1 is involved in the formation of •H2AX and protects against DNA damage in response to topoisomerase 1 inhibition. We further demonstrate that the catalytic domain, but not activity, is necessary to support •H2AX formation, decrease DNA breaks, and promote cell death in response to camptothecin. This result was unexpected, as TET1 expression has been shown primarily to support gene expression through demethylation. For example, active demethylation by TET1 was shown to be functionally involved and necessary in activity-induced FGF and BDNF expression in mouse brain. Additionally, the hydroquinone-induced expression of cytoprotective genes GCLC and 14-3-3σwere TET1-dependent and were shown to be demethylated in a mechanism involved 5hmC. TET1 knock down rendered cells susceptible to a significant increase in DNA breaks, yet relatively resistant to cell death. These seemingly paradoxical outcomes could lead to aneuploidy or accumulation of mutations and translocations. Interestingly the increase in DNA breaks was observed in TET1-deficient cells even in the absence of an exogenous stressor, suggesting that TET1 plays a role in maintaining routine repair and apoptosis in the face of endogenous and random genomic insults.

Noting that decreasing levels of TET1 in HEK293 cells led to an increase in DNA breaks and resistance to cell death induced by CPT, we sought to determine the effect of knocking down TET1 in human glioblastoma cells known for aggressiveness and stem-like qualities. We found that knocking down TET1 in glioblastoma sphere-forming cells led to an increase in both the number and size of spheres formed, and that TET1-deficient

cells had higher mRNA levels of the pluripotency-related transcription factor Oct4, suggesting that TET1 levels seem to be linked to stemness of glioblastoma cells.

Interest in TET proteins has increased dramatically with the discovery that they contributed to active demethylation. Claims of *bona fide* demethylase enzymes in mammals have not been verified and, until the discovery of TET enzymes, passive demethylation via inhibition of DNMTs was the only known mechanism of DNA demethylation. The biology of TET proteins is also potentially important in cancer biology, as their downregulation has been observed in numerous cancers. For example, Orr *et al.* studied 5hmC marks in 225 human gliomas and found that genomic levels were lower in high grade tumors and low levels of 5hmC were negatively correlated with survival in glioblastoma. Additionally, Liu *et al.* reported 5hmC levels in hepatocellular carcinoma (HCC). Low 5hmC correlated with tumor size and poor prognosis, and TET1-3 genes were downregulated in HCC. These studies are provocative and suggest that downregulation of TET expression or activity may provide advantages to tumor cells. Our study demonstrates that TET1 plays a role in the formation of γH2AX, an important signal of DNA damage, as well as protection against DNA breaks and the promotion of cell death following DNA damage. While the loss of TET1 in our model led to characteristic features of cancer cells – increased DNA breaks and decreased cell death in response to DNA damage – it remains to be seen whether the loss of TET proteins or activity play a significant role in tumorigenesis or cancer biology.

It is unclear why formation of γH2AX in response to CPT is blunted in TET1-deficient cells. Kinases known to phosphorylate H2AX could be involved, though we show that there is no difference in activated (phosphorylated) ATM levels, a major player

in H2AX phosphorylation in response to CPT. It is possible that the catalytic domain of TET1 interacts with a kinase, recruiting it to close proximity to damaged sites to phosphorylate H2AX. In this way, loss of TET1 could lead to loss of γH2AX without a loss of phosphorylation of other ATM targets, such as p53. Consistent with this idea is the fact that our studies show no difference in phospho-ATM or serine-15 phosphorylation of p53, a known target of ATM. An additional possibility is that TET1 affects ATM activity through its binding partner O-linked N-acetylglucosamine transferase (OGT). TET1 has been shown to interact with OGT, which catalyzes serine/threonine glycosylation of numerous proteins. A major function of OGT is to regulate enzyme activity by competing with kinases for serines and threonines on target proteins. Interestingly, ATM is known to be activated by OGT-mediated glycosylation. Future studies should test the ability of TET1 to directly or indirectly regulate ATM activity. A third possibility is that binding partners of the catalytic domain of TET1 bring about global changes in chromatin architecture, opening chromatin to allow kinases and repair proteins to access sites of DNA damage. Studies have reported that heterochromatic regions are refractory to phosphorylation of H2AX, though the mechanisms are not well-understood.

In summary, we highlight a novel, non-enzymatic role for TET1 in the response to DNA damage by CPT. We demonstrate that the catalytic domain of TET1 is sufficient to rescue γH2AX formation, and that TET1 protects against endogenous and exogenous DNA damage, suggesting it may be involved in diseases involving altered DNA repair and apoptosis. We also show that decreasing levels of TET1 in stem-like glioblastoma

cells increased sphere-forming capacity, which is consistent with the idea that TET1 plays a protective role over the genome.

3.5 METHODS

Cells and Lentivirus Production

HEK293 cells were obtained from ATCC and grown in DMEM supplemented with 10% FBS. pLKO shRNA vectors targeting TET1 (Thermo Scientific) or the empty vector were co-transfected with Pax2 and MDG2 plasmids for packaging and envelop production, respectively, at a ratio of 1:3:2 into HEK293FT cells. Lipofectamine was used for transfections at 30μL/10mL in 10cm dishes. DMEM medium was replaced after an overnight incubation with transfection reagents. 48 hours following transfection, the medium was collected and centrifuged. Supernatant was collected and filtered using 22μm filter cap. HEK293 target cells were seeded at low density in 6 well plates and incubated for 30 minutes with 5μg/mL polybrene. Supernatant containing lentiviral paricles was applied to HEK293 cells overnight. Following 24 hours incubation, infection medium was removed and DMEM containing 10% FBS and 1μg/mL puromycin was applied to select transduced cells. Puromycin concentration was determined as the lowest dose capable of killing untransfected HEK293 cells. Cells with integrated pLKO encoding empty vector (shEV) or shRNA against TET1 (shTET1) were cultured in the

presence of puromycin until plated for an experiment. 20913 Glioblastoma sphere-forming cells were maintained in defined medium supplemented with EGF and FGF growth factors. Transduction with lentiviral particles encoding empty vector or shRNA against TET1 was accomplished as noted for HEK293 cells, yet virus was harvested in DMEM medium lacking FBS.

Western Blotting and Antibodies

Whole cell lysates were prepared using radioimmunoprecipitation assay buffer and included addition of protease inhibitors. Protein concentrations were measured using the Bradford assay, and 30μg of whole cell lysates was separated on a 4–20% Tris-glycine gradient gel (Invitrogen). Protein was transferred to a nitrocellulose membrane, blocked with 0.5% casein, and incubated overnight at 4 °C with antibodies against pATM (Cell Signaling, 1:1000), TET1 (Abnova, 1:1000), pSer15-p53 and total p53 (Cell Signaling, 1:1000), GAPDH (Sigma, 1:5000), FLAG M2 (Sigma, 1:1000), or ϒH2AX (Abcam, 1:1000). Blots were incubated with secondary antibody (1:10,000) for 1 h before visualization on the Licor Odyssey Imager.

Immunofluorescence

HEK293 cells were grown on glass coverslips and allowed to adhere overnight.Cells were exposed to camptothecin or DMSO control for 14 hours and fixed in 3.7% formaldehyde for 20 minutes. Following 3 washes in cold PBS, cells were stored at 4˚C

in PBS until staining. Cells were permeablized and blocked in PBS with 0.2% TritonX-100 and 2.5% BSA for 2 hours at room temperature. Cells were incubated in PBS with 2.5% BSA and rabbit anti- ɣH2AX antibody (1:200) overnight at 4˚C. Following incubation with primary antibody, cells were washed for 5 minutes three times in PBS at room temperature. Cells were incubated with Texas Red anti-rabbit secondary antibody for 2 hours at room temperature, followed by 3 washes in PBS. Cells were mounted and coverslipped using ProLong Gold antifade reagent with DAPI (Life Technologies) and allowed to dry overnight at room temperature. Cells were imaged using a *Zeiss* Axioplan microscope.

Immunodotblotting of Genomic DNA

Genomic DNA was isolated using GenElute Mammalian Genomic DNA kit (Sigma) and measured using a NanoDrop (Thermo Scientific). 250 ng was denatured with 0.4 M NaOH at 95 °C for 10 min. DNA was immobilized on nitrocellulose membrane using a 96-well vacuum apparatus and then dried and fixed by vacuum baking at 80 °C. The membrane was then rehydrated in room temperature PBS and blocked with 0.5% casein for 1 hour prior to probing with antibodies against 5-hmC (Active Motif; 1:8000). Mouse anti-single-stranded DNA (Abcam; 1:1500) antibody was used simultaneously for normalizing 5hmC. Blots were then washed in PBS three times and then incubated with IR-cojugated secondary antibodies (Ms 600, Rb 800). Binding was measured using the Licor Odyssey.

Cloning of Human TET1 Catalytic Domain

mRNA was prepared from HEK293 cells using Trizol and cDNA was prepared using Superscript III Reverse Transcriptase (Life Technologies). The catalytic domain of TET1 was amplified with Taq DNA polymerase and pfu Taq High Fidelity Taq polymerase (Promega) using the following primers:

Forward:

ATACCGGAATTCATGGACTACAAGGACGACGATGACAAGGGAGTATTTCCAC

CACTCACTCAG

Reverse:

ATACCGGGTACC.TCATCAGACCCAATGGTTATAGGG

PCR product was run on a 1% agarose gel to confirm expected size of 2700bp and restriction digested with EcoR1 and Kpn1 prior to insertion into pcDNA3.1(-) mammalian expression vector under control of CMV promoter. Plasmids were sequenced to validate correct orientation and sequence of the insert (JHU DNA Analysis Facility). TET1 antibody (Abnova) recognizes the c-terminus of the peptide.

TUNEL Assay for Flow Cytometry

HEK293 shEV and shTET1 cells were exposed to indicated concentrations of camptothecin for 24 hours. Cells were trypsinized and harvested along with any non-adherent cells and washed with cold PBS. Labeling was accomplished according to Phoenix Flow Systems (San Diego, CA) recommended protocol. In brief, to ensure

smaller DNA fragments were included, cells were resuspended in 1% (w/v) paraformaldehyde in PBS at $1x10^6$ cells/mL for 60 minutes. Cells were then washed in PBS and resuspended in 70% ethanol overnight. Following washes, $1x10^5$ cells were labeled in 100µL of solution containing provided buffer, terminal deoxynucleotidyl transferase enzyme, BrdU, and distilled H_2O in a 37˚C water bath for 4 hours. Cells were agitated every 20 minutes throughout the labeling incubation. Following the reaction, cells were washed and then labeled with FITC-conjugated anti-BrdU antibody for 30 minutes, then diluted with propidium iodide/RNase A solution to label DNA. Cells were analyzed using a FACSCalibur flow cytometer and analyzed using Cell Quest software (BD Biosciences).Single-cells were gated using FL3A v. FL3W and the R3 gate consisted of positively labeled cells in FL1, set by included negative and positive control cells provided by the manufacturer.

Annexin V Labeling for Cell Viability

HEK293 shEV and shTET1 cells were exposed to indicated concentrations of camptothecin for 24 hours and washed in cold PBS. Cells were resuspended in binding buffer: 10mM HEPES, 140mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4 at $1x10^5$cells/100µL. 5µL 488-conjugated annexin V in the dark for 15 minutes. After the incubation, 400µL binding buffer was added and cells were kept on ice until analysis using a FACSCalibur flow cytometer and analyzed using Cell Quest softward (BD Biosciences).

Sphere Formation Assay

Cells were incubated in neurosphere culture medium 5 days and embedded in low-melting agar. Spheres were stained overnight at 4˚C in Wright's solution (1%) and de-stained in PBS. Sphere formation was analyzed by computer-assisted morphometry (MCID) by measuring the number of neurospheres > 50 μM or > 100μM in diameter in four random microscopic fields per well.

IV. CONCLUSIONS, PERSPECTIVES, AND FUTURE STUDIES

4.1 Toxicant-induced DNA demethylation of cytoprotective genes through increased TET1 activity.

Environmental exposures have been known to affect DNA methylation. Bollati *et al.,* for example, studied methylation levels of specific genes in participants with variable exposures to benzene (9). 78 gas station attendants, 77 traffic officers, and 58 "unexposed" control participants were included in the study. For every 10-fold increase in airborne benzene levels, the authors observed significant decreases in methylation of LINE-1 and Alu1, suggesting a genome-wide decrease. In addition, hypomethylation of MAGE-1 and hypermethylation of p15 were also observed. Studies in TK6 lymphoblastoid cells also show that numerous compounds, including benzene and hydroquinone, lead to widespread demethylation of DNA. While associations between

environmental and pharmaceutical chemicals and DNA methylation have been widely known, mechanistic data, which could be used to develop biomarkers and predict effects of chemically similar compounds, were not available.

In chapter 2, we investigated the ability of the major benzene metabolite, hydroquinone, to change DNA methylation using an *in vitro* model system. We found that hydroquinone exposures that did not affect viability, but which did affect gene expression, led to decreases in genomic DNA methylation in a mechanism involving ROS. We further demonstrated the involvement of the 5-methylcytosine dioxygenase TET1 in the active demethylation of LINE-1, GCLC, and 14-3-3σ, implicating TET1 in a functional response to ROS. The proposed mechanism involves no single demethylase, but a multi-step process of demethylation involving hydroxylation of the methyl group, followed by deamination to form 5-hydroxymethyluracil (5hmU). Base excision repair (BER) would be expected to replace 5hmU with cytosine. Indeed, Guo *et al.* demonstrated an accumulation of 5hmU with TET1 overexpression and small molecule inhibitors of BER (7). When we overexpressed the human deaminase AP2, we observed an enhanced demethylation in cells exposed to hydroquinone, suggesting that the chemical leads to demethylation through the same or a similar pathway. Finally, we were able to link TET1 to changes in the cell cycle following hydroquinone exposure, which have been reported as early as 1953. It is possible that chemical exposures affecting G_2 to M progression, like hydroquinone, may involve TET1, and cells lacking TET1 continue to cycle, leaving them vulnerable to mutations. While TET1 is clearly affected by hydroquinone and a similar compound, menadione, it is unclear how ROS affects TET1 protein levels and activity (58). TET1 contributed to most of the 5mC hydroxylase

activity in HEK293 cells, but it is not know whether chemicals affect the other TET members.

While each of the three TET proteins catalyzes the same reaction, their temporal and tissue-specific distributions vary greatly. While TET3 is highly expressed in germ cells, TET1 is highly expressed in the brain, lung, heart, and liver and TET2 is expressed in bone marrow, lymphocytes, brain, and spleen. It is remarkable how little is known about their regulation, both at the transcriptional and activity levels. For example, TET1 and TET3 contain a CXXC-domain toward the amino terminus which is very similar to DNMT1. In DNMT1, the domain is known to bind DNA with high affinity, yet the domain binds DNA with very little affinity in TET1 and TET3. In fact, the CXXC domain is dispensable for enzymatic activity *in vivo*. Our data, in agreement with numerous other studies, show that overexpression of the human or mouse catalytic domain is sufficient to cause global increases in 5hmC. Interestingly, the amino terminus of TET2 underwent an inversion during evolution and is now transcribed as a separate gene, *IDAX2,* leaving TET2 as a shorter gene with no CXXC domain (51). *IDAX* is shown to bind DNA and recruit TET2 to DNA, where it is then targeted for caspase cleavage. It is not yet known whether TET1 or TET3 are regulated in a similar manner, or if the amino terminus plays any important role in its activity or destruction. The carboxy terminus contains a cysteine-rich region close to the catalytic site, though a function of the region is not known. Tahiliani *et al*,. in a landmark publication describing the function of TET1, demonstrated that the protein had a tendency to form dimers when run on a non-denaturing, non-reducing gel, presumably due to disulfide bond formation at the cysteine-rich regions (12). A possibility could be that perturbations in redox status from a

reducing environment to a localized region of ROS in the nucleus affects the cysteine-rich regions of TET1, leading to retention of the protein in the nucleus.

Future studies need to be conducted to determine whether DNA demethylation involving TET1 is unique to hydroquinone or common to chemicals that cause ROS. Our observation of increased 5hmC following exposure to menadione suggests that the effect is not unique to hydroquinone, and a recent publication reported that numerous quinone compounds were able to decrease DNA methylation in a TET1-dependent manner.

While benzene is a ubiquitous environmental chemical, there are specific populations exposed to significantly higher exposures, such as those living in urban environments and workers exposed to high concentrations of hydrocarbon fuels and combustion. It should be noted, however, that airborne benzene, while impossible to eliminate, can be monitored and regulated. Benzene is also a component of one fracturing fluid (gel) that has been used in hydraulic fracturing, and the mixture of compounds that are liberated following hydraulic fracturing are complex mixtures potentially containing benzene. The effects of environmental chemicals on the human epigenome should continue to be studied in order to better characterize risks to health. In addition, it may be possible to develop biomarkers of exposures and risks if we can determine genetic "hotspots" likely to change in methylation.

Preliminary Study into Alzheimer's Disease and Global 5hmC

Noting that hydroquinone caused a ROS-dependent increase in 5hmC (58), we were curious to know whether a disease that may involve ROS (59), environmental toxicants, and changes in DNA methylation may show changes in global 5hmC as well

(49,50). We obtained 5 post-mortem samples each of Hippocampus, Broadmann Area 22 (BA22), and Broadmann Area 46 (BA46) from patients who had varying stages of Alzheimer's disease (AD) (60). Using immunodotblotting, we measured the global levels of 5hmC and found 5hmC levels in the hippocampus (Fig. 1*A*) increased from Braak Stages 1 and 2 (least severe and associated aging) to Stages 3 and 4, and further still to Stages 5 and 6 (most severe). 5hmC levels decreased from Stages 1 and 2 to all other stages in Broadmann Area 22 (Fig. 1*B*), and were unchanged in Broadmann Area 46 (Fig. 1*C*).

While preliminary, our data show that changes in 5hmC increase with severity of Alzheimer's disease in hippocampus. The involvement of ROS in the disease is well-accepted and future studies should involve identifying differences in specific gene methylation that occur in AD as well as potential biomarkers that could be measured in blood or sputum. A prospective study with regularly sampled blood or sputum collection could provide a rich data set that could be used to develop methylation marks that are predictive changes in risk for AD.

Figure 1: Global 5hmC increases with severity of AD in hippocampus. Immunodotblotting was used to measure global 5hmC levels in hippocampus (*A*), Broadmann Area 22 (*B*), and Broadmann Area 46 (*C*). 5hmC was normalized to ssDNA. Samples were measured in triplicate and immunodotblotting was replicated twice. * indicate difference from Braak Stages 1+2 using 1-way ANOVA with Dunnett's post-test.

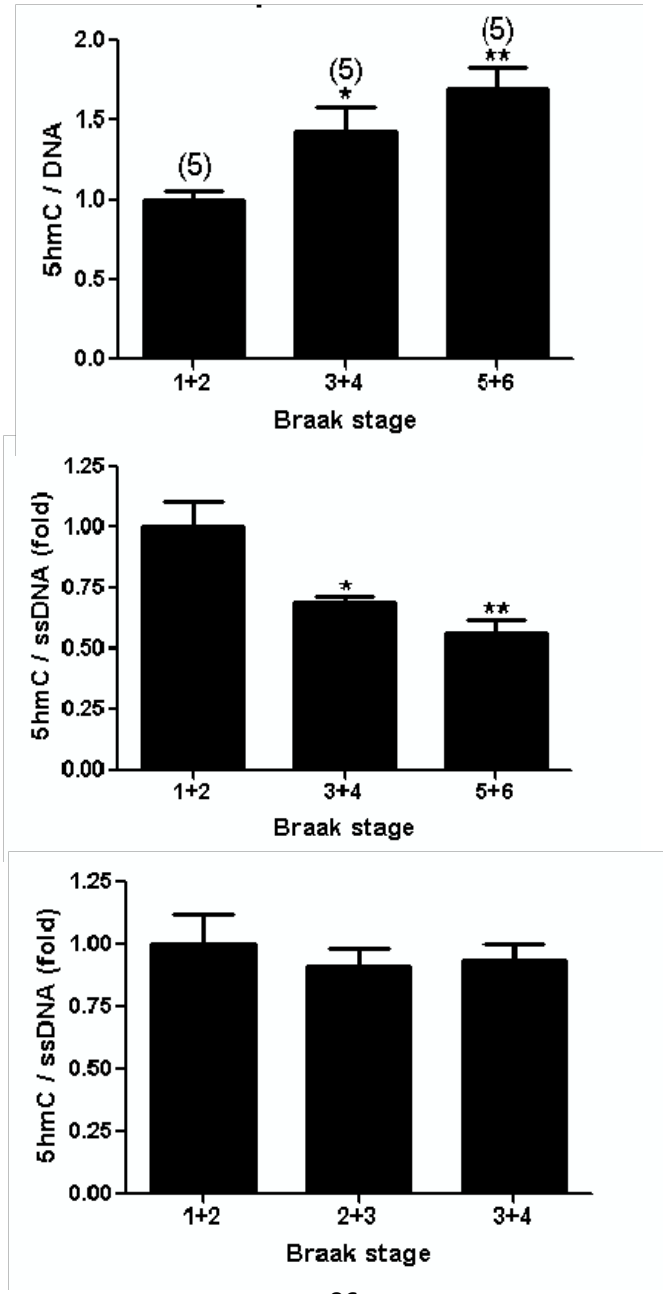


Table 1. Case characteristics of Alzheimer's disease tissue samples.

Diagnosis	**Age**	**Sex**	**PMI**
Braak 1	65	M	18.57
Braak 1	59	M	18.43
Braak 1	72	M	24.87
Braak 2	72	M	6.83
Braak 2	74	M	25
average	**68.4**		**18.74**
Braak 3	87	F	22.32
Braak3	86	F	10.6
Braak 4	90	M	24
Braak 4	77	F	27.07
Braak 4	72	M	23.72
Average	**82.4**		**21.542**
Braak 5	82	M	16.92
Braak 5	82	F	17.18
Braak 6	86	F	27.83
Braak 6	82	F	12.16
Braak 6	82	F	21.5
Average	**82.8**		**19.118**

4.2 Activity-independent protection against DNA damage by TET1.

DNA methylation and the molecular components establishing, erasing, and interpreting the marks are crucial elements in cellular functions. Highlighting their importance are the growing lists of diseases and disorders involving changes in DNA methylation. Alzheimer's disease (global increases and decreases in both 5mC and 5hmC), cancers (low global 5mC, high promoter 5mC, low 5hmC), and Lupus (decreased promoter 5mC), for example, each have significant epigenetic components and contribute greatly to worldwide morbidity and mortality. While aberrant DNA methylation has been described and linked to gene expression in many diseases, the overarching mechanisms leading to changes are not well-understood, and represent potential targets for biomarkers and intervention. Moreover, defects in the molecular machinery involved in establishing and interpreting epigenetic marks also lead to profound disease and disability. For example, Rett Syndrome, caused by mutations in the gene encoding the 5mC-binding MeCP2 protein, is a debilitating neurodevelopmental disorder characterized by loss of purposeful hand movements and severe cognitive defects. Rett Syndrome can be cause by mutations throughout the gene, however, and not all mutations in MeCP2 disrupt binding to 5mC, indicating additional important roles for the protein.

In Chapter 3 we report a non-enzymatic role for TET1 in responding to DNA damage induced by the topoisomerase inhibitor CPT. Loss of TET1 rendered cells more sensitive to DNA breaks, yet relatively resistant to cell death. Re-introducing the catalytic domain of the conserved mouse TET1 protein rescued the formation of γH2AX, an important recognition signal of DNA damage, in response to CPT. While transient

overexpression of human or mouse TET1 catalytic domain led to fold-increases in global 5hmC, catalytically inactive mouse TET1 was sufficient to rescue γH2AX formation and sensitize cells to CPT. The lack of enzymatic activity required to rescue TET1-deficient cells suggests a novel role for the protein, perhaps involving novel binding partners. We also report that knocking down TET1 in glioblastoma sphere-forming cells led to an increase in both number and size of spheres, further implicating the protein as a relevant player in cancer biology.

Future studies should be directed toward understanding regulation of TET1 in different contexts. For example, despite a lack of change in TET1 mRNA, we observed a significant increase in its activity when exposed to hydroquinone. The protein could be translocated from the cytoplasm to the nucleus, cleaved by proteases, or inhibited from being translated by microRNA's in certain environments or cell types. Recent interest in microRNA-mediated regulation of genes has surged, and these non-protein coding RNAs may serve cancer cells by inhibiting TET1-mediated protection, thereby providing chemo- and radio-resistance. A better understanding of the regulation of TET1 is critical in providing a mechanistic explanation of diseases involving epigenetic changes. It is our hope that this work may serve as part of that foundation.

Novel Roles for TET1 in Response to Stress

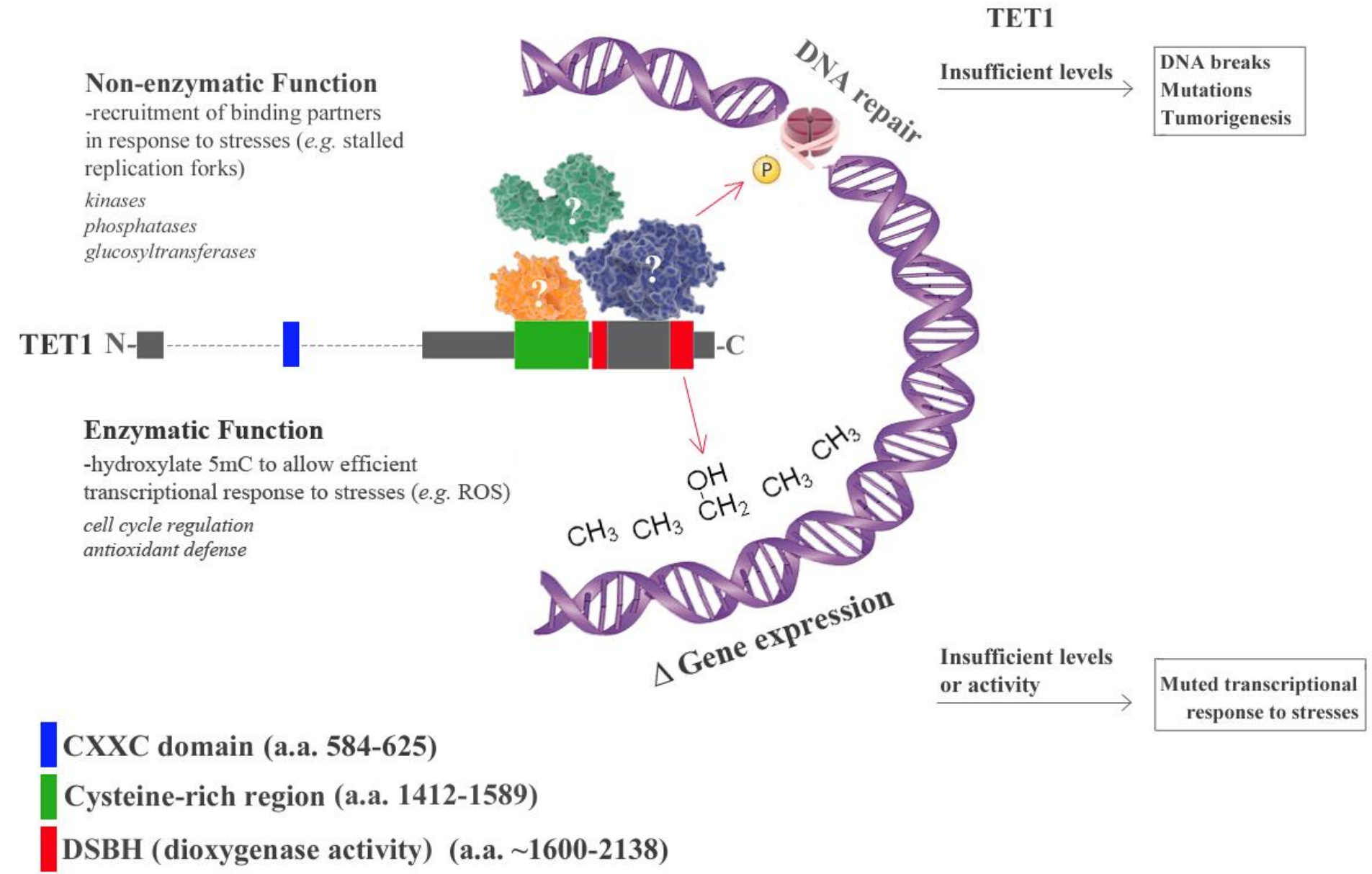


Figure 2: Proposed novel roles for TET1 in response to stress. Stresses such as ROS lead to increased TET1 activity, resulting in the efficient expression of genes involved in cell cycle regulation and antioxidant defense. TET1 promotes phosphorylation of histone variant H2AX in an activity-independent manner, resulting in recognition of DNA strand breaks.

REFERENCES

1. Jaenisch, R., and Bird, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat.Genet.* 33, 245–254

2. Tsai, H. C., and Baylin, S. B. (2011) Cancer epigenetics: linking basic biology to clinicalmedicine. *Cell Res.* 21, 502–517

3. Millan, M. J. (2013) An epigenetic framework for neurodevelopmental disorders: from pathogenesis to potential therapy. *Neuropharmacology* 68, 2–82

4. Ishida, K., Kobayashi, T., Ito, S., Komatsu, Y., Yokoyama, T., Okada, M., Abe, A., Murasawa, A., and Yoshie, H. (2012) Interleukin-6 gene promoter methylation in rheumatoid arthritis and chronic periodontitis. *J. Periodontol.* 83, 917–925

5. Ponferrada-Marín, M. I., Martínez-Macías, M. I., Morales-Ruiz, T., Roldán-Arjona, T., and Ariza, R. R. (2010) Methylation-independentDNA binding modulates specificity of repressor of silencing 1 (ROS1) and facilitates demethylation in long substrates. *J. Biol. Chem.* 285, 23032–23039

6. Christman, J. K. (2002) 5-Azacytidine and 5-aza-2_-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 21, 5483–5495

7. Guo, J. U., Ma, D. K., Mo, H., Ball, M. P., Jang, M. H., Bonaguidi, M. A., Balazer, J. A., Eaves, H. L., Xie, B., Ford, E., Zhang, K., Ming, G. L., Gao, Y., and Song, H. (2011) Neuronal activity modifies the DNA methylation

landscape in the adult brain. *Nat. Neurosci.* 14, 1345–1351

8. O'Driscoll, C. M., Coulter, J. B., and Bressler, J. P. (2013) Induction of a trophoblast-like phenotype by hydralazine in the P19 embryonic carcinoma cell line. *Biochim. Biophys. Acta* 1833, 460–467

9. Bollati, V., Baccarelli, A., Hou, L., Bonzini, M., Fustinoni, S., Cavallo, D., Byun, H. M., Jiang, J., Marinelli, B., Pesatori, A. C., Bertazzi, P. A., and Yang, A. S. (2007) Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Res.* 67, 876–880

10. Lee, B. H., Yegnasubramanian, S., Lin, X., and Nelson, W. G. (2005) Procainamide is a specific inhibitor of DNAmethyltransferase 1. *J. Biol. Chem.* 280, 40749–40756

11. Lee, W. J., and Zhu, B. T. (2006) Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols. *Carcinogenesis* 27, 269–277

12. Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L. M., Liu, D. R., Aravind, L., and Rao, A. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324, 930–935

13. Ito, S., D'Alessio, A. C., Taranova, O. V., Hong, K., Sowers, L. C., and Zhang, (2010) Role of TET proteins in 5mC to 5hmC conversion, ES cell

self-renewal and inner cell mass specification. *Nature* 466, 1129–1133

14. Guo, J. U., Su, Y., Zhong, C., Ming, G. L., and Song, H. (2011) Hydroxylation

of 5-methylcytosine by TET1 promotes activeDNAdemethylation in the adult brain. *Cell* 145, 423–434

15. Ong, C. N., Lee, B. L., Shi, C. Y., Ong, H. Y., and Lee, H. P. (1994) Elevated levels of benzene-related compounds in the urine of cigarette smokers. *Int. J. Cancer* 59, 177–180

16. International Agency for Research on Cancer (1987) Overall evaluations of carcinogenicity: an updating of IARC monographs volumes 1 to 42. *IARC Monogr. Eval. Carcinog. Risks Hum. Suppl.* 7, 1–440

17. Parke, D. V., and Williams, R. T. (1953) The metabolism of benzene containing C14 benzene. *Biochem. J.* 54, 231–238

18. Snyder, R., and Hedli, C. C. (1996) An overview of benzene metabolism. *Environ. Health Perspect.* 104, 1165–1171

19. Kolachana, P., Subrahmanyam, V. V., Meyer, K. B., Zhang, L., and Smith, M. T. (1993) Benzene and its phenolic metabolites produce oxidative DNAdamage in HL60 cells *in vitro* and in the bone marrow *in vivo*. *Cancer Res.* 53, 1023–1026

20. Shen, D. X., Shi, X., Fu, J. L., Zhang, Y. M., and Zhou, Z. C. (2003) The role of thiol reduction in hydroquinone-induced apoptosis in HEK293 cells. *Chem. Biol. Interact.* 145, 225–233

21. Parmentier, R., and Dustin, P., Jr. (1948) Early effects of hydroquinone on mitosis. *Nature* 161, 527

22. Ji, Z., Zhang, L., Peng, V., Ren, X., McHale, C. M., and Smith, M. T. (2010)

A comparison of the cytogenetic alterations and global DNA hypomethylation induced by the benzene metabolite, hydroquinone, with those induced by melphalan and etoposide. *Leukemia* 24, 986–991

23. Muotri, A. R., Marchetto, M. C., Coufal, N. G., Oefner, R., Yeo, G., Nakashima, K., and Gage, F. H. (2010) L1 retrotransposition in neurons is modulated by MeCP2. *Nature* 468, 443–446

24. Westfall, M. D., Mays, D. J., Sniezek, J. C., and Pietenpol, J. A. (2003) The Np63d phosphoprotein binds the p21 and 14-3-3σ promoters *in vivo* and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. *Mol. Cell. Biol.* 23, 2264–2276

25. Du, W., Rani, R., Sipple, J., Schick, J., Myers, K. C., Mehta, P., Andreassen, P. R., Davies, S. M., and Pang, Q. (2012) The FA pathway counteracts oxidative stress through protection of antioxidant defense gene promoters. *Blood* 119, 4142–4151

26. Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., and Gregory, S. (2001)

Initial sequencing and analysis of the human genome. *Nature* 409, 860–921

27. Hermeking, H., Lengauer, C., Polyak, K., He, T. C., Zhang, L., Thiagalingam, S., Kinzler, K. W., and Vogelstein, B. (1997) 14-3-3σ is a p53-regulated inhibitor of G2/M progression. *Mol. Cell* 1, 3–11

28. Lodygin, D., and Hermeking, H. (2005) The role of epigenetic inactivation of 14-3-3σ in human cancer. *Cell Res.* 15, 237–246

29. Ferguson, A. T., Evron, E., Umbricht, C. B., Pandita, T. K., Chan, T. A., Hermeking, H., Marks, J. R., Lambers, A. R., Futreal, P. A., Stampfer, M. R., and Sukumar, S. (2000) High frequency of hypermethylation at the 14-3-3σlocus leads to gene silencing in breast cancer. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6049–6054

30. Yi, B., Tan, S. X., Tang, C. E., Huang, W. G., Cheng, A. L., Li, C., Zhang, P. F., Li, M. Y., Li, J. L, Yi, H., Peng, F., Chen, Z. C., and Xiao, Z. Q. (2009) Inactivation of 14-3-3• by promoter methylation correlates with metastasis in nasopharyngeal carcinoma. *J. Cell. Biochem.* 106, 858–866

31. O'Hagan, H. M., Wang, W., Sen, S., Destefano Shields, C., Lee, S. S., Zhang, Y. W., Clements, E. G., Cai, Y., Van Neste, L., Easwaran, H., Casero, R. A., Sears, C. L., and Baylin, S. B. (2011) Oxidative damage targets complexes containing DNA methyltransferases, SIRT1, and polycomb members to promoter CpG islands. *Cancer Cell* 20, 606–619

32. Lertratanangkoon, K., Wu, C. J., Savaraj, N., and Thomas, M. L. (1997) Alterations of DNA methylation by glutathione depletion. *Cancer Lett.* 120, 149–156

33. Cerda, S., and Weitzman, S. A. (1997) Influence of oxygen radical injury on DNA methylation. *Mutat. Res.* 386, 141–152

34. Castro, G. D., Díaz Gómez, M. I., and Castro, J. A. (1996) 5-Methylcytosine attack by hydroxyl free radicals and during carbon tetrachloride promoted liver microsomal lipid peroxidation: structure of reaction products. *Chem. Biol. Interact.* 99, 289–299

35. Wagner, J. R., and Cadet, J. (2010) Oxidation reactions of cytosine DNA components by hydroxyl radical and one-electron oxidants in aerated aqueous solutions. *Acc. Chem. Res.* 43, 564–571

36. Orr, B. A., Haffner, M. C., Nelson, W. G., Yegnasubramanian, S., and Eberhart, C. G. (2012) Decreased 5-hydroxymethylcytosine is associated with neural progenitor phenotype in normal brain and shorter survival in malignant glioma. *PLoS One* 7, e41036

37. Liu, C., Liu, L., Chen, X., Shen, J., Shan, J., Xu, Y., Yang, Z., Wu, L., Xia, F., Bie, P., Cui, Y., Bian, X. W., and Qian, C. (2013) Decrease of 5-hydroxymethylcytosine is associated with progression of hepatocellular carcinoma through down-regulation of TET1. *PLoS One* 8, e62828

38. Vavouri, T. and Lehner, B. (2012) Human genes with CpG island promoters have a distinct transcription-associated chromatin organization. *Genome Biol.* 13, R110

39. Robertson, K.D. (2005) DNA methylation and human disease. *Nature Genetics Rev.* 6, 597-610

40. Baylin, S.B. (2005) DNA methylation and gene silencing in cancer. *Nature Clin. Practice Oncol.* 2, S4-S11

41. Smith, Z.D., Meissner, A. (2013) DNA methylation: roles in mammalian development. *Nature Rev Genetics.* 14, 204-220

42 Nishino, K., Toyoda, M., Yamazaki-Inoui, M., Fukawatase, Y., Chikazawa, E., Sakaguchi, H., Akutsu, H., and Umezawa, A., (2011) DNA methylation dynamics in human induced pluripotent stem cells over time. *PLOS Genetics.*7, e100285

43. Feng, J., Fouse, S., and Fan, G. (2007) Epigenetic regulation of neural gene expression and neuronal function. *Pediatric Res.* 61, 58-R-63R

44. Muotri, A.R., Zhao, C., Marchetto, M.C., and Gage, F.H. (2009) Environmental influence on L1 retrotransposons in the adult hippocampus. *Hippocampus.* 10, 1002-7

45. Billam, M., Sobolewski, M.D., Davidson, N.E. (2010) Effects of a novel DNA methyltransferase inhibitor zebularine on human breast cancer cells. *Breast Can Res and Treatment. 120, 581-592*

46. Phillips, C.L., Davies, S.M., McMasters, R., Absolon, M., O'Brien, M., Mo, J., Broun, R.,Moscow, J.A., Smolarek, T., Garzon, R., Blum, W., Schwind, S., Marcucci, G., and Perentesis, J.P. (2013) Low dose decitabine in very high risk relapsed or refractory acute myeloid leukemia in children and young adults. *Br J Haematol.* 161, 406-410

47. Sutcliffe, J.S., Nelson, D.L., Zhang, F., Pieretti, M., Caskey, C.T., Saxe, D., and Warren, S.T. (1992) DNA methylation represses FMR-1 transcription in fragile X syndrome. *HumMol Genet.* 6, 397-400

48. Stoger, R., Kajimura, T.M., Brown, W.T., Laird, C.D. (1997) Epigenetic variation illustrated by DNA methylation patterns of the fragile-x gene FMR-1. *Hum Mol Genet. 6, 1791-1801*

49. Rao, J.S., Keleshian, V.L., Klein, S., and Rapoport, S.I. (2012) Epigenetic modifications in frontal cortex from Alzheimer's disease and bipolar disorder patients. *Translational Psychiatry.* 2, e132.

50. Chouliaras, L., Mastroeni, D., Delvaux, E., Grover, A., Kenis, G., Hof, P.R., Steinbusch, H.W., Coleman, P.D., Rutten, B.P., van den Hove, D.L. (2013) Consistent decrease inglobal DNA methylation and hydroxymethylation in the hippocampus of Alzheimer's disease patients. *Neurobiology of Aging.* 34, 2091-2099

51. Pastor, W.A., Aravind, L., and Rao, A. (2013) TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. *Nat Rev Mol Cell Biol.* 14, 341-356

52. Wu, H., and Zhang, Y. (2011) Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. *Genes and Dev.* 25, 2436-2452

53. Arioka, Y., Watanabe, A., Saito, K., and Yamada, Y. (2012) Activation-induced cytidine deaminase alters the subcellular localization of Tet family proteins. *PLOS One.* 7, e45031

54. Orita, H., Coulter, J., Tully, E., Kuhajda, F.P., and Gabrielson, E. (2008) Inhibiting fatty acid synthase for chemoprevention of chemically induced lung tumors. *Clinical Cancer Res.* 14, 2458-64

55. Lodygin, D., and Hermeking, H. (2005) The role of epigenetic inactivation of 13-3-3σ in human cancer. *Cell Research.* 15, 237-246

MGMT promoter methylation correlates with survival benefit and sensitivity to temozolomide in pediatric glioblastoma. *Pediatric Blood Cancer.* 48, 403-7

56. Roberston, K.D. DNA methylation and chromatin – unraveling the tangled web. *Oncogene.* 21, 5361-79

57. Suva, M.L., Rheinbay, E., Gillespie, S.M., Patel, A.P., Wakimoto, H., Rabkin, S.D., Riggi, N., Chi, A.S., Cahill, D.P., Nahed, B.V., Curry, W.T., Martuza, R.L., Rivera, M.N., Rossetti, N., Kasif, S., Beik, S., Kadri, S., Tirosh, I., Wortman, I., Shalek, A.K., Rozenblatt-Rosen, O., Regev, A., Louis, D.N., Bernstein, B.E. (2014) Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. *Cell. In press.*

58. Coulter, J.B., O'Driscoll, C.M., and Bressler, J.P., (2013) Hydroquinone increases 5-hydroxymethylcytosine formation through Ten Eleven Translocation 1 (TET1) 5-methylcytosine dioxygenase. *J. Biol. Chem.* 288, 28792-28800

59. Norris, P.J., Faull, R.L., and Emson, P.C. (1996) Neuronal nitric oxide synthase nNOS) mRNA expressin and NADPH-diaphorase staining in the frontal cortex, visual cortex and hippocampus of control and Alzheimer's disease brains. *Brain Res Mol Brain Res.* 41, 36-49

60. Harvard Brain Tissue Resource Center, PHS grant R24 MH068855

61. Hewitson, K.S., Granatino, N., Welford, R.W.D., McDonough, M.A., and , C.J. (2005) Oxidation by 2-oxoglutarate oxygenases: non-haem iron systems in catalysis and signalling. *Phil. Trans. R. Soc. A.* 363, 807-828

62. Ozer, A., and Bruick, R.K. (2007) Non-heme dioxygenases: cellular sensors and regulators jelly rolled into one? *Nature Chemical Biology*, 3, 144-152

63. Berg, R.A., and Prockop, D.J. (1973) Affinity column purification of protocollagen proline hydroxylase from chick embryos and further characterization of the enzyme. *J. Biol. Chem.* 4, 1175-1182

64. Semenza, G.L. (2003) Targeting HIF-1 for cancer therapy. *Nature Rev. Cancer*, 3, 721- 732

65 Ye, D., Ma, S., Xiong, Y., and Guan, K-L. (2013) R-2-hydroxyglutarate as the key effector of IDH mutations promoting oncogenesis. *Cancer Cell*, 3, 274-276

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EDUCATION AND TRAINING

Doctor of Philosophy 2009 - 2014

Program in Molecular and Translational Toxicology

Department of Environmental Health Sciences

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Advisor: Joseph P. Bressler, Ph.D

Master of Health Science, Environmental Health 2008 - 2009

Specialty track in Human Toxicology and Pathophysiology

Advisor: Joseph P. Bressler, Ph.D

Department of Environmental Health Sciences

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Bachelor of Science, Biology May 2004

James Madison University Harrisonburg, VA

The CITY School Program 1998-1999

Virginia Western Community College and Patrick Henry High School

Roanoke, VA

Patrick Henry High School May 1999

Roanoke, VA

EMPLOYMENT

Part-time (during MHS program)

Research Technologist 2008 to June 2009

Gabrielson Laboratory Baltimore, MD

Full-time

Research Technologist 2004-2008

Laboratory of Edward Gabrielson, M.D. Baltimore, MD

Division of Molecular Pathology, Department of Pathology

Johns Hopkins University School of Medicine

PROFESSIONAL SOCIETIES

Society of Toxicology, National Capital Area Chapter, 2011-present.

American Society for Biochemistry and Molecular Biology, 2013-present

AWARDS

2nd Place, Bern Schwetz Student Travel Award. National Capital Area Regional Chapter of Society of Toxicology. Annual Meeting, San Antonio, TX, 2013.

Honorable Mention, Bern Schwetz Student Travel Award. National Capital Area Regional Chapter of Society of Toxicology Annual Meeting, San Francisco, CA, 2012.

National Institute of Environmental Health Sciences Training Program Appointment ESO7141 (2009-14)

TEACHING

Lectures and Laboratory Instruction:

Bio-Trac Program, Foundation for Advanced Education in the Sciences,

National Institutes of Health. Bethesda, MD.

Director: Mark Nardone

1 "*Basic Techniques in the Study of Cancer Biology and Experimental Therapeutics*." Cytotoxicity assay lab instruction. May 24, 2012. Track 7: Animal and Human Cell Culture: Methods and Applications.

2 "*Roles of Cytosine Modifications in Active DNA Demethylation.*" December 6, 2012. Track 39: Epigenetics.

3 "*Roles of Cytosine Modifications in Active DNA Demethylation.*" April 2, 2013. Track 39: Epigenetics.

4 *"Basic Techniques in the Study of Cancer Biology and Experimental Therapeutics."* Immunocytochemistry lab instruction. May 2, 2013. Track 7: Animal and Human Cell Culture: Methods and Applications.

5 *"Culture and Use of Stem and Progenitor Cells."* May 2, 2013. Track 7: Animal and Human Cell Culture: Methods and Applications.

6 "*Mechanisms of DNA Demethylation.*" December 2, 2013. Track 39: Epigenetics.

Teaching Assistant: Toxicological Pathology, 4th Term 2011

Department of Environmental Health Sciences

Johns Hopkins University Bloomberg School of Public Health

Course Director: Kathleen Gabrielson, DVM, Ph.D

ABSTRACTS AND POSTERS

Jonathan B. Coulter, Cliona M. O'Driscoll, Joseph P. Bressler. ROS-generation leads to 5-hydroxymethylcytosine formation and DNA demethylation. Society of Toxicology Annual meeting, Epigenetics Section, San Antonio, TX. March 11, 2013.

Jonathan B. Coulter, Cliona M. O'Driscoll, John J. Laterra, Walter E. Kaufmann, and Joseph P. Bressler. MeCP2 expression in differentiating P19 mouse embryocarcinoma and human glioblastoma neurosphere cells. Society of Toxicology Annual meeting, Epigenetics Section, San Francisco, CA. March 14, 2012.

Jonathan B. Coulter, Cliona M. O'Driscoll, John J. Laterra, and Joseph P. Bressler. Potential role of MeCP2 in the regulation of 5-hydroxymethylcytosine levels in stem cell differentiation. NIEHS Training Grant Workshop. Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD. March, 2012.

Jonathan B. Coulter, Cliona M. O'Driscoll, John J. Laterra, Walter E. Kaufmann, and Joseph P. Bressler. MeCP2 in the differentiation of P19 mouse embryocarcinoma and human glioblastoma neurosphere cells. NIEHS Training Grant Scientific Workshop. Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD. February, 2011.

PEER-REVIEWED PUBLICATIONS

Jonathan B. Coulter, Cliona M. O'Driscoll, and Joseph P. Bressler. Hydroquinone increases 5-hydroxymethylcytosine formation through Ten Eleven Translocation 1 (Tet1) 5-methylcytosine dioxygenase. **Journal of Biological Chemistry.** 2013 Oct 4;288(40):28792-800.

Cliona M. O'Driscoll, Jonathan B. Coulter, and Joseph P. Bressler. Induction of a trophoblast-like phenotype by hydralazine in the P19 embryonic carcinoma cell line. **Biochimica et Biophysica Acta - Molecular Cell Research.** 2012 Nov 26.

Jewel Daniel, Jonathan Coulter, Ju-Hyung Woo, Kathleen Wilsbach, and Edward Gabrielson. High levels of the Mps1 checkpoint protein are protective of aneuploidy in breast cancer cells. **Proceedings of the National Academy of Sciences of the USA.** 2011 Mar 29;108(13):5384-9.

Hajime Orita, Jonathan Coulter, Ellen Tully, Masaaki Abe, Elizabeth Montgomery, Hector Alvarez, Koichi Sato, Okio Hino, Yoshiaki Kajiyama, Masahiko Tsurumaru, and Edward Gabrielson. High levels of fatty acid synthase expression in esophageal cancers represent a potential target for therapy. **Cancer Biology and Therapy.** 2010 Sept 15; 10(6).

Anju Singh, Swetlana Boldin-Adamsky, Rajesh K Thimmulappa, Srikanta K Rath, Hagit Ashush, Jonathan Coulter, Amanda Blackford, Steven N Goodman, Fred Bunz, Walter H Watson, Edward Gabrielson, Elena Feinstein, and Shyam Biswal. RNAi mediated silencing of Nrf2 gene expression in non-small cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy. **Cancer Research.** 2008 Oct 1; 68 (19): 7975–84.

Hajime Orita, Jonathan Coulter, Ellen Tully, Francis P. Kuhajda, and Edward Gabrielson. Inhibiting fatty acid synthase for chemoprevention of chemically induced lung tumors. **Clinical Cancer Research.** 2008 April 15. 14(8): 2458-2464.

Ju-Hyung Woo, Li Dapeng, Kathleen Wilsbach, Hajime Orita, Jonathan Coulter, Ellen Tully, Shi Xu, and Edward Gabrielson. Coix seed extract, a commonly used treatment for cancer in China, inhibits NFkB and protein kinase C signaling. **Cancer Biology and Therapy.** 2007 Dec. 6(12): 2005–2011. 3

Hajime Orita, Jonathan Coulter, Colleen Lemmon, Ellen Tully, Michael L. Pinn, Aravinda Vadlamudi, Susan M. Medghalchi, Francis P. Kuhajda, and Edward Gabrielson. Selective inhibition of fatty acid synthase for lung cancer treatment. **Clinical Cancer Research.** 2007 Dec 1. 13(23): 7139-7145.

EXTRACURRICULAR ACTIVITIES

Student Leadership and Activity

Secretary and Division Representative, Environmental Health Sciences Student Organization Baltimore, MD
Department of Environmental Health Sciences
Johns Hopkins University Bloomberg School of Public Health 2012-2013

Biological Embedding Journal Club 2012-2013

Johns Hopkins University Interdepartmental Journal Club Baltimore, MD

Board Member, Environmental Health Sciences Student Organization 2011-2012

Department of Environmental Health Sciences Baltimore, MD

Johns Hopkins University Bloomberg School of Public Health

MHS class Student Representative 2008-2009

Environmental Health Sciences Student Organization Baltimore, MD

Johns Hopkins University Bloomberg School of Public Health

Volunteering, Community Service and Fundraising

Peer Mentor March 9-11, 2013

Committee for Diversity Initiatives Undergraduate Mentoring Program

Society of Toxicology 2013 Annual Meeting San Antonio, TX

Baltimore Running Festival Half-Marathon and Fundraiser October 2012, 2013

International Center for Spinal Cord Injury

Kennedy Krieger Institute Charity Team, Baltimore, MD

Incentive Mentoring Program Gala May 2009

Coordinator and volunteer Baltimore, MD

Believe in Tomorrow Children's Foundation	March 2008,
2009	
Port to Fort 6k Run and Fundraiser, Baltimore, MD	
Maryland Susan G. Komen Race for the Cure, Towson, MD	2008
B. Frank Polk HIV/AIDS Unit Volunteer	2005-2007
The Johns Hopkins Hospital Baltimore, MD	
English Tutor to foreign medical school graduates	2005 -2006
USMLE Step 2 Exam Prep, Baltimore, MD	