

TET1-Mediated Hydroxymethylation Facilitates Hypoxic Gene Induction in Neuroblastoma

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<http://dx.doi.org/10.1016/j.celrep.2014.04.040>

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SUMMARY

The ten-eleven-translocation 5-methylcytosine dioxygenase (TET) family of enzymes catalyzes the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), a modified cytosine base that facilitates gene expression. Cells respond to hypoxia by inducing a transcriptional program regulated in part by oxygen-dependent dioxygenases that require Fe(II) and α -ketoglutarate. Given that the TET enzymes also require these cofactors, we hypothesized that the TETs regulate the hypoxia-induced transcriptional program. Here, we demonstrate that hypoxia increases global 5-hmC levels, with accumulation of 5-hmC density at canonical hypoxia response genes. A subset of 5-hmC gains colocalize with hypoxia response elements facilitating DNA demethylation and HIF binding. Hypoxia results in transcriptional activation of *TET1*, and full induction of hypoxia-responsive genes and global 5-hmC increases require *TET1*. Finally, we show that 5-hmC increases and *TET1* upregulation in hypoxia are HIF-1 dependent. These findings establish TET1-mediated 5-hmC changes as an important epigenetic component of the hypoxic response.

INTRODUCTION

The TET proteins are dioxygenases that convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). Whereas 5-mC represses transcription (Jones, 2012), elevated 5-hmC levels are associated with increased gene expression (Gan et al., 2013; Madzo et al., 2014; Szulwach et al., 2011). Colocalization of 5-hmC with regulatory regions such as transcription factor binding sites, promoters, and enhancers suggests that 5-hmC has

important regulatory functions (Madzo et al., 2014; Pastor et al., 2011; Stroud et al., 2011; Williams et al., 2011; Wu et al., 2011). 5-hmC also serves as an intermediate in demethylation pathways (Branco et al., 2012). Three TET enzymes have been identified: TET1, TET2, and TET3. *TET1* is highly expressed in embryonic stem cells and is upregulated in the generation of induced pluripotent stem cells (Ito et al., 2010; Koh et al., 2011; Piccolo et al., 2013). TET2 and TET3 are required for normal hematopoiesis and early reprogramming of the mammalian zygote, respectively (Ficz et al., 2011; Gu et al., 2011; Iqbal et al., 2011; Madzo et al., 2013; Wossidlo et al., 2011). These studies demonstrate that TET-mediated conversion of 5-mC to 5-hmC is an important epigenetic component of transcriptional regulation.

Hypoxia is a pervasive stimulus that affects a wide variety of biological processes. In tumor biology, rapid cellular proliferation and abnormal tumor vasculature result in highly hypoxic regions that confer an aggressive phenotype by upregulating angiogenic, metabolic, survival, proliferative, and metastatic pathways (Majmundar et al., 2010). Study of the transcriptional response to hypoxia has largely focused on the hypoxia inducible factors (HIFs) HIF-1 and HIF-2, which are α - β heterodimer transcription factors. HIF α subunits are targeted for degradation by O₂, Fe(II), and α -ketoglutarate-dependent prolyl hydroxylases. These hydroxylases have reduced activity in hypoxia, resulting in HIF α accumulation (Kaelin and Ratcliffe, 2008; Prabhakar and Semenza, 2012).

Epigenetic regulation plays an important role in regulating transcriptional changes in hypoxia. Genes encoding for the O₂, Fe(II), and α -ketoglutarate-dependent jumonji domain (JmJ) histone demethylases are transcriptionally upregulated in hypoxia (Beyer et al., 2008; Pollard et al., 2008; Schödel et al., 2011), and global changes in many histone modifications, such as H3K4me3, have also been reported (Johnson et al., 2008). Site-specific changes in histone modifications have been observed at hypoxia-induced genes including *CA9*, *LDHA*, and *PDK1* (Luo et al., 2012; van den Beucken et al., 2009). In addition, hypoxia affects DNA methylation (Liu et al., 2011; Shahrzad et al., 2007; Skowronski et al., 2010) and regulates noncoding

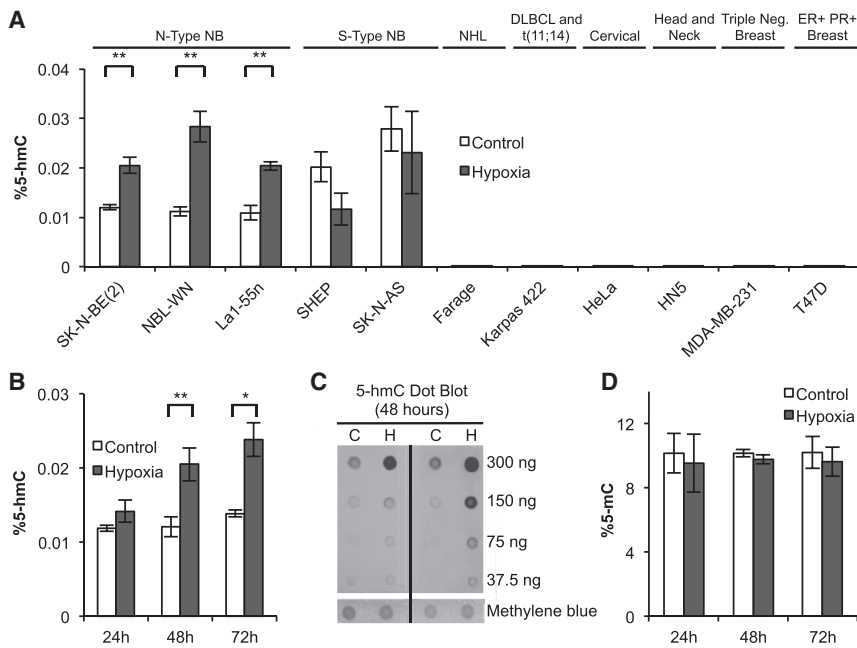


Figure 1. Global 5-hmC Levels Increase in Hypoxia

(A) Quantitation of 5-hmC by HPLC-MS/MS in various cancer cell lines exposed to 48 hr of hypoxia (NB, neuroblastoma; NHL, non-Hodgkin lymphoma; DLBCL, diffuse large B cell lymphoma; ER, estrogen receptor; PR, progesterone receptor). Percentage 5-hmC is calculated relative to the sum of all cytosine species ($n \geq 3$).

(B) Quantitation of 5-hmC by HPLC-MS/MS in SK-N-BE(2) cells exposed to 24, 48, or 72 hr of hypoxia ($n \geq 3$).

(C) Dot blot quantification of 5-hmC in SK-N-BE(2) cells exposed to 48 hr of hypoxia.

(D) Quantitation of 5-mC by HPLC-MS/MS in SK-N-BE(2) cells. Percentage 5-mC is calculated relative to the sum of all cytosine species ($n \geq 3$).

Data represent means \pm SEM. p values calculated by Student's t test, * $p < 0.05$, ** $p < 0.01$. See also Figure S1.

RNAs providing additional layers of epigenetic regulation (Choudhry et al., 2014). Despite the evidence that hypoxia induces epigenetic alterations, whether hypoxia also affects global or site-specific 5-hmC levels is not known. We hypothesized that the TETs play a role in regulating the transcriptional response to hypoxia, because, like the JmJ histone demethylases and the HIF α prolyl hydroxylases, the TETs require O₂, Fe(II), and α -keto-glutarate for their activity.

RESULTS

Hypoxia Increases Global 5-hmC Levels

We explored the role of TET-mediated 5-hmC changes in regulating the hypoxia-induced transcriptional program by measuring changes in global 5-hmC levels by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) in a variety of cancer cells after treatment with 1% O₂ for 48 hr: SK-N-BE(2), NBL-WN, La1-55n, SHEP, and SK-N-AS (neuroblastoma); Farage (non-Hodgkin B cell lymphoma), Karpas 422 (diffuse large B cell lymphoma with t[11;14]), HeLa (cervical cancer), HN5 (head and neck cancer), MDA-MB-231 (triple-negative breast cancer), and T47D (ER⁺ PR⁺ breast cancer). Although many cancer cell lines had low levels of 5-hmC in both hypoxia and normoxia, we found that 5-hmC increased after hypoxia in tumorigenic N-type neuroblastoma cells, but not in nontumorigenic S-type cells, despite the fact the TET enzymes depend on oxygen (Figures 1A and S1A). We further pursued the functional significance of 5-hmC increases in neuroblastoma cells for several reasons. First, neuroblastomas are derived from neural crest cells and neuronal cell types have high levels of 5-hmC (Globisch et al., 2010; Szulwach et al., 2011). Second, neuroblastoma cells dedifferentiate in hypoxia (Bhaskara et al., 2012; Jögi et al., 2002, 2004), suggesting that altered differentiation could be driven by 5-hmC changes because the TET enzymes

regulate transitions between pluripotent and differentiated states (Ito et al., 2010; Koh et al., 2011; Madzo et al., 2014).

To further characterize the timeline of 5-hmC increases, SK-N-BE(2) cells were exposed to 24, 48, and 72 hr of hypoxia, and 5-hmC levels were analyzed by HPLC-MS/MS. HIF-1 α protein levels were elevated in hypoxia exposed cells at all time points, demonstrating that cells were oxygen deprived (Figure S1B). 5-hmC levels were elevated in cells exposed to 48 and 72 hr of hypoxia (Figures 1B and S1C). Increased 5-hmC levels in hypoxic SK-N-BE(2) cells were also observed by dot blot assay after labeling 5-hmC with biotin (Figure 1C) (Song et al., 2011). In contrast to the observed changes in 5-hmC, 5-mC levels remained unchanged at all time points (Figures 1D and S1D). Hypoxia-induced 5-hmC gains were largely reversed following reoxygenation (Figure S1E).

5-hmC Gains Occur at Hypoxia-Regulated Genes

To determine the genomic positions gaining 5-hmC in hypoxia, we purified and sequenced 5-hmC-enriched DNA fragments from SK-N-BE(2) cells exposed to 48 hr of hypoxia using the hMe-Seal method (Song et al., 2011) in two independent biological replicates (Figures S2A and S2B). RNA sequencing (RNA-seq) was performed to quantify transcriptional changes. To determine the distribution of 5-hmC gains across the genome, we summed gained hMe-Seal sequencing tags over various genomic elements (exons, introns, untranslated regions [UTRs], CpG islands/shores, and intergenic regions). To test for potential regulatory function of 5-hmC, the positions of previously published HIF binding sites (Schödel et al., 2011), and the locations of DNase-hypersensitive sites (DHSs) (Bernstein et al., 2012) were included. In absolute terms, 5-hmC gains occurred to the greatest extent over introns (Figure 2A). Enrichment of 5-hmC gains at each element was then calculated by normalizing for element length. 5-hmC gains were enriched at or near known HIF-1 binding sites, at DHSs, and at CpG shores (Figures 2B, S2C, and S2D). Enrichment of 5-hmC gains was

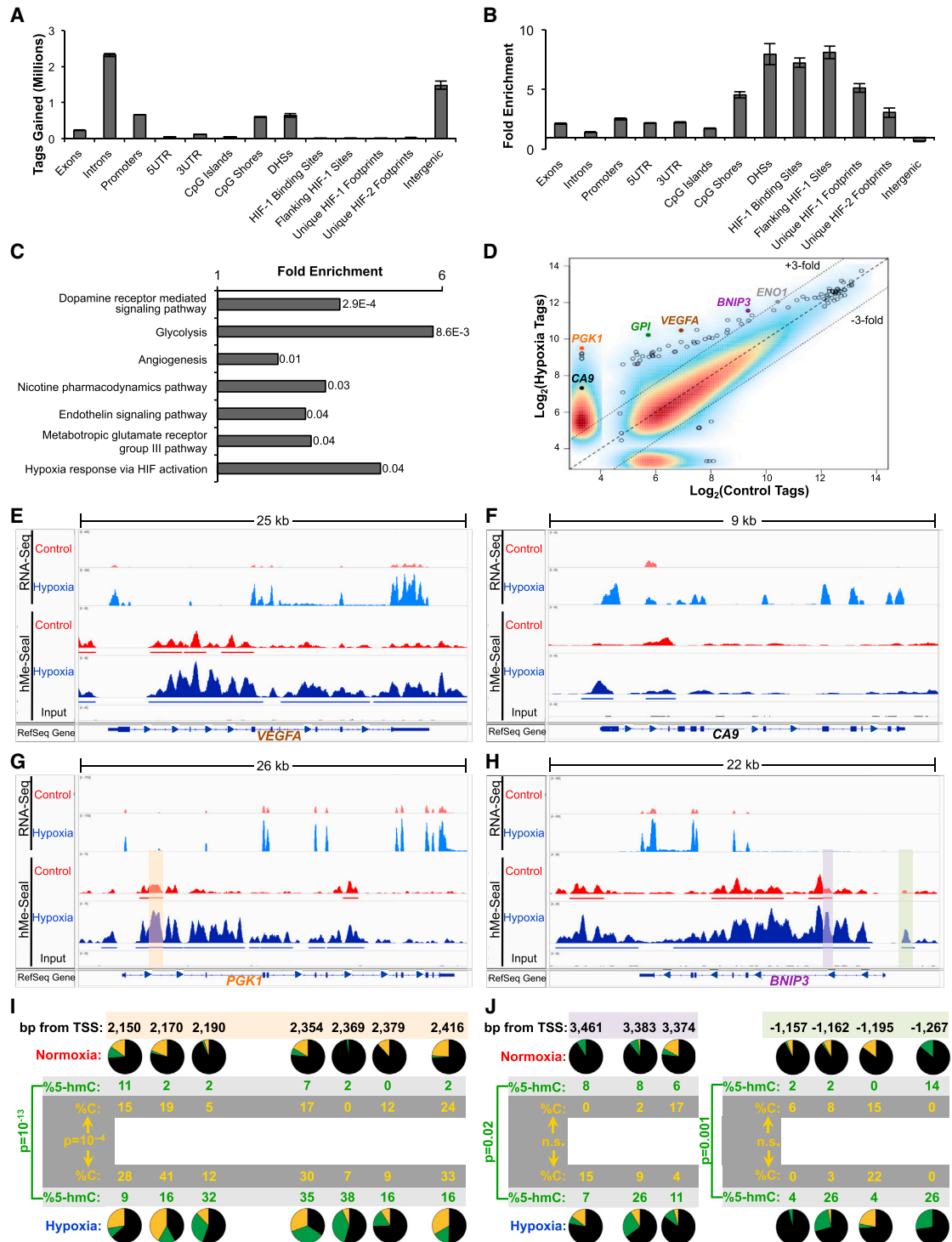


Figure 2. 5-hmC Gains Occur at Hypoxia Regulated Regions in SK-N-BE(2) Cells

(A and B) Absolute 5-hmC gains (A) and enrichment for 5-hmC gains (B) were calculated at genomic elements. Regions flanking HIF binding sites are regions ± 1 kb from the ChIP-seq peak. Footprints include the ChIP-seq peak and flanking region (Schödel et al., 2011). Data represent means \pm SD for two biological replicates.

(C) PANTHER pathway analysis of genes with regions gaining at least 3-fold 5-hmC. p values are Bonferroni corrected results of binomial tests (Mi et al., 2013).

(D) Regions gaining the most 5-hmC map to the HIF-1 target genes CA9, PGK1, GPI, VEGFA, BNIP3, and ENO1. Plotted points represent combined data from two experiments.

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greater at or near unique HIF-1 binding sites (sites that bind HIF-1, but not HIF-2) than at unique HIF-2 binding sites (sites that bind HIF-2, but not HIF-1) (Schödel et al., 2011).

We performed PANTHER pathway analysis on the genes with a 3-fold increase of 5-hmC density in hypoxia and identified pathways such as dopamine receptor mediated signaling, glycolysis, angiogenesis, and hypoxia response via HIF activation (Figure 2C). For each genomic position, we plotted the number of sequencing tags mapping to the region for the control and hypoxia samples. This analysis demonstrated that regions gaining the most 5-hmC map to canonical hypoxia response genes, including *CA9*, *PGK1*, *GPI*, *VEGFA*, *BNIP3*, and *ENO1* (Figure 2D). 5-hmC gains occurred over gene bodies and/or promoter regions of these genes (Figures 2E–2H and S2E–S2H). To resolve 5-hmC, 5-mC, and unmodified cytosine at single-base resolution at loci that gain 5-hmC in hypoxia, we performed Tet-assisted bisulfite sequencing (TAB-seq) (Yu et al., 2012) at the *PGK1* first intron, and the *BNIP3* promoter and first intron (Figures 2I and 2J). Given previous reports that 5-hmC is associated with transcription factor binding sites (Gan et al., 2013; Madzo et al., 2014), we used Homer motif analysis to calculate enrichment for putative transcription factor binding sites at positions gaining 5-hmC in hypoxia. We identified these regions as enriched for binding sites of ZFX, Eomes, ZNF711, NeuroD1, N-Myc, Foxo1, Max, c-Myc, Stat3, and the androgen receptor (Table S1). Finally, we repeated hMe-Seal and RNA-seq experiments in a second neuroblastoma cell line, NBL-WN. NBL-WN cells gained 5-hmC at similar genomic annotations and hypoxia-responsive genes (Figures S2I–S2L). These results implicate 5-hmC as an important regulator of the hypoxia-induced transcriptional program.

TET1 Expression Increases in Hypoxia and Is Necessary for Hypoxic Gene Induction

Given the dependence of TET enzymes on oxygen, we were surprised to find global increases of 5-hmC in hypoxia. Therefore, we hypothesized that transcriptional increases in one or more *TET* genes could compensate for potentially lower enzymatic activity in hypoxia, as has been shown for the HIF α prolyl hydroxylases (Stiehl et al., 2006). We found that *TET1* transcription increased in SK-N-BE(2) cells in hypoxia as measured by quantitative PCR (qPCR) (Figure 3A), whereas no changes in *TET2* or *TET3* mRNA levels were observed (Figure 3B). These *TET* expression results were also observed by RNA-seq (Figure 3C). Like 5-hmC levels, the increase in *TET1* mRNA was reversed after reoxygenation (Figure S3A) and was observed in other N-type, but not S-type, cell lines (Figure 3D). To determine whether the *TETs* are induced in hypoxia in other cancer cell lines, we quantified *TET1*, *TET2*, and *TET3* expression after 48 hr of hypoxia in Farage, Karpas 422, HeLa, HN5, MDA-MB-231, and T47D cells. We only observed induction of *TET2* by hypoxia in Karpas 422 cells (Figures S3B–S3D).

Given the increase of *TET1* in hypoxia, we hypothesized that TET1 was responsible for global 5-hmC gains. We tested this by knocking down *TET1* with small interfering RNA (siRNA) and noted that this treatment did not interfere with HIF-1 α accumulation (Figure 3E). We found that TET1 depletion inhibited global 5-hmC gains (Figure 3F). We then tested if TET1 activity is necessary for full induction of genes gaining 5-hmC in hypoxia using cells transiently depleted of *TET1* with siRNA or cells stably expressing a *TET1*-targeting short hairpin RNA (shTET1) (Figure S3E). In addition to genes identified as gaining large amounts of 5-hmC in Figure 2, we included *HK2* in our analysis, because it gained narrow 5-hmC peaks over its promoter and first intron in our hMe-Seal experiments (Figure S3F). *TET1* depletion blunted hypoxia-induced induction of *CA9*, *BNIP3*, and *VEGFA* at 16 hr of hypoxia (Figures S3G and S3H). After 48 hr of hypoxia, *TET1* depletion attenuated *PGK1* and *HK2* expression, in addition to *CA9*, *BNIP3*, and *VEGFA* (Figures 3G and 3H). These experiments show that TET1 is required for full induction of the hypoxic transcriptional program.

Interestingly, *CA9* expression is delayed in hypoxic neuroblastoma cells relative to other hypoxia-induced genes (Figures S3I and S3J) and closely mirrors the time course of global 5-hmC gains in hypoxia (Figures 1B and S1C), suggesting that *CA9* expression could be particularly dependent on epigenetic remodeling. Therefore, we further investigated the role of 5-hmC gains in inducing *CA9* expression. *CA9* gains 5-hmC over its transcriptional start site (TSS) where there is a well-characterized hypoxia response element (HRE) (Wykoff et al., 2000). Because HIF-1 binding to HREs is methylation sensitive (Wenger et al., 1998), we tested the effect of hydroxymethylation on HIF-1 binding within the HRE using an electrophoretic mobility shift assay (EMSA). Like 5-mC, we found that 5-hmC inhibited HIF-1 binding (Figure 3I). We therefore hypothesized that 5-hmC may accumulate near the HRE to facilitate an open chromatin state, and that 5-hmC within the HRE would be a transient intermediate facilitating demethylation and HIF binding. To test this hypothesis, we performed TAB-seq (Yu et al., 2012) at the *CA9* TSS. Consistent with our hypothesis and the observed slow induction kinetics of *CA9*, we observed small but insignificant accumulation of 5-hmC at the *CA9* TSS by 16 hr of hypoxia, accompanied by an increase of unmodified cytosine at the HRE (Figure S3K). By 48 hr of hypoxia, significant accumulation of 5-hmC at a CpG 74 bp upstream of the TSS was observed and further demethylation of multiple CpGs in this region, including at the HRE, was observed (Figure 3J). To determine if 5-hmC gains at HIF-1 chromatin immunoprecipitation sequencing (ChIP-seq) peaks are associated with HRE demethylation at other sites, we performed TAB-seq at two additional HIF-1 ChIP-seq peaks associated with 5-hmC gains in hypoxia (Figures S2C and S2D). Similar to our findings at *CA9*, we found that these hMe-Seal peaks mark HREs that gained unmodified cytosine over 48 hr of hypoxia (Figures S3L and S3M).

(E–H) RNA-seq and hMe-Seal sequencing data are plotted in the Integrated Genomics Viewer (IGV) genome browser at *VEGFA*, *CA9*, *PGK1*, and *BNIP3* genes. RefSeq gene tracks represent the position of known genes in the hg19 build of the human genome. Arrows represent the direction of transcription. (I and J) TAB-seq data for the corresponding shaded regions of *PGK1* (G) and *BNIP3* (H). For each CpG, black, green, and gold represent the percentage of 5-mC, 5-hmC, and unmodified cytosine, respectively. Results represent pooled data over three independent experiments. p values calculated by chi-square tests. See also Figure S2 and Table S2.

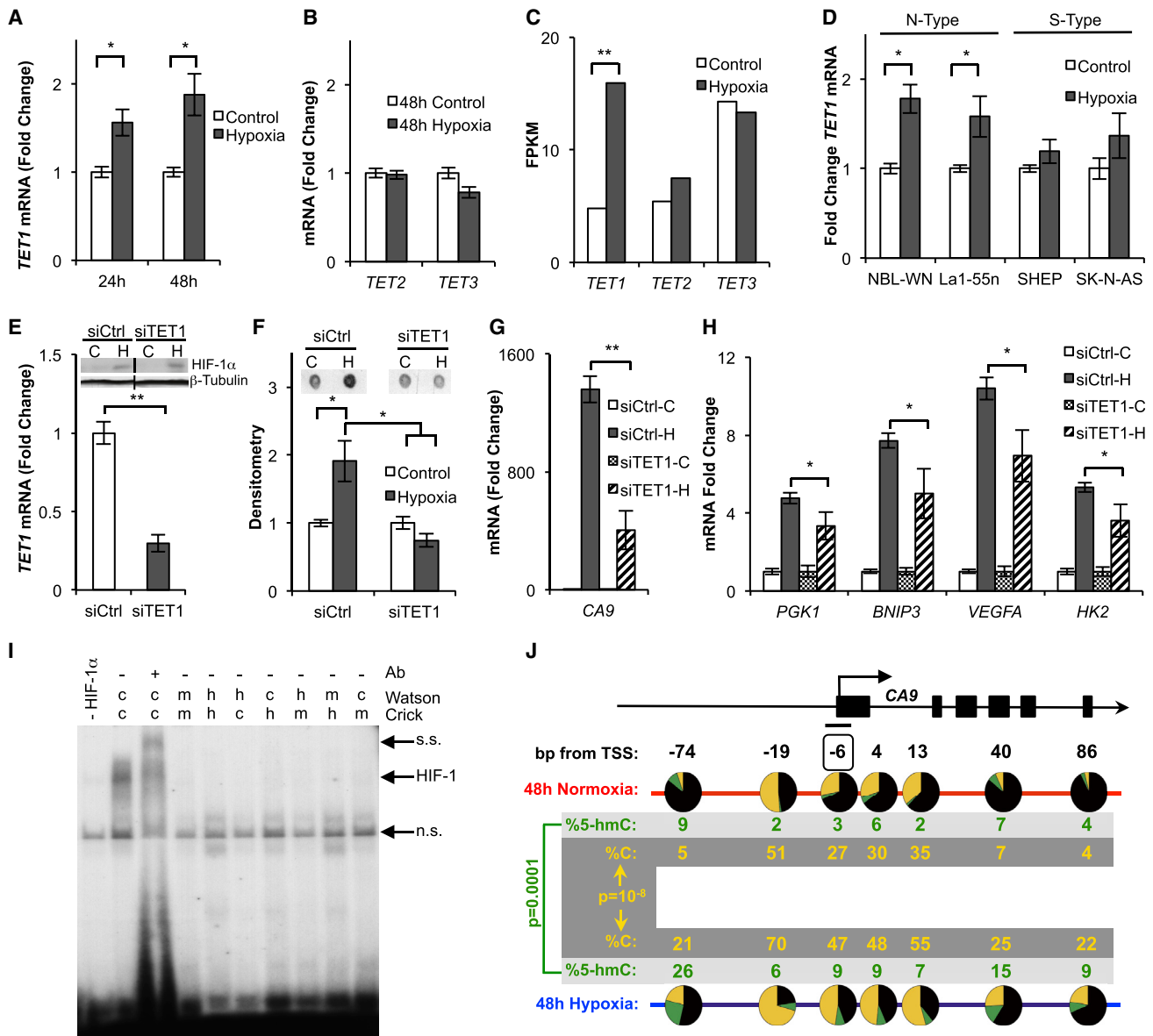


Figure 3. TET1 Is Required for Full Hypoxic Gene Induction

(A) *TET1* mRNA quantified by qPCR from SK-N-BE(2) cells (n ≥ 4). (B) *TET2* and *TET3* mRNA quantified by qPCR from SK-N-BE(2) cells exposed to 48 hr of hypoxia (n = 3). (C) *TET* mRNA levels measured in fragments per kilobase of transcript per million mapped reads (FPKM) by RNA-seq. Data represent Cuffdiff output from two biological replicates of SK-N-BE(2) cells exposed to 48 hr of hypoxia. p values were false discovery rate corrected. (D) *TET1* mRNA measured by qPCR in neuroblastoma cell lines exposed to 48 hr of hypoxia (n ≥ 4). (E) *TET1* mRNA determined by qPCR, and HIF-1α expression determined by immunoblot, in SK-N-BE(2) cells transfected with siCtrl or siTET1 (n = 3). (F) 5-hmC quantified by dot blot in SK-N-BE(2) cells transfected with siCtrl or siTET1 and exposed to 48 hr of hypoxia. Quantification represents means ± SEM (n = 3). (G and H) qPCR quantification of hypoxia-induced gene expression in *TET1* depleted SK-N-BE(2) cells after 48 hr of hypoxia (n = 3). (I) EMSA binding studies of HIF-1 to the *CA9* HRE. c, cytosine; h, 5-hmC; m, 5-mC. (J) TAB-seq of the *CA9* TSS. The boxed CpG 6 bp upstream of the TSS resides within the HRE. Results are pooled over three replicates. p values calculated by chi-square tests.

All graphs represent means ± SEM. p values for expression analysis calculated by Student's t test, *p < 0.05, **p < 0.01. See also Figure S3.

TET1 and Global 5-hmC Increases Are HIF-1 Dependent

We then determined the mechanisms underlying *TET1* induction and 5-hmC increases in hypoxia. We hypothesized that these changes may be HIF-1 regulated, because (1) pathways and

genes identified in our hMe-Seal analysis showed a strong HIF-1 signature (Figure 2); and (2) SK-N-BE(2) and NBL-WN cells selectively expressed *HIF-1α* over *HIF-2α* (Figure S4A), consistent with a previous report (Qing et al., 2010). We generated

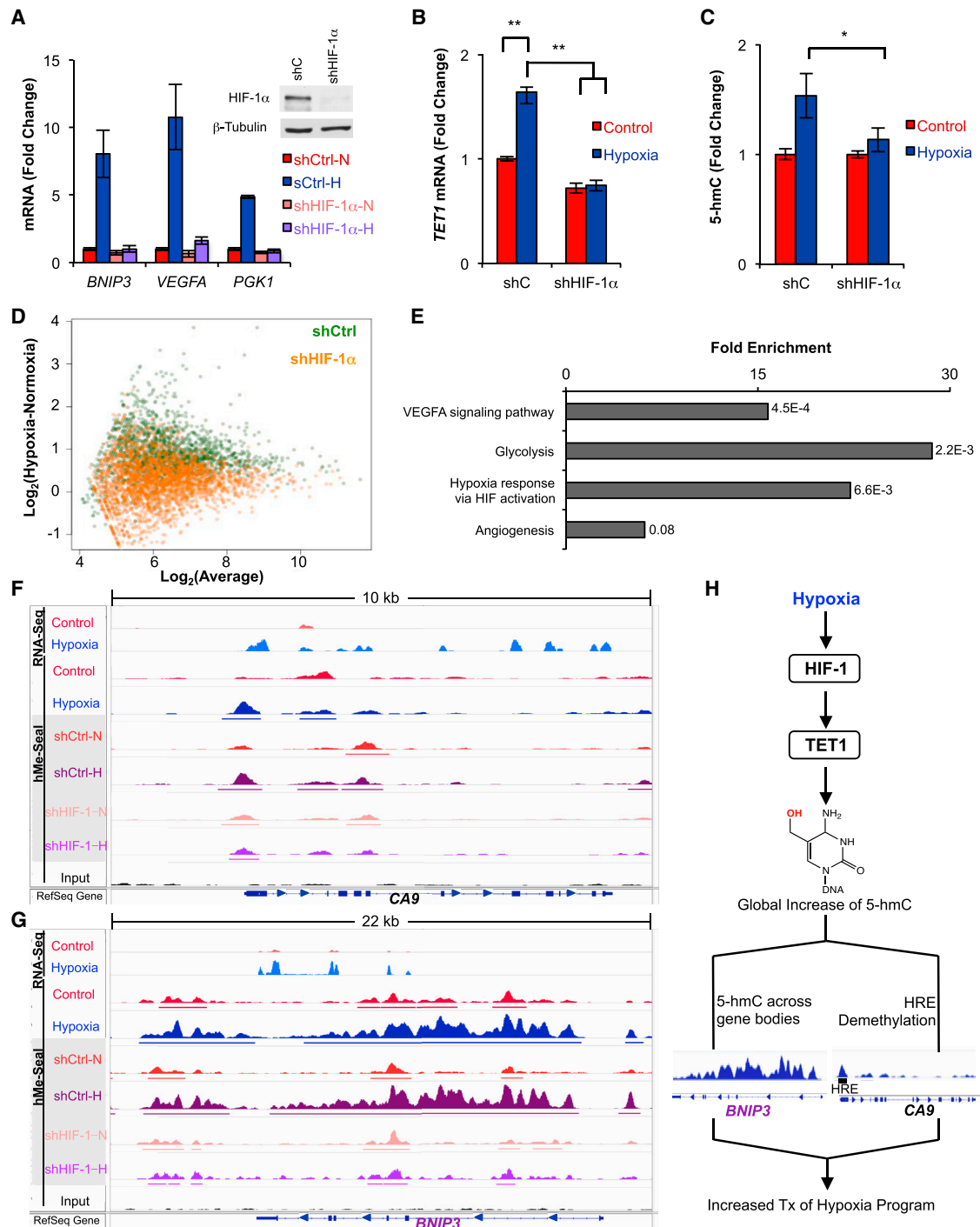


Figure 4. HIF-1 Regulates *TET1* and Is Necessary for Genome-wide and Locus-Specific 5-hmC Gains in SK-N-BE(2) Cells

(A) qPCR quantification of HIF-1 target gene expression in shHIF-1 α or shCtrl-transduced cells after 48 hr of hypoxia (n = 3).

(B and C) *TET1* mRNA quantification by qPCR (B) and 5-hmC quantification by HPLC-MS/MS (C) in shCtrl or shHIF-1 α -transduced SK-N-BE(2) cells exposed to 48 hr of hypoxia (n = 3).

(D) Regions gaining 5-hmC from Figure 2D are plotted in shCtrl or shHIF-1 α -expressing cells. Gains of 5-hmC in hypoxia are represented as an upward shift on the y axis.

(E) PANTHER pathway analysis of regions losing 5-hmC in hypoxic shHIF-1 α cells relative to hypoxic shCtrl cells. p values are Bonferroni corrected results of binomial tests (Mi et al., 2013).

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SK-N-BE(2) cells expressing a previously validated short hairpin RNA targeting either HIF-1 α (shHIF-1 α) (Stiehl et al., 2012) or a nontargeting control (shCtrl). shHIF-1 α efficiently reduced HIF-1 α protein levels in hypoxia and inhibited transcriptional upregulation of HIF-1 target genes (Figure 4A). HIF-1 α knockdown also prevented the upregulation of *TET1* and global 5-hmC increases in hypoxia (Figures 4B, 4C, and S4B). These results demonstrate that HIF-1 is required for hypoxic induction of *TET1* and global increases in 5-hmC.

To investigate whether site-specific 5-hmC gains are HIF-1 regulated, we repeated hMe-Seal with cells expressing shCtrl and shHIF-1 α . We analyzed genomic positions identified as gaining 5-hmC in our initial hMe-Seal experiment and found that shCtrl-transduced cells gained 5-hmC at these positions, but shHIF-1 α -transduced cells did not (Figure 4D). PANTHER pathway analysis of genes losing 5-hmC in hypoxic shHIF-1 α cells relative to hypoxic shCtrl cells demonstrated that 5-hmC depletion in hypoxic shHIF-1 α cells is specific to genes regulated by HIF-1 (Figure 4E). *CA9*, *BNIP3*, *VEGFA*, *PGK1*, *GPI*, and *ENO1* gained large amounts of 5-hmC in shCtrl-transduced cells but showed little to no 5-hmC gains in shHIF-1 α -transduced cells (Figures 4F, 4G, and S4C–S4F). In contrast, HIF-1 α knockdown had no effect on 5-hmC levels at genes that are not regulated by hypoxia (Figures S4G and S4H). These findings indicate that induction of *TET1* and gain of 5-hmC-density at hypoxia-induced genes are HIF-1 dependent.

DISCUSSION

Our results demonstrate a hitherto uncharacterized role for the *TET1* dioxygenase and 5-hmC in regulating the hypoxia-induced transcriptional program in neuroblastoma cells. We propose a model (Figure 4H) in which *TET1* is upregulated in hypoxia by HIF-1. This is associated with *TET1* and HIF-1-dependent 5-hmC gains clustered at hypoxia-induced genes. At these genes, *TET1* facilitates expression. We find 5-hmC gains in hypoxia at promoters, exons, introns, UTRs, and other regulatory regions, where 5-hmC may have distinct functions. These findings raise numerous questions that will be the subject of future work, including the following: determining whether *TET1* is a direct or indirect transcriptional target of HIF-1; how *TET1* is directed to specific genes induced in hypoxia; and the function of 5-hmC at different genomic elements.

We observe induction of *TET1* and increase in 5-hmC within tumorigenic, N-type neuroblastoma cells. The low levels of 5-hmC in other cancer cells are consistent with previous reports that (1) 5-hmC levels are reduced in numerous cancers (Mariani et al., 2013) and (2) neuronal cells have high levels of 5-hmC (Globisch et al., 2010; Szulwach et al., 2011). It will be important for us and others to test an even wider range of cancer and other cell types in the future to understand if our observations are broadly applicable.

Our work is consistent with previous observations that hypoxia induces global and site-specific epigenetic changes (Beyer et al., 2008; Johnson et al., 2008; Liu et al., 2011; Pollard et al., 2008; Shahrzad et al., 2007; Skowronski et al., 2010). Our finding that *TET1* is transcriptionally upregulated in hypoxia and subsequently facilitates hypoxia-induced gene expression is analogous to previous findings regarding the JmJ histone demethylases: genes encoding for JmJ histone demethylases are HIF targets (Beyer et al., 2008; Pollard et al., 2008; Schödel et al., 2011), and the protein products of these genes facilitate hypoxic gene induction by remodeling chromatin at other HIF targets (Luo et al., 2012). Previous work has also demonstrated that treatment of cells with 5-azacytidine augments induction of the hypoxia transcriptional program, particularly by demethylation of HREs (Koslowski et al., 2011). This is consistent with our observation that HIF-1 binding sites gaining 5-hmC in our hMe-Seal experiments exhibit an increase in the amount of unmodified cytosine at the HRE in TAB-seq experiments, which facilitates gene induction. The fact that very little 5-hmC accumulates at the CpG within these HREs suggests that demethylation at these CpGs occurs quickly. Our results further highlight the importance of chromatin remodeling in the hypoxic response and identify 5-hmC changes as a previously unknown component of this process.

Two recent studies have demonstrated that most HIF-target genes have promoter-proximally paused RNA PolII in normoxia, and that HIF binding to these genes releases PolII from the paused state (Choudhry et al., 2014; Galbraith et al., 2013). Nonetheless, a small subset of hypoxia regulated genes recruit PolII de novo on hypoxic exposure (Choudhry et al., 2014). Broad 5-hmC gains across the bodies of genes, such as *BNIP3*, suggest a potential role for 5-hmC in PolII elongation, whereas narrow 5-hmC peaks gained at or near the TSS of genes like *CA9* may facilitate PolII recruitment. Determining if 5-hmC serves to regulate either de novo PolII recruitment or release from the promoter-proximal paused state may further our molecular understanding of how hypoxia-induced genes are regulated.

Finally, our work emphasizes the importance of employing techniques that can distinguish 5-mC from 5-hmC. A previous study examined the effect of hypoxia on covalent cytosine modifications using Illumina 450K methylation arrays (Yuen et al., 2013). These arrays use sodium bisulfite treatment of DNA to distinguish modified and unmodified cytosines but cannot distinguish between 5-mC and 5-hmC (Mariani et al., 2013). Using these arrays, cytosine modification levels in 147 CpGs were found to differ between cytotrophoblastic cells treated with 24 hr of normoxia or hypoxia (Yuen et al., 2013). Only one of the CpGs identified in this study correlates with a location gaining 5-hmC in our data (Illumina cg 23097499, Chr20: 62330618, associated with *TNFRSF6B*) (Yuen et al., 2013). This is most likely because this previous study would be blind to any transitions between 5-mC and 5-hmC due to the

(F and G) hMe-Seal data for parental and transduced SK-N-BE(2) cells exposed to 48 hr of hypoxia or normoxia.

(H) Hypoxia stimulates HIF-1-dependent upregulation of *TET1*, which is necessary for genome-wide 5-hmC increases. Site-specific 5-hmC gains are clustered at hypoxia response genes that require *TET1* for induction.

All graphs represent means \pm SEM. See also Figure S4.

limitations of sodium bisulfite based techniques. Combined with recent work that used techniques specific for 5-hmC to examine lineage commitment of hematopoietic stem cells (Madzo et al., 2014), our work suggests that 5-hmC may play a broad role in regulating cellular responses that require dynamic and wide-spread transcriptional changes. Future work should continue to test this hypothesis using techniques specific for 5-hmC.

EXPERIMENTAL PROCEDURES

Detailed procedures are provided in the [Supplemental Experimental Procedures](#).

Hypoxia Exposures

Hypoxic cells were incubated under 1% O₂ and 10% CO₂ in modular incubator chambers (Billups-Rothenberg). Normoxic cells were incubated at 10% CO₂ and 20% O₂.

Detection of 5-hmC and 5-mC by HPLC-MS/MS

Genomic DNA was hydrolyzed to nucleosides and run on a Zorbax XDB-C18 2.1 3 50 mm column (1.8 mm particle size) attached to an Agilent 1200 Series liquid chromatography machine coupled to an Agilent 6410 Triple Quad Mass Spectrometer (Madzo et al., 2014; Vasanthakumar et al., 2013).

5-hydroxymethylcytosine Selective Chemical Labeling

5-hydroxymethylcytosine selective chemical labeling (hMe-Seal) was performed as previously described (Song et al., 2011). Briefly, 20 μg of sonicated genomic DNA was labeled with UDP-6-N3-glucose and biotinylated using DMCO-S-S-PEG3-Biotin Conjugate (Click Chemistry Tools). Biotinylated DNA was affinity purified and sequenced.

Sequence Mapping and Analysis

DNA reads were mapped to hg19 with the Burrows-Wheeler Aligner. The BWA-mem algorithm was used for 100 bp reads and BWA-backtrack for 50 bp reads. Peak calling was performed using MACS1.4. RNA reads were aligned using TopHat. Cufflinks and Cuffdiff were used for expression analysis.

Enrichment Calculations for 5-hmC Gains at Genomic Elements

Regions gaining 5-hmC in hypoxia were intersected with the genomic element being analyzed. Intersections were weighted by the length of the intersection and the amount of 5-hmC being gained over the intersection and then summed genome-wide to calculate an observed value of 5-hmC gained at each element. Expected values were calculated by scrambling 5-hmC gains over the genome and performing the same calculation. Mean values from 1,000 scrambling experiments were used as the expected value in enrichment calculations.

Generation of Lentivirally Transduced Cells

Production of lentivirus and subsequent infection of neuroblastoma cells followed the protocol found at <http://www.addgene.org/tools/protocols/plko/>. pLKO.1 plasmids coding for *HIF-1α* and *TET1* targeting hairpins were previously described (Stiehl et al., 2012; Sun et al., 2013).

Transfection with *TET1* siRNA

TET1 targeting siRNA (Thermo ON-TARGETplus SMARTpool D-014635) and nontargeting control siRNA (Thermo ON-TARGETplus Non-targeting pool L-001810) were transfected into neuroblastoma cells using Mirus TransIT-siQUEST reagent (MIR 2114).

HIF-1 EMSA Assay

HIF-1α and ARNT were produced by IVTT using the TNT T7 Coupled Coupled Reticulocyte Lysate System (Promega). Nucleotide probes were designed based off of the previously characterized CA9 HIF1 binding site (Wykoff et al., 2000): 5'-CCAATGCACTACAGCCC-3' and 5'-GGGCTGTACGTGCA

TTGG-3' with the underlined cytosine being unmodified, methylated, or hydroxymethylated.

TAB-seq

TAB-seq was performed using the Wisegene 5-hmC Tab-seq kit. Ten micrograms of DNA was sonicated to 500 bp and conjugated to UDP-glucose with β-glycosyltransferase. After reaction cleanup, conjugated DNA was oxidized using mTet1, and bisulfite treated. Glycosylated DNA without mTet1 oxidation was used for quantification of modified and unmodified cytosines. After PCR amplification, products were TOPO TA cloned (Life Technologies K4575-02) and sequenced.

ACCESSION NUMBERS

Next generation sequencing data sets are available at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55391> (GEO accession number: GSE55391).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.04.040>.

AUTHOR CONTRIBUTIONS

C.J.M. designed and performed experiments, analyzed data, and wrote the manuscript. A.V. and A.Y. performed experiments. J.M. analyzed data and helped perform experiments. T.B., Y.Y., and S.B. performed library building and next generation sequencing. R.H.W., S.L.C., and J.N. helped design experiments. A.V. supervised next-generation sequencing. N.R.P. and L.A.G. conceived the study, analyzed the data, and edited the manuscript.

ACKNOWLEDGMENTS

We would like to thank Marsha Rosner for the *TET1* shRNA plasmid and Chuan He for UDP-6-N3-glucose. We thank Jeremy Marks and Angela Stoddart for comments on the manuscript and insightful discussions. This work was supported in part by NIH grants T32-HD007009-38 (to C.J.M.) and P01-HL090554-06 (to N.R.P.).

Received: March 20, 2014

Revised: April 14, 2014

Accepted: April 25, 2014

Published: May 15, 2014

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