5-hydroxymethylation of the EBV genome regulates the latent to lytic switch

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Latent Epstein–Barr virus (EBV) infection and cellular hypermethylation are hallmarks of undifferentiated nasopharyngeal carcinoma (NPC). However, EBV infection of normal oral epithelial cells is confined to differentiated cells and is lytic. Here we demonstrate that the EBV genome can become 5-hydroxymethylated and that this DNA modification affects EBV lytic reactivation. We show that global 5-hydroxymethylcytosine (5hmC)-modified DNA accumulates during normal epithelial-cell differentiation, whereas EBV+ NPCs have little if any 5hmC-modified DNA. Furthermore, we find that increasing cellular ten–eleven translocation (TET) activity [which converts methylated cytosine (5mC) to 5hmC] decreases methylation, and increases 5hmC modification, of lytic EBV promoters in EBV-infected cell lines containing highly methylated viral genomes. Conversely, inhibition of endogenous TET activity increases lytic EBV promoter methylation in an EBV-infected telomerase-immortalized normal oral keratinocyte (NOKs) cell line where lytic viral promoters are largely unmethylated. We demonstrate that these cytosine modifications differentially affect the ability of the two EBV immediate-early proteins, BZLF1 (Z) and BRLF1 (R), to induce the lytic form of viral infection. Although methylation of lytic EBV promoters increases Z-mediated and inhibits R-mediated lytic reactivation, 5hmC modification of lytic EBV promoters has the opposite effect. We also identify a specific CpG-containing Z-binding site on the BRLF1 promoter that must be methylated for Z-mediated viral reactivation and show that TET-mediated 5hmC modification of this site in NOKs prevents Z-mediated viral reactivation. Decreased 5-hydroxymethylation of cellular and viral genes may contribute to NPC formation.

EBV | 5hmC | lytic reactivation | NPC

Epstein–Barr virus (EBV) is a gamma-herpesvirus that is the causative agent of infectious mononucleosis. It also contributes to the development of epithelial- and B-cell malignancies such as nasopharyngeal carcinoma (NPC) and Burkitt lymphoma (1, 2). Like all herpesviruses, EBV has both latent and lytic forms of infection. Latent EBV infection occurs in normal B lymphocytes, as well as in EBV-associated B-cell and epithelial-cell malignancies, and promotes transformation of EBV-infected tumor cells (1, 2). Lytic EBV infection, which is required for horizontal spread of the virus from host to host, occurs in differentiated oropharyngeal epithelial cells, B-cell receptor-activated B cells, and plasma cells (3–8).

The EBV genome becomes highly methylated following infection of normal B cells and within B-cell and epithelial-cell tumors (9). CpG methylation of the EBV genome is detectable within 2 wk postinfection in B cells (10) and plays a critical role in promoting the most stringent (and least immunogenic) form of viral latency (reviewed in refs. 11, 12). In addition, methylation of the viral genome is required for the ability of the EBV BZLF1 (Z) immediate-early protein to induce the latent to lytic switch, because Z preferentially binds to and activates the methylated forms of lytic EBV promoters (reviewed in refs. 11, 13). Z (also known as EB1, ZEBRA, and Zta) is a bZip protein homologous to AP-1 and binds to AP-1–like sites (Z-responsive elements, ZREs) that often contain CpG motifs (11, 13–15). Once established, EBV genome methylation is maintained during latent viral genome replication (licensed and mediated by host cell replication machinery) via the enzymatic activity of DNA methyltransferases (1, 2). However, the virally encoded replication machinery mediating the lytic form of viral DNA replication does not preserve viral genome methylation (9, 11), and therefore, packaged EBV genomes are always unmethylated.

EBV infection of normal differentiated epithelial cells is completely lytic, and the viral genome does not become methylated in these cells (1–5, 7, 9, 10). Lytic viral gene expression following EBV infection of normal epithelial cells likely reflects the ability of the other EBV immediate-early (IE) protein, BRLF1 (R), to efficiently activate unmethylated lytic viral promoters. We recently showed that overexpression of R, but not Z, induces lytic EBV gene expression and replication in a latently infected telomerase-immortalized normal oral keratinocyte (NOKs) line, where the lytic viral promoters remain largely unmethylated (16, 17). R activates lytic gene expression by binding to R-response elements (RREs) with a consensus sequence of GNCCN$_9$GGNG (N$_9$ is a nine-nucleotide spacer region that can be any sequence) (18) or indirectly by interacting with cellular transcription factors (13). Z and R activate one another's promoters, and once both IE proteins are expressed, they cooperate to induce fully lytic infection regardless of whether the viral genome is methylated or unmethylated (13, 16).

Given the profound effect that cytosine methylation plays on the ability of Z versus R to activate lytic EBV gene expression, we have now explored whether another more recently described

Significance

Epstein–Barr virus (EBV) normally establishes a lytic infection in differentiated epithelial cells. However, in the abnormal context of nasopharyngeal carcinoma (NPC), EBV latently infects undifferentiated epithelial cells. Here we demonstrate that the EBV genome can become 5-hydroxymethylated and that this DNA modification affects EBV lytic reactivation. We find that 5-hydroxymethylcytosine accumulates during differentiation of normal epithelial cells but not in EBV+ NPCs. Furthermore, we show that ten–eleven translocation (TET) enzymes dysregulate lytic viral reactivation by altering the 5-methylcytosine and 5-hydroxymethylcytosine state of lytic promoters. These data suggest that loss of TET activity may promote cellular hypermethylation and alter EBV gene regulation in NPC tumors.

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cytosine modification, 5-hydroxymethylcytosine (5hmC), also affects the ability of Z and/or R to regulate lytic EBV gene promoters. The 5hmC modification occurs as an intermediate during active demethylation of cytosines in vivo, especially during early zygote development (reviewed in ref. 19). Removal of CpG methylation begins when one of the three ten–eleven translocation (TET) enzymes (TET1, TET2, or TET3) hydroxylates 5-methylcytosine (5mC), producing 5hmC (20, 21). 5hmC can then be lost passively through multiple rounds of DNA replication, as the mark is not recognized by DNMT1 (19). Alternatively, 5hmC and further oxidized forms of this modification can be actively removed through multiple pathways, often involving base excision repair (reviewed in refs. 19, 22). Although 5hmC is rare in most cells, it is relatively abundant in certain cell types such as Purkinje neurons, embryonic stem cells, and others (19, 22). Additionally, 5hmC accumulates during differentiation of many cell types and is extremely low in undifferentiated cancer cells (23, 24). When 5hmC is maintained on the cellular genome, it is usually associated with gene activation (19, 25).

Global reduction of 5-hydroxymethylation via various mechanisms is commonly found in myeloid cancers, glioblastoma, and melanoma, and recently 9% of NPC tumors were found to have a mutation in TET1, TET2, or TET3 (22, 26–29). In addition to mutations in the TET family members, mutations that disrupt α-ketoglutarate production, which is required for the activity of all TET enzymes, can also decrease 5hmC accumulation. Such mutations are found in over 70% of secondary glioblastomas and are common in acute myeloid leukemia (22, 26). In particular, the isocitrate dehydrogenase (IDH) 1 and 2 enzymes, which normally convert isocitrate into α-ketoglutarate, are mutated to forms [including IDH1(R132H) and IDH2(R172K)] that instead convert isocitrate into the oncometabolite 2-hydroxyglutarate, which inhibits the function of all three TET enzymes (29).

Here we show that TET-mediated 5hmC modification of lytic EBV promoters inhibits Z binding and activation of these promoters, while promoting R activation. Furthermore, we identify a CpG-containing Z-binding site in the R promoter that is 5hmC-modified in EBV-infected NOKs and demonstrate that inhibition of endogenous TET activity converts the 5hmC mark into a 5mC mark and restores the ability of Z to induce lytic EBV reactivation in this cell line. Finally, using 5hmC-specific immunohistochemistry (IHC), we confirm that global 5hmC-modified DNA is very low or undetectable in EBV+ NPCs but accumulates during differentiation of normal tonsil epithelium. These results reveal that TET-mediated 5hmC modification of lytic EBV promoters regulates lytic viral reactivation and suggest that decreased 5hmC modification of both cellular and viral genes may contribute to NPC tumors.

Results

5hmC-Modified DNA Accumulates in Differentiated Normal Tonsil Epithelium and in Both Differentiated and Undifferentiated NOKs but Not in NPC Tumors. Given the finding that the methylation status of the EBV genome determines which viral-immediate early protein can induce lytic reactivation, we examined the level of global 5hmC-modified DNA using IHC. 5hmC-modifed DNA was not detectable in undifferentiated normal tonsillar epithelium but was easily detected in the more differentiated layers of the tonsillar epithelium (Fig. 1*A*). In contrast, 5hmC-modified DNA was either very low or not detected in nine undifferentiated NPC tumor specimens examined (Fig. 1*B* and Fig. S1), although staining was visible in the normal surrounding cells. Interestingly, NOKs (telomerase-immortalized NOKs), which support an unusually low level of lytic EBV promoter methylation in comparison with other EBV-infected epithelial cell lines (16, 17), had easily detectable 5hmC-modified DNA, even in the less differentiated basal cells (Fig. 1*C*). These results suggest that high TET activity may explain the low level of lytic EBV promoter methylation in stably EBV-infected NOKs. Furthermore, because global 5hmC-modified cellular DNA is strongly increased by differentiation of normal tonsillar epithelial cells, the EBV genome may likewise be more likely to become 5hmC-modified in differentiated (versus undifferentiated) normal epithelial cells. Conversely, the low level of global 5hmC-modified DNA in NPC tumor specimens, consistent with the undifferentiated state of these tumors as well as the possible presence of TET gene mutations, suggests that 5hmC modification of the EBV genome would be unlikely to occur in these tumors.

Z Binding to ZREs in Vitro Is Inhibited by 5hmC, but R Binding to RREs Is Unaffected. Because cytosine methylation greatly enhances the ability of Z to bind to certain CpG-containing ZREs, we next examined how 5hmC modification of two different CpG-containing ZREs affects Z binding in vitro. As shown in Fig. 2, oligonucleotide probes containing ZRE motifs from either the ZRE2 site in the EBV R promoter or the ZRE1 site in the EBV BRRFl (Na) promoter were commercially synthesized to contain

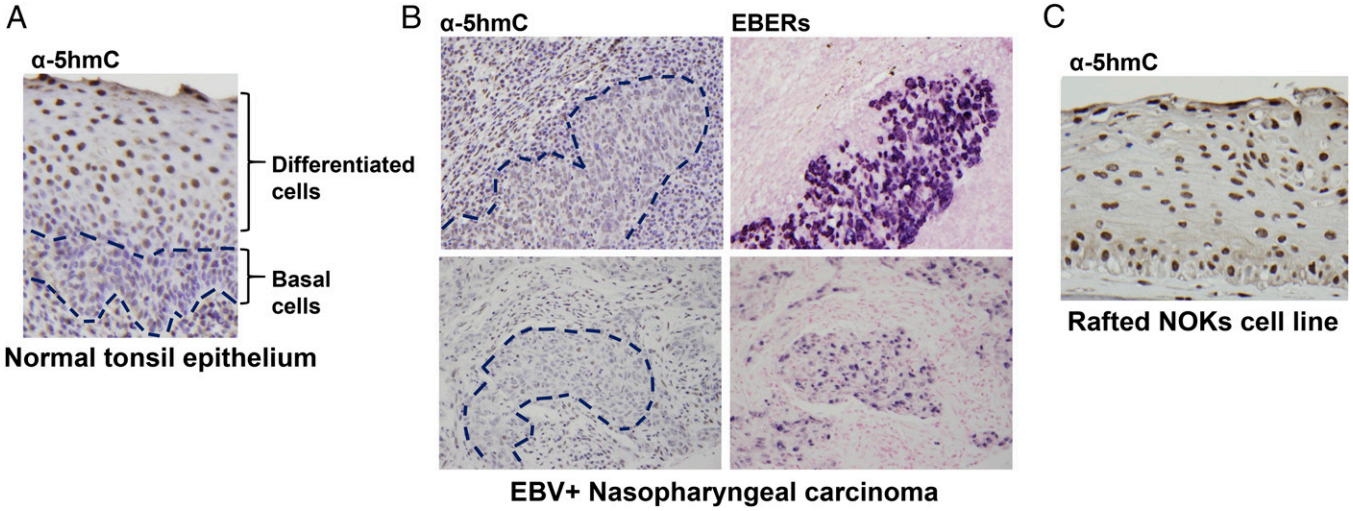

Fig. 1. 5hmC accumulates in differentiated tonsil epithelium but not in NPCs. (*A*) 5hmC level was assessed by IHC in normal tonsil epithelium; the undifferentiated basal cell layer is outlined. (*B*) 5hmC IHC (*Left*) and EBER in situ hybridization (*Right*) staining of two representative EBV+ NPCs. EBV-positive cells are outlined on *Left*. (*C*) 5hmC staining of telomerase-immortalized NOKs cells differentiated in air-interface cultures. Original magnification of all figures, 40×.

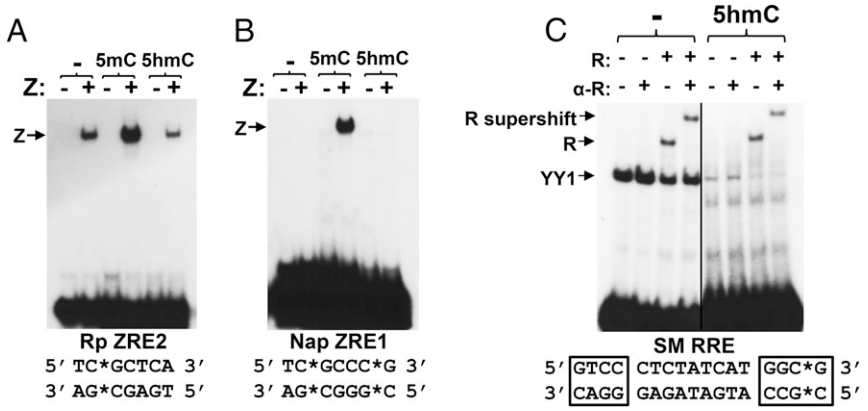

Fig. 2. 5hmC modification of CpG-containing binding motifs inhibits Z but not R binding in vitro. Z binding to unmodified, 5mC-modified, or 5hmC-modified CpG-containing ZREs (*A* and *B*) or R binding to an unmodified or 5hmC-modified RRE (*C*) was examined using EMSA as described in *Materials and Methods*. Z–DNA and R–DNA complexes are indicated with arrows, and binding of the cellular protein YY1 to the RRE is also denoted. ZRE and RRE sequences are shown below each EMSA, with the modified cytosines indicated by asterisks and nucleotides where R directly contacts DNA boxed. R-binding studies were performed with or without a supershifting anti-R antibody as indicated. (*A*) ZRE2 site from the BRLF1 (R) promoter. (*B*) ZRE1 site from the BRRF1 (Na) promoter. (*C*) RRE motif from the SM promoter.

an unmodified cytosine, 5mC, or 5hmC at the nucleotide positions indicated by an asterisk. Binding was assayed using reticulocyte lysate-synthesized Z protein, compared with lysate as a control. As previously described (30), Z bound to the unmethylated form of the R promoter (Rp) ZRE2 motif, although binding was clearly increased by methylation (Fig. 2*A*). When the methylation mark on the Rp ZRE2 motif was converted to a 5hmC mark, Z binding was reduced to a level similar to that of the unmethylated probe. In the case of the Na promoter ZRE1 motif, which we previously showed is almost totally methylation-dependent in regard to Z binding (31), conversion of the methylated cytosine into a 5hmC-modified cytosine abrogated Z binding. These data suggest that Z "reads" 5hmC-modified ZREs similarly as unmodified ZREs and indicate that TET-mediated 5hmC modification of methylated ZREs in vivo, should it occur, would reverse the ability of Z to bind to these promoters.

We next explored how 5hmC modification affects R DNA binding, as many R-binding sites also contain CpG motifs. We previously showed that CpG cytosine methylation does not affect the ability of R to bind to lytic EBV promoters in vitro or in vivo, although methylation of lytic promoters decreases R-mediated transcriptional activation (16). A probe encoding an SM promoter R-binding motif [RRE sequence shown below electrophoretic mobility shift assay (EMSA) image] was commercially synthesized to contain 5hmC or unmodified cytosine (denoted with an asterisk) within one of the core regions (boxed) required for R binding to DNA (18). As demonstrated in Fig. 2*C*, R bound similarly to both the unmodified and 5-hydroxymethylated probes (indicated with arrows), although binding of cellular protein YY1 to the motif (18) was decreased by 5hmC modification. These results suggest that R binds equally well to unmodified and 5-hydroxymethylated CpG-containing binding motifs, even when they are located in the core part of the motif where R makes direct contact with DNA. These results, along with our previous findings (16), suggest that neither 5mC nor 5hmC modification of R-binding motifs affects R binding.

TET2 Reduces Z- but Not R-Mediated Activation of Methylated CpG-Containing Lytic EBV Promoters. Next we performed luciferase assays to determine if cotransfection with a highly active TET2 expression vector (containing the catalytic domain of mouse *Tet2*) inhibits the ability of Z to activate lytic EBV promoters with methylated CpG-containing ZREs. TET2 converts methylated CpGs (but not unmodified CpGs) into 5hmC-modified CpGs (20, 21). Three different lytic EBV promoters [BRLF1 (R), BRRF1 (Na), and BHLF1] were cloned upstream of the luciferase gene in the pCpGL-basic vector, which lacks CpG motifs in the vector backbone (32). The promoter vectors were methylated or mock-treated in vitro using CpG methyltransferase (M.SssI) and transfected with and without the TET2 vector into EBV-negative HONE.1 cells. As we and others previously reported (16, 30, 31, 33), methylation greatly enhances Z activation of the R and Na promoters, both of which contain ZREs with CpGs (Fig. 3*A*). Of note, consistent with the finding that Z does not bind well to 5hmC-modifed ZREs (Fig. 2), cotransfected TET2 inhibited Z activation of the methylated R and Na promoters (Fig. 3*A*), presumably by converting the methylated CpG motifs into 5hmC-modified CpG motifs. Consistent with this interpretation, TET2 did not decrease (and in fact enhanced) Z activation of the methylated BHLF1 promoter construct, in which all promoter ZREs are CpG-free and hence cannot be 5mC- or 5hmC-modified. We have previously shown that the BHLF1 promoter is exceptional in that methylation of the promoter at CpG sites outside of ZREs inhibits Z-mediated transactivation (16, 33). Immunoblot analysis confirmed that similar levels of Z were expressed in the presence and absence of TET2 (Fig. S2).

We likewise examined the effect of cotransfected TET2 on the ability of R to activate three different lytic promoters (BALF2, SM, and BHLF1) that have CpG-containing R-binding motifs. We previously showed that although R binds equally well to the unmethylated and methylated forms of these promoters, CpG methylation greatly reduces the ability of R to activate these promoters (16). As previously reported, we found that methylation substantially inhibited R activation of each promoter (Fig. 3*B*). Importantly, cotransfected TET2 restored the ability of R to activate methylated lytic promoters (Fig. 3*B*). Immunoblot analysis confirmed that the level of R expression was similar in the presence and absence of TET2 (Fig. S2).

Z Binding to the Endogenous Viral Genome Is Inhibited by TET-Mediated 5-Hydroxymethylation. To determine if 5-hydroxymethylation of the intact EBV genome affects the ability of Z to bind to lytic promoters in vivo, we transfected latently infected cells with the highly active TET2 expression vector, which was previously shown to greatly increase the global level of 5hmC-modified cellular DNA when transfected into cells (21). Using DNA immunoprecipitation (DIP) assays, we confirmed that the transfected TET2 vector greatly increases the amount of 5hmC-modified and greatly decreases the amount of 5mC-modified EBV BRLF1 (R) promoter DNA

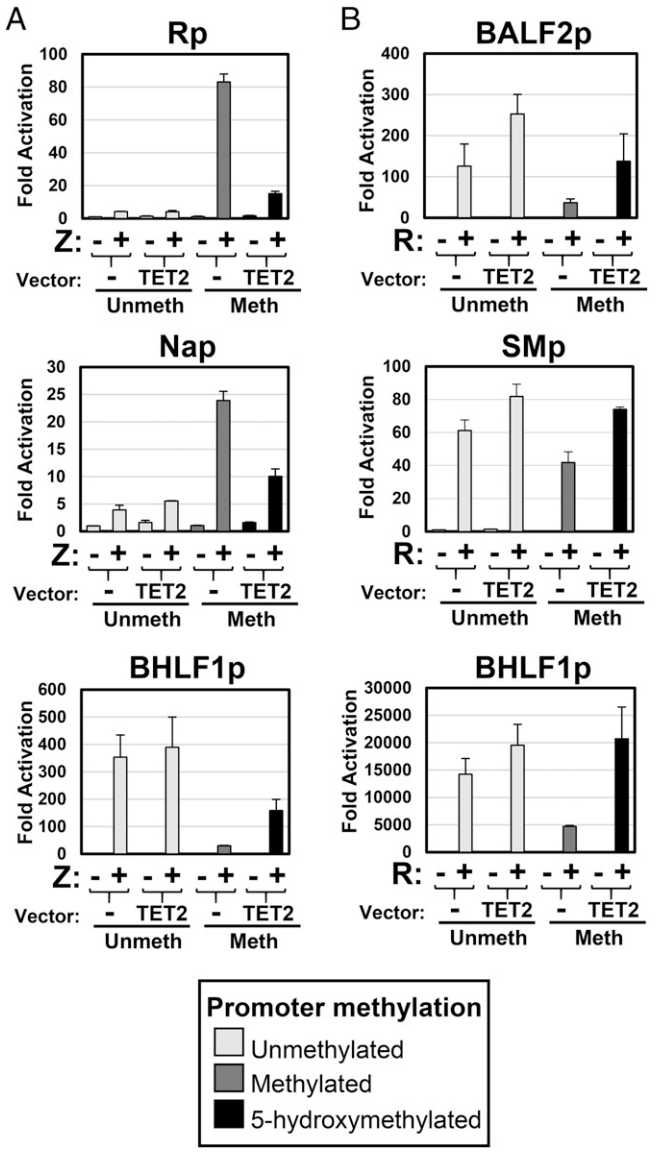

Fig. 3. TET2 differentially affects Z versus R transactivation of lytic EBV promoters. The ability of Z and R to induce expression of unmethylated, methylated, and 5-hydroxymethylated lytic viral promoters was assessed by luciferase assays. EBV promoter-luciferase constructs (containing various lytic EBV promoters inserted upstream of the luciferase gene in a CpG-free vector) were methylated or mock-treated in vitro using M.SssI as indicated and transfected into EBV-negative HONE.1 cells with either vector controls, a Z expression vector (with or without a TET2 expression vector) (*A*), or an R expression vector (with or without a TET2 expression vector) (*B*). Luciferase assays were performed 2 d posttransfection; the fold luciferase activation is shown relative to the activity of the unmethylated promoter transfected with control vectors (set at 1). The error bars indicate +1 SD calculated from three replicate experiments.

in latently infected EBV-293T cells (Fig. 4*A*). We next examined the effect of cotransfected TET2 on the ability of Z to bind to various different lytic EBV promoters in a 293 cell line stably infected with an R-deleted EBV mutant; this line was chosen so that Z binding to lytic EBV promoters could be examined in the absence of any TET2 effect on R function. Importantly, in vivo ChIP assays demonstrated that cotransfected TET2 greatly decreased Z binding to three different lytic EBV promoters [BRLF1 (Rp), BALF2, and BMRF1] with CpG-containing ZREs that are preferentially bound by Z in the methylated form but did not substantially affect Z binding to an EBV promoter (BHLF1) that has only CpG-free ZREs (Fig. 4*B*) (16, 33). Consistent with the EMSA result shown in Fig. 2*C*, cotransfected TET2 had little if any effect on R binding to five different lytic viral promoters on the endogenous EBV genome in 293 cells infected with a Z-deleted EBV mutant (Fig. 4*C*). Immunoblot analysis confirmed that similar levels of Z or R were expressed in the presence and absence of TET2 (Fig. S3).

TET2 Differentially Affects Z- Versus R-Induced Lytic Reactivation in EBV-Infected Cell Lines. To determine if TET2 expression alters the ability of the Z or R proteins to activate early lytic viral protein expression in latently infected cell lines, we transfected cell lines with either Z or R expression vectors, in the presence or absence of cotransfected TET2, and performed immunoblots to examine the level of various lytic viral proteins. Consistent with the ability of TET2 to decrease Z binding to lytic viral promoters (Fig. 4*B*), TET2 inhibited the ability of Z to induce lytic viral protein expression in EBV-infected 293 cells, C666.1 cells, and HONE-Akata cells (Fig. 5 *A*–*D*). In contrast, cotransfected TET2 enhanced the ability of R to induce lytic viral protein expression from the endogenous viral genome in the same three cell lines (Fig. 5 *B*–*D*). Cotransfected TET2 also differentially affected the ability of Z versus R to induce virion release in HONE-Akata cells (Fig. 5*D*). These results are similar to those observed in the reporter gene assays (Fig. 3) and suggest that 5hmC modification of lytic EBV promoters, like CpG methylation, has different effects on the ability of Z versus R to activate lytic EBV gene expression.

Inhibition of Endogenous TET Activity via Expression of the Mutant IDH1(R132H) Protein Allows Z to Initiate Lytic Reactivation in EBV-Infected NOKs. To date, the only EBV-infected cell line known to be lytically reactivated by overexpression of R, but not Z, is the NOKs line (16). We and others previously showed that a number of lytic viral promoters, particularly the BRLF1 (Rp) promoter, have relatively low methylation levels in EBV-positive NOKs compared with other EBV-infected cell lines (16, 17). This suggests that the inability of Z to activate lytic EBV promoters (particularly the BRLF1 promoter) in NOKs may be due to inadequate methylation of CpG-containing ZREs. Given our finding that NOKs have a high level of global 5hmC-modified DNA (Fig. 1*C*), we hypothesized that TET enzymes may inhibit Z binding to the EBV genome in this cell type by converting 5mC-modified cytosines in the viral genome into 5hmC-modified cytosines, which might then be converted into unmodified cytosines following cellular replication.

To determine if constitutive TET activity contributes to the low level of lytic EBV promoter methylation in EBV-infected NOKs (which express both TET2 and TET1), we infected EBV-positive NOKs with a retroviral vector expressing a mutant form of IDH1 [IDH1(R132H)], which inhibits the activity of all three cellular TET enzymes, or a control retroviral vector and selected the cells in puromycin for at least 1 mo. A DNA dot blot, using antibodies that specifically recognize only 5hmC-modified DNA, confirmed that by 1 mo after infection with the IDH1 mutant protein, the level of 5hmC-modified DNA was clearly decreased (Fig. 6*A*).

To determine if loss of TET activity results in enhanced methylation of the EBV Rp, we performed a DIP assay to compare the levels of 5mC at various promoters in the control vector versus IDH1(R132H)-expressing lines. These assays revealed that the EBV R promoter had increased methylation when TET activity was inhibited (Fig. 6*B*). In contrast, the methylation status of the EBV Cp promoter (a viral latency promoter that drives EBNA gene expression in B cells but is not used in EBV-infected NOKs) (17) was not affected, suggesting that this promoter is not a major target for TET-mediated demethylation in this cell line. The cellular UBE2B promoter, which contains an unmethylated CpG

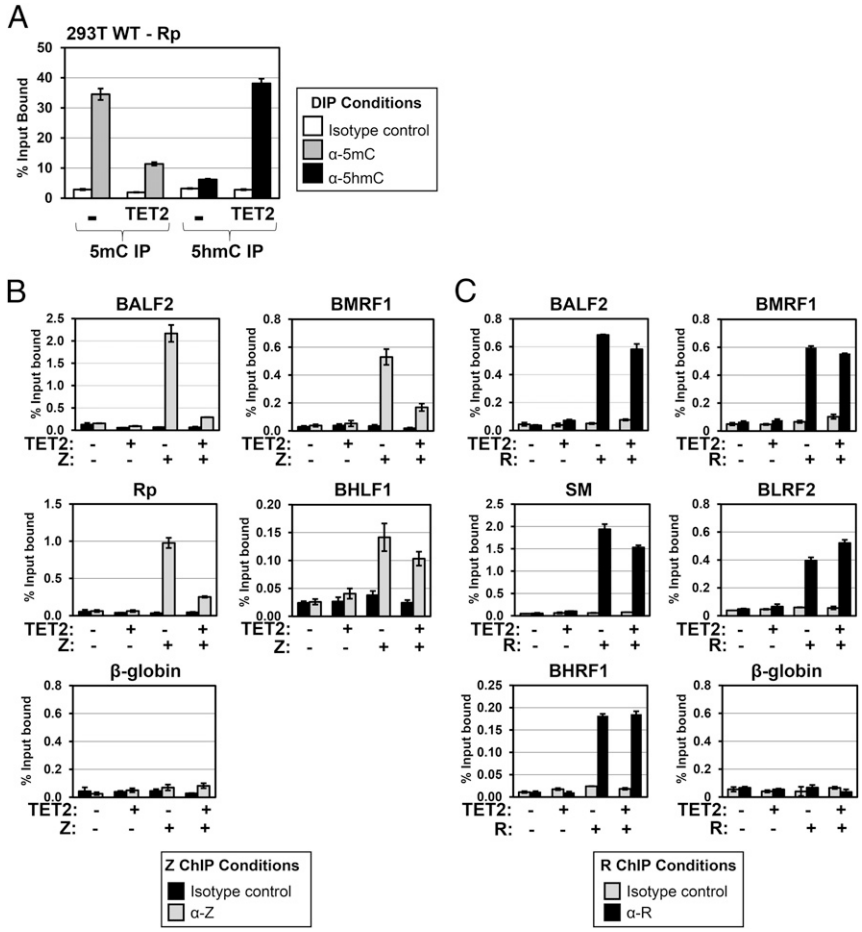

Fig. 4. Z binding to lytic promoters in vivo is inhibited by 5hmC modication of the EBV genome, whereas R binding is unaffected. (*A*) DIP assays were performed in EBV-infected 293T cells transfected with pcDNA control vector (–) or a TET2 catalytic domain expression vector, using IgG isotype control ab (white bars), anti-5mC ab (gray bars, *Left*), or anti-5hmC ab (black bars, *Right*), and then quantitative PCR (qPCR) amplification was done using primers spanning the EBV R promoter (as described in *Materials and Methods* and listed in Table S1) to determine the effect of TET2 on the amount of 5mC-modified versus 5hmC-modified Rp. The percent immunoprecipitated DNA (compared with input DNA) is shown for each condition. (*B*) The 293 cells infected with an R-deleted EBV mutant were transfected with control vector, Z alone, TET2 alone, or Z + TET2 as indicated, and ChIP assays were performed using mouse IgG isotype control (black bars) or anti-Z ab (gray bars). (*C*) The 293 cells infected with a Z-deleted EBV mutant were transfected with control vector, R alone, TET2 alone, or R + TET2 as indicated, and ChIP assays were performed using rabbit IgG isotype control (gray bars) or anti-R ab (black bars). (*B* and *C*) The percent input DNA bound by Z and R was determined with qPCR using primers spanning various EBV promoters as well as the negative control β-globin cellular promoter. (*A*–*C*) The error bars indicate +1 SD calculated from three replicate experiments.

island in many cell types, served as a negative control for methylation and as expected was not significantly methylated in the vector control cells, although its methylation status was increased by expression of the IDH1(R132H) mutant. Thus, the UBE2B promoter may also be a target for TET activity in NOKs, consistent with a previous report showing 5hmC on the UBE2B promoter in human embryonic stem cells (34).

To examine whether inhibition of TET activity restores the ability of Z to induce lytic EBV gene expression in NOKs, cells were transfected with a Z expression vector or a control vector, and the level of R expression induced by Z was examined by immunoblot. As previously reported by our group, transfected Z did not activate R expression in EBV-positive NOKs infected with the control retroviral vector (Fig. 6*C*). However, transfected Z activated R expression in NOKs when TET activity was inhibited for over 1 mo using the mutant IDH1(R132H) protein (Fig. 6*C*). In addition, inhibition of TET function restored the ability of transfected Z to produce infectious virions in NOKs (Fig. 6*D*). Similar results were obtained in three separate independently generated lines. Together, these results suggest that TET activity plays an important role in preventing methylation of the lytic EBV R promoter in EBV-infected NOKs and thereby inhibits Z-mediated lytic viral reactivation. Interestingly, inhibition of TET2 function in EBV-infected NOKs also increased the amount of constitutive virion production as well as the amount of virions produced following R transfection (Fig. 6*D*). This latter result can be explained by the fact that the EBV genomes were still largely unmethylated after 1 mo of TET inhibition (thus still providing a viral template for R-initiated lytic expression), whereas R-induced Z protein was now able to induce lytic gene expression from the newly methylated EBV genomes.

TET Activity Enhances 5hmC Modification and Decreases 5mC Modification at a Specific ZRE CpG Motif in the EBV R Promoter. To determine if TET2 globally modifies 5hmC and 5mC levels on CpG-containing ZREs in Rp or only affects specific CpG motifs, CpG methylation was quantified using bisulfite treatment of DNA followed by pyrosequencing (Fig. 7*A*). To distinguish between 5mC- versus 5hmC-modified sites, the DNA was pretreated with an oxidizing agent, $KRuO_4$ (Oxi), or mock-treated (Mock), as oxidation of 5hmC, but not 5mC, results in conversion of the base into uracil such that 5-hydroxymethylated sites are read like unmodified cytosines after this treatment (35). Therefore, comparison of the

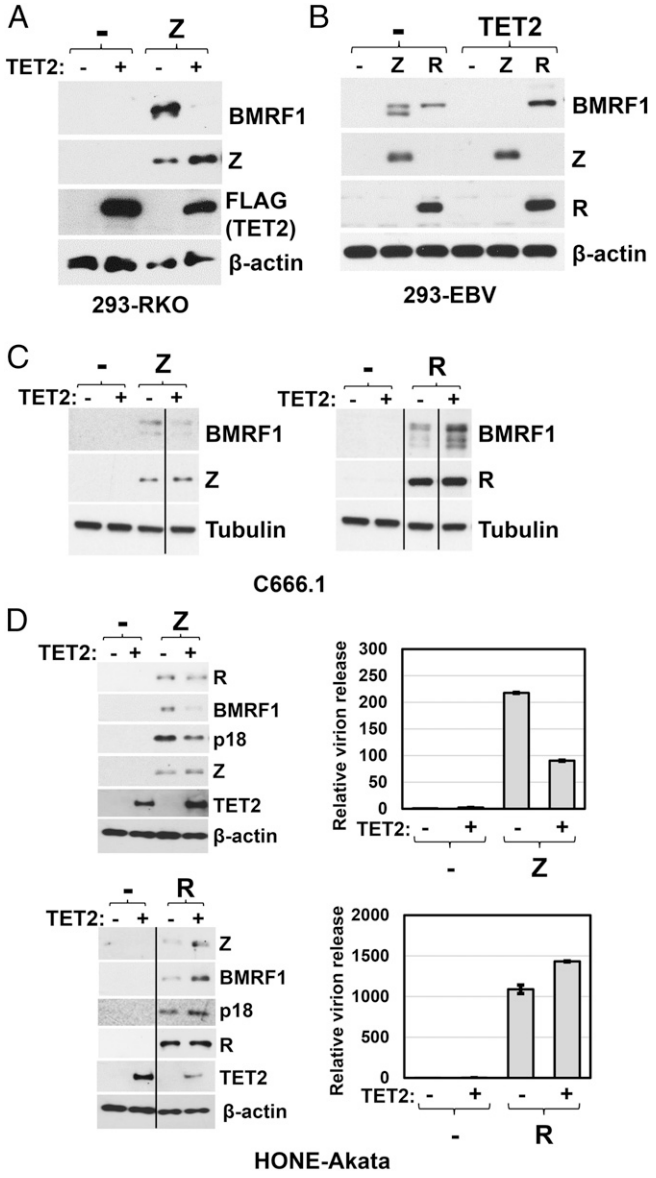

Fig. 5. 5hmC modification of the EBV genome inhibits Z-mediated but not R-mediated lytic reactivation. The effect of TET2 overexpression on Z- and R-induced lytic gene expression from the endogenous viral genome was analyzed using immunoblots. EBV+ (*A*) 293-RKO cells were transfected with control vector, Z alone, TET2 alone, or Z and TET2 together. (*B*) 293-EBV cells (B95.8 strain), (*C*) C666.1 cells, and (*D*, *Left*) HONE-Akata cells were transfected with Z or R expression vectors in the presence or absence of cotransfected TET2. Immunoblot analysis was performed 2 d posttransfection to compare the levels of Z- or R-induced EBV lytic expression of the following proteins: BMRF1 (*A*–*D*), R (*D*), Z (*D*), and late viral protein p18 (*D*), as well as transfected Z or R and TET2 (*A*–*D*). β-actin or tubulin served as a loading control. (*D*, *Right*) HONE-Akata cells were transfected with Z or R expression vectors in the presence and absence of cotransfected TET2. Media was harvested 3 d posttransfection, and released EBV virions were quantified by qPCR as described in *Materials and Methods*. The error bars indicate SE calculated from three replicate experiments.

reference DNA sequence to the mock and oxidized samples reveals which sites are methylated versus 5-hydroxymethylated.

We previously showed that the Rp contains two different CpG-containing ZREs (ZRE2 and ZRE3), both of which are required for efficient Z activation of the methylated form of the promoter (30). Pyrosequencing results revealed that methylation of the single CpG site within the Rp ZRE2 motif was increased approximately twofold in the presence of the mutant IDH1(R132H) protein; however, 5hmC modification of this site could not be detected, suggesting that this modification is very transient at this site, and presumably rapidly removed by cellular DNA replication and/or via the base excision repair pathway (19, 22) (Fig. 7*A*). Nevertheless, the increased methylation at this site that occurs following inhibition of TET activity suggests that the 5hmC pathway plays a role in preventing 5mC modification at this site.

Interestingly, in the case of the Rp ZRE3 site (TCG**CG**A), which contains two adjacent CpG motifs, low-level constitutive 5hmC modification was detectable on the second CpG motif (shown in bold), but not the first, in EBV-infected NOKs, and this modification was lost when TET activity was inhibited by the IDH1 mutant protein. Furthermore, loss of TET function increased the level of 5mC modification at the second CpG (which can be 5hmC-modified), but not significantly affecting the amount of 5mC modification at the adjacent CpG motif (Fig. 7*A*). Of note, in silico modeling of Z binding to the Rp ZRE3 motif predicts that Z interacts directly with the second (potentially 5hmC-modified) CpG motif but not the adjacent first CpG motif (31). To confirm that methylation of the second CpG motif in the Rp ZRE3 site is specifically required for Z binding, we performed EMSA assays using oligonucleotide probes containing different combinations of methylated and unmethylated CpG motifs in the Rp ZRE3 sequence (Fig. 7*B*). These results confirmed that methylation of the second (bolded) CpG motif in RpZRE3 (TCG**CG**A), but not the first CpG motif, is specifically required for Z binding to this motif. In addition, 5hmC modification of the Rp ZRE3 probe abrogated Z binding. Together, these results suggest that TET-mediated 5hmC modification of the second (but not first) CpG motif in the Rp ZRE3 site inhibits Z binding to this motif and prevents Z-mediated activation of R expression. These results also show that the effect of TET loss on the EBV genome CpG methylation state is extremely context-dependent.

Discussion

The undifferentiated form of NPC is almost universally associated with EBV infection, and a recently published genomic landscape of NPC demonstrated that mutations in the cellular TET1, TET2, or TET3 genes occur in 9% of NPC tumors (28). TET enzymes convert methylated cytosines into 5-hydroxymethylated cytosines, which can then lead to cytosine demethylation following DNA replication. Here we show that 5hmC modification of the EBV genome differentially affects the ability of the two EBV IE proteins to activate lytic gene expression and demonstrate that endogenous TET activity regulates EBV lytic reactivation in EBV-infected NOKs. In addition, we find that differentiation of normal epithelial cells leads to increased global 5hmC. These results are the first, to our knowledge, to show that constitutive 5hmC modification of a viral genome substantially alters viral gene regulation and furthermore suggest that loss of 5hmC in NPC tumors (via TET gene mutations or other mechanisms) not only affects cellular gene expression but also alters EBV gene regulation.

We previously showed that although cytosine methylation is commonly required for the ability of Z to activate lytic EBV promoters, it has the opposite effect on the ability of R to activate the same promoters (16). As a "pioneer" factor, once Z is bound to DNA, it can activate promoters even in the presence of inhibitory chromatin modifications normally associated with 5mC modification (36). In the case of R, CpG methylation does not affect R binding to RREs on lytic promoters but decreases R-mediated acetylation of histone 3 lysine 9 (16). Therefore, R, but not Z, appears to require open chromatin to induce lytic gene expression.

Here we show that 5hmC modification also produces different effects on Z- versus R-mediated activation of lytic EBV promoters. Although 5hmC modification of CpG-containing Z-binding motifs prevents efficient Z binding to, and activation of, lytic EBV promoters, the 5hmC modification does not inhibit R binding to RREs

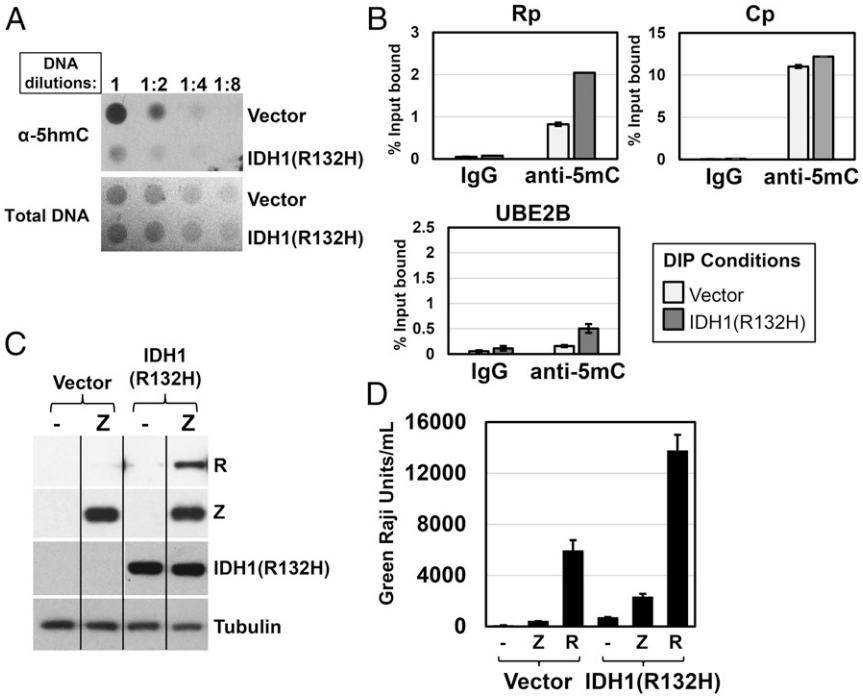

Fig. 6. Inhibition of TET activity enhances methylation of the EBV R promoter and restores Z-mediated activation of R expression in EBV-infected NOKs. EBV-positive NOKs were infected with control or IDH1(R132H)-expressing retrovirus vectors and selected with puromycin for 1 mo. (*A*) A dot blot was performed to measure global levels of 5hmC-modified DNA in control versus IDH1(R132H)-expressing cells as described in *Materials and Methods*. (*B*) DIP assays were performed on DNA isolated from EBV-positive NOKs infected with retrovirus control vector (white bars) or the IDH1(R132H)-expressing retrovirus (gray bars) using mouse IgG isotype control or anti-5mC antibody, and then qPCR was performed using primers spanning the EBV R or C promoters, or the cellular UBE2B promoter. The error bars indicate +1 SD calculated from three replicate experiments. (*C*) EBV+ NOKs infected with control retrovirus or IDH1(R132H)-expressing retrovirus were transfected with a Z expression vector (or vector control), and immunoblot analysis was performed to examine the level of transfected Z, induced R expression (from the latent EBV genome), mutant IDH1(R132H) expression, and tubulin (loading control). (*D*) EBV+ NOKs infected with control retrovirus or IDH1(R132H)-expressing retrovirus were transfected with vector control, a Z expression vector, or an R expression vector. Media was harvested 3 d posttransfection, and production of infectious virions was quantified using a green Raji assay as described in *Materials and Methods*. The error bars indicate SE calculated from three replicate experiments.

and in fact enhances R-mediated activation of promoters. 5hmC modification may increase R-mediated activation of methylated lytic promoters both by removing the inhibitory cytosine methylation mark and by inducing an open chromatin conformation. In glioblastoma cells, 5hmC recruits a complex that methylates arginine 3 of histone 4, thus activating the expression of genes involved in glioblastomagenesis (37). Another recent report by Mendonca et al. showed that 5hmC-modified DNA may convert chromatin to a more open and active state by weakening the DNA–H2A–H2B dimer interaction (38).

EBV-infected NOKs are so far unique among stably EBV-infected cell lines with regard to their dependence upon R, but not Z, expression to convert to a lytic form of infection, and our results here suggest that this may reflect the unusually high level of constitutive 5hmC modification and TET activity in this cell line. This phenotype may reflect the relatively "normal" state of the NOKs line, which can differentiate in air–interface cultures (Fig. 1) and in response to calcium/serum. We previously suggested that insufficient methylation of the R promoter in NOKs inhibits Z-mediated EBV reactivation (16, 17). Both Z and R transcriptional function are required to activate many lytic EBV promoters, and thus activation of R expression is the essential first step in Z-mediated lytic reactivation. We demonstrate here that global inhibition of TET activity in NOKs [using the IDH1(R132H) mutant] reverses low-level constitutive 5hmC modification of a specific CpG motif (CpG2) within an essential Z-binding motif (ZRE3) in the R promoter (Fig. 7). Furthermore, we show that this specific CpG motif switches from having no detectable methylation to having detectable methylation (5%) when TET activity is inhibited (Fig. 7). Additionally, we can detect the "footprint" of transient 5hmC modification at the Rp ZRE2 site, as inhibition of TET activity also results in a twofold gain of 5mC at this site, although 5hmC was not detected at this site by pyrosequencing. Even though the level of Rp methylation at the ZRE3 CpG2 motif remains relatively low (5%) following 1 mo of TET inhibition, the fact that EBV-infected cells generally have many copies of the genome per cell, combined with an absolute necessity for ZRE3 CpG2 methylation for Z binding (Fig. 7), presumably allows even this low level of methylation to substantially increase Z-mediated viral reactivation in NOKs, where this site normally has no detectable methylation.

At this point, it is not clear how TET proteins direct 5hmC to a specific CpG within the Rp ZRE3 motif, while sparing the adjacent CpG motif. Increasing evidence suggests that cellular transcription factors can interact with TET proteins and tether them to promoters (reviewed in ref. 22), and we suspect this is likewise the case for the EBV Rp, although the exact transcription factor playing this role remains unknown. Importantly, we show that the ZRE3 CpG2 motif targeted by 5hmC must be methylated for Z to bind to ZRE3, whereas methylation of the adjacent CpG1 motif (not modified by 5hmC) is not required.

Our results here suggest the following model in regard to how loss of TET activity in EBV-infected epithelial cells, or the presence of other modifications that decrease the amount of global 5hmC, might promote NPC. First, we predict that absence of TET activity in EBV-infected normal differentiated epithelial cells initially enhances EBV latency by increasing 5mC modification of lytic viral promoters. In normal differentiated epithelial cells, R, rather than

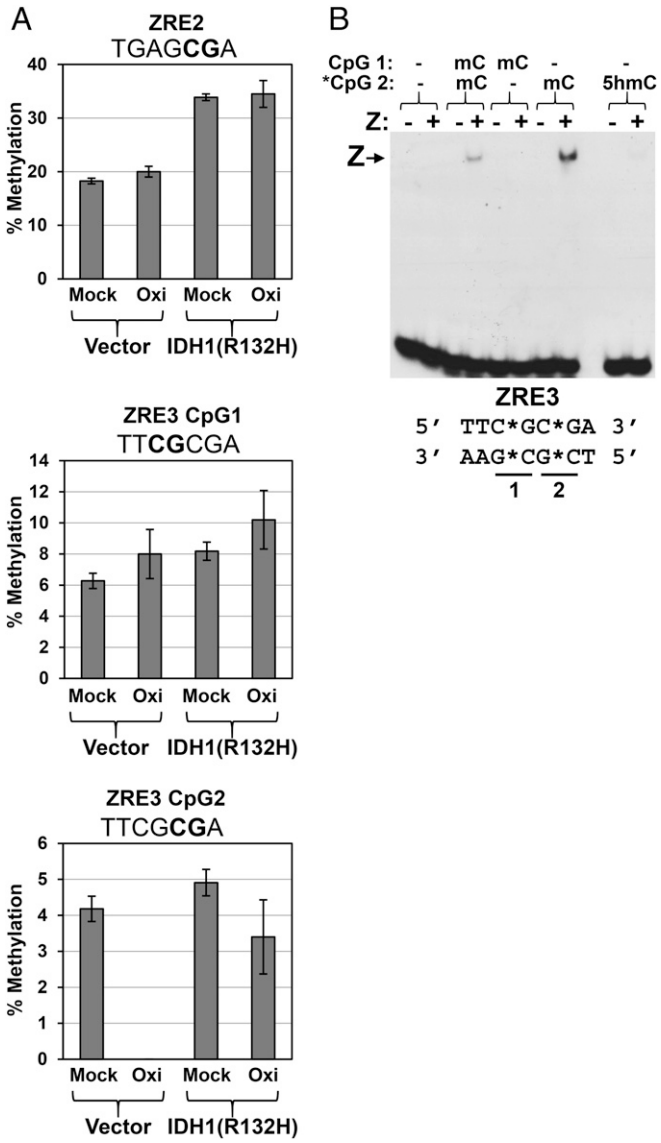

Fig. 7. The R promoter ZRE3 site is 5-hydroxymethylated in EBV-infected NOKs via a TET-dependent mechanism. (*A*) DNA was isolated from EBV-positive NOKs infected with either the retrovirus control vector or the IDH1(R132H)-expressing retrovirus, and then the DNA was either mock-treated (Mock) or oxidized with KRuO4 (Oxi), followed by bisulfite conversion and pyrosequencing as described in *Materials and Methods*. The sequences of each ZRE motif are shown above the respective graph, with the CpG sites bolded; the two adjacent CpGs in ZRE3 are numbered 1 and 2. The error bars indicate SE calculated from two replicate experiments where each sample was pyrosequenced in triplicate. (*B*) The ability of Z to bind to the Rp ZRE3 site containing various patterns of methylation and 5-hydroxymethylation was examined using EMSA; the cytosine modification status in each lane is shown above the EMSA image. Z–DNA complexes are indicated with an arrow.

Z, is likely to drive lytic gene expression, as the incoming viral genome is unmethylated, and viral latency does not normally occur in these cells. As shown here, 5mC modification of lytic EBV promoters inhibits the ability of R to activate lytic gene expression (Figs. 3 and 5), and TET loss promotes 5mC modification of lytic EBV promoters (Figs. 6 and 7). Given our finding that normal undifferentiated tonsillar epithelial cells have very little 5hmC modification even in the absence of TET mutations (Fig. 1), TET mutations in NPC may initially occur in the differentiated epithelium, perhaps even before EBV infection. Loss of TET function would likely result in a selective growth advantage in newly EBV-infected differentiated epithelial cells not only by inducing methylation of cellular tumor suppressor genes but also by reducing R-mediated lytic EBV infection (which eventually results in cell death) and decreasing viral immunogenicity. Whether loss of TET function and/or the establishment of latent EBV infection also promotes dedifferentiation of epithelial cells is an important unanswered question.

Subsequently, as the viral genome becomes progressively more methylated in TET-deficient epithelial cells, we predict EBV acquires susceptibility to Z-mediated lytic reactivation, as methylation of lytic viral promoters greatly increases Z binding to, and activation of, lytic EBV promoters (Figs. 2–7). Furthermore, as shown here (Figs. 2 and 3), TET-induced 5hmC modification of lytic viral promoters inhibits Z binding to, and activation of, these promoters, and the 5hmC modification would no longer occur when TET activity is lost. Consistent with this part of the model, the EBV genome is highly methylated in NPC tumors, and NPC patients characteristically have unusually high IgA antibody titers to lytic EBV antigens even before the tumors become clinically apparent (39).

Finally, later on during NPC evolution, we propose that Z expression in tumors must be largely turned off to reduce the immunogenicity associated with lytic viral protein expression and to prevent virally induced lytic cell death. Consistent with this part of the model, NPC tumors commonly have only rare cells expressing lytic viral proteins, despite the high level of antibody titers to lytic viral proteins in NPC patients. Loss of Z expression in NPCs may reflect the absence of differentiation-dependent cellular transcription factors (BLIMP1 and KLF4) that synergistically activate the Z and R promoters (40). In conclusion, our results here suggest that loss of TET function in EBV-infected epithelial cells, or the presence of other mutations that globally inhibit 5hmC, may initiate a series of events that promote EBV-induced NPC not only by enhancing methylation of cellular tumor suppressor genes but also by altering 5mC and 5hmC modification of lytic EBV promoters.

Materials and Methods

IHC and EBER studies were performed with formalin-fixed, paraffin-embedded tissue sections and cells. Samples were deparaffinized, hydrated, and treated with 10 mM citrate buffer (0.05% Tween 20, pH 6.0) for 20 min in a water bath at 98 °C. To detect 5hmC, slides were treated with 2N HCl for 30 min after antigen retrieval, rinsed in distilled water, and then treated with 100 mM Tris·HCl (pH 8.5) for 10 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase solution, and nonspecific labeling was blocked in a 2.5% (vol/vol) normal horse serum blocking solution (Vector Labs). Sections were incubated with the primary antibody for 1 h at room temperature. The anti-5hmC polyclonal primary antibody (Active Motif, 39769; 1:500–1:3,000) was used. An ImmPRESS Anti-Rabbit Ig (Peroxidase) Polymer Detection Kit (Vector Labs) was used by following the manufacturer's instructions. Colors were developed with the diaminobenzidine tetrachloride substrate kit (Vector Laboratories Inc.) by following the manufacturer's instructions. EBER in situ hybridization studies were conducted using the Peptide Nucleic Acid (PNA) Probe/Fluorescein Detection Kit (DakoCytomation) according to the manufacturer's protocol as previously described (41). Human normal tonsil tissue slides (IHC World TS-H5024) and NPC panel slides (US Biomax NPC111 and NPC481) were commercially purchased. NOKs slides were prepared and sectioned as described in *SI Materials and Methods* (kindly provided by Paul Lambert and Dennis Lee, University of Wisconsin-Madison, Madison, Wisconsin).

A detailed description of all experimental methods is provided in *SI Materials and Methods*.

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