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Repression of Hox genes by LMP1 in nasopharyngeal carcinoma and modulation of glycolytic pathway genes by HoxC8

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Abstract

Epstein-Barr virus (EBV) causes human lymphoid malignancies, and the EBV product latent membrane protein 1 (LMP1) has been identified as an oncogene in epithelial carcinomas such as nasopharyngeal carcinoma (NPC). EBV can epigenetically reprogram lymphocyte specific processes and induce cell immortalization. However, the interplay between LMP1 and the NPC host cell remains largely unknown. Here, we report that LMP1 is important to establish the Hox gene expression signature in NPC cell lines and tumor biopsies. LMP1 induces repression of several Hox genes in part via stalling of RNA Pol II. Pol II stalling can be overcome by irradiation involving the epigenetic regulator TET3. Furthermore, we report that HoxC8, one of the genes silenced by LMP1, plays a role in tumor growth. Ectopic expression of HoxC8 inhibits NPC cell

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growth *in vitro* and *in vivo*, modulates glycolysis and regulates the expression of TCA-cycle related genes. We propose that viral latency products may repress via stalling key mediators that in turn modulate glycolysis.

Keywords

LMP1; Hox; RNA polymerase II; Stalling; DNA methylation; TET3; 5hmC; tricarboxylic acid; Warburg effect

Introduction

Recruitment of RNA polymerase II (Pol II) to the promoter region is regarded as one of the rate-limiting steps prior to the initiation of transcription [23]. The arrest of Pol II at promoter-proximal regions is known as Pol II stalling, and has been detected at many developmentally regulated genes, such as Hox genes [2, 9, 16, 37]. Pol II stalling is part of the signaling network that controls epigenetic modifications and the transition from silent to activate genes [23, 29].

Cytosine methylation is a key epigenetic mechanism [14, 57, 69] that regulates transcription together with other chromatin modifications[36]. One mechanism of transcriptional control by DNA methylation is provided by Pol II stalling [56, 58, 63]. Recently, members of the Ten-eleven translocation methylcytosine dioxygenase (TET) enzymes have been shown to catalyze 5-hydroxymethylcytosine (5hmC) [55, 59] and to be involved in the regulation of gene expression [20, 64].

Epstein-Barr virus (EBV) was the first human tumor virus causally associated with the development of numerous tumors, including Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma (NPC) [21, 43]. Pol II stalling at the viral C promoter (Cp) of the EBV genome promotes the transcription of immortalizing genes during EBV infection [41].

LMP1 (latent membrane protein 1) is the first oncogene of EBV viral products and is expressed in many malignancies, including NPC, which are prevalent and predominant in Southeast China [34, 43, 47, 70]. The frequency of LMP1 expression in NPC patients points to a critical role for this protein in carcinogenesis [27, 30]. Several EBV products are involved in the epigenetic dysregulation of host genes [38, 52]. Whether LMP1 plays a role in the epigenetic regulation of NPCs remains largely unknown.

Homeobox (Hox) genes play crucial roles in development and regulate numerous processes in a temporal-spatial and tissue-specific manner. 39 Hox genes are organized into four paralogous clusters, Hox-A to Hox-D. Hox genes are in part controlled by Pol II stalling in *Drosophila*[8] and mice [58]. Hox genes are strictly regulated to control growth and development [50], and aberrant expression may play a role in oncogenesis or tumor suppression, depending on the cellular context [50].

Here, we addressed the question if and how LMP1 is involved in transcriptional regulation of Hox genes in nasopharyngeal carcinoma. We report Pol II stalling of several Hox gene family members by LMP1. Furthermore, we demonstrate a role of the epigenetic modulator

TET3 in the release of Pol II stalling at Hox genes after irradiation. Lastly, we show that one of the Hox genes, HoxC8 modulates tumor growth and that ectopic expression of HoxC8 is associated with alterations of glycolytic pathway genes. Our results provide novel insights how an oncovirus controls via Hox gene regulation the expression of genes involved in energy metabolism genes.

Results

LMP1 affects Hox gene expression signature in NPC

First, we addressed the question if LMP1 affects Hox gene expression in NPC. We examined the signature of Hox gene expression in normal immortalized nasopharyngeal (NP) cells NP69, CNE1 NPC cells and CNE1 NPC cells that stably express LMP1 (CNE1-LMP1). While most Hox genes were silenced in immortalized normal nasopharyngeal cells (Figure 1A), many Hox genes were expressed in NPC cells (Supplementary Table S1). Five genes showed increased expression in the presence of LMP1, whereas 15 genes were down-regulated by LMP1, including HoxB3, HoxB13, and HoxC8 (Figure 1B and supplementary Figure S1A). Seventeen genes did not change in dependence of LMP1. Approximately 38% of Hox genes were decreased and about 13% increased in the presence of LMP1 in NPC cells (Supplementary Figure S1B).

Next, we asked if an inverse correlation occurs between the expression of LMP1 and the expression of HoxB3, HoxB13 and HoxC8. We analyzed 73 human nasopharyngeal carcinoma tissues by immunohistochemistry. Hox proteins were mainly detected in cellular nuclei, whereas the LMP1 protein was found in the membrane of tumor cells (Figure 1C and 1D). Remarkably, Hox proteins were only expressed when LMP1 was not present (Figure 1C). Conversely, Hox gene expression was almost completely silenced when LMP1 was highly expressed (Figure 1D and Table 1). The expression of HoxB3, HoxB13 and HoxC8 proteins shows a moderate, but significant negative correlation with the expression of LMP1 (Table 1).

Taken together, the expression of LMP1 and Hox genes shows an inverse relationship, suggesting the possibility that LMP1 induces repression of Hox genes in NPCs.

LMP1 represses Hox genes via RNA Pol II stalling

To further assess the role of LMP1 on Hox gene expression, we examined mRNA expression of four selected genes by real-time PCR analysis. The mRNA of HoxA5, HoxB3, HoxB13 and HoxC8 genes was down-regulated in CNE1-LMP1 cells compared to CNE1 cells (Figure 2A), confirming the results of the array data. The same Hox genes showed also high expression in HK1 cells, a cell line which is LMP1 negative, whereas the cell line C666-1, which expresses the endogenous LMP1 and EBV, showed decreased Hox gene expression (Figure 2B). Using another LMP1-positive NPC cell line, HNE2-LMP1, we could further confirm the inverse relationship between LMP1 expression and Hox gene expression (Figure 2C).

To test for a direct role of LMP1 expression on Hox promoter activity, we generated two Hox promoter reporter plasmids and transiently transfected HEK293 human primary kidney

cells. The HoxC8 promoter activity was significantly decreased at high dosage of the LMP1 expression vector (Figure 2D). Likewise, the HoxB13 promoter activity was significantly decreased in a dose-dependent manner by LMP1 (Figure 2E). Furthermore, the NPC cell line HK1 showed also a dose-dependent repression of HoxB13 after LMP1 transfection (Figure 2F). Finally, Western Blot analysis, confirmed reduced HoxC8 protein levels after LMP1 transfection in two different NPC cell lines (supplementary Figure S2A) and an increase of HoxC8 protein level after reduction of LMP1 protein using shLMP1 RNA interference in EBV positive cells (supplementary Figure S2B). Taken together, the data indicates that LMP1 negatively regulates Hox promoter activity.

We next investigated the molecular mechanism of Hox gene repression and examined if RNA polymerase II (Pol II) stalling was involved. Using ChIP assays with antibodies directed against the unphosphorylated and phosphorylated form of Pol II, we assessed Pol II association at the transcriptional start site (TSS) (Region 1) and the gene body (Region 2), since stalled genes typically show a strong accumulation of Pol II around the TSS compared to the gene body. As expected, Pol II binding was present in LMP1 negative NPC cells (Figure 2G-J) and Pol II amounts were similar at the TSS and the gene body. This finding is consistent with the active transcription of Hox genes in LMP1 negative NPCs. Pol II was also found at repressed Hox genes in LMP1 positive NPCs. However, the Pol II occupancy was greater at the TSS compared to the gene body, consistent with a stalled Pol II. This suggests that at least part of the mechanism by which repression occurs in LMP1 positive cells is mediated via Pol II stalling.

Irradiation overcomes Hox gene repression induced by LMP1

Irradiation therapy is the predominant treatment regimen for NPC. Therefore, we asked whether irradiation could influence Hox genes silencing in NPCs. NPC cells (with or without LMP1) were irradiated for different lengths of time. We have found that LMP1 positive cells are more resistant to irradiation than LMP1-negative cells [65]. The DNA damage response was assessed by γ-H2Ax staining, a marker for double-stranded DNA breaks, and by using the comet assay, a single cell assay to determine DNA damage/repair (supplementary Figure S3A, B, C). After irradiation, we detected an increase in Hox gene expression (normalized to actin expression) in LMP1 positive CNE1 cells that peaked at 24 hours (Figure 3A-D). In addition, the Hox protein levels were increased (Figure 3E). Likewise, another NPC cell line expressing LMP1 showed an increase of Hox gene expression after irradiation (supplementary Figure S4). Moreover, the expression of several Hox genes was only enhanced in the presence of LMP1 (Figure 3A,B,D and supplementary Figure S4B,D). This indicates that irradiation can reactivate this Hox gene signature, at least in part, in a LMP1 dependent manner.

Next, we addressed the question if irradiation could overcome Pol II stalling which was induced by LMP1. Using a ChIP assay for Pol II binding, we observed reduced Pol II occupancy around the TSSs and enhanced enrichment at the gene body in irradiated LMP1 positive cells after radiation (Figure 4A-4D). The altered distribution of Pol II (away from TSS toward the gene body) indicates that Pol II stalling is overcome.

Taken together, our data suggests that LMP1 induces Pol II stalling at several Hox genes, which can be a released by irradiation.

TET3 is involved in the reactivation of Hox genes

Pol II stalling can be regulated by epigenetic changes [56, 58, 63]. In particular, changes in DNA methylation can release Pol II stalling [58, 59] and, furthermore, irradiation causes changes in DNA methylation [44]. For this reason, we examined the possibility that alterations of DNA methylation may be involved in irradiation induced Hox gene re-activation. Using hMeDIP with a 5hmC antibody, we detected a decrease of 5hmC at the HoxA5, HoxB13 and HoxC8 promoters after irradiation in CNE1-LMP1 cells compared to untreated CNE1-LMP1 cells (Figure 4E). Furthermore, we used methylation sensitive PCR-analysis, employing a restriction enzyme (PvuRts1I) that specifically digests 5-hmC DNA (and not 5-methylcytosine residues or unmethylated DNA). Genomic DNA derived from CNE1-LMP1 cells treated with irradiation was resistant to PvuRts1I digestion in comparison to CNE1-LMP1 cells that were not irradiated (Figure 4F). This indicates that irradiation causes an alteration of 5hmC level at several loci within the HoxA5 and HoxB3 promoter regions.

Next, we wanted to investigate whether TET proteins that catalyze 5-hmC mediate Hox gene reactivation after irradiation. Since only TET2 and TET3 are expressed in CNE1-LMP1 cells (Supplementary Figure S5A and Figure 5A), we selected TET2 and TET3 for further study. Using shRNA we could successfully create TET2 and TET3 knockdown cells (supplementary Figure S5B and Figure 5A). While TET2 knockdown did not show any effect (Supplementary Figure S5C-F), TET3 knockdown in CNE1-LMP1 cells inhibited Hox activation after irradiation (Figure 5B-5E). For example, irradiation increased HoxC8 expression more than 15 fold after 24 hours, but two independently derived stable TET3 knockdown cell lines showed a more than seven fold inhibition (Figure 5E). This indicates that TET3 is critical for the establishment of the Hox gene signature in response to irradiation in LMP1 positive NPCs.

Ectopic expression of HoxC8 decreased glycolytic metabolism and up-regulated TCA cycle-related genes

Our results had indicated that LMP1 represses Hox genes and that Hox genes serve also as targets for irradiation therapy. Therefore we wanted to address the question, whether Hox genes play a functional role in NPC growth and tumorigenic characteristics.

A preliminary screen of published data using the Cell Miner tool hinted to a potential role for HoxC8 as tumor suppressor in epithelial cell-derived cancers (Table S2). Furthermore, it suggested a potential link between HoxC8 and the regulation of glycolysis. Our previous studies had demonstrated that LMP1 promotes the Warburg effect, describing the use of aerobic glycolysis in cancer cells [65]. For this reason we selected HoxC8 for further studies and we first addressed the question, whether HoxC8 had an effect on glycolytic metabolism. After transient expression of HoxC8 in CNE1-LMP1 cells, we determined the concentration of glucose and lactic acid in the medium (Figure 6A,B). Both, glucose consumption and lactic acid production, decreased in a dose-dependent manner demonstrating that overexpression of HoxC8 can reduce energy metabolism. Furthermore, the expression of

HK2 and Glut1, key regulators of glycolysis, are down-regulated after the transient transfection of HoxC8 into CNE1-LMP1 cells (Figure 6C and supplementary Figure S6). This indicated that HoxC8 attenuated glycolysis. Since glycolytic metabolites enter the TCA-cycle which shows reduced function in cancer cells, and since TCA genes can act as tumor suppressor genes [12], we examined the role of HoxC8 on several TCA-cycle related genes. After transient transfection of HoxC8 in CNE1-LMP1 cells, we detected a significant increase of 2-hydroxyglutarate dehydrogenase (2-HGDH), SDHD, α-ketoglutarate dehydrogenase (α-KDH), OGDH, DLST, DLD, fumarate hydratase (FH), aconitase 1 (ACO1) and aconitase 2 (ACO2) (Figure 6D). The same genes (except DLST and ACO1) are downregulated in LMP1 positive NPCs compared to LMP1 negative NPCs (supplementary Figure S7). Moreover, several genes (SDHD, OGDH, FH and ACO2) are repressed after transfection of LMP1, and the repression can be overcome by HoxC8 overexpression in CNE1-LMP1 cells (supplementary Figure S8). Taken together, our results indicate that HoxC8 impairs glycolysis and concomitantly promotes the TCA cycle by up-regulating TCA-cycle related genes.

The ectopic expression of HoxC8 represses tumor progression in NPC cells

Since transient expression of HoxC8 seemed to counteract the Warburg effect in LMP1 positive NPCs, we aimed to reveal the functional role of HoxC8 in the growth and tumor cell characteristics of LMP1 positive NPC.

For this purpose, we generated CNE1-LMP1 cells that stably expressed ectopic HoxC8 and validated HoxC8 protein levels by Western analysis (inset of Figure 7A). Ectopic expression of HoxC8 in CNE1-LMP1 cells resulted in reduced growth in vitro compared to control cells without HoxC8 (Figure 7A). Furthermore, NPCs with ectopic HoxC8 expression displayed decreased formation of colonies in soft agar compared to controls (Figure 7B and 7C). In addition, HoxC8 overexpression impaired growth as measured in a plate colony formation assay (Figure 7D and 7E). Finally, we tested xenograft tumor formation in nude mice after injection of CNE1-LMP1 cells and CNE1-LMP1 cells with ectopic HoxC8 expression. HoxC8 overexpression resulted in a significant reduction of tumor size (Figure 7F, G) suggesting reduced growth of tumor cells with ectopic HoxC8 expression. In addition, HoxC8 significantly reduced the tumor weight (Figure 7H), while the body weight did not change significantly in either group (supplementary Figure S9). Taken together, our data indicates that ectopic HoxC8 expression can function as tumor suppressor in vitro and in vivo of LMP1 positive NPC.

Finally, we examined expression of HoxC8 protein in a NPC tissue array by immunohistochemical analysis. While HoxC8 was almost completely silenced in non-cancerogenic inflamed nasopharyngeal tissue, HoxC8 protein expression was greatly increased in nasopharyngeal carcinoma tissues (Figure 8A and Figure 8B). Lastly, NPC tissues were grouped in EBV negative, EBP positive (+) and EBV positive (++) based on the expression of EBER using *in situ* hybridization (ISH) (Figure 8A). HoxC8 expression was decreased in EBV positive NPCs compared to EBV negative NPCs (Figure 8A, B). Moreover, the higher the EBER marker was expressed, the more HoxC8 expression was reduced, indicating a reverse correlation between HoxC8 expression and EBV infection in

NPC. Furthermore, the Kaplan-Meier plot showed a statistically significant difference in the overall survival between NPC patients with high expression of HoxC8 and those with low expression of HoxC8 (supplementary Figure S10). Our results confirm an inverse relationship between the presence of LMP1/EBV and HoxC8 in cancer biopsies. In addition, the data indicates that high HoxC8 expression is associated with a poor prognosis in NPC patients.

Discussion

This study provides several novel mechanistic insights into the role of the oncoprotein LMP1 in NPC, a prevalent cancer in China. Firstly, we report that LMP1 regulates Hox gene expression via Pol II stalling and that the epigenetic TET3 signaling axis is involved in Hox gene repression. Irradiation, a common treatment procedure for NPC, can overcome Pol II stalling and leads to Hox gene reactivation. Furthermore, this report is the first to demonstrate that HoxC8 acts as a modulator of glycolysis, down-regulates energy-related genes, such as Glut1 and HK2, and up-regulates TCA-related genes that are well-known tumor suppressor genes. These findings demonstrate that HoxC8 plays an important role in the regulation of energy metabolism (Figure 8C). Finally, we provide evidence that HoxC8 in NPC reduces tumor growth *in vitro* and *in vivo*, and higher HoxC8 expression in NPC tissues is associated with better survival in patients.

Aberrant Hox gene expression is a frequent characteristic in cancer cells [50]. In this study, we report that LMP1 can reduce the expression of Hox genes, including HoxA5, HoxB3, HoxB13 and HoxC8, and that repression was in part mediated by Pol II stalling. These findings are consistent with previous reports showing that Hox genes are regulated by Pol II stalling [7, 8, 58]. Other viral products, for example, derived from the murine Moloney leukemia virus and human papillomavirus, encode oncoproteins that regulate Hox genes [3, 19, 32]. Furthermore, it was reported, that LMP1 induced Pol II stalling at the Cp promoter of the EBV genome [41].

In this study, we report that LMP1 induced silencing leads to a Hox gene expression signature that is associated with nasopharyngeal carcinoma. Immunohistochemistry demonstrated that HoxB3, HoxB13 and HoxC8 were downregulated in EBV LMP1-positive NPC tissue sections. The search for biomarkers to improve diagnosis, to give a better prediction of the prognosis and to individualize treatments has become a focus of cancer studies. It has been reported that reduced expression of HoxA5 in oral squamous cell carcinoma [48] and loss of nuclear HoxB13 expression in non-muscle-invasive bladder transitional cancer [31] is significantly correlated with patient survival, suggesting a potential prognostic role for Hox gene expression. Recently, Chen *et al.* [6] found that HoxB3 can bind as a transcription factor to the promoter region of cell division cycle associated 3 (CDCA3) to promote its expression, resulting in LNCaP cell proliferation and migration. Knocking down HoxB3 in PC-3 cells inhibited proliferation in a CDCA3-dependent manner, suggesting that Hox genes may serve as a potential novel target in cancer therapy. The Hox gene expression profile in NPC, as shown in our study, may provide useful information to improve early diagnosis of NPC and may promote the development of novel molecular strategies for NPC treatment.

Our study suggests that HoxC8 may be regarded as a tumor suppressor, which is consistent with previous findings [1, 13]. HoxC8 expression is inversely related to the progression and metastasis of pancreatic ductal adenocarcinoma and is used as a prognostic marker in patients with esophageal squamous cell carcinoma [1, 13]. HoxC8 is a transcription factor for cadherin 11 (CDH11) and both genes are positive regulators of breast tumorigenesis [25]. CDH11 is regarded as a tumor suppressor in many tumors including NPC [5, 24], further indicating that HoxC8 may be involved in reduced tumor growth.

In our study, we discovered that Hox gene expression was significantly suppressed by the EBV-encoded oncoprotein LMP1 in NPC cell lines. LMP1 becomes aggregated in the plasma membrane or at the Golgi compartment where LMP1 may act as a constitutively activated receptor to mediate its signal transduction activity [61, 62]. Although LMP1 is initially expressed on the membrane of T cells, subsequently, its expression occurs solely in the nuclei of these T cells, it hints that LMP1 might not play the same role in the lymphomagenesis of T cells as it does in B cells [67]. Immunofluorescence assay shows that LMP1 could also localize in the nuclei after transiently transfection of LMP1 [18]. It is not clear whether LMP1 can exert directly an effect within the nucleus.

We observed that irradiation overcomes Pol II stalling at Hox genes leading to an increase in Hox gene transcripts. This suggests that irradiation at least in part modulates Hox gene expression via release of Pol II stalling. However, there may be other contributing factors, since we noticed that Hox gene expression in LMP1 positive NPCs after irradiation was in some cases above that of LMP1 negative cells. Irradiation induces several DNA damage response pathway genes [22, 28], as well as other genes (kinases and phosphatases) that may have in combination with LMP1 a specific effect on transcriptional initiation, mRNA splicing or mRNA stability [60]. With respect to Pol II stalling our data suggests that an alteration of DNA methylation may play a functional role. It is possible that the activation of DNA repair processes may involve the base excision repair pathway which changes 5mC or 5hmC into C leading to DNA demethylation[10, 11, 17, 45]. DNA demethylation in turn can release stalled Pol II at tumor suppressor genes [59]. Alternatively, irradiation leads to epigenetic alterations beyond the specific chromatin changes present at the site of DNA damage [35]. Widespread epigenetic alterations after irradiation are well documented and encompass changes in DNA methylation and histone modifications, e.g. loss of H4K20me3, and may also affect small non-coding RNA [33, 53]. DNA methylation changes may be induced by alterations in DNA methyltransferase protein levels or may involve Tet3 expression changes, as reported here, epigenetic changes in turn can alter transcription.

TETs are associated with malignancy and tumorigenesis, and research in this field has mainly focused on TET2 [20, 42]. Moreover, the loss of 5hmC is an epigenetic hallmark in cancer whereas the TET2 gene is usually a bona fide tumor suppressor [26, 54, 68]. Here, we found that TET1 was completely silenced in NPC cells and irradiation could not reactivate its expression, as evidenced by RT-PCR (data not shown). Moreover, TET2 did not contribute to the reactivation of stalled genes in response to irradiation after knocking down TET2 expression. We found that irradiation increased TET3 expression, the reason for this remains unclear and requires further investigation. Our findings show that TET3 is involved

in the reactivation of Hox genes after irradiation. Since Pol II stalling is overcome by irradiation, our data suggests that TET3 is required for irradiation induced Pol II release.

We report here that LMP1 represses HoxC8. Ectopic expression of HoxC8 in turn can modulate NPC growth in vitro and in vivo, and leads to a concomitant modulation of several genes involved in glycolysis and TCA-cycle. It requires further investigation whether HoxC8 mediates its effect on growth via the modulation of energy metabolism genes, but the well documented important role of energy metabolism in cancer suggests a possible explanation how HoxC8 may affect tumor growth. Abnormal energy metabolism, including glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation and many other aspects of metabolism, is a prominent feature of tumors that has a tremendous impact on tumorigenesis and tumor progression [4, 12, 15]. The alteration of tumor cell metabolism is known as metabolic reprogramming. This process involves complex regulatory mechanisms including, the transcriptional regulation of genes encoding metabolic enzymes [4]. We report here, that glucose uptake and lactate production were both remarkably reduced after HoxC8 overexpression, indicating that HoxC8 may inhibit glycolysis. Moreover, we found that HoxC8 upregulates TCA cycle genes including hydroxy glutarate dehydrogenase (2-HGDH), succinate dehydrogenase complex II D (SDHD), α-ketoglutarate dehydrogenase (α-KGDH), fumaric acid (FH) and aconitases (ACOs), which are regarded as tumor suppressors [12, 39, 66]. These results suggest that Hox gene reactivation upon irradiation may reverse the Warburg effect and promote TCA cycles by increasing TCA related genes, which, in turn, inhibits tumor growth. Recently, the first *in vitro* proof of reversing Warburg effect was reported as a novel strategy for cancer therapy [46], indicating that the reactivation of stalled genes may be potential strategy for cancer therapy and prevention.

In conclusion, EBV may negatively regulate HOX gene expression at the transcriptional level through Pol II stalling in nasopharyngeal carcinoma. DNA methylation changes, induced by irradiation, may contribute to the release of Pol II stalling and result in the reactivation of HOX gene transcription by the TET3/5hmC pathway, which plays an important role in glycolysis of tumors.

Materials and methods

Cell lines and cell culture

NP69 is an immortalized normal nasopharyngeal epithelial cell line. CNE1 and HK1 are LMP1-negative nasopharyngeal squamous carcinoma cell lines. CNE1-LMP1 is a stable LMP1-integrated integrated nasopharyngeal squamous carcinoma cell line. HNE2-pSG5 is an EBV-LMP1-negative human NPC cell line produced through transfection with the pSG5 vector into HNE2 cells. HNE2-LMP1 is a cell line with constitutive expression of LMP1 after HNE2 transfected with pSG5 vector inserted with LMP1 full-length cDNA. C666-1 is a NPC cell line consistently harbouring Epstein-Barr virus. HEK293 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). CNE1, CNE1-LMP1, HNE2-pSG5, HNE2-LMP1, HK1 and C666-1 were cultured in RPMI-1640 (GIBCO, Life Technologies, Basel, Switzerland) medium with fetal bovine serum (FBS) to a final concentration of 10%. HEK293 (ATCC® CRL1573™) was cultured in DMEM (GIBCO, Life Technologies, Basel, Switzerland) medium with FBS to a final concentration of 10%.

AGS-EBV was cultured in F-12 medium with FBS to a final concentration of 10%. NP69 cell line was propagated in defined keratinocyte-SFM (KSFM, GIBCO, Life Technologies, Basel, Switzerland) medium supplemented with growth factors. All cell lines were maintained at 37°C with 5% CO_2.

Construction of expression vectors

The pcDNA 3.1(-)B-HOXC8 expression plasmid was constructed by cloning the entire HoxC8 coding sequence into the pcDNA 3.1(-)B vector. The HoxC8 coding sequence was also inserted into the lentivirus vector pLJM1-EGFP (Addgene plasmid 19319[49]). The HoxB13 promoter luciferase reporter construct (-5.2 kb to +0.2 kb) was a generous gift from Dr. Samson T. Jacob, Ohio State University, USA. The HoxC8 promoter luciferase reporter construct was created by cloning the HOXC8 promoter (-764bp to +212bp) into the multiple cloning sites of the pGL4.16 vector, driving the expression of firefly luciferase. The pSG5-based expression vector for wild-type LMP1 was derived from the B95.8 EBV strain. Lentiviral plasmids containing GV298-shTET3 (#3,27621-1; #5,27623-1) were obtained from GeneChem.

Lentiviral Infection

To generate knockdown TET3 cells, GV298-shTET3 lentivirus plasmid was transfected into 293T cells with psPAX2 and pMD2.G. Viral supernatant factions were collected at 48 h after transfection and filtered through a 0.45μm filter followed by infection into cells together with 5μg/ml polybrene and incubation for 16 h. At 48 h after infection, replace with fresh medium with puromycin. To generate stable CNE1-LMP1 cells expressing HoxC8 (HoxC8-EGFP), the pLJM1-Mock or pLJM1-HoxC8 was cotransfected into 293T cells together with pCMV-VSV-G, pMDLg-pRREand pRSV-Rev (Addgene). Viral supernatant factions were collected at 48 hours after transfection and filtered through a 0.45μm filter followed by infection into cells together with 5μg/ml polybrene and incubation for 16 hours. At 48 hours after infection, replace with fresh medium with 1μg/ml puromycin and incubated for another 6 days.

Reverse transcription and real-time PCR

Cells were harvested with Trizol (Invitrogen). cDNAs were synthesized with SuperScript II (Invitrogen) according to the manufacturer's protocol. Real-time PCR analysis was performed using the Applied Biosystems 7500 Real-Time PCR System, according to the manufacturer's instructions. The reactions were performed in triplicate for three independent experiments: the results were normalized to β-actin. The primer sequences used are in Supplemental Table S3. The mean± SD of three independent experiments is shown.

Immunohistochemistry (IHC) analysis and In situ hybridization of NPC biopsies

NPC biopsies, validated by pathologist Dr. Desheng Xiao (Xiangya Hospital), were obtained from Pathology Department of Xiangya Hospital. The NPC tissue array was purchased from Pantomics (Richmond, CA, USA). Paraffin sections from NPC patient samples were firstly dewaxed and antigen retrieved in citrate buffer using a microwave for 15min. After cooling of the citrate buffer to room temperature, the sections were incubated with PBS (containing

5% bovine serum albumin (BSA), and 3% FBS) for 30 min, and subsequently incubated with HoxB3 (ab83404, Abcam), HoxB13 (ab53931, Abcam), HoxC8 (HPA028911, Sigma) or LMP1 (M0897, DAKO) primary antibody for 1 h. The slides were thoroughly washed three times with PBS (5%BSA, 3%FBS) solution for 10 min each and then incubated for 30 min with HRP- conjugated secondary antibody for 30 min at room temperature. The slides were thoroughly washed three times with PBS before using DAB. The images were surveyed and captured using a CX41 microscope (OLYMPUS, Tokyo, Japan) with the Microscope Digital Camera System DP-72 (OLYMPUS, Tokyo, Japan) and differentially quantified by two pathologists, Dr. Bo Li and Dr. Songqing Fan (The Second Xiangya Hospital, Changsha, China).

In situ hydridization (ISH) was performed using the EBERs HRP conjugated probe and DAB as substrate from ISH kit (Life technologies), according to the instructions of the manufacturers.

Transient transfection and luciferase reporter assays

To study the effect of LMP1 on Hox gene repression, we transfected HoxB13 or HoxC8 promoter reporter constructs and the pSG5-LMP1 expression vector into HEK293 or HK1 cells. The transfections were conducted by using Lipofectamine 2000 (Invitrogen), according to the manufacture's recommendations. Cells were harvested for analysis of luciferase activity using the Dual Luciferase Reporter assay (Promega) and the GloMax™ Microplate Luminometer (Promega). The luciferase reporter plasmids were co-transfected with pRL-TK to correct for variations in transfection efficiency. The data represents the mean ± SD of three independent experiments performed in triplicates.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were essentially performed as previously described[51] with modifications: 5×10^6 cells were fixed with formaldehyde (1% final volume concertration, Sigma), 10 min at room temperature. Fixation was stopped by addition of 1/10 volume 1.25M glycine and incubated for 5min at room temperature. The sonication step was performed in a Qsonica sonicator (5min, 20s on, 20s off), and 200μg of protein-chromatin complex was used for each immunoprecipitation. Antibody-protein complex was captured with preblocked dynabeads protein G (Invitrogen). ChIP DNA was analyzed by qPCR with SYBR Green (Biorad) in ABI-7500 (Applied Biosystems) using the primers specified in Supplemental Table S4. The antibodies used are as followed: 8WG16 (ab817, Abcam), normal mouse IgG (12-371, Millipore).

Irradiation treatment

NPC cells covered with 1.0 cm thick tissue glue were treated with 10 Gy irradiation using a Siemens Primus instrument (Siemens, German) at the Department of Clinical Oncology of Xiangya Hospital (Changsha, China).

Comet assay and immunofluorescence staining

Comet assay—We carried out comet assays following the instruction of Olive PL et al [40]. All data were normalized to the untreated group that was assigned a value of 1. The data represent the mean ± SD of three independent experiments performed in triplicate.

Immunofluorescence staining—CNE1-LMP1 cells were treated with 10 Gy irradiation and then cultured and fixed in ice-cold methanol for 10 min. To identify the presence of γ-H2Ax protein, cells were incubated with an anti-γ-H2Ax antibody (2212-1, Epitomics), followed by incubation with Alexa Fluor 488-conjugated anti-IgG (A-11008, Invitrogen). To visualize the nuclei, the cells were stained with DAPI. Fluorescent images were observed and analyzed with a laser scanning confocal microscope (Leica, TCS-SP5).

Methylated/5-hydroxymethylated DNA Immunoprecipitation (MeDIP/hMeDIP)

hMeDIP was performed according to the manual's protocol (Diagenode). The antibodies used are as follows: 5hmC antibody (633HMC-100, Diagenode), Normal mouse IgG (12-371, Millipore). The primers for MeDIP/hMeDIP were presented in Supplemental Table S5.

5hmC restriction digestion assay

Whole genome DNA was obtained and then digested by PvuRts1 I enzyme (Active Motif), which is capable of specifically digesting 5-hydroxymethylcytosine residues and cut off double stranded DNA but not 5-methylated or unmethylated cytosine residues. The digested DNA fragments were detected by PCR. The primers for 5hmC restriction digestion assay were presented in Supplemental Table S6

Cell proliferation assay and glucose consumption/lactate production detection assay

Details of the cell proliferation assay was described previously[51]. Glucose consumption/ lactate production detection assay: CNE1-LMP1 cells transfected with HoxC8 expressing plasmid, were planted 5×10^5 per-well in 6-well plate then cultured in 8 ml RPMI-1640 medium with 10% fetal bovine serum, 37°C, 5%CO_2. After 72 hrs incubation, supernatant medium was collected to examine glucose and lactate content using an Automatic Biochemical Analyzer (7170A, HITACHI, Tokyo, Japan) at the Clinical Biochemical Laboratory of Xiangya Hospital (Changsha, China). The assay was independently performed three times.

In vivo tumor growth

Athymic nude mice were divided into two groups and injected in the armpit with Mock or HoxC8 expressing CNE1-LMP1 cells (2×10^6). Tumors were measured by caliper every 3 days. All studies were performed following guidelines approved by the Experimental Animal Ethics Committee of Central South University.

Glucose uptake and lactate production measurement

Cells (5×10^5) were seeded in 6-well plates and after incubation for 4 h, medium was discarded and cells were incubated in fresh medium for 8 h. Glucose and lactate levels were

measured (Automatic Biochemical Analyzer, 7170A; HITACHI) at the Clinical Biochemical Laboratory of Xiangya Hospital (Changsha, China).

Soft agar-colony formation and plate-colony formation assay

For soft agar-colony formation assay, cells (8×10^3/mL/well) were seeded into 6-well plates with 0.3% Basal Medium Eagle agar containing 10% FBS and cultured, colonies were scored using a microscope and Image J software (1.47V, NIH, USA). For plate-colony formation assay, cells (2×10^3/mL/well) were seeded into 6-well plates and culture in RPMI-1640 medium supplemented with 10% FBS, colonies were fixed by methanol and stained by viola crystallina then scored using a microscope and Image J software (1.47V, NIH, USA).

Statistical Analysis and Bioinformatics

Quantitative data was expressed as mean and standard deviation of at least three biologically independent experiments. Statistical analyses were performed using the Student's t-test. A p value of < 0.05 was considered statistically significant. For survival analysis, Kaplan–Meier curves were drawn and differences between the curves were calculated by the log-rank test. *$P < 0.05$ was considered statistically significant. To search for a potential role of Hox genes in the regulation of metabolism-related genes in 60 cancer cell lines we used the CellMiner tool (http://discover.nci.nih.gov/cellminer/analysisi.do).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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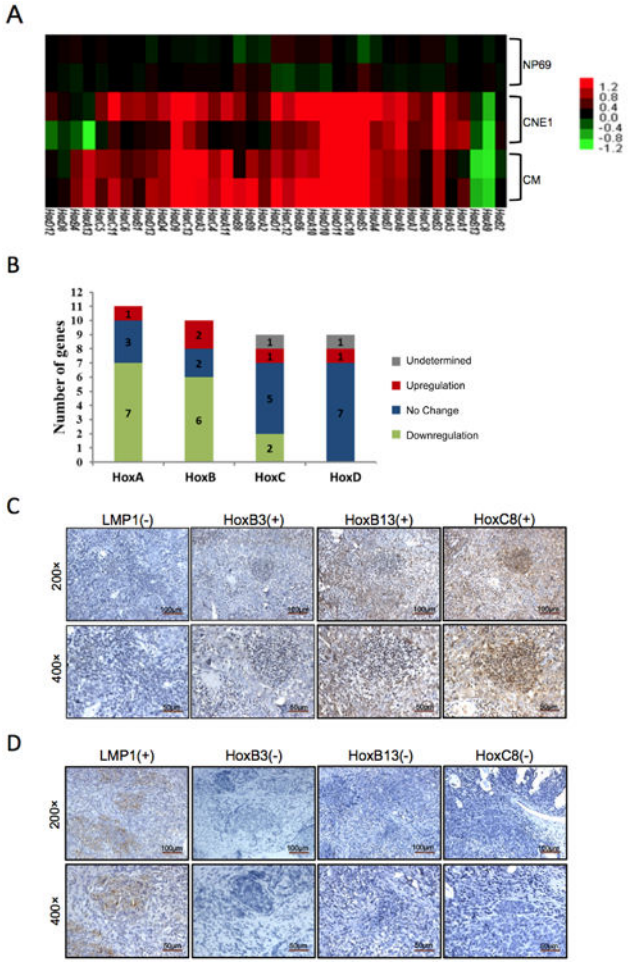

Figure 1. The signature of Hox gene expression in nasopharyngeal carcinoma cell lines and biopsies

(A) Heat map illustrating Hox gene mRNA levels in immortalized nasopharyngeal epithelial cells (NP69), LMP1-negative NPC cells (CNE1) and LMP1 overexpressing NPC cells (CNE1-LMP1, CM). The fold change in expression level is represented as a relative mean value increase (red) or decrease (green). (B) Summary of differentially expressed Hox genes. Each bar represents a Hox cluster (1 through 13). The colors represent the relative fold change in Hox gene expression in CNE1-LMP1 cells compared to CNE1 cells (Green, <1; Blue, between 1 and 1.5; Red, >1.5; Gray, mRNA below detection). Immunohistochemical analysis for detection of HoxB3, HoxB13 and HoxC8 proteins located in the nucleus and partially located in the cytoplasm. (C) In LMP1-negative tissue, HoxB3, HoxB13 and HoxC8 showed nuclear staining (up, ×200; down, ×400). (D) In LMP1-positive tissue, all three Hox genes show less staining (up, ×200; down, ×400).

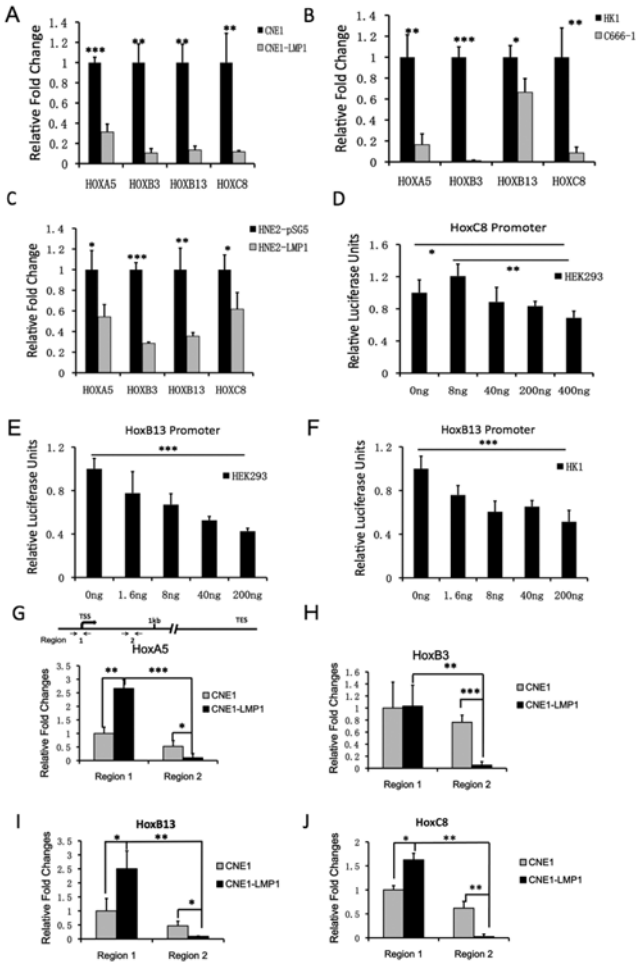

Figure 2. Pol II stalling at repressed Hox genes in LMP1-positive NPC cells

RT-PCR analysis for the detection of HoxA5, HoxB3, HoxB13, and HoxC8 using total RNA derived from LMP1-negative cells such as CNE1 (A), HK1 (B), and HNE2 cells (C) and matching LMP1-positive cell lines. The level of gene expression was normalized against the house keeping gene β-actin and is represented as fold change compared to LMP1-negative cells. A luciferase reporter assay was carried out to evaluate Hox promoter regulation in HEK293 cells using increasing does of a LMP1 expression vector. HoxC8 promoter activity in HEK293 cells (D) and HoxB13 promoter activity in HEK293 (E) and HK1 (F) cell lines. All promoter luciferase intensity was normalized to the pRL Renilla Luciferase Control Reporter and is represented as the fold change compared to the control group. ChIP analysis of CNE1 and CNE1-LMP1 cells was performed to detect Pol II around the TSSs. Schematic representation of the primer positions used for Pol II ChIP analysis (top of Figure 2G). TES, transcriptional end site. The bar graphs show the relative fold change of Pol II enrichment at the Region 1 and Region 2 of HoxA5 (G), HoxB3 (H), HoxB13 (I), and HoxC8 (J) in CNE1 cells expressing LMP1 and was normalized against the CNE1 cell line without LMP1. Error bars indicate the standard deviation of three to four independent ChIP assays. * p <0.05, ** p <0.01, *** p <0.001.

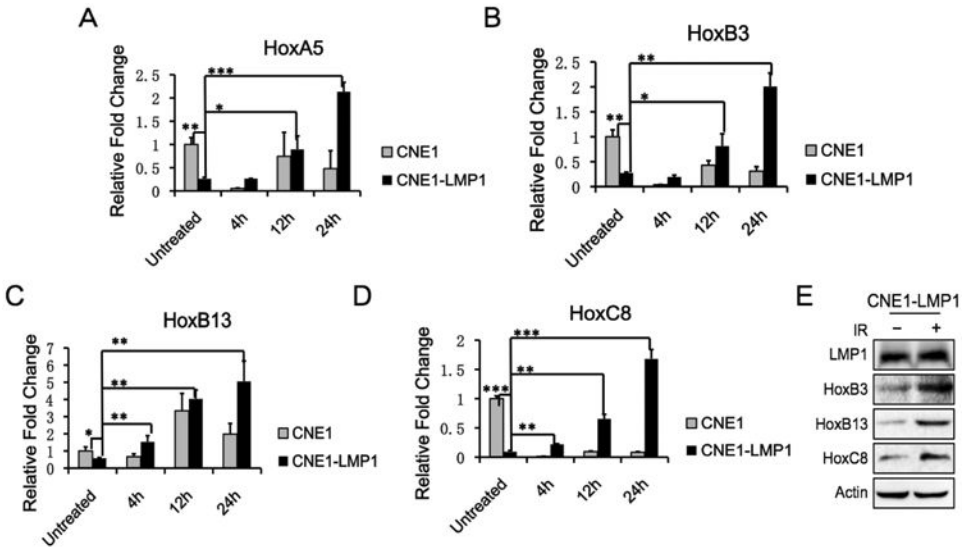

Figure 3. Hox gene reactivation after irradiation-induced DNA damage response

RT-PCR analysis was conducted to detect selected Hox genes (A for HoxA5, B for HoxB3, C for HoxB13 and D for HoxC8) using total RNA obtained from CNE1-LMP1 at 4 h, 12 h, and 24 h after 10Gy IR treatment. The level of gene expression was normalized against β-actin and is represented as the fold change compared to untreated CNE1 cells (LMP1 negative) that is set to one. The means and S.D. values were derived from three independent experiments. * p <0.05, ** p <0.01, *** p <0.001. (E) Immunoblotting was used to analyze the expression level of Hox genes as indicated and LMP1 protein in CNE1-LMP1 cells after irradiation for 24 hours.

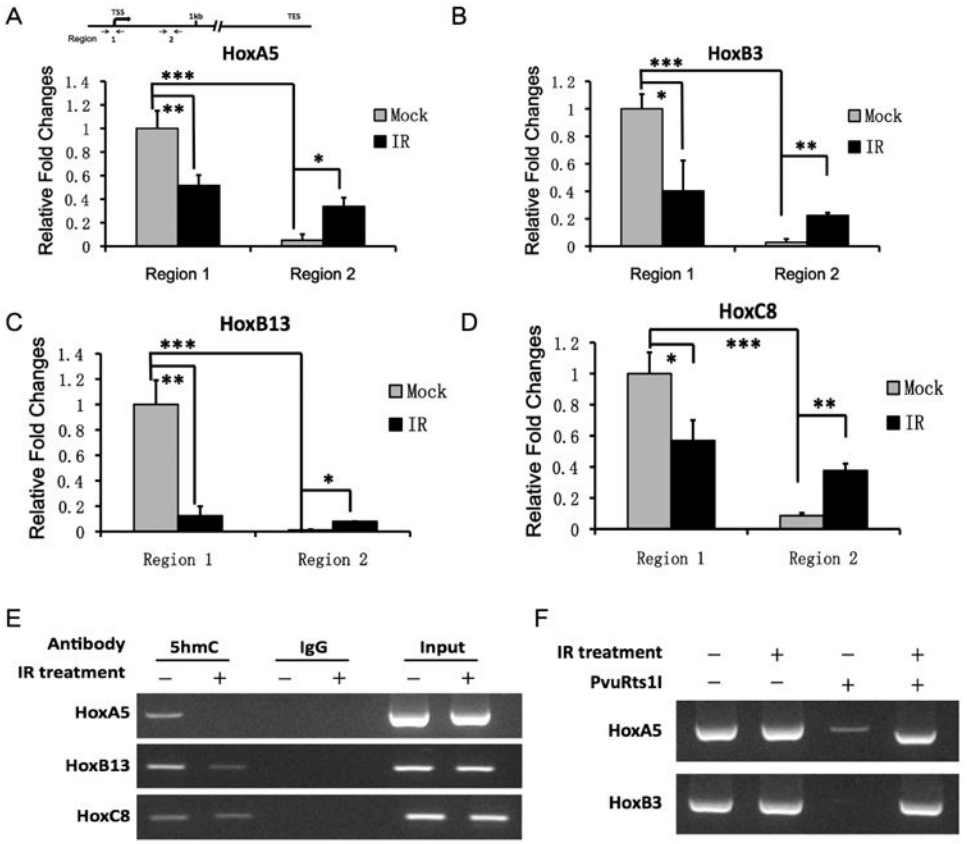

Figure 4. Pol II was released and 5hmC levels decreased around the TSS regions of Hox genes after IR treatment

Schematic representation of the primer positions used for Pol II ChIP analysis. TES, transcriptional end site (up in A). ChIP assays were conducted to assess Pol II enrichment at the TSS of HoxA5, HoxB3, HoxB13 and HoxC8 after irradiation in CNE1-LMP1 cells. The bar graphs shows the relative fold change of Pol II enrichment at the Region 1 and Region 2of HoxA5 (A), HoxB3 (B), HoxB13 (C), and HoxC8 (D) and was normalized against CNE1 cells. The means and S.D. values were derived from three independent experiments. * p <0.05, ** p <0.01, *** p <0.001. (E) hMeDIP assays to assess 5hmC levels at the TSS of HoxA5, HoxB13 and HoxC8 before and after irradiation (IR) treatment. (F) PvuRts1I restriction enzyme digestion to assess 5hmC level at the TSS of HoxA5 and HoxB3 genes before and after IR treatment.

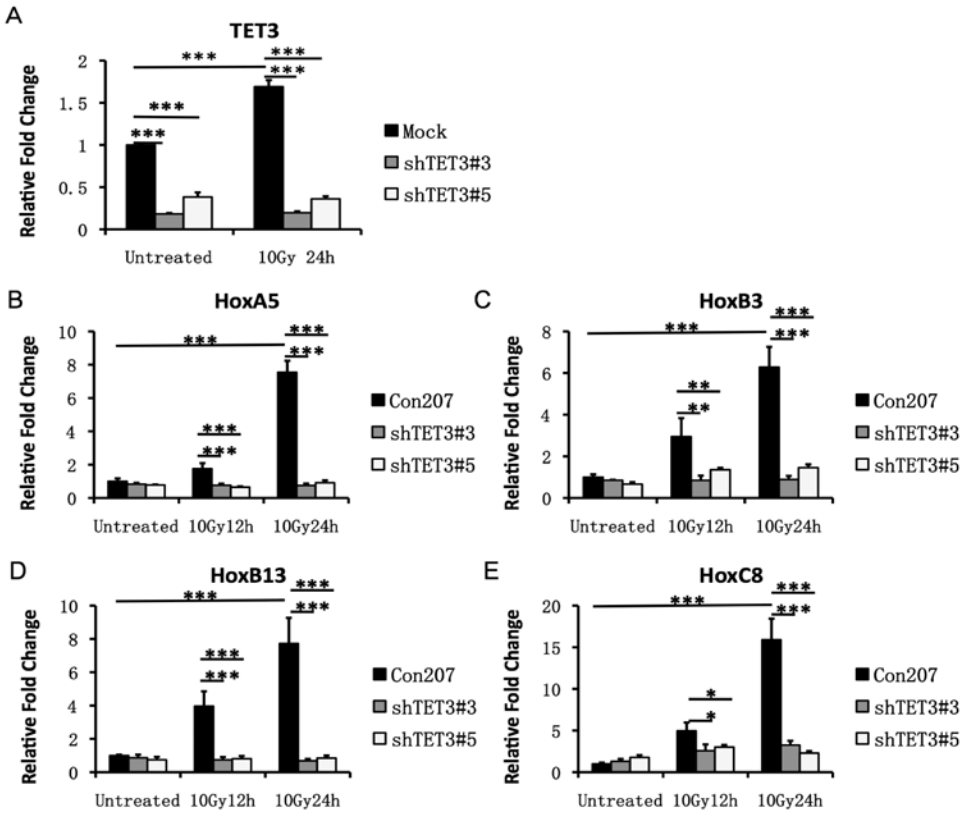

Figure 5. TET3 knockdown inhibited Hox gene reactivation after IR treatment

(A) Two stable TET3 knockdown cell lines (shTET3#3 and shTET3#5) were established by transferring shTET3 sequences into CNE1-LMP1 cells. RT-PCR analysis for detection of TET3 mRNA before and after IR. The expression of HoxA5 (B), HoxB3 (C), HoxB13 (D) and HoxC8 (E) was examined after IR treatment in shTET3 treated CNE1-LMP1 cells and in control treated group. The means and S.D. values were derived from three to four independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

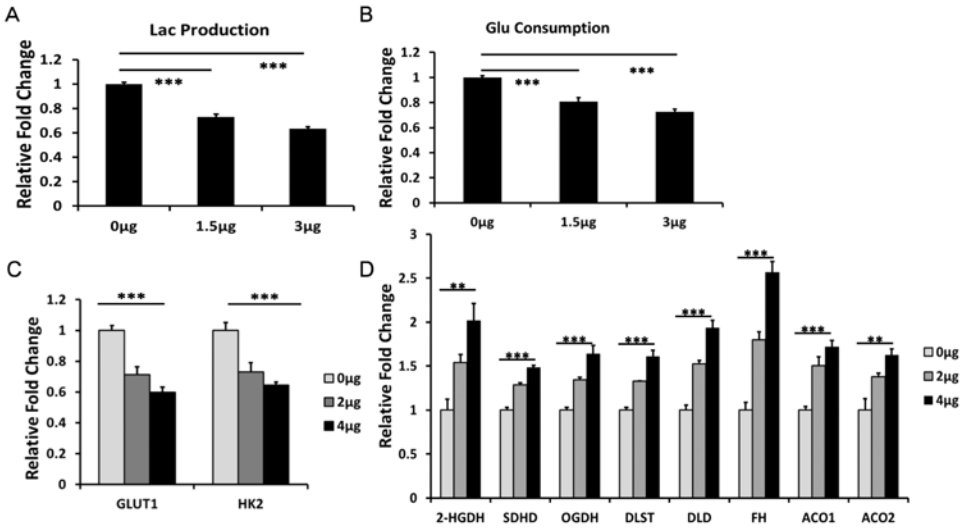

Figure 6. HoxC8 affected glycolytic metabolism and TCA cycle-related genes

Ectopic expression of HoxC8 in CNE1-LMP1 cells reduced lactate production (A) and glucose consumption (B) in a dosage dependent manner. The expression of glycolysis (C) and TCA cycle (D) associated genes was analyzed by RT-PCR. The means and S.D. values were derived from four independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

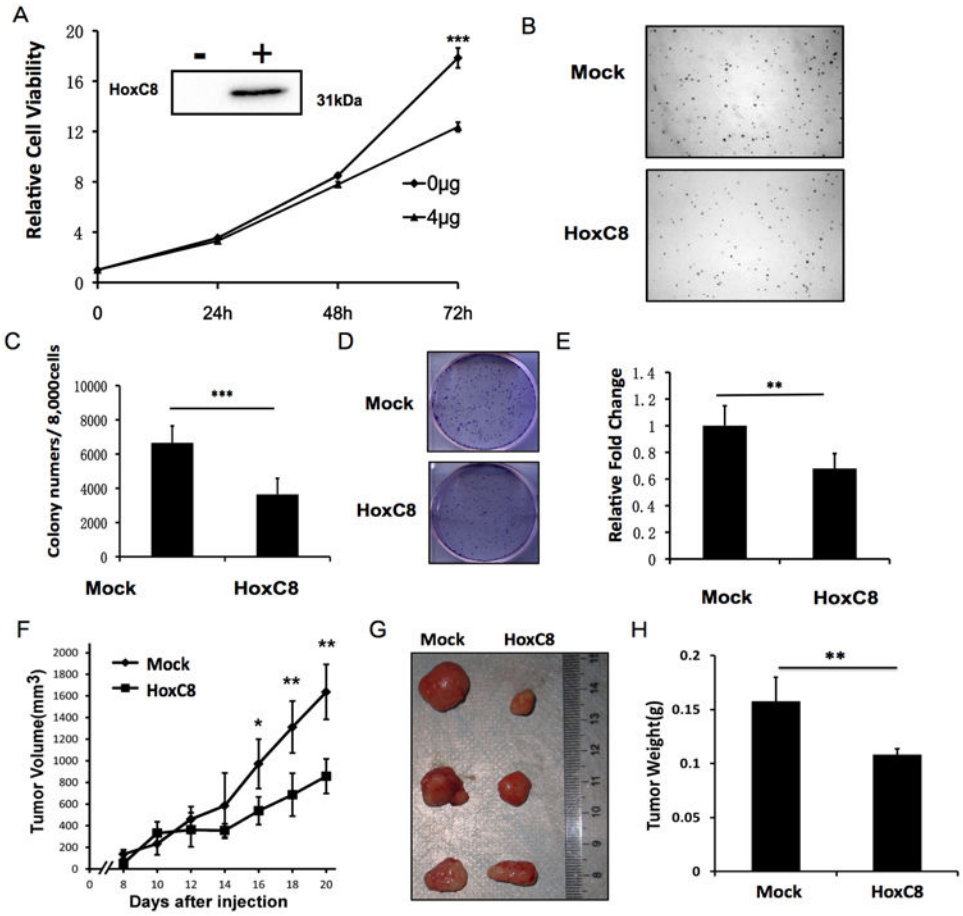

Figure 7. HoxC8 attenuated cell growth and tumorigenesis

(A) MTS assay was applied to assess cell viability in CNE1 NPCs that were mock transfected or transfected with 4μg of a HoxC8 expression vector. Growth in soft agar (B and C) and plate colony formation (D and E) was measured in CNE1-LMP1 cells that stably overexpressed HoxC8. The means and S.D. values were derived from three independent experiments. * p <0.05, ** p <0.01, *** p <0.001. (F, G and H). A xenograft model of tumor growth was established in nude mice to evaluate the ability of HoxC8 overexpression cells and Mock cells to form tumors.

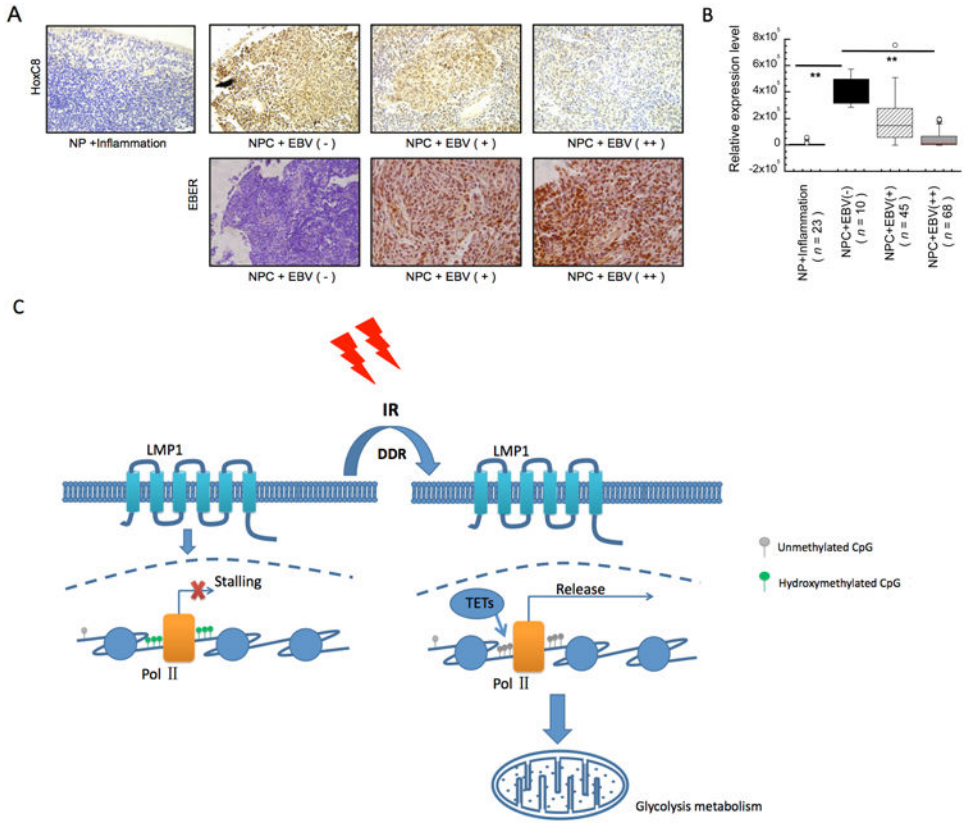

Figure 8. HoxC8 is inversely expressed to EBV in NPC biopsies from a tissue array, and a schematic diagram illustrating a model how stalled Hox genes are linked to glycolysis

(A) Immunohistochemical analysis was used to examine the level of HoxC8 in an NPC tissue array from NPC patients. HoxC8 was expressed at low levels in inflamed nasopharyngeal tissues, whereas it was expressed at high levels in NPC tissues (up). The level of EBER, an infection marker of EBV, was analyzed by ISH in NPC tissues. (B). Expression level of HoxC8 in inflamed nasopharyngeal and NPC tissues. The level of EBER, was analyzed as companied with Hox gene expression level. *n*, number of analyzed samples, * $p < 0.05$, ** $p < 0.01$. (C) A schematic model. Hox genes are aberrantly silenced in LMP1-positive cells via RNA Pol II stalling and 5hmC may contribute to Pol stalling. Irradiation reactivated Hox genes through TET3, which mediates DNA demethylation of 5hmC. The reactivated Hox genes such as HoxC8 inhibit glycolysis and tumorigenesis by inhibiting glycolysis genes and upregulating TCA related genes.

Table 1

Correlation between LMP1 and Hox genes in 73 patient biopsies (n=73)

Gene	Parameter	LMP1
HoxB3	Correlation coefficient	-.574
	Significant (2-tailed)	.016
HoxB13	Correlation coefficient	-.443
	Significant (2-tailed)	.041
HoxC8	Correlation coefficient	-.536
	Significant (2-tailed)	.032