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Epigenetic regulation of cancer stem cell and tumorigenesis

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Abstract

As a unique subpopulation of cancer cells, cancer stem cells (CSCs) acquire the resistance to conventional therapies and appear to be the prime cause of cancer recurrence. Like their normal counterparts, CSCs can renew themselves and generate differentiated progenies. Cancer stem cells are distinguished among heterogenous cancer cells by molecular markers and their capacity of efficiently forming new tumors composed of diverse and heterogenous cancer cells. Tumor heterogeneity can be inter- or intra-tumor, molecularly resulting from the accumulation of genetic and non-genetic alterations. Non-genetic alterations are mainly changes on epigenetic modifications of DNA and histone, and chromatin remodeling. As tumor-initiating cells and contributing to the tumor heterogeneity in the brain, glioblastoma stem cells (GSCs) attract extensive research interests. Epigenetic modifications confer on tumor cells including CSCs reversible and inheritable genomic changes and affect gene expression without alteration in DNA sequence. Here, we will review recent advances in histone demethylation, DNA methylation, RNA methylation and ubiquitination in glioblastomas and their impacts on tumorigenesis with a focus on CSCs.

1. Introduction

Glioma is an umbrella term to describe brain tumors of glial cell origin, accounting for 80% of all malignant brain tumors (Goodenberger & Jenkins, 2012; Schwartzbaum, Fisher, Aldape, & Wrensch, 2006). These encompass astrocytic tumors (World Health Organization classification grades I, II [low grade astrocytoma], III [anaplastic astrocytoma], and IV [glioblastoma]), oligodendrogliomas, ependymomas and mixed glioma. Glioblastoma, also called glioblastoma multiforme (GBM), is the most frequently occurring glioma. The majority of primary glioblastomas (approximately 80%), are de novo diagnosed brain tumors without antecedent history, while secondary glioblastomas slowly progress from lower grade astrocytomas (WHO grade II and III) (Kleihues, Soylemezoglu, Schauble,

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Scheithauer, & Burger, 1995; Thakkar et al., 2014). While there has been progress in treatment modalities, there is still no cure for glioblastomas with a median survival of about 15 months (Koshy et al., 2012; Tran & Rosenthal, 2010).

The standard treatment for glioblastoma is surgical resection, followed by radiation therapy and adjuvant chemotherapy with temozolomide (TMZ). In general, the invasiveness of glioblastoma makes it difficult to remove tumor bulk surgically from normal brain tissue. TMZ is an alkylating agent and can penetrate the blood-brain-barrier (BBB) after oral administration. Intracellular TMZ leads to the transfer of methyl groups to DNA, producing N^3-methyladenine, N^7-methylguanine, and O^6-methylguanine DNA adducts (Newlands, Stevens, Wedge, Wheelhouse, & Brock, 1997). Even though only 5% of alkylation take place on the O^6 position of guanine, it is primarily responsible for the cytotoxicity of TMZ by triggering apoptosis (Roos et al., 2007). O^6-methylguanine-DNA-methyltransferase (MGMT) repairs O^6-alkylguanine lesions stoichiometrically and then self-inactivates. Thus the expression levels of MGMT regulated by the methylation status on its promoter fundamentally impact the therapeutic efficacy of alkylating reagents like TMZ (Brandes et al., 2009; Lee, 2016). Frequent resistance of residual CSCs to TMZ chemotherapy inevitably leads to the recurrence of glioblastomas.

The integrated deregulation of several cellular processes drives malignant transformation. Genetic instability is a key factor for heterogeneity, which is subsequently exacerbated by oxygen and nutrient availability as well as selective pressure from therapies. Also, hierarchical differentiation, a process where CSCs give rise to specialized descendants, leads to heterogeneity. The diversity and heterogeneity of cancer cells underlie diverse responses to therapeutic agents, the emergency of resistant subclones and eventually tumor relapse. Even though the cellular vulnerabilities are defined later, evolution properties of cancer cells under selective pressure confer the ability to escape from the next round therapeutic intervention.

Tumor heterogeneity can be inter- or intra-tumoral, resulting from the accumulation of genetic mutation and non-genetic alterations (Easwaran, Tsai, & Baylin, 2014; Wainwright & Scaffidi, 2017), e.g., epigenetic modification of DNA and histone, and chromatin remodeling. Tumor-initiating cells, including GSCs, contribute to the extent of heterogeneity (Chen et al., 2010; Tang, 2012). Glioblastoma is the first tumor that is systematically analyzed at a multi-dimensional genomic level by The Cancer Genome Atlas Research Network (TCGA) (McLendon et al., 2008). There are three core pathways with biologically relevant alterations in glioblastomas: receptor tyrosine kinase (RTK)/Ras/phosphoinositide 3-kinase (PI3K), p53 and Rb signaling (Brennan et al., 2013; McLendon et al., 2008). Transcription factors and chromatin regulators mediating chromatin remodeling are implicated in many malignant settings (Flavahan, Gaskell, & Bernstein, 2017; Suva, Riggi, & Bernstein, 2013). These epigenetic modifications can enact reversible and inheritable genomic changes and affect gene expression without altering DNA sequence. Here, we will review recent advances in histone demethylation, DNA methylation, RNA methylation and ubiquitination in GSCs and their impacts on tumorigenesis.

2. Histone demethylation

Genomic DNA is packaged with histones in the form of nucleosomes, the fundamental subunit of chromatin, and accessibility of DNA is orchestrated by chromatin structure which can be regulated by posttranslational modifications of histones, i.e., histone code. Histone modifications include methylation, acetylation, phosphorylation, ubiquitination, sumoylation and ADP ribosylation (Bannister & Kouzarides, 2011). Lysine side chains and arginine side chains on histones can be mono-, di- or tri-methylated, and mono or di-methylated, respectively (Black, Van Rechem, & Whetstine, 2012; Greer & Shi, 2012). Several histone methyltransferases and demethylases (KDM) have been identified.

Isocitrate dehydrogenase (IDH1) and IDH2 are frequently mutated in low grade gliomas and secondary glioblastomas, acute myeloid leukemias and other malignancies, resulting in DNA and histone hypermethylation through producing 2-hydroxyglutarate (2-HG) (Dang et al., 2009; Lu et al., 2012; Turcan et al., 2012; Ye, Ma, Xiong, & Guan, 2013). The cellular 2-HG can act as a competitive inhibitor of α-ketoglutarate (α-KG), a product of WT IDH1/2, and antagonize α-KG-dependent dioxygenases, including histone demethylases, KDM4A, KDM4C and KDM2A, and the TET family of 5-methlycytosine (5mC) hydroxylases (Chowdhury et al., 2011; Xu et al., 2011).

In response to RTK signaling inhibition, GSCs can adopt a slow-cycling state (Liau et al., 2017). The survival of GSClines acutely decrease after exposure to inhibitors of oncogenic signaling pathways. However, a viable and slowly proliferating subpopulation of GSCs arise after sustained exposure, and recover to their initial state upon withdrawal of inhibitors, indicating an epigenetic mechanism of drug resistance. Transcriptional profiling demonstrates that cell cycle and proliferation genes are depleted, and genes related to signatures of quiescent neural stem cells and stemness are enriched in persistent GSCs. Among the enriched genes, Notch signaling is demonstrated to drive GSC to switch to a persistent state through Notch1 intra-cellular domain (N1ICD) binding to elements of Notch pathway and neurodevelopmental genes. Chip-seq reveales that activation of the Notch pathway and neurodevelopmental program is accompanied by an increase of histone H3 lysine 27 acetylation and probably facilitated by a global reduction of histone H3 lysine 27 trimethylation (H3K27me3). At the same time, H3K27 demethylases, KDM6A and KDM6B, are up-regulated in drug tolerant GSCs. Knockout of KDM6A or KDM6B inhibits the growth of the persistent GSCs, with no apparent effect on native GSCs. In addition, KDM6A or KDM6B deficient GSCs have less potential to acquire tolerance to drug treatments. GSKJ4, a small molecule inhibitor of KDM6 enzymatic activity, dampen the proliferation of GSC persisters and drug-induced H3K27me3 loss of naive GSCs. Therefore, KDM6 reshaped H3K27me3 landscape of neurodevelopmental genes is required for the transition of GSC to the drug tolerant state.

Lysine-specific histone demethylase 1A (KDM1A, or LSD1) can demethylate histone H3 lysine 4(K3K4), K3K4me2 and K3K4me1 (Shi et al., 2004). KDM1A expression is negatively associated with MYC expression through reducing its H3K4me3 levels in glioblastoma cell lines (Kozono et al., 2015). Depletion of KDM1A promotes the expression of MYC, which in turn activates a set of transcription factors: oligodendrocyte lineage

transcription factor 2 (OLIG2), SRY (sex determining region Y) box 2 (SOX2), and POU class 3 homeobox 2 (POU3F2). These genes confer stem-like traits on glioblastoma cells, thereby promoting tumorigenesis. However, another report establishes that KDM1A is highly expressed in gliomas and positively correlates with the degree of malignancy (Sareddy et al., 2013). KDM1A is also shown to be enriched in glioblastoma cells, especially in GSCs (Zhou et al., 2016). In addition, the nuclear accumulation of GSK3β in GSCs prompts an exploration on whether GSK3β regulates KDM1A protein turnover through the ubiquitin proteasome system. GSK3β is shown to reduce KDM1A ubiquitination and to stabilize KDM1A. Given that several GSK3β consensus phosphorylation motifs are found in KDM1A, point mutation experiments show that serine 683 of KDM1A is a substrate of GSK3β. Moreover, serine 687 phosphorylation mediated by CK1α is a prerequisite for serine 683 phosphorylation in KDM1A by GSK3β. Because GSK3β-induced phosphorylation compromises the ubiquitination of KDM1A, a deubiquitylase (DUB) is predicted to trim ubiquitins and then protect KDM1A from degradation. A screen for DUBs that could stabilize KDM1 demonstrates USP15, USP21, USP22 and USP28 as potential hits, among which ectopic expression of USP22 reduced KDM1A ubiquitination levels strikingly. In vitro deubiquitination assays also demonstrate that USP22 is a deubiquitylase of KDM1A. In GSCs, Knockdown of USP22 causes a decrease in KDM1A protein without affecting KDM1A mRNA expression. Experiments with knockdown of GSK3β or serine 683 mutation of KDM1A prove that GSK3β-mediated phosphorylation of KDM1A is essential for KDM1A binding to UPS22 as well as deubiquitination of KDM1A by USP22 (Zhou et al., 2016). Consistent with previous reports, KDM1A regulates the expression of BMP2, CDKN1A and GATA6 by suppressing the H3K4me2 and the permissive H3K4me3 in their promotors. Depletion of GSK3β or UPS22 weakens the binding of KDM1A to the promoters of BMP2, CDKN1A and GATA6, but enhances the expression of BMP2, CDKN1A and GATA6. These results suggest that GSK3β and UPS22 restrain BMP2, CDKN1A and GATA6 expression upstream of KDM1A. Functionally, KDM1A is required for neurosphere formation of GSCs, and depletion of KDM1A decreases the expression of stem cell markers, Nestin, Oct4 and CD133, while increases the expression of differentiation marker, Tuj1. Likewise, GSK3β and UPS22 are shown to be required for KDM1A-mediated GSCs stemness. Therefore, this signaling axis of GSK3, USP22 and KDM1A facilitates GSC tumorigenesis.

KDM2B is a H3K36me2 demethylase (He, Kallin, Tsukada, & Zhang, 2008), which is crucial for the maintenance of GSCs and its inhibition impairs the expression of Sox2, a transcription factor for self-renewal and tumorigenicity of GSCs (Staberg et al., 2018). The expression of KDM2B in glioblastoma samples is much higher than that in normal brain tissue. In contrast to non-GSC glioblastoma cells, the expression of KDM2B is generally higher in GSCs, and is essential for GSCs stemness. KDM2B silencing induces DNA damage and apoptosis, impairs cell viability and proliferation and sensitizes glioblastoma cells to chemotherapy.

KDM4A, KDM4B, KDM4C and KDM4D belong to KDM4 subfamily of histone demethylases. KDM4A and KDM4C can demethylate H3K9Me3, H3K9me2 and K3K36me3, while KDM4B and KDM4D can only demethylate H3K9Me3 (Cloos et al., 2006; Klose et al., 2006; Whetstine et al., 2006). The function of KDM4 subfamily in

glioblastomas was not reported until recently (Chen et al., 2020). Among these four histone demethylases, KDM4D is the most divergent, due to its lack of both the plant homeodomain (PHD) and Tudor domain. Thus, only the expression levels of KDM4A, KDM4B and KDM4C are usually examined in human glioblastoma specimens. In contrast to KDM4A and KDM4B, KDM4C is highly expressed in glioblastoma samples, verified by comparing KDM4C expression in glioblastomas to that in WHO grade III gliomas. In line with previous reports (Zhang et al., 2011), Wnt signaling is activated in the majority of GSCs and glioblastomas, as verified by phosphorylation of LRP6, a marker of Wnt signaling, and relatively higher mRNA levels of Wnt target genes (Chen et al., 2020). These results imply that KDMC positively correlates with the activity of Wnt signaling in malignant gliomas. Depletion of KDMC abolishes the elevated expression of Wnt signaling target genes in the prescence of Wnt3a, a ligand of the Wnt signaling pathway. In addition, a mRNA array of Wnt signaling targets shows that the expression of almost all Wnt target genes induced by Wnt3a is abrogated by KDM4C knockdown. To confirm whether the catalytic activity of KDM4C is required for Wnt signaling, small hairpin RNA (shRNA)-resistant forms of wild type (WT) KDM4C or a catalytically dead mutant is introduced into KDM4C shRNA expressing cells. WT KDM4C rescues the up-regulation of Wnt3a downstream target genes, whereas the enzymatically dead mutant of KDM4C does not. These results indicate that the histone demethylase activity of KDM4C facilitates the activation of Wnt signaling target genes.

In response to the ligation of Wnt signaling receptors on cell membrane, β-catenin proteins accumulate, translocate to the nucleus from cytoplasm and finally mediate transcriptional activation in cooperation with LEF/TCF transcription factors, for example TCF4 (Clevers, 2006; Clevers & Nusse, 2012). Through Chip-PCR and co-immunoprecipitation assay, KDM4C is shown to bind to TCF4-associated promoters and TCF4 in the presence of Wnt3a. Moreover, the inability of KDM4C to bind to the promoter of the Wnt3a target gene in the absence of TCF4 suggests that TCF4 is essential for recruitment of KDM4C to the promoters of Wnt3a target genes. In cells stimulated with Wnt3a, KDM4C proteins accumulate, bind to β-catenin and co-localize with β-catenin in the nucleus. Although having no effect on Wnt3a-induced up-regulation of KDM4C, ablation of β-catenin inhibits the binding of KDM4C to TCF4 and to the promoter of Wnt target genes in the presence of Wnt3a. These results indicate that β-catenin promotes the recruitment of KDM4C to TCF4 and to promoters of Wnt target genes.

To explore the epigenetic changes of Wnt activation, ChIP assays coupled with western blot analysis demonstrate that Wnt3a stimulation reduces the levels of H3K9me3 and H3K9me2 and increases H3K9 acetylation at TCF-associated region of chromatin. In contrast, Wnt3a stimulation does not affect global H3K9me3 and H3K9me2. These epigenetic alternations induced by Wnt3a are abolished by KDM4C knockdown, suggesting that demethylation of H3K9me3 and H3K9me2 is dependent on KDM4C in Wnt signaling. Heterochromatin protein 1 (HP1) binds to the methylated H3K9 (Cloos et al., 2006; Loh, Zhang, Chen, George, & Ng, 2007; Vakoc, Mandat, Olenchock, & Blobel, 2005). HP1γ, a member of HP1, is shown to occupy TCF4-associated loci, which is suppressed by Wnt3a stimulation. The adverse effect of Wnt3a on chromatin occupation by HP1γ is eliminated by KDM4C or β-catenin deficiency (Chen et al., 2020). These results indicate that KDM4C recruited by β-

catenin to chromatin demethylates H3K9me3, leading to dissociation of HP1γ form Wnt target gene loci.

As mentioned above, Wnt3a stimulation elevates the expression of KDM4C protein but not KDM4C mRNA, probably due to an increased stability of KDM4C protein. β-catenin can be phosphorylated by GSK3β, subsequently recognized and ubiquitinated by β-TrCP and finally degraded through the proteasome (Clevers, 2006; Clevers & Nusse, 2012). These processes can be suppressed by ligation of Wnt signaling receptors. Similar to β-catenin, KDM4C also can be ubiquitinated by β-TrCP and degraded by the proteasome, which can be promoted by GSK3β and restrained by Wnt3a. Through searching for a putative degron of β-TrCP and genetic mutation, it was found that a sequence near serine 918 (S918) of KDM4C was required for the interaction between KDM4C and β-TrCP. S918 of KDM4C is predicted to be phosphorylated by protein kinase R (PKR). An experiment using specific antibodies against phosphorylated S918 of KDM4C proves that KDM4C is a substrate of PKR and this S918 phosphorylation can be inhibited by Wnt3a. PKR depletion also diminishes the interaction between KDM4C and β-TrCP as well as the ubiquitination of KDM4C. Thus, PKR-mediated phosphorylation of KDM4C S918 is essential for the degradation of KDM4C through regulating the interaction between β-TrCP and KDM4C. Because Wnt3a stimulation can inhibit GSK3β-dependent activation of PKR kinase activity, Wnt3a blocks the ubiquitination of KDM4C through suppressing PKR kinase activity.

Given that KDM4C promotes Wnt signaling-dependent transcription and Wnt signaling is activated in GSCs (Zhang et al., 2011), the function of KDM4C was examined. Depletion of KDM4C impedes the growth of GSCs, as well as β-catenin-dependent tumorigenesis of GSCs in an intracranial mouse model. Tumorigenicity of grade III glioma cell lines conferred by Wnt3a overexpression is abolished by KDM4C knockdown. Immunohistochemical analysis of glioblastoma samples also shows the correlation between KDM4C expression and the Wnt signaling marker, phosphorylated LRP6, as well as the expression of a Wnt target gene, c-MYC. Collectively, these results indicate that KDM4C is required for oncogenic activity of Wnt signaling in glioblastomas.

In summary, histone modifications are important epigenetic modifications and several histone methyltransferase and demethylases (KDM) have been identified and implicated in GSC stemness and drug resistance, such as KDM6, KDM1A, KDM2B and KDM4C. Notably, the signaling axis of GSK3, USP22 and KDM1A facilitates GSC tumorigenesis; KDM2B promotes glioblastoma chemoresistance; and KDM4C facilitates the activation of Wnt signaling target genes. Therefore, histone methylation has profound impacts on the properties and gliomagenesis of GSCs.

3. N^6-mA DNA methylation

DNA methylation is one of the most extensively studied epigenetic mechanisms that regulates a plethora of biological processes (Smith & Meissner, 2013). N^6-methyladenine (N^6-mA) is usually found in genomic DNA of bacteria, protists and other lower eukaryotes (Wion & Casadesús, 2006), and has been extensively characterized in metazoans (Fu et al., 2015; Greer et al., 2015; Liang et al., 2018; Liu et al., 2016; Wu et al., 2016; Xiao et al.,

2018; Yao et al., 2017; Zhang et al., 2015). N^6-mA is associated with epigenetic silencing in mouse (Wu et al., 2016), whereas N^6-mA in exons is associated with active expression of genes in human cells (Xiao et al., 2018).

The genomic N^6-mA level is positively associated with the prognosis of cancer and decreased DNA N^6-mA promotes tumorigenesis (Xiao et al., 2018). The function of the N^6-mA DNA modification in glioblastomas was reported (Xie et al., 2018). Dot blot analysis of genomic DNA with antibodies specifically against N^6-mA shows that N^6-mA is significantly increased in GSCs and primary human glioblastoma specimens compared to normal human astrocytes. Higher levels of N^6-mA DNA modification were also detected in GSCs by mass spectrometry and immunostaining relative to normal human astrocytes. The elevation of DNA N^6-mA levels in glioblastomas is histologically verified by a tissue microarray containing normal brain and glioblastoma samples. To investigate the genomic distribution of N^6-mA modification, DNA immunoprecipitation with anti-N^6-mA antibodies followed by next-generation sequencing (N^6-mA DIP-seq) indicates that each chromosome is occupied by N^6-mA modification generally in regions between genes. Gene ontology (GO) analysis reveals that genes adjacent to N^6-mA peaks are enriched in pathways of neurogenesis and neuronal development. Furthermore, the similarity of the N^6-mA modification motif to satellite repeats enriched in constitutive heterochromatic regions indicates that genomic N^6-mA modifications probably suppress neurodevelopmental pathways through facilitating heterochromatin formation.

To elucidate the mechanism of N^6-mA suppressing gene expression, chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) was conducted against two heterochromatin marks, H3K9me3 and H3K27me3, and one euchromatin mark (H3K4me3). More than 80% of N^6-mA peaks overlapped with H3K9me3 and H3K27me3 occupied regions, while only 3.5% of N^6-mA peaks were modified by H3K4me3. Echoing the correlation of N^6-mA modification and heterochromatin histone modification, N^6-mA seems to attenuate the expression of several tumor suppressor genes.

Human ALKBH1 is then shown directly demethylates N^6-mA in oligo-nucleotides as its homolog in murine ESCs (Wu et al., 2016). Moreover, DNA N^6-mA levels are up-regulated by shRNA-mediated ALKBH1 silencing in GSCs. To understand the pathways regulated by ALKBH1 in glioblastomas, RNA sequencing (RNA-seq) and N^6-mA DIP-seq were conducted in the presence or absence of ALKBH1. There are 321 genes down-regulated and 37 genes up-regulated by more than two fold upon ALKBH1 knockdown. Furthermore, 68% of the down-regulated genes overlap with genomic regions with elevated N^6-mA upon ALKBH1 depletion. These results indicate that ALKBH1 knockdown induces gene repression resulting from installation of N^6-mA. Results from an assay for transposase-accessible chromatin using sequencing (ATAC-seq) also demonstrated that the number of genomic loci with decreased chromatin accessibility (1389 sites) is much higher than that with increased accessibility (243 sites). Open chromatin regions shown by ATAC-seq peaks are negatively associated with N^6-mA peaks, suggesting that N^6-mA deposition reduces the chromatin accessibility. Moreover, N^6-mA peaks regulated by ALKBH1 are strongly associated with the heterochromatin histone modifications, H3K9me3. Consistently, genes enriched with N^6-mA modification overlap with those enriched with H3K9me3. H3K9me3

ChIP-seq also shows that after ALKBH1 depletion in GSCs there are more increased H3K9me3 peaks than decreased ones. In addition, the expression of hypoxia response genes are repressed by ALKBH1 depletion. These results indicate that ALKBH1 primarily activates transcription through removing N^6-mA modification and impairing heterochromatin histone modifications.

To evaluate the biological relevance of N^6-mA in glioblastomas, ALKBH1 was investigated in different ways. ALKBH1 knockdown mediated by either shRNAs or single guide RNAs (sgRNAs) blocks proliferation of GSCs. Depletion of ALKBH1 in GSCs compromises their ability to form spheroids, a marker of self-renewal. The survival of animals transplanted with ALKBH1-depleted GSCs is longer than that of animals transplanted with control GSCs. The expression of ALKBH1 in glioblastomas is higher than that in non-tumor brain tissue and positively correlates with the grade of gliomas. Furthermore, the high expression of ALKBH1 in glioblastomas predicts a poor patient survival.

Collectively, as one of the most important epigenetic mechanisms, DNA methylation, particularly N^6-mA, plays an important role in tumorigenicity and CSC stemness. ALKBH1 inhibits heterochromatin formation through erasing N^6-mA modification and promotes oncogenic transcription. Consistently, ALKBH1 is required for the growth and tumorigenicity of GSCs and dysregulation of N^6-mA DNA modification is implicated in glioblastomas.

4. m^6A RNA methylation

Apart from 5′ cap and 3′ polyadenylation, internal modifications of mRNA have also been identified recently, especially after the technical advances in detection of RNA modifications (Dominissini et al., 2012; Meyer et al., 2012). Modified nucleosides on mRNA include but are not limited to N^6-methyladenosine (m^6A), N^1-methyladenosine (m^1A), 5-methylcytosine (5mC) and 5mC oxidized derivative, hydro-xymethylcytosine (hm5C) (Roundtree, Evans, Pan, & He, 2017). Among those modifications, m^6A is the most prevalent internal modification of mRNA, and is enriched around stop codons and in 3′ untranslated regions (3′ UTRs) (Frye, Harada, Behm, & He, 2018; Zhao, Roundtree, & He, 2017). RNA methyltransferases, METTL3, METTL14 and METTL16, act as writers to catalyze m^6A (Liu et al., 2014; Wang, Li, Toth, Petroski, Zhang, & Zhao, 2014; Warda et al., 2017). FTO and ALKBH5 are erasers to remove m6A (Jia et al., 2011; Zheng et al., 2013). Binding proteins of m6A act as readers, such as YTHDF1, YTHDF2, YTHDF3, YTHDC1, YTHDC2 and eIF3 (Li, Chen, et al., 2017; Meyer et al., 2015; Shi et al., 2017; Wang, Lu, et al., 2014; Wang et al., 2015; Wojtas et al., 2017; Xiao et al., 2016; Xu et al., 2014). These writer, eraser and reader proteins regulate mRNA stability, splicing and translational efficiency, implicating m^6A in T cell homeostasis, tumorigenesis, metabolism, reproduction and development (Batista et al., 2014; Cui et al., 2017; Geula et al., 2015; Ivanova et al., 2017; Li, Tong, et al., 2017; Li, Weng, et al., 2017; Mendel et al., 2018; Paris et al., 2019; Tang et al., 2018; Wang et al., 2018; Weng et al., 2018; Zhang et al., 2017; Zhong et al., 2018).

ALKBH5, a RNA demethylase of the m^{6}A modification, regulates nuclear mRNA export and metabolism (Zheng et al., 2013). ALKBH5 contributes to the maintenance and tumorigenicity of GSCs (Zhang et al., 2017). Consistent with the finding that high expression of ALKBH5 is associated with poor prognosis, the expression of ALKBH5 is much higher in glioblastoma cell lines and primary glioblastoma cultures containing GSCs than that in normal astrocyte and low grade gliomas. Histologically, ALKBH5 expression correlates with a transcription factor of self-renewal, Sox2, and a neural stem cell marker, Nestin. These correlations between ALKBH5 and Sox2, as well as Nestin, are also observed in TCGA glioblastoma datasets. These results imply that ALKBH5 is important for properties of GSCs. Malfunction of ALKBH5 attenuates the capacities of tumor-spheroid formation and the expression of transcription factor for GSCs self-renewal, while promoting the differentiation of GSCs. Ablation of ALKBH5 also restrains the proliferation and tumorigenesis of GSCs. FOXM1, a transcription factor known to regulate cell cycle and GSCs self-renewal, is differentially detected by both microarray and m^{6}A sequencing in the presence or absence of ALKBH5. Interestingly, knockdown of ALKBH5 results in reduced expression of FOXM1 at mRNA and protein levels, and the decrease of FOXM1 precursor mRNA levels. Furthermore, ALKBH5 directly interacts with nascent transcripts of FoxM1. Consistent with the demethylase activity of ALKBH5, the m^{6}A levels of FOXM1 pre-mRNA are higher in the ALKBH5 depleted GSCs than that in normal GSCs. There is a unique m^{6}A peak on the 3′-UTR of FOXM1 mRNA and it can be removed by ALKBH5, mirroring the importance of this region. Analysis of the function of 3′-UTR of FOXM1 mRNA using the constructs of FOXM1 CDS with or without 3′-UTR suggestes that 3′-UTR is essential for the regulation of FOXM1 by ALKBH5, as only the expression of FOXM1 construct with 3′-UTR is reduced by ALKBH5 silencing. On the other hand, a long non-coding RNA (lncRNA), referred to as FOXM1-AS, is found to partially complement FOXM1 mRNA. FOXM1-AS directly interacts with ALKBH5, proved by the facts that FOXM1-AS is enriched in ALKBH5 immunoprecipitants and that probes corresponding to FOXM1-AS other than the FOXM1 mRNA complementary region could pull-down ALKBH5 proteins. In addition, FOXM1 pre-mRNA can bind to the FOXM1-AS. Functional studies indicate that FOXM1-AS is required for the interaction between FOXM1 pre-mRNA and ALKBH5, the m^{6}A modification and expression of FOXM1 and tumor growth of GSCs. Therefore, FOXM1 promotes tumorigenesis of GSCs downstream of ALKBH5 and FOXM1-AS.

Coincidently, another group also reported the critical roles of m^{6}A Methylation in GSCs tumorigenesis (Cui et al., 2017). Elevation of m^{6}A levels is observed in differentiated GSCs in contrast to undifferentiated counterparts, indicating that m6A modification may play a part in GSCs differentiation and self-renewal. Knockdown of either METTL1 or METTL4, components of m^{6}A methyltransferase complex, boosts self-renewal and tumorigenicity of GSCs. Conversely, Inhibition of FTO, an RNA demethylase, represses tumor progression and stemness of GSCs. Experiments using RNA sequencing and m^{6}A-seq demonstrate that RNA m^{6}A modification mediated by METTL3 and METTL14 can reprogram the transcript profiles of cancer-associated genes and eventually regulate GSCs-initiated tumorigenicity.

5. Ubiquitination and deubiquitination

Ubiquitin, a conserved small protein consisting of 76 amino acids, is widely expressed in eukaryotic cells. Ubiquitination is a reversible process where ubiquitins are covalently attached to target proteins by a cascade of E1, E2 and E3 enzymes (Scheffner, Nuber, & Huibregtse, 1995; Swatek & Komander, 2016). Ubiquitination regulates protein degradation, cellular trafficking, DNA repair, transcription and signaling transduction (Grabbe, Husnjak, & Dikic, 2011; Mukhopadhyay & Riezman, 2007; Schwertman, Bekker-Jensen, & Mailand, 2016; Weake & Workman, 2008). Ubiquitin can be cleaved from modified protein by deubiquitinating enzymes (DUBs).

PTEN is a tumor suppressor that dephosphorylate phosphatidylinositol-3,4,5-triphosphate (PIP3) and antagonize PI3K/AKT signaling pathway (Lee, Chen, & Pandolfi, 2018). The stability and cellular location of PTEN was tightly regulated by posttranslational modification, including ubiquitination. NEDD4–1 is a E3 ligase for PTEN, promoting the polyubiquitination and the proteasome mediated degradation of PTEN (Wang et al., 2007). Nuclear PTEN is required for DNA repair, genomic stability, cell cycle and tumor suppression (Bassi et al., 2013; Chung & Eng, 2005; Gong et al., 2015; Shen et al., 2007; Song et al., 2011). Monoubiquitination of PTEN at K23 and K289 is essential for PTEN nuclear import (Trotman et al., 2007). PTEN is frequently mutated or deleted in glioblastoma (McLendon et al., 2008). Two mutation sites of PTEN were identified that could regulate its stability and subcellular location in glioblastoma cells (Yang et al., 2017). Through surveying the expression in 16 patient-derived glioblastoma cell lines, PTEN was found to be barely expressed at protein levels in two cell lines, GBM651 and GBM276 which harbored L320S and T277A mutations, respectively, within phosphatase domain and C2 domain of PTEN (Yang et al., 2017). However, L320S and T277A mutations have no effects on lipid phosphatase activity of PTEN. Ectopically expressed PTEN L320S and PTEN T277A have lower protein levels and impaired ability to antagonized PI3K signaling compared to WT PTEN. Even though expressed at similar levels, the capability of reducing PIP3 by PTEN L320S and PTEN T277A is weaker relative to WT PTEN. Because cellular location is important for PTEN activity, and the introduction of L320S or T227A mutations into PTEN compromises its localization on cell membrane and in nucleus, a myristoylation sequence fused to the N-terminal of PTEN L320S could tether PTEN to the cell membrane and alleviated the defect of PTEN L320S in blocking PIP3 signaling. These results indicate that L320S and T227A mutations impair the activity of PTEN through destabilizing PTEN and disrupting its location. Both L320S and T277A mutations alter the conformations and increase the susceptibility of PTEN to ubiquitination. Co-expression of K48R ubiquitin, but not WT ubiquitin, increased the abundance and promoted the nuclear location of PTEN L320S, indicating that mono-ubiquitination of PTEN stabilizes and promotes nuclear localization of PTEN (Yang et al., 2017).

TRIM33 is a member of tripartite motif (TRIM) family proteins that contain a RING-finger domain and belong to the RING-type E3 ubiquitin ligase (Hatakeyama, 2011). TRIM33 mediates the mono-ubiquitination of Smad4 to restrict the TGFβ signaling in the nucleus (Dupont et al., 2009; He et al., 2006). TRIM33 plays an important role in gliomas (Xue et al., 2015). Specifically, TRIM33 constitutively interacts with β-catenin in the nucleus, which

is enhanced by Wnt3a treatment. Overexpression of TRIM33 reduces the protein levels of β-catenin in the nucleus, whereas TRIM33 depletion results in accumulation of nuclear β-catenin. However, TRIM33 knockdown has no effect on the mRNA levels of β-catenin. TRIM33 silencing also increases the half-life of nuclear β-catenin as well as the transcription of β-catenin target genes, cyclin D1 and Axin2. It's known that β-catenin is located on the cell membrane, in the cytoplasm and in the nucleus. Knockdown of TRIM33 increases the protein levels of β-catenin in the cytoplasm and nucleus, but not on the cell membrane. Overexpression of TRIM33 reduces the protein levels of cytosolic and nuclear β-catenin, while overexpression of TRIM33 N-terminally fused to a nuclear export sequence does not. Therefore, TRIM33 regulates the protein levels of nuclear β-catenin directly and cytosolic β-catenin indirectly, presumably through the shuttling of β-catenin between the cytoplasm and the nucleus. Because the proteasome inhibitor, MG132, blocks the reduction of β-catenin protein levels elicited by overexpression of TRIM33, TRIM33 may promote the degradation of β-catenin by the proteasome.

TRIM33 is shown to be an E3 ubiquitin ligase of β-catenin (Xue et al., 2015). Specifically, knockdown of TRIM33 by small interfering RNA (siRNA) or shRNA impairs ubiquitination of nuclear β-catenin. Both deletion of the RING-finger domain and mutation of active sites, C125A/C128A, compromise the ability of TRIM33 to promote ubiquitination of β-catenin in the nucleus. Moreover, K48-only ubiquitin is conjugated to β-catenin in cells overexpressing TRIM33, consistent with that TRIM33 promotes the degradation of β-catenin. Indeed, WT TRIM33 promotes the ubiquitination of β-catenin in vitro, but not TRIM33 lacking the RING-finger domain. It is well known that β-TrCP promotes ubiquitination and degradation of β-catenin in the cytoplasm, which is blocked by Wnt signaling activator. In the presence of Wnt3a, ubiquitination of β-catenin in the cytoplasm is negligible, and β-catenin ubiquitination in the nucleus is inhibited by knockdown of TRIM33, but not by knockdown of β-TrCP. When Wnt signaling is deactivated, the ubiquitination level of nuclear β-catenin decreases, and ubiquitination of cytosolic β-catenin is blocked by depletion of β-TrCP, but not by depletion of TRIM33. These results indicate that TRIM33 is an E3 ubiquitin ligase for β-catenin in the nucleus.

Experiments using truncational mutants of β-catenin suggest that its C-terminal domain is required for β-catenin to bind to TRIM33. Because proteins are usually phosphorylated before being recognized by their E3 ligases, mutations of several potential phosphorylation sites were made in the C-terminal domain of β-catenin. Overexpression of TRIM33 significantly blocks the activity of a Wnt signaling reporter in cells transfected with WT β-catenin, or β-catenin mutants, except the S715A/S718A mutant. The S715A mutation, but not S718A mutation, disables β-catenin to bind to TRIM33. Compared to WT β-catenin or β-catenin S718A, β-catenin S715A displays a longer half-life and little TRIM33-mediated ubiquitination. Bioinformatic analysis indicates that S715 of β-catenin is potentially phosphorylated by PKCδ. The general PKC inhibitor bisindolylmaleimide I, but not the PKCα/β inhibitor Go6976 abolishes the inhibitory effects of TRIM33 on β-catenin-dependent transcription. Among constitutively active and kinase-dead mutants of PKCα, PKCδ and PKCε, only the ectopic expression of constitutively active PKCδ reduces the protein levels of nuclear β-catenin. PKCδ knockdown increases the half-life of nuclear β-catenin and abolishes the interaction between TRIM33 and β-catenin. In vitro kinase assays

demonstrate that constitutively active PKCδ directly phosphorylates WT β-catenin but not β-catenin S715A. Phosphorylation of β-catenin at Ser715 is also detected by a specific antibody, which is abolished by depletion of PKCδ. Furthermore, prolonged Wnt3a treatment induces the phosphorylation of PKCδ at Ser643, an autophosphorylation site (Stempka et al., 1999), and β-catenin. These results indicate that activation of PKCδ serves as a feedback mechanism of prolonged Wnt signaling through promoting the ubiquitination of β-catenin by TRIM33.

There is no tumor formed in the brain injected intracranially with U87 cells, whereas TRIM33 knockdown renders U87 cells tumorigenic, which is abolished by concomitant β-catenin depletion. Moreover, the tumorigenicity of U87/EGFRvIII cells is impaired by ectopic expression of TRIM33, which is rescued by the overexpression of β-catenin S715A, but not WT β-catenin. TRIM33 also inhibits the growth and colony formation of U87/EGFRvIII cells through enhancing the degradation of β-catenin. In addition, tumor tissue derived from U87/EGFRvIII cells exhibits lower protein levels of TRIM33 and higher protein levels of nuclear β-catenin when compared to normal brain tissue. The levels of TRIM33 protein are negatively associated with the levels of the active form of β-catenin in human glioblastomas. The expression of TRIM33 is much lower in tumors relative to the adjacent normal tissues, and is negatively associated with Ser715 phosphorylation of β-catenin. These results indicate that TRIM33 acts as a tumor suppressor in glioblastomas by targeting β-catenin for degradation.

Wnt signaling activation increases the protein levels of FOXM1 (Zhang et al., 2011), and ubiquitination is critical for FOXM1 turnover (Chen et al., 2016). Pulse chase assays demonstrate that the half-life of FOXM1 protein increases in the presence of Wnt3a. However, the mRNA is insensitive to acute Wnt3a treatment. Because GSK3β promotes the degradation of β-catenin in a kinase dependent manner, it is explored if GSK3β participates in regulation of FOXM1 proteins. The protein levels and the stability of FOXM1 significantly increases in GSKα and GSKβ double-knockout cells as compared to WT cells. FOXM1 can bind to GSK through its C-terminal domain. Also, FOMX1 contains three motifs resembling putative substrates for GSK, in which a S474A mutation substantially reduces the ubiquitination levels of FOMX1 in the presence of proteasome inhibitor, MG132. Consistently, phosphorylation of FOXM1 at S474 is enhanced by kinase active GSK3β and the phosphorylated FOXM1 is enriched with ubiquitination in contrast to WT FOXM1. It is known that Axin is a scaffold for the interaction between β-catenin and GSK3 (Clevers, 2006; Clevers & Nusse, 2012). Ablation of Axin disrupts the interaction between Foxm1 and GSK3, abolishes the phosphorylation of FoxM1 at S474 and reduces the ubiquitination of FOXM1. Axin can also bind to FOXM1, which is inhibited by Wnt3a stimulation. Thus, Axin mediates the interaction between FOXM1 and GSK3, which is blocked by Wnt3a. Because FoxM1 can be phosphorylated by GSK3 and ubiquitinated for degradation, degrons in FOXM1 indicate that FBXW7 may be the E3 ligase. Ubiquitination of FOXM1 is increased by overexpression of FBXW7, which is further enhanced by constitutively active GSK3β. Taken together, FOXM1 is phosphorylated at S474 and further ubiquitinated by FOXW7, which is facilitated by GSK3β and Axin1.

To identify the DUB of FOXM1, a panel of ubiquitin-specific proteases (USPs) was screened and USP5 was found to increase the protein level of FOXMB1 in the presence of Wnt3a. USP5 can bind to FOXM1, which is enhanced by administration of Wnt3a and attenuated by expression of constitutively active GSK3β. Overexpression of USP5 impairs the ubiquitination of FOXM1 and depletion of USP5 diminishes the ability of Wnt3a to reduce the ubiquitination of FOXM1. Thus, FOXM1 is deubiquitinated and stabilized by USP5 upon wnt3a stimulation.

Inhibitor of β-catenin and TCF4 (ICAT) is a negative regulator of Wnt signaling by disrupting the interaction between β-catenin and TCF4 (Tago et al., 2000). Previous reports indicate that FoxM1 and ICAT bind to the same region of β-catenin (Tago et al., 2000; Zhang et al., 2011). Indeed, FoxM1 and ICAT compete for binding to β-catenin (Chen et al., 2016). Consistent with a previous report (Daniels & Weis, 2002), ICAT suppresses the recruitment of β-catenin to TCF-binding elements of Wnt target genes. However, FoxM1 promotes the binding of β-catenin to TCF-binding elements. Moreover, the transactivation of Wnt signaling reporter by overexpression of FOXM1 is blocked by overexpression of ICAT, and vice versa. These results suggest that FOXM1 activates β-catenin dependent transcription by competing with ICAT.

Overexpression of USP5 accelerates the proliferation of glioblastoma cells, which is abrogated by knockdown of FOXM1. Ectopic expression of FOXM1 promotes cell growth, which is blocked by β-catenin depletion or ICAT overexpression. In a panel of glioblastoma samples, the expression levels of USP5, FOXM1 and a Wnt signaling target gene, cyclin D1, appeare to correlate with one another. In conclusion, these results demonstrate that deubiquitination of FoxM1 by USP5 can activate Wnt/β-catenin signaling and is clinically relevant to glioblastomas.

As the enzymatic subunit of the Polycomb repressive complex 2 (PRC2), the enhancer of Zeste homolog 2 (EZH2) catalyzes the di- and trimethylation of H3K27, which contributes to chromatin compaction and gene repression (Margueron & Reinberg, 2011). EZH2 overexpression is associated with several types of cancers, including glioblastomas (Kim & Roberts, 2016; Lee et al., 2008; Suva et al., 2009). In breast cancer cells, EZH2 can physically bind to estrogen receptor α and β-catenin, promote transcriptional activation of estrogen and Wnt pathways, and accelerate cell cycle progression (Shi et al., 2007). Reduction of EZH2 protein levels and its stability are initially observed in β-catenin-knockout mouse embryonic fibroblasts (MEFs) as compared to WT MEFs (Ma et al., 2019). Knockdown of β-catenin also leads to the decline of EZH2 protein levels in GSCs. Conversely, overexpression of constitutively active β-catenin increases the protein levels and the stability of EZH2, while reducing the ubiquitination of EZH2. However, manipulating the expression of β-catenin does not affect the mRNA levels of EZH2. These results indicate that β-catenin suppresses ubiquitination and promotes stabilization of EZH2.

Among DUBs, only USP1 can stabilize EZH2 and restrain its ubiquitination. USP1 knockdown results in EZH2 protein levels decline in GSCs. USP1 and EZH2 interact with each other and overexpression of USP1 diminishes the ubiquitination of EZH2, which is further enhanced by the overexpression of USP1 binding partner, WDR48. However, both

catalytically inactive USP1 and WDR48 alone fail to decrease the ubiquitination of EZH2. Moreover, EZH2 is deubiquitinated in vitro by USP1/WDR48. Thus, USP1 is a deubiquitinating enzyme of EZH2.

Then it was examined whether β-catenin could enhance the stability of EZH2 through USP1. According to TCGA database, USP1 correlates with β-catenin and TCF4 at transcriptional level in glioblastomas. Gain of function and loss of function experiments indicate that β-catenin positively regulates the expression of USP1. In addition, β-catenin directly binds to the promoter of USP1, which is enhanced by Wnt3a stimulation. Luciferase reporter assays using USP1 promoter or mutants lacking TCF4 binding sites demonstrate that the activity of WT USP1 promoter is increased by Wnt3a treatment and reduced by β-catenin inhibition or deficiency. However, the activity of mutant promoter is insensitive to Wnt3a stimulation and β-catenin inhibition or deficiency. The reduced expression of EZH2 caused by β-catenin deletion can be restored by USP1 overexpression. These results indicate that β-catenin transcriptionally activates USP1 which in turn stabilizes EZH2.

Knockdown of USP1 reduces the binding of EZH2 to the promoters of its target genes, as well as H3K27me3 levels of these promoters (Ma et al., 2019). The expression of EZH2 target genes is increased by depletion of EZH2, USP1 or β-catenin in GSCs. Moreover, elevated expression of EZH2 target genes triggered by knockdown of USP1 is abolished by overexpression of K421R/Y641F mutant of EZH2 which is resistant to degradation by the proteasome (Sahasrabuddhe et al., 2015; Yu et al., 2013). The proliferation of GSCs is suppressed by depletion of β-catenin, USP1 or EZH2, which is rescued by overexpression of EZH2 K421R/Y641F mutant. Furthermore, the tumorigenicity of GSCs is abolished by knockdown of β-catenin, USP1 or EZH2. The expression of USP1 is also found to histologically correlate with the expression of nuclear β-catenin and EZH2 in human glioma specimens. The protein levels of β-catenin, USP1 and EZH2 are enriched in glioblastomas in contrast to that in low grade gliomas. These results indicate that EZH2 stabilization mediated by USP1 contributes to β-catenin driven tumorigenesis of gliomas.

In summary, FOXM1-β-catenin signaling is important for tumorigenesis, including glioblastomas. Both FOXM1 and β-catenin are regulated by ubiquitination. TRIM33 promotes the degradation of β-catenin in the nucleus by acting as an E3 ubiquitin ligase. Activation of PKCδ serves as a feedback mechanism of prolonged Wnt signaling through promoting the ubiquitination of β-catenin by TRIM33. Thus, TRIM33 acts as a tumor suppressor in glioblastomas by targeting β-catenin for degradation. On the other hand, FOXM1 is phosphorylated at S474 by GSK3 and further ubiquitinated by FOXW7, which restrains β-catenin transcription activity. In contrast, USP5 deubiquitinates and stabilizes FOXM1 upon Wnt3a stimulation. Furthermore, β-catenin could transcriptionally activate USP1 which in turn deubiquitinates and stabilizes EZH2. At last, EZH2 contributes to gliomagenesis driven by β-catenin. Therefore, the ubiquitination and deubiquitination play critical roles in tumorigenesis of gliomas.

6. Outlook

CSCs underlie cancer development, progression and recurrence. Their maintenance and tumorigenicity are regulated at multiple levels including genetic and epigenetic mechanisms. Included in important epigenetic modifications are methylation of histone, DNA and RNA, and ubiquitination. Further figuring out these epigenetic changes in CSCs will be fundamentally of importance to understand cancer pathogenesis, paving the way to identify molecular biomarkers and design effective targeted therapies for cancers, including glioblastoma.

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