G9a, a multipotent regulator of gene expression

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Abbreviations

ANK, Ankyrin; ATRA, All Trans retinoic acid; β^{maj}, β globin major; β^{minor}, β globin minor; BAT8, HLA-B-Associated Transcript 8; BDNF, Brain-derived neurotrophic factor; CA1, Cornu ammonis 1; Camk2a, Calcium/calmodulin-dependent protein kinase II alpha; CARM1, Coactivator-associated arginine methyltransferase 1; Cdc 14B, CDC14 cell division cycle 14 homolog B; Cdk 5, Cyclin Dependent Kinase 5; CDP/cut, CCAAT displacement protein/cut homolog; CDYL1, Chromodomain Y-Like protein; CEBP β,CCAAT/ Enhancer Binding Protein beta; CLBC, Claudin low breast cancer; CSB, Cockayne syndrome group B protein; CSF-2, Colony-stimulating factor-2; CST7, Cystatin-7; COMT, Catechol O Methyltransferase; DIM-5, Defective In Methylation-5; DNMT1, DNA Methyltransferase 1; E^{y}, Embryonic □ like globin; EC, Entorhinal cortex; EMT, Epithelial to mesenchymal; Ep-CAM, Epithelial Cell Adhesion Molecule; FosB, FBJ murine osteosarcoma viral oncogene homolog B; Gfi1, Growth factor independent 1 transcription repressor; GLP, G9a-like-protein; GR, Glucocorticoid Receptor; GRIP1 , Glutamate receptor interacting protein 1; HDAC1, Histone Deacetylase 1; HIV1, Human immunodeficiency virus 1; HP1, Heterochromatin Protein; IL6, Interleukin 6; IL8, Interleukin 8; JAK2, Janus Kinase 2; JARID 1a, Jumonji, AT rich interactive domain 1; KLF-12, Kruppel-Like factor-12; LIF, Leukemia inhibitory Factor; L-LTP, Late phase long-term potentiation; LSH, Lymphoid-specific helicase; LTM, Long term memory formation; Magea, Melanoma antigen family A, 1; mAM, mouse ATFa-associated Modulator; MHC, Major Histocompatibility Complex; MMP-9, Matrix metalloproteinsase-9; Msx1, Msh homeobox 1; NAc, Nucleus accumbens; NF-kB, Nuclear Factor Kappa light chain enhancer of activated B Cells; NLS, Nuclear localization signals; NRSF, Neuron restrictive silencing factor; PCNA, Proliferating cell nuclear antigen; PIC, Pre initiation Complex; PPARγ, Peroxisome proliferator-

activated receptor gamma; Runx2, Runt-related transcription factor 2; Runx3, Runt related transcription factor 3; SAM, S-adenosyl-L-methionine; SASP, Senescent Associated Secretory Phenotype; SDF-1, Stromal differentiating factor-1; SET, **S**u(var)3-9, **E**nhancer of Zeste, **T**rithorax ; SETDB1, SET domain, bifurcated 1; SETDB2, SET domain, bifurcated 2; Sharp1, Enhancer-of-split and hairy-related protein; SIAH1, Seven In Absentia Homolog; SNF2, Sucrose NonFermentable 2; TA-CA1, Temporo ammonis–cornu ammonis 1; UHRF1, Ubiquitin-like with PHD and ring finger domains 1; WIZ, Widely interspaced zinc finger motifs protein; YY1,Ying and Yang 1.

Abstract

Lysine methylation of histone and non-histone substrates by the methyltransferase G9a is mostly associated with transcriptional repression. Recent studies, however, have highlighted its role as an activator of gene expression through mechanisms that are independent of its methyltransferase activity. Here we review the growing repertoire of molecular mechanisms and substrates through which G9a regulates gene expression. We also discuss emerging evidence for its wide-ranging functions in development, pluripotency, cellular differentiation and cell cycle regulation that underscore the complexity of its functions. The deregulated expression of G9a in cancers and other human pathologies suggests that it may be a viable therapeutic target in various diseases.

G9a structure and activity

In an attempt to identify genes related to autoimmune disorders in the unexplored regions of MHC class III cluster on human chromosome 6, an undefined gene G9a or BAT8 was found using a combination of chromosome walking with overlapping cosmid genomic clones and pulsed field gel electrophoresis[1,2]. The human G9a/EHMT2 protein which was initially thought to contain 1001 amino acids was later found to lack NG36 transcripts[3]. Further investigations revealed that full length G9a protein (isoform a) is a product of both NG36 (encoded by 4 exons) and G9a (encoded by 24 exons) transcripts[4] (Fig 1). In addition, a shorter isoform has been characterized (isoform b) which lacks exon 10. The mouse homolog, found on chromosome 17, has two alternatively spliced isoforms as well. The long isoform (G9a L) lacks exon 1 and includes all of exon 2, while the short isoform (G9a S) contains exon 1 but splices out part of exon 2 to result in a N-terminal region resembling the human homolog. A closely related paralog GLP/EHMT1[5] has been identified which exhibits 45% identity with human G9a and differs primarily in the N-terminus[6].

G9a belongs to the SET domain containing Su(var)3-9 family of proteins which include Suv39h1/h2, SETDB1, SETDB2, and DIM-5[7–11]. G9a contains an evolutionarily conserved SET domain which contributes to its methyltransferase activity, a domain containing ankyrin repeats which is involved in protein-protein interactions[3,7] and nuclear localization signals that have been identified at the N-terminal region[12]. The catalytic SET domain with variable I-SET insert is flanked by pre- and post-SET regions consisting of a pseudo knot. The substrate binding groove is formed by I-SET and post-SET domains[13] . When transiently expressed, G9a and GLP form homomeric and heteromeric complexes via their SET domain; however, endogenously they function exclusively as a heteromeric complex in a wide variety of human and mouse cells[14].

Biochemical studies have revealed that G9a and GLP possess the same substrate specificities on histones. Both enzymes have histone 3 (H3) lysine 9 (K9) methyltransferase activity *in vitro*[5,7]. G9a transfers methyl groups from S-adenosyl-L-methionine (SAM) to ε-amino group of the target lysine residue, resulting in mono- and di-methylation of K9 (H3K9me1 and H3K9me2 respectively), and to a lesser extent, K27of H3[7]. Two tyrosine (Y) residues of G9a are of particular importance for catalytic activity and H3K9 di-methylation specificity. Y1154, participates in catalysis and Y1067 is involved in hydrogen bonding with the nitrogen atom of the ε-amino group of the substrate lysine residue. Loss of Y1154 renders G9a catalytically inactive, whereas replacement of Y1067 to phenylalanine allows G9a to tri-methylate H3K9 (H3K9me3). The proposed model of G9a action involves interaction of the peptide substrate at the rigid I-SET domain[12]. Concurrent binding of SAM at the flexible post-SET domain stimulates domain folding and orients the remaining peptide-binding residues. As such the post-SET domain can "close" onto the substrate to complete the substrate-binding groove and allow catalysis to occur. This concurs with the random order of substrate and co-factor binding as previously suggested[15]. Commercially available compounds BIX-01294 and UNC0638 act as competitive inhibitors of G9a activity by binding to the substrate binding groove and render it inaccessible to substrates[16,17].

Substrate recognition and specificity: G9a interacts with amino acid residues 6-11 of H3, and the minimum substrate recognition criterion *in vitro* is a seven amino acid heptapeptide with RK/ARK consensus e.g. 6-T**ARK**STG-12 of H3[18–20]. In the H3 peptide, arginine (R) residue at position 8 adjacent to a lysine residue is an absolute requirement for G9a activity; any other amino acid substituted at this position abolishes methylation of the H3 peptide substrate. Other than the RK specificity, G9a exhibits a preference for hydrophilic amino acids at position 6;

smaller amino acids such as alanine (A) are preferred at position 7, whereas hydrophilic and hydrophobic amino acids respectively are favoured at positions 10 and 11[20]. Apart from these positional preferences, modification of H3 proximal amino acids serine (S)10 and threonine (T)11 by phosphorylation impairs methylation by G9a[18,20]. In addition to H3, the G9a recognition motif is found in several non-histone proteins. In fact, G9a itself has such a motif at its non-catalytic N-terminus which is automethylated[18,21]. Auto-methylation of K239 (mouse) does not result in a loss of its catalytic activity indicating that methylation does not render G9a inactive. The auto-methylated G9a peptide resembles tri-methylated H3K9 (H3K9me3). Similar to H3K9me3 associated HP1 interaction, auto-methylated G9a peptide interacts with HP1 which might stage recruitment of other repressors such as HDAC1 and DNMT1[18]. G9a also methylates other methyltransferases such as mAM**,** an activator protein of SetDB1, and GLP, suggesting a complex internal regulation of these histone methyl transferases[18,21]. Several additional non-histone substrates have been identified including WIZ, CDYL1, CSB, ACINUS, HDAC1, DNMT1, and KLF12[20]. While the functional relevance of methylation of non-histone proteins has not been established in each case, G9a-mediated methylation of Reptin at K67 under hypoxic conditions results in Reptin-dependent inhibition of hypoxia responsive genes, providing a mechanism to adjust the cellular response to hypoxia[22]. Many transcription factors are methylated by G9a which appears to negatively impact their transcriptional activity. For instance, G9a methylates CEBP β at K39 which interferes with activation of myeloid genes[23,24]. Similarly, MyoD methylation at K104 blocks its transcriptional activity and expression of muscle differentiation genes[25], and G9a/GLP complex methylates p53 at K373 rendering it transcriptionally inactive[26].

Changing partners and switching roles: from repressor to activator

Whilst the vast number of studies have implicated its function in mediating H3K9me2 marks that is associated with transcriptional repression, in erythroid cells, G9a exhibits a more complex role in regulating transcription of the human β globin gene[27]. G9a is recruited to the β globin locus in a NF-E2/p45-dependent manner. Interestingly, G9a represses embryonic globin E^y expression, whereas it activates the adult β globin β^{maj} gene. Through pull-down assays followed by mass spectrometry, G9a was found to associate with the H3K4 demethylase Jarid1a at the embryonic E^y globin promoter resulting in silencing of its expression. On the other hand, G9a recruits Mediator only to the β^{maj} promoter, activating its expression[28]. Thus differential interaction with Jarid1a and Mediator in distinct co-repressor and co-activator complexes accounts for its opposing impact at the E^y and β^{maj}-globin promoters. Similar to these findings, other studies have also reported G9a to act as a co-activator together with GRIP1, CARM1 and p300 for nuclear receptors in a methyltransferase activity-independent manner[29]. G9a is recruited by ligand-activated GR bound to GR-binding sites where it functions as a scaffold for recruitment of p300 and CARM1 to positively regulate gene expression. The N-terminal region of G9a is sufficient to bring about recruitment/stabilization of this activator complex in response to dexamethasone treatment[30]. G9a is also recruited by Runx2 to MMP-9, CSF-2, SDF-1, and CST7 promoters to activate their expression independent of its methyltransferase activity[31]. From these studies, it appears that the role of G9a as an activator does not require its methyltransferase activity; and suggest its function as a repressor or activator depends upon its interacting partners (Fig 2).

Besides regulation of RNA polymerase II transcription, G9a has also been found to regulate RNA polymerase I-mediated transcription. This occurs through interaction of G9a with CSB, resulting in H3K9 methylation at the coding region of ribosomal RNAs (rRNAs)[32].

Moreover, both ATPase activity of CSB and methyltransferase activity of G9a are required for association of RNA polymerase I to rDNA. In view of this, a role for G9a has been proposed in the inactivation of actively-transcribed rRNAs in preparation for transcriptional re-initiation.

Function in development and differentiation

G9a is widely expressed in most tissues including fetal liver, bone marrow, peripheral blood leukocytes, thymus, lymph node, spleen and developing skeletal muscles[4,33]. Loss of G9a in mice results in embryonic lethality between embryonic (E) days E9.5-E12.5. Even though no abnormalities are seen, G9a$^{-/-}$ embryos at E9.5 morphologically resemble wild type embryos at E8.0-E8.5. This growth retardation is attributed to increased apoptosis rather than growth arrest. Interestingly, G9a$^{-/-}$ embyronic stem (ES) cells do not show overt growth defects in culture conditions, but exhibit severe differentiation defects, suggesting a role for G9a in commitment and differentiation[34]. Consistent with this notion, there is substantial evidence that G9a plays a prominent role in early development by repression of Oct-3/4, Nanog and DNMT3L which are required for maintenance of pluripotency in ES cells. Thus Oct-3/4 and Nanog expression is sustained until E7.5 in G9a-null embryos, indicating the importance of G9a in the silencing of pluripotent genes at early differentiation stages[35]. Post-implantation repression of these pluripotency genes is achieved through establishment of methylated H3K9 heterochromatinization via the SET domain, as well as through *de novo* DNA methylation by recruitment of DNMT3a/3b that associate with the ANK repeats of G9a. The establishment of these two separate epigenetic marks prevents reprogramming of ES cells to an undifferentiated state. Consistently, in contrast to wild type cells, G9a$^{-/-}$ ES cells differentiated with retinoic acid revert back to a pluripotent state by expressing Oct-3/4 and Nanog when re-cultured in LIF-containing medium[36,37]. In addition to control of ES cell pluripotency, G9a has also been

implicated in genomic imprinting. While G9a is not required for X-chromosome inactivation[38], it is recruited by non-coding RNAs *Kcnq1ot1* and *Air* to respective target domains in a lineage-specific manner and stimulate the formation of heterochromatin to cause monoallelic expression of specific genes[39,40].

G9a is recruited by LSH, a member of SNF2 family of ATP-dependent chromatin remodelling enzymes, to generate normal DNA methylation patterns and stable gene silencing during lineage commitment and differentiation of distinct cell types[41]. Progressive H3K9me2 patterning occurs in a G9a/GLP dependent manner during lineage specification of hematopoietic stem and progenitor cells (HSPCs)[42]. H3K9me2 marks are deficient in primitive HSCs and are formed *de novo* during lineage commitment, with highest levels in megakaryocytes and T cells. Treatment of HSPCs with UNC0638 results in preservation of stem cell-like phenotype and function during *in vitro* expansion. An increase in G9a-mediated H3K9me2 also occurs during retinoic acid-induced differentiation of the leukemic cell line HL-60, concomitant with decreased JAK2 expression. G9a is recruited to the JAK2 promoter to repress its transcription in a YY1-dependent manner[43]. G9a is also important in Th2 cell differentiation. $CD4^+$ T cells from conditional knockout mice in which G9a is specifically deleted in T cells fail to differentiate into Th2 cells resulting in impaired cytokine production[44].

In contrast to these studies, G9a expression levels and H3K9me2 repression marks decline during differentiation of skeletal muscle precursor cells[25]. Overexpression of G9a in myoblasts negatively regulates differentiation, and this is accompanied by elevated H3K9me2 marks on muscle promoters as well as methylation of MyoD, a key regulator of skeletal myogenesis. MyoD methylation inhibits its transcriptional activity and the consequent activation of its downstream targets involved in differentiation[25,45]. Recruitment of G9a to muscle

promoters is mediated by the bHLH transcription factor Sharp-1, as well as by the homeoprotein Msx1[33,46]. Similar to its impact on skeletal myogenesis, G9a represses differentiation of adipocytes, which is dependent upon H3K9me2-mediated inhibition of PPARγ expression. In addition, G9a enhances Wnt10b expression, an inhibitor of adipogenesis, in a SET-domain independent manner[47].

In the nervous system, G9a/GLP complex functions as master regulator of lineage specific gene expression in the brain. Recruitment of G9a by NRSF is important in the repression of neuronal genes outside of the nervous system[48]. Similarly, conditional ablation of GLP/G9a in postnatal neurons of the forebrain using the Camk2a-Cre mice results in expression of early neuronal progenitor genes in various regions of the brain[49]. These changes led to brain impairment with reduced exploratory behaviour and locomotor activity, which manifested as a mental retardation-like syndrome in adult mice. The phenotype closely resembles human 9q34 mental retardation syndrome that involves the sub-telomeric deletion of human chromosome 9(9q34), including the GLP gene. Recently, G9a/GLP were also found to play a role in memory consolidation[50]. Inhibition of GLP/G9a activity with BIX-01294 in the hippocampus disrupted LTM formation, and in the EC, enhanced memory consolidation, by increasing expression of memory permissive genes. This differential regulation of genes in the hippocampus and EC highlights the function of G9a in molecular cross talk between the two brain regions during memory consolidation. G9a also plays an important role in cocaine-induced structural and behavioural plasticity[51]. Repeated administration of cocaine decreases G9a mRNA levels and global H3K9me2 repressive marks in NAc, resulting in increased gene expression. Repression of G9a and H3K9me2 in the NAc neurons promotes cocanine addiction and conversely, its overexpression has a reciprocal effect. Together these studies highlight the relevance of

H3K9me2 in maintaining transcriptional homeostasis in adult neurons, and underscore the function of GLP/G9a in mental retardation, defects in learning, environmental adaptation and motivation in mice and humans.

Role in cellular proliferation, senescence and cancer

A large body of evidence has indicated a role for G9a in cellular proliferation, quiescence, senescence and replication. Recruitment of G9a by CDP/cut, UHRF1 and Gfi1 to the $p21^{Cip/Waf1}$ promoter results in repression of its expression[52–54]. Consistent with these studies, pharmacological inhibition of G9a activity with BIX-01294 reduces proliferation of foetal pulmonary arterial smooth muscle cells that is accompanied by G_1 cell cycle arrest and elevated $p21^{Cip/Waf1}$ expression. Moreover, migration and contractility was also inhibited with altered global methylation levels[55]. In addition to its role in G_1 arrest, G9a was reported to be present in a complex with E2F6 and other polycomb group proteins on E2F responsive promoters in G_0 cells[5]. These studies indicate the involvement of G9a in repression of E2F responsive genes in quiescent cells. Similarly, G9a has also been shown to repress HIV1 gene expression and maintain quiescence of the latent HIV1 provirus by mediating H3K9me2 on the HIV1 long terminal repeat promoter[56].

DNMT1 levels are reduced in senescent cells and knockdown of DNMT1 in human diploid fibroblasts correlates with a decrease in G9a/GLP expression. During Ras-induced senescence, proteosomal degradation of G9a and GLP occurs through APC/C^{cdh1}, an ubiquitin ligase. Cdc14B and p21 activate APC/C^{cdh1}, thereby degrading G9a/GLP and causing decreased H3K9 di-methylation globally as well as on IL-6 and IL-8 promoters, which are major components of senescence-associated secretory phenotype. Knockdown of Cdh1 abolishes IL-6

and IL-8 expression in senescent cells, indicating the importance of both G9a/GLP and APC/C^{cdh1} in senescence[57].

During replication, DNMT1 complexes with G9a at replication foci, coordinating DNA and H3K9 methylation, indicating that both molecules work together to restore heterochromatin at replication forks during the S phase[58]. Gene expression analysis performed on G9a conditional knockout mouse ESCs revealed a strong correlation between H3K9me2 and late replication genes including Magea1 and Dub1a. This study indicates that G9a creates facultative heterochromatin by mediating H3K9me2 within late replicating chromatin at the nuclear periphery[59]. Consistent with its role during replication, $G9a^{-/-}$ ESCs exhibited significant delay in progression through S phase. Interestingly, G9a mono-methylates H3K56 (H3K56me1), which acts as a docking site for PCNA during G_1 phase, and thus facilitates PCNA functionality during the S phase[60].

Given its role in cellular proliferation, it is not surprising that G9a expression is high in many cancers compared to normal tissue. Cancer transcriptome analysis has revealed high G9a expression in many tumors including hepatocellular, colon, prostate, lung and invasive transitional cell carcinomas, and in B cell chronic lymphocytic leukemia[26]. In a number of human bladder and lung carcinoma patients[61], G9a expression is up-regulated. Knockdown of G9a in both bladder and lung cancer cell lines causes growth suppression and apoptosis with an increase in sub-G_1 population. By microarray and chromatin immunoprecipitation experiments on G9a knockdown cells, the tumor suppressor SIAH1[62,63] was identified to be transcriptionally regulated by G9a[61]. Studies on prostate cancer further corroborate the role of G9a in carcinogenesis, where downregulation of G9a causes centrosome disruption, chromosomal instability, inhibition of cell growth and increased cellular senescence in cancer cells[64]. G9a is

also involved in repression of Runx3 expression which is inactivated in gastric cancer[65]. In Runx3-expressing gastric cell lines, hypoxia increases the accumulation of G9a and H3K9me2 which leads to Runx3 gene silencing. Overexpression of G9a in gastric cancer cells grown under hypoxic conditions leads to further repression of Runx3 expression and deletion of the SET domain in G9a results in a failure to repress Runx3 under both normoxic and hypoxic conditions.

In aggressive lung cancer, high levels of G9a correlate with poor prognosis with increased cell migration and invasion *in vitro*, and metastasis *in vivo*. The metastatic function of G9a in lung cancer is due to repression of Ep-CAM[66]. Knockdown of G9a in lung cancer cells leads to de-repression in Ep-CAM expression and decrease in H3K9me2 levels along with reduced levels of DNMT1, HP1 and HDAC1 recruitment at the Ep-CAM promoter. Similar studies in CLBC have uncovered the role of G9a in promoting EMT by repression of E-cadherin expression[67]. Repression of E-cadherin is brought about by the interaction of Snail with G9a through its ANK and SET domain. HP1 and other DNA methyltransferases are recruited to the E-cadherin promoter required for EMT-induced E-cadherin promoter DNA methylation and H3K9me2. Knockdown of G9a reduced H3K9me2 and DNA methylation marks and rescued the expression of E-Cadherin, preventing cell migration and invasion *in vitro* and decreased tumor growth and metastatic potential of these cells to colonize the lungs *in vivo* in CLBC metastasis models. Thus by targeting and blocking the association of the Snail-G9a-DNMTs complex, novel therapies could be developed to target CLBCs.

Future perspectives

The abundance of data on G9a and its associated H3K9 methylation activity has led to the view that G9a is a co-repressor that silences genes found at euchromatic regions. Nevertheless, the fact that G9a has been shown to interact with the Mediator complex and exists in distinct co-activator complexes highlight a new role for G9a in transcriptional activation. It remains to be seen whether, similar to the control of embryonic and adult β-globin expression in differentiating erythroid cells, G9a exerts such dual activities on regulation of other cell fate decisions. As such more work has to be done to determine the significance of this newly discovered function of G9a. Regardless, these studies highlight the importance of elucidating G9a interacting partners and relevant complexes that dictate its activity. In addition, the transcription factors that recruit G9a to target promoters in various cell types need to be identified.

The fact that G9a is over-expressed in many different types of cancers, and has been shown to be responsible for various aspects of tumorigenesis, including cellular differentiation, proliferation and EMT, indicates that G9a may be a feasible target for cancer therapy. This supports the prevailing perception that de-regulation of the epigenome in cells is a contributing, and possibly causative, factor of oncogenesis. Given its myriad roles, it would also be important to investigate the role of G9a in fear-related memory disorders, mental retardation, addictive disorders, metabolic disease, as well as skeletal muscle development and regenerative potential. This will lead to a greater understanding of the pathologies in which G9a is involved, and, as with cancer, the potential for using G9a inhibitors as therapeutic avenues. The efficacy of commercially available G9a inhibitors in cancer and other disease models however remain to be tested.

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Figure Legends

Figure 1: Schematic representation of G9a domain structure. Human and mouse G9a isoforms are shown with their respective NCBI accession ID (left). The domain structure was constructed using DOG 1.0 software. The Cysteine (Cys) rich region, ankyrin repeats (ANK) and the catalytic SET domain with flanking Pre SET and post SET regions are shown. The site for methylation (Me),nuclear localization signal (NLS), and the glutamic acid (E) rich region are denoted. Numbers indicate amino acid residues.

Figure 2: Transcriptional repression and activation by G9a. G9a recruitment by transcription factors and association with distinct co-factor complexes leads to opposing outcomes on gene expression. Transcriptional repression of genes such as myogenin, p21, JAK2 and embryonic β-globin is dependent on G9a methyltransferase activity (SET domain). On the other hand, G9a recruitment by GR, Runx2, and NF-E2/p45 leads to activation of target genes in a SET-domain independent manner. This occurs through association of G9a with co-activators such as p300 and CARM1, as well as the Mediator complex.