A post-translational modification switch controls coactivator function of histone methyltransferases G9a and GLP

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Abstract

Like many transcription regulators, histone methyltransferases G9a and G9a-like protein (GLP) can act gene-specifically as coregulators, but mechanisms controlling this specificity are mostly unknown. We show that adjacent post-translational methylation and phosphorylation regulate binding of G9a and GLP to heterochromatin protein 1 gamma (HP1 γ), formation of a ternary complex with the glucocorticoid receptor (GR) on chromatin, and function of G9a and GLP as coactivators for a subset of GR target genes. HP1 γ is recruited by G9a and GLP to GR binding sites associated with genes that require G9a, GLP, and HP1 γ for glucocorticoid-stimulated transcription. At the physiological level, G9a and GLP coactivator function is required for glucocorticoid activation of genes that repress cell migration in A549 lung cancer cells. Thus, regulated methylation and phosphorylation serve as a switch controlling G9a and GLP coactivator function, suggesting that this mechanism may be a general paradigm for directing specific transcription factor and coregulator actions on different genes.

Keywords Aurora kinase B; G9a; glucocorticoid receptor; methylation; phosphorylation

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Post-translational Modifications, Proteolysis & Proteomics

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Introduction

DNA-binding transcription factors activate and repress transcription of their target genes by recruiting coregulator proteins to the promoter/enhancer regions of their target genes. Coregulators remodel chromatin structure and promote or inhibit the assembly of an active transcription complex. Most of the known coregulators were discovered either for their roles in transcriptional activation

or repression. However, many coregulators, including the lysine methyltransferases G9a and G9a-like protein (GLP), function in both activation and repression of transcription, depending on the specific gene and cellular environment [1–5]. The factors that determine whether transcription factors and coregulators positively or negatively regulate a specific target gene are mostly unknown.

Many coregulators regulate local chromatin structure by adding post-translational modifications (PTM) to histones. While methylation of histone H3 at lysine 9 (H3K9) is an extremely abundant repressive histone mark in heterochromatin made by several different coregulators, it is also found in euchromatin at repressed promoter/enhancer regions and in the gene bodies of actively transcribed genes [6]. Histone methyltransferases G9a (also known as EHMT2 or KMT1C) and G9a-like protein (GLP, also known as EHMT1 or KMT1D) are the major H3K9 methyltransferases in euchromatin and are responsible for the majority of mono- and dimethylation of H3K9 in most if not all mammalian cell types [7]. G9a and GLP repress many genes involved in a variety of cellular processes in embryonic development and adult tissues [8,9] and are overexpressed in a variety of human cancers, where they repress important tumor suppressor genes [10]. However, G9a functions also as a coactivator for several transcription factors, including steroid hormone receptors (SR) [4,11,12], RUNX2 [13], and hematopoietic activator NF-E2 [14]. G9a coactivator function has been implicated in physiological processes, such as adult erythroid cell differentiation [14] and T helper cell differentiation and function [15].

Whether transcription factors and coregulators act positively or negatively on a specific gene target presumably depends upon signals, such as protein–protein interactions and PTM, arising from the unique local regulatory environment of each target gene. Here, we investigate the role of PTM in controlling whether G9a and GLP act as coactivators, using as our model system genes regulated by the glucocorticoid receptor (GR, also known as NR3C1), a steroid hormone-activated transcription factor, in A549 lung cancer cells. In addition to histones, G9a also methylates some non-histone proteins involved in transcriptional regulation

[10], including itself. G9a is auto-methylated on lysine 185 (K185) and phosphorylated, at least *in vitro*, by Aurora kinase B on threonine 186 (T186) in the N-terminal domain of the protein [16,17]. Heterochromatin protein 1 gamma (HP1 γ , also known as CBX3) specifically binds the K185-methylated form of G9a, and this binding is inhibited by T186 phosphorylation [17], but the biological function of these two PTMs and of the G9a interaction with HP1 γ is unknown.

G9a forms heterodimers with its paralogous partner GLP in cells. As they share a similar sequence in their N-terminal domain, we tested whether methylation and phosphorylation occur at the homologous sites on GLP. Moreover, in these cells, G9a potentiates gene activation and gene repression on distinct subsets of GR target genes and is selectively recruited to GR binding regions (GBR) associated with GR target genes that require G9a as a coregulator, indicating that G9a acts directly on these target genes [4]. As we previously showed that the N-terminal domain of G9a, which includes these two PTM sites, is required for the coactivator function of G9a in the context of SR [12], and since HP1 γ has previously been shown to act as a coactivator as well as a corepressor [18], we hypothesized that these PTMs and HP1 γ could be involved in the regulation of the coactivator function of G9a and GLP. Here, we report the effects of point mutations at the PTM sites and of inhibitors of methylation and phosphorylation on the ability of G9a and GLP to form ternary complexes with GR and HP1 γ and to cooperate with HP1 γ as coactivators for glucocorticoid regulation of transient reporter genes and a subset of endogenous GR target genes that require both G9a and GLP as coactivators. Additional endogenous genes that are activated by GR but do not require G9a or GLP for this activation serve as important internal controls to demonstrate the gene-specific mechanisms of the coactivator functions and gene-specific requirements for G9a, GLP, and HP1 γ . The results support an important role for these G9a and GLP PTMs and HP1 γ in G9a and GLP coactivator function and thus provide key insights into the mechanisms that control whether G9a exerts positive regulation on specific target genes. At the physiological level, we also explore the involvement of G9a and GLP as coactivators for GR regulation of genes that control cell migration and other cellular functions.

Results

G9a and GLP methylation is required for recruitment of HP1 γ to a complex with GR

To study possible effects of G9a and GLP methylation in cells, we first confirmed sites of G9a methylation and identified sites of GLP methylation. The sequence in the N-terminal domain of human G9a (hG9a) containing the methylation site is highly conserved with hGLP (Fig 1A). Purified N-terminal domains of hG9a and hGLP or the mutant version with substitutions for the putative methylated lysines (K185R and K205R, respectively) were incubated with [3 H-methyl]S-adenosylmethionine (SAM) and a recombinant hG9a C-terminal fragment (amino acids 735–1,210, hG9a Δ N) containing the enzymatic activity. Fluorography showed that N-terminal fragments of both hGLP and hG9a are methylated by hG9a Δ N (Appendix Fig S1A). Substitution of K185 of hG9a or K205 of hGLP

with arginine strongly decreased methylation. These data indicate that hG9a methylates hG9a and hGLP primarily on K185 and K205, respectively, *in vitro*.

In order to determine if G9a and GLP are methylated in cells, we found a pan-methyllysine antibody (developed to recognize methyl-lysine on a variety of methylated proteins) that did not recognize an unmethylated recombinant hG9a N-terminal fragment (amino acids 1–280) but interacted strongly with the G9a N-terminal fragment after *in vitro* methylation by hG9a Δ N (Fig 1B, upper left panel). In contrast, the same N-terminal hG9a fragment with a K185R mutation was not recognized by the pan-methyllysine antibody after incubation in the methylation reaction, confirming K185 as the methylation site. Using the same approach, we found that hGLP is also auto-methylated on K205 (Fig 1B, lower right panel). The N-terminal fragments of both G9a and GLP were methylated by the C-terminal fragment of either G9a or GLP (Fig 1B, upper and lower panels). Thus, while intramolecular auto-methylation is possible, G9a and GLP methylation can occur *in trans*.

The pan-methyllysine antibody also recognized (by immunoprecipitation or immunoblot) wild-type full-length hG9a transiently expressed in Cos-7 cells, but not full-length hG9a with the K185R mutation (Fig 1C, left panel), confirming that G9a in cells is methylated on K185. Similarly, full-length hGLP transiently expressed is methylated on the K205 (Fig 1C, right panel). In addition, the signal from this antibody was strongly decreased when cells expressing wild-type hG9a or hGLP were treated with small molecule inhibitors (UNC0646, UNC0638, UNC0642) specific for G9a and GLP methyltransferase activity [19,20] (Fig 1D, Appendix Figs S1B and C), or treated with the general SAM-dependent methylation inhibitor adenosine dialdehyde (Adox) (Appendix Fig S1D), confirming that the signal detected on G9a and GLP in cells by the pan-methyllysine antibody is due to methylation. We also detected methylation of endogenous G9a and GLP in A549 human lung adenocarcinoma cells (Fig 1E), which were the primary cells used for G9a and GLP functional analyses in this study; in multiple experiments, there was no consistent change in the G9a or GLP methylation level in response to dexamethasone (dex), the synthetic GR agonist used in this study. When A549 cells were treated with G9a/GLP methyltransferase inhibitor UNC0646, the endogenous level of G9a and GLP increased, but the proportion of G9a and GLP that was methylated decreased substantially (Appendix Fig S1E). The decreased methylation signal further validates the methylation of endogenous G9a and GLP, while the increased levels of G9a and GLP indicate that methylation somehow influences G9a and GLP protein production or turnover, but additional experiments are required to test the latter possibilities.

To explore the role of G9a/GLP methylation in binding to GR and coregulators HP1 γ , GRIP1, p300, and CARM1 in the context of GR signaling, we first performed co-immunoprecipitation experiments with wild type and methylation site mutants of G9a and GLP. GR interacts in a hormone-independent manner with G9a via its N-terminal domain [4] and also with GLP (Appendix Fig S2A). Mutation of the methylation site (K185) did not affect GR binding to G9a as determined by co-immunoprecipitation (Appendix Fig S2B), indicating that G9a methylation is not involved in its interaction with GR. Similarly, mutation of the G9a methylation site did not affect its previously described interaction with coregulators GRIP1, p300, and CARM1 [4,11] (Appendix Fig S2C). It was

Figure 1. G9a and GLP are methylated on their N-terminal domain in cells.

A Schematic representation of the related proteins GLP (EHMT1) and G9a (EHMT2). N: N-terminal coactivator domain, E: polyglutamate domain, Cys: cysteine-rich region, ANK: six ankyrin repeats, SET: SET-domain containing methyltransferase activity. Partial protein sequence of hG9a and hGLP homologs shows the hypothetical methylated lysine residues (K) in red.

B After protein methylation reactions, *in vitro* methylated proteins were detected by immunoblot with pan-methyllysine antibody (pan met-K). The corresponding Coomassie-stained gels are shown as loading controls. SAM, S-adenosylmethionine.

C Cos-7 cells were transfected with plasmids encoding full-length HA-hG9a wild type or K185R mutant, or full-length HA-hGLP wild type or K205R mutant. Lysates were immunoprecipitated (IP) with pan met-K antibody and immunoblotted with HA antibody (top), or the usage of the two antibodies was reversed (bottom). Expression of HA-tagged proteins and β -actin (loading control) in the unfractionated extracts is shown at the bottom (Input).

D Cos-7 cells were transfected with a plasmid encoding full-length HA-hG9a and treated with 2 μ M UNC0646 or vehicle DMSO for 24 h. Lysates were immunoprecipitated with pan met-K antibody and immunoblotted with HA antibody (top), or the usage of the two antibodies was reversed (bottom).

E Methylation and phosphorylation of endogenous G9a and GLP in A549 cells treated with 100 nM dex for 4 h were analyzed by immunoprecipitation with control IgG antibody, anti-G9a (top), or anti-GLP (bottom), followed by immunoblot with antibodies listed. Expression of G9a, GLP, and β -actin (loading control) in the unfractionated extracts is shown at the bottom (Input).

previously shown that G9a methylation is essential for its interaction with HP1 γ [17]. Likewise, when wild-type G9a or GLP or the corresponding methylation site point mutants were overexpressed in Cos-7 cells, the methylation site mutations almost eliminated co-immunoprecipitation of G9a and GLP with HP1 γ (Fig 2A and B). Interestingly, GR also co-precipitated with HP1 γ in these

experiments, but only very weakly unless wild-type G9a or GLP was coexpressed (Fig 2A and B, Appendix Fig S2B), indicating that the auto-methylation site is important for the formation of a ternary complex (GR-G9a/GLP-HP1 γ), with either G9a or GLP binding HP1 γ via the methylated lysine site and binding GR through a different site.

Figure 2. G9a and GLP methylation is required for HP1γ-G9a/GLP-GR ternary complex formation.

- A** Cos-7 cells were transfected with plasmids encoding hGR and full-length HA-hG9a wild type or the K185R mutant. Lysates supplemented with 15 U/ml of DNase I were immunoprecipitated with HP1γ antibody and immunoblotted using antibodies listed.
- B** Cos-7 cells were transfected with plasmids encoding hGR and full-length HA-hGLP wild type or the K205R mutant and were treated and analyzed as in (A).
- C** To analyze interaction of endogenous GR and HP1γ by PLA, A549 cells were treated with 100 nM dex or the equivalent volume of vehicle ethanol (Eth) for 2 h. After cell fixation, PLA with antibodies against GR and HP1γ was performed. The detected interactions are indicated by red dots. The nuclei were counterstained with DAPI (blue). The number of interactions detected by ImageJ analysis is shown as the mean $\pm$ SEM of three independent experiments. P -value was determined using a paired t -test. $**P \leq 0.01$. Scale bar represents 10 μ m.
- D** PLA was conducted as in (C) after transfection of A549 cells with siRNA for G9a (siG9a), GLP (siGLP), or non-specific siRNA (siNS) and treatment of cells with 100 nM dex for 2 h. Detected interactions are shown as the mean $\pm$ SEM of three independent experiments. P -value was determined using a paired t -test. $***P \leq 0.001$. Scale bar represents 10 μ m. Whole-cell extracts were analyzed for G9a, GLP, GR, HP1γ, and β -actin expression by immunoblot.
- E** PLA was conducted as in (C) after treatment of cells with 2 μ M UNC0646 or equivalent volume of vehicle DMSO for 24 h and with 100 nM dex for the final 2 h. Detected interactions are shown as the mean $\pm$ SEM of three independent experiments. P -value was determined using a paired t -test. $**P \leq 0.01$. Scale bar represents 10 μ m.

Importantly, we confirmed these observations for the endogenous proteins in A549 cells using proximity ligation assay technology (PLA). With this technique, protein–protein interactions are visualized by immunofluorescence, where each red dot represents a single molecular complex [21]. HP1γ interacted with G9a in nuclei of A549 cells in a hormone-independent manner (Appendix Fig S2D); depletion of either protein with siRNA eliminated most of the signal, validating the interaction and the antibodies used to detect it (Appendix Fig S2E). Moreover, we established stable cell lines where expression of wild-type or K/R mutant G9a or GLP (containing an N-terminal HA-tag) is doxycycline inducible. In this system, HP1γ interacted significantly less with G9a/GLP methylation site mutants than with wild-type G9a/GLP (Appendix Fig S3A and B). Moreover, HP1γ also associated with GR, and this interaction was highly dependent on treatment of cells

with dex (Fig 2C), presumably due to the nuclear localization of GR caused by dex; depletion of HP1γ further validated the detection of the complex by PLA (Appendix Fig S2F). The dex-induced GR-HP1γ interaction was also inhibited by the depletion of G9a or GLP (Fig 2D), thus validating the ternary complex GR-G9a/GLP-HP1γ. Depletion of GLP also caused depletion of G9a protein (Fig 2D), since the stability of G9a protein depends on the presence of GLP [22]. Therefore, while G9a is clearly required for the association between GR and HP1γ, we cannot conclude whether GLP is also directly involved. GR-HP1γ interaction in PLA was also strongly decreased when cells were treated with G9a/GLP methyltransferase inhibitor UNC0646 (Fig 2E), consistent with our observation that G9a/GLP methylation is crucial for GR-G9a/GLP-HP1γ ternary complex formation. Moreover, overexpression of the methylation site mutant of G9a or GLP (but not overexpression of

wild-type G9a or GLP) inhibited the GR-HP1 γ interaction (Appendix Fig S3C and D). Thus, G9a and/or GLP nucleates a ternary complex with GR and HP1 γ , and methylation of G9a K185 or GLP K205 is required for their interactions with HP1 γ .

G9a and GLP phosphorylation by Aurora kinase B antagonizes HP1 γ recognition

Since Aurora kinase B (also known as AURKB) was previously shown to phosphorylate G9a at T186 in a cell-free reaction [17], we tested whether this occurred in cells. Using an approach similar to that described above for detecting methylation, we validated a pan-phosphothreonine antibody to detect G9a phosphorylation at T186 in cells. The pan-phosphothreonine antibody recognized overexpressed wild-type G9a but not the T186A mutant in immunoprecipitation and immunoblot experiments, thus indicating that G9a is phosphorylated on T186 in Cos-7 cells (Fig 3A, left panel). Likewise, we demonstrated for the first time that GLP is phosphorylated in cells on T206 (Fig 3A, right panel). Depletion of Aurora kinase B from Cos-7 cells with siRNA strongly decreased the phosphorylation detected by immunoprecipitation with the pan-phosphothreonine antibody followed by immunoblot with antibody against the HA epitope-labeled G9a or GLP (Fig 3B, upper panel), confirming that Aurora kinase B phosphorylates G9a and GLP in cells. Using the same detection strategy, we demonstrated that endogenous G9a and GLP are phosphorylated in A549 cells, in a hormone-independent manner (Fig 1E). Interestingly, inhibition of G9a and GLP phosphorylation by depleting Aurora kinase B from cells increased the interaction between HP1 γ and G9a or GLP (Fig 3B, lower panels).

Consistent with this result, inhibition of Aurora kinase B activity with a specific inhibitor (ZM443979) decreased G9a and GLP phosphorylation signals (Appendix Fig S4C) and increased the interaction between HP1 γ and G9a (Appendix Fig S4D). However, inhibition of Aurora kinase B activity did not affect GR or HP1 γ phosphorylation (Appendix Fig S4E). Overexpression of Aurora kinase B had the opposite effect, decreasing the HP1 γ -G9a interaction (Appendix Fig S4F). Furthermore, we found that mutations of either the methylation site (K185) or phosphorylation site (T186) of G9a inhibited co-immunoprecipitation of G9a and GR with HP1 γ (Appendix Fig S4G). A phospho-mimic mutation T186E prevented co-immunoprecipitation of G9a and GR with HP1 γ , confirming the effect of the phosphorylation on the binding of HP1 γ to G9a. Also, we observed that the T186A mutation decreased the interaction between G9a and HP1 γ , presumably because unmodified T186 is part of the recognition sequence of HP1 γ . As a control, we showed that the methylation site mutation did not prevent phosphorylation of G9a in cells, and the phosphorylation site mutation did not prevent methylation (Appendix Fig S4A). Similarly, in cell-free methylation reactions the phosphorylation site mutation did not prevent G9a methylation (Appendix Fig S4B). We conclude that G9a or GLP phosphorylation by Aurora kinase B in cells prevents HP1 γ recognition.

G9a and GLP coactivator function requires HP1 γ and is regulated by auto-methylation and phosphorylation

As G9a and GLP PTMs occur in the N-terminal domain that is required for the coactivator function, we investigated the role of

Figure 3. G9a and GLP phosphorylation in cells by Aurora kinase B antagonizes HP1 γ recognition.

- A** Cos-7 cells were transfected with plasmids encoding full-length HA-hG9a wild type or T186A mutant, or full-length HA-hGLP wild type or T206A mutant. Lysates were immunoprecipitated with pan phospho-threonine antibody (IP pan ph-T) and immunoblotted with HA antibody (top), or the usage of the two antibodies was reversed (bottom).
- B** Cos-7 cells were transfected with a plasmid encoding HA-hG9a or HA-hGLP and siRNA against Aurora kinase B (siAuroraB) or non-specific siRNA (siNS). Lysates were immunoprecipitated with pan ph-T antibody and immunoblotted with HA antibody (top). Then, lysates were immunoprecipitated with HP1 γ antibody and immunoblotted with indicated antibodies (bottom).

G9a and GLP PTMs in the regulation of their coactivator function, first using transient luciferase reporter genes. As shown previously [11], G9a is not a very effective coactivator for steroid receptors by itself but acts cooperatively with coactivator GRIP1. Thus, when GR and coregulator GRIP1 were overexpressed by transient transfection of CV-1 cells, dex-induced expression of a GR-regulated reporter gene was enhanced by coexpression of full-length G9a (Fig 4A, bars 4–5). In contrast, the K185A and K185R mutants of full-length G9a were significantly less active (Fig 4A, bars 6–9), although mutant and wild-type hG9a were expressed at similar levels. Similar results were obtained when the N-terminal fragment of hG9a (amino acids 1–280 with wild-type sequence or substitutions for K185) was used instead of full-length G9a (Appendix Fig S5A), consistent with our previous finding that this N-terminal fragment is necessary and sufficient for G9a coactivator function in these transient reporter gene assays [12]. Thus, the lysine at residue 185 is required for the full coactivator function of G9a in this assay. Likewise, in the same system, dex-induced expression of the GR-regulated reporter gene was enhanced by coexpression of full-length GLP (Fig 4B, bars 4–5), indicating that GLP, as well as G9a, can act as a coactivator of GR. In contrast, the K205R mutant of GLP is less active (Fig 4B).

If K185 methylation is necessary for G9a coactivator function, then we would expect that the N-terminal fragment must be methylated in order to function as a coactivator; but G9a catalytic activity is localized in the C-terminal domain, suggesting that methylation of the N-terminal fragment would need to occur *in trans*. We found that the N-terminal fragment of G9a is indeed methylated when overexpressed in Cos-7 cells, but at a lower efficiency compared with overexpressed full-length G9a (Appendix Fig S5B), and treatment of the cells with the G9a/GLP inhibitor UNC0646 decreased methylation of the N-terminal fragment (Appendix Fig S5C) as well as full-length G9a (Fig 1D). This result indicates that G9a and GLP can be methylated *in trans* in cells. Consistent with this, methyltransferase assays *in vitro* with G9a and GLP fragments also demonstrated that methylation of G9a or GLP can happen *in trans* (Fig 1B).

Since phosphorylation of G9a on T186 or GLP on T206 inhibits binding to HP1 γ (Fig 3), we next studied the impact of G9a and GLP phosphorylation on its coactivator function. In transient luciferase reporter gene assays, the coactivator function of G9a and GLP, in cooperation with GRIP1, was significantly enhanced by the specific Aurora kinase enzyme inhibitor ZM447439 (Fig 4C and D, bars 6–7 in comparison with bars 4–5). This finding further supports the roles of G9a/GLP PTMs and HP1 γ in G9a and GLP coactivator function.

To characterize the effect of these PTMs on the endogenous target genes that are induced by dex-activated GR, we used gene expression microarray profiling to identify genes that require G9a and GLP for activation by dex and GR. The subset of GR target genes positively regulated by G9a in A549 cells was already identified by comparing cells expressing shRNA against G9a (shG9a) with cells expressing a non-specific shRNA (shNS) [4]. A similar analysis with shGLP was performed in parallel with the previously published shG9a analysis and is reported here (Dataset EV1). As indicated above (Fig 2D), both GLP and G9a were depleted by shGLP in the samples analyzed by microarray (Fig 5A). We identified 1,254 genes for which mRNA level was significantly different

(no fold cutoff was imposed) in the 24-h dex-treated shGLP cells versus the dex-treated shNS control cells (Fig 5B). The expression of 2,271 genes was significantly changed by at least 1.5-fold after 24 h of dex treatment, and 415 of the total 2,271 dex-regulated set of genes also belonged to the GLP-regulated gene set (Fig 5B). By comparison, 122 of the 2,271 dex-regulated genes were also significantly regulated by G9a [4], and the majority of the G9a-regulated gene set overlapped with the GLP-regulated gene set. Of the 415 genes significantly regulated by dex and GLP, 240 (116 + 124 in Appendix Fig S6A) were repressed by dex and 175 (67 + 108 in Appendix Fig S6A) were activated by dex. Interestingly, from the 175 genes that were activated by dex and significantly regulated by GLP, 108 were induced less upon GLP depletion, indicating a putative coactivator function for GLP on these genes (Appendix Fig S6A and Fig 5C, darker bars). Moreover, the great majority among these 108 genes that required GLP for their dex-induced expression also required G9a for optimal dex-induced expression (Fig 5C, lighter bars), as indicated by the negative fold change in expression due to GLP or G9a depletion (by comparing gene expression profiles in the dex-treated cells expressing shNS and the dex-treated shGLP or shG9a cells). Even if they were not always significantly regulated by G9a, the effect of G9a depletion in the previous microarray analysis [4] was in the same direction as that for GLP depletion. However, there were a few GR target genes that were strongly dependent on GLP as a coactivator for their dex-induced expression, but were affected little or not at all by depletion of G9a (Fig 5C). This demonstrates that although G9a and GLP largely supported the same genes, there was a smaller number of GR target genes that required GLP but not G9a for dex-induced expression.

As validation of the microarray results, quantitative RT-PCR showed that depletion of G9a or GLP by siRNAs (Appendix Fig S6B) significantly decreased dex-induced expression of specific G9a- and GLP-dependent GR target genes (Fig 5D, left panel), but had little or no effect on dex-induced expression of genes that do not require G9a or GLP (Fig 5D, right panel). The GR target genes selected for validation and further mechanistic studies included three genes that were significantly dependent on GLP for dex-induced expression in the microarray analysis of 24-h-dex-treated cells (CDH1, CDH16, and PPL), one gene that was not quite significant in the above shGLP microarray but required GLP significantly after shorter periods of dex treatment (HSD11B2), and one gene that was previously shown to be G9a-dependent for dex-induced expression (ENaC α , also called SCNN1A) [4]; three GR target genes that were not dependent on G9a or GLP for dex-induced expression were also chosen, to serve as controls in various functional studies. In addition to these properties, these genes were selected because of strong response to dex, making it easier to observe effects of coregulator depletion, and well-documented GR binding sites associated with them [23].

As we previously demonstrated that methylation of G9a K185 and GLP K205 facilitates recruitment of HP1 γ (Fig 2), we next analyzed the importance of HP1 γ for dex-induced expression of endogenous GR target genes that are positively regulated in A549 cells by G9a or GLP. We depleted HP1 γ using a pool of four siRNAs (Appendix Fig S6C) and measured mRNA levels of the same eight endogenous GR target genes. Dex-induced levels of mRNAs for the G9a- and GLP-dependent genes, CDH16, ENaC α ,

Figure 4. G9a and GLP PTMs regulate their coactivator function.

- A CV-1 cells were transfected with MMTV-LUC reporter plasmid (200 ng) and plasmids encoding GR (1 ng), Grip1 (100 ng), and HA-labeled full-length (FL) hG9a wild type or K185A or K185R mutants (150 or 400 ng) as indicated. Cells were grown with 100 nM dex or the equivalent amount of ethanol for 48 h and assayed for luciferase activity. Relative luciferase units are normalized to sample 3 and represent mean $\pm$ SEM for eight independent experiments. *P*-value was calculated using a paired *t*-test. **P* $\leq$ 0.05, ***P* $\leq$ 0.01. Whole-cell extracts were analyzed for G9a expression by immunoblot with anti-HA antibody.
- B Transient reporter gene assays were performed as in (A) with HA-labeled hGLP WT or hGLP K205R (150 or 400 ng) as indicated. Relative luciferase units are normalized to sample 3 and represent mean $\pm$ SEM for six independent experiments. *P*-value was calculated using a paired *t*-test. **P* $\leq$ 0.05.
- C Transient reporter gene assays were performed as in (A) after transfected cells were treated or not with 100 nM dex and 2 μ M ZM447439 (ZM) or equivalent volume of DMSO for 48 h as indicated. Relative luciferase units are normalized to sample 3 and represent mean $\pm$ SEM for four independent experiments. *P*-value was calculated using a paired *t*-test. ****P* $\leq$ 0.001.
- D Transient reporter gene assays were performed as in (C), except with hGLP instead of hG9a. Relative luciferase units are normalized to sample 3 and represent mean $\pm$ SEM for four independent experiments. *P*-value was calculated using a paired *t*-test. **P* $\leq$ 0.05.

PPL, HSD11B2, and CDH1 were significantly reduced by HP1 γ depletion (Fig 5E, left panel), indicating a positive regulatory effect of HP1 γ . However, induction of mRNAs from G9a- and

GLP-independent GR target genes, FKBP5, FOXO1, and CITED2, by dex was not affected by HP1 γ depletion (Fig 5E, right panel). Depletion of pairs or all three of the G9a, GLP, and HP1 γ

Figure 5. G9a and GLP act as coactivators for a subset of endogenous GR target genes.

- A Immunoblot showing GLP, G9a, and tubulin protein levels in whole-cell extracts from A549 cells that were transduced with a control lentivirus encoding a non-specific shRNA (shNS) or lentivirus encoding an shRNA targeting GLP (shGLP).
- B Large black Venn diagram represents the total number of dex-regulated genes from the microarray analysis (q -value ≤ 0.01 and at least 1.5-fold increase or decrease) for cells transfected with siNS and treated with 100 nM dex for 24 h compared with ethanol. Blue Venn diagram represents the number of GLP-regulated genes with significantly different expression (q -value ≤ 0.05) in dex-treated cells expressing shGLP versus dex-treated cells expressing siNS. Small purple Venn diagram represents the number of G9a-regulated genes with significantly different expression (q -value ≤ 0.05) in dex-treated cells expressing shG9a versus dex-treated cells expressing siNS [4]. Overlap areas indicate the number of genes shared among sets.
- C For all 108 dex-induced genes that require GLP as a coactivator according to microarray analysis (x-axis), the $\log_2$ fold change due to GLP depletion for the 24-h-dex-induced mRNA levels is shown by blue bars (y-axis). The $\log_2$ fold change for the same genes caused by G9a depletion [4] is shown by superimposed purple bars.
- D A549 cells transfected with non-specific siRNA (siNS) or with SMART-pool siRNA targeting G9a (siG9a) or GLP (siGLP) were treated with 100 nM dex for the indicated times (0-h dex indicates ethanol treatment for 8 h). mRNA levels for the indicated GR target genes were measured by reverse transcriptase followed by qPCR and normalized to β -actin mRNA levels. Results shown are mean $\pm$ SEM for four independent experiments. P -value was calculated using a paired t -test. * $P \leq 0.05$, ** $P \leq 0.01$.
- E mRNA levels for the indicated GR target genes were determined as in (D), using A549 cells transfected with non-specific siRNA (siNS) or with SMART-pool siRNA targeting HP1 γ (siHP1 γ). Results shown are mean $\pm$ SEM for five independent experiments. P -value was calculated using a paired t -test. * $P \leq 0.05$, ** $P \leq 0.01$.
- F mRNA levels for the indicated GR target genes were determined as in (D), using A549 cells, which were not transfected with siRNA 1 h prior to hormone or ethanol treatment, 2 μ M ZM447439 or equivalent volume of DMSO was added. Results shown are mean $\pm$ SEM for at least four independent experiments. P -value was calculated using a paired t -test. * $P \leq 0.05$, ** $P \leq 0.01$.

coregulators did not have a greater effect than individual depletion of any of them, indicating that these coregulators all function in the same pathway (Appendix Fig S6D).

As HP1 γ is part of the HP1 family of proteins, we analyzed the involvement of the other two family members, HP1 α and HP1 β , in the dex-induced expression of these genes. Depletion of HP1 α or HP1 β did not affect the dex-induced expression of the G9a/GLP-dependent or G9a/GLP-independent GR target genes (Appendix Fig

S6E). These results indicate that endogenous HP1 γ is selectively required for full induction by dex of the endogenous GR target genes that are positively regulated by G9a and GLP and thus is required for G9a and GLP coactivator function.

To explore the role of G9a and GLP phosphorylation on G9a and GLP coactivator function, we analyzed the expression of the same eight endogenous GR target genes after treatment of the A549 cells with ZM447439 inhibitor. We observed significant increases in

dex-induced CDH16, ENA α , PPL, HSD11B2, and CDH1 mRNA levels in comparison with cells not treated with the inhibitor (Fig 5F, left panel). However, induction of mRNAs for the G9a- and GLP-independent GR target genes, FKBP5, FOXO1, and CITED2, by dex was not significantly altered by inhibition of the kinase activity of Aurora kinase B (Fig 5F, right panel). As G9a phosphorylation is reduced by inhibition of Aurora kinase B in cells, we conclude that the selective increase in the dex-induced expression of GR target genes that required G9a, GLP, and HP1 γ as coactivators is due to enhanced binding of HP1 γ to G9a and/or GLP.

HP1 γ is recruited to GR binding regions associated with G9a/GLP-dependent GR target genes and facilitates recruitment of RNA polymerase II

G9a is selectively recruited to GR binding regions (GBR) associated with GR target genes that require G9a for their dex-induced expression [4]. To test whether the GR-G9a-HP1 γ complex we observed by co-immunoprecipitation and PLA assay (Fig 2) forms on the GBR associated with G9a/GLP-dependent GR target genes, we tested for dex-induced occupancy of HP1 γ on GBR associated with the same G9a/GLP-dependent and G9a/GLP-independent GR target genes that were analyzed above for expression. In chromatin immunoprecipitation (ChIP) analyses, we observed dex-induced HP1 γ occupancy on the GBRs closely associated with the CDH16 and ENA α genes, which are G9a/GLP-dependent GR target genes (Appendix Fig S7A). However, little or no dex-induced enhancement of HP1 γ occupancy was observed at other sites in and around the CDH16 and ENA α genes, except for a modest enhancement at the transcription start sites (TSS) where some GR occupancy was also observed. Dex-induced enhancement of HP1 γ occupancy was also observed on GBRs associated with three other genes (PPL, HSD11B2, and CDH1) that require G9a and GLP for their dex-induced expression (Fig 6A, left panel, darker bars). Importantly, when HP1 γ was depleted with a pool of four siRNAs (Appendix Fig S7B), hormone-induced HP1 γ occupancy at the GBRs of all five of these G9a/GLP-dependent GR target genes was abolished (Fig 6A, left panel, lighter bars), validating the specificity of the HP1 γ ChIP enrichment using this antibody. GR occupancy at the GBRs of the G9a/GLP-dependent GR target genes was not affected by HP1 γ depletion (Appendix Fig S7C).

In contrast to the G9a- and GLP-dependent GR target genes, no dex-induced enhancement of HP1 γ occupancy was observed at GBRs associated with the FKBP5, CITED2 and FOXO1 genes (Fig 6A, right panel, darker bars), which do not require G9a or GLP for their dex-induced expression (Fig 5D) and exhibit no dex-induced occupancy of G9a [4] (Appendix Fig S8D) on the associated GBRs. It is interesting to note that some HP1 γ occupancy was observed on most of the above eight GBRs even in the absence of dex, as indicated by the reduction in the ChIP signal observed in the cells treated with ethanol (the vehicle for dex) after HP1 γ depletion (Fig 6A, lighter bars). Similarly, higher-than-background HP1 γ ChIP signals on some non-GBR sites associated with the CDH16 and ENA α genes in ethanol-treated cells indicate constitutive HP1 γ occupancy (Appendix Fig S7A). Thus, HP1 γ occupancy was observed on all of the eight GBRs (and some other sites in and around these genes) prior to dex treatment, but was enhanced after

dex treatment only on the GBRs of G9a/GLP-dependent GR target genes (Fig 6A).

Since dex-induced occupancy of HP1 γ (Fig 6A) corresponded to dex-induced occupancy of G9a [4] (Appendix Fig S8D), we tested whether G9a is required for dex-induced HP1 γ occupancy on the GBRs of the GR target genes. Indeed, depletion of G9a using a pool of four siRNAs (Appendix Fig S7D) essentially eliminated the dex-dependent HP1 γ recruitment specifically on GBRs of GR target genes that are positively regulated by G9a and GLP (Fig 6B); in contrast, the constitutive, non-dex-inducible HP1 γ occupancy observed on the GBRs of the G9a/GLP-independent GR target genes (Fig 6A, right panel) was not affected by G9a depletion (Fig 6B).

As it was previously shown that HP1 α and HP1 β , in addition to HP1 γ , bind methylated G9a [16], we analyzed HP1 α and HP1 β recruitment on the GBRs of the GR target genes previously studied. There was no dex-induced enrichment of HP1 α or HP1 β on the GBRs of the G9a/GLP-dependent or G9a/GLP-independent GR target genes (Appendix Fig S7E). However, when HP1 α or HP1 β was depleted with a pool of four siRNAs, their occupancy at the GBRs decreased, showing there was some constitutive occupancy and validating the ChIP signals from the antibodies used (Appendix Fig S7F).

A similar PTM switch (adjacent methylation and phosphorylation sites) exists on histone H3; that is, H3K9me3 recruits HP1 γ and H3S10ph opposes this effect [24,25]. Since these histone H3 PTMs could also affect the expression of the GR target genes, we analyzed H3K9me3 and H3S10ph levels at the GBR associated with the GR target genes of interest. ChIP experiments showed that H3K9me3 levels at these GBR were near background levels and did not increase with dex treatment (Appendix Fig S7G). A region with high H3K9me3 occupancy served as a positive control. H3S10ph levels varied at the different GR binding sites but also did not change with dex treatment (Appendix Fig S7H). Depletion of Aurora kinase B reduced the signals at all of these sites and thus validated the ChIP signal (Appendix Fig S7I). Since H3K9me3 and H3S10ph were not increased by dex, they are not responsible for the dex-dependent binding of HP1 γ to these sites or the dex-induced expression of these genes.

To study the role of G9a/GLP methylation in HP1 γ recruitment to GBR of G9a/GLP-dependent GR target genes, we established stable cell lines where expression of wild-type or K/R mutant G9a or GLP (containing an N-terminal HA-tag) is doxycycline inducible (Appendix Fig S8A and B). We first validated the fact that overexpression of G9a/GLP wild type, K/R, and T/A mutants does not have any impact on total cellular H3K9me3 or H3S10ph levels (Appendix Fig S8A–C). In ChIP experiments using HA antibody, mutation of the methylation site did not reduce dex-induced G9a and GLP occupancy on the GBRs of G9a/GLP-dependent GR target genes (Appendix Fig S8D and E). As expected, there was no dex-induced G9a and GLP occupancy on the GBRs of G9a/GLP-independent GR target genes. Dex-dependent HP1 γ recruitment observed in cell lines overexpressing wild-type G9a was eliminated in the cell lines that overexpress the unmethylatable mutant G9a (Appendix Fig S8F), indicating that methylation of this lysine is a prerequisite for dex-induced HP1 γ occupancy on the GBRs of G9a/GLP-dependent GR target genes. Altogether, these results indicate that dex-induced HP1 γ recruitment requires G9a/GLP methylation and is specific for the subset of GR target genes where G9a is recruited by GR and is

Figure 6. Occupancy of HP1γ on GBR of GR target genes.

- A** A549 cells were transfected with non-specific siRNA (siNS, dark blue bars) or with SMART-pool siRNA targeting HP1γ (siHP1γ, light blue bars) and treated with 100 nM dex or ethanol for 4 h. Immunoprecipitated DNA was analyzed by qPCR using primers that amplify the GBRs associated with the indicated GR target genes. Results are normalized to input chromatin and shown as mean $\pm$ SEM for four independent experiments. P -value was calculated using a paired t -test. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.
- B** A549 cells transfected with non-specific siRNA (siNS, dark blue bars) or with SMART-pool siRNA targeting G9a (siG9a, light blue bars) were treated with 100 nM dex or ethanol for 4 h. ChIP was performed with HP1γ antibody and immunoprecipitated DNA was analyzed by qPCR using primers specific for the GBRs associated with the indicated genes. Results are normalized to input chromatin, and the mean $\pm$ SEM of the ratio between 4-h dex or ethanol treatment for three independent experiments is shown. P -value was calculated using a paired t -test. $^{**}P \leq 0.01$.
- C** Cos-7 cells were transfected with plasmids encoding full-length HA-hG9a wild type or K185R mutant. Lysates were immunoprecipitated (IP) with HA antibody and immunoblotted with phospho-S93-HP1γ (pS93-HP1γ) or HA antibodies. Expression of HA-tagged G9a, HP1γ, and β -actin (loading control) in the unfractionated extracts is shown at the bottom (Input).
- D** A549 cells transfected with non-specific siRNA (siNS, dark blue bars) or with SMART-pool siRNA targeting HP1γ (siHP1γ, light blue bars) were treated with 100 nM dex or ethanol for 4 h. ChIP was performed with phospho-S93-HP1γ antibody, and immunoprecipitated DNA was analyzed by qPCR using primers that amplify the GBRs associated with the indicated GR target genes. Results are normalized to input chromatin and shown as mean $\pm$ SEM for three independent experiments. P -value was calculated using a paired t -test. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.
- E** A549 cells were treated as in (D). ChIP was performed with antibodies against RNA polymerase II phosphorylated on S5 of the C-terminal domain repeats (pS5(CTD)-Rpb1), and immunoprecipitated DNA was analyzed by qPCR using primers that amplify the TSS associated with the indicated GR target genes. Results are normalized to input chromatin and shown as mean $\pm$ SEM for three independent experiments. P -value was calculated using a paired t -test. $^*P \leq 0.05$, $^{**}P \leq 0.01$.

required as a coactivator. In contrast, the constitutive HP1γ occupancy does not require G9a.

To explore the mechanism by which HP1γ contributes to dex-induced expression of G9a/GLP-dependent target genes, we used possible clues from previous reports that HP1γ is phosphorylated by Pim-1 and PKA [26,27], that phosphorylation of HP1γ on S93

impaired its repression activity [26,27], that HP1γ interacts with RNA polymerase II [6], and that phospho-S93-HP1γ interacts with RNA polymerase II that is phosphorylated on S5 of the C-terminal repeat domain [27]. We observed that wild-type G9a or GLP, but not the unmethylatable mutants, co-immunoprecipitated with phospho-S93-HP1γ (Fig 6C and Appendix Fig S7J). In ChIP experiments,

occupancy of phospho-S93-HP1 γ on GBRs of G9a/GLP-dependent GR target genes (but not on G9a/GLP-independent GR target genes) was significantly induced by dex, and the dex-induced ChIP signal was eliminated by depletion of HP1 γ (Fig 6D). G9a was previously reported to be important for dex-induced RNA polymerase II occupancy of TSS associated with G9a-dependent GR target genes [12]; and we observed that dex-induced occupancy by RNA polymerase II at TSS of G9a/GLP-dependent GR target genes (but not at a G9a/GLP-independent GR target gene) was significantly reduced and essentially eliminated by depletion of HP1 γ (Fig 6E). Thus, recruitment of HP1 γ by G9a or GLP methylation facilitates recruitment of RNA polymerase II to the TSS for efficient transcriptional activation.

G9a and GLP methylation and coactivator function drive dex-induced inhibition of cell migration

A pathway analysis of the genes from the microarray data that require GLP for their dex-induced expression indicated that genes involved in cell movement were enriched (Appendix Fig S9A and B), including *CDH1*, which encodes E-cadherin, a key component of adherens junctions. Loss of E-cadherin expression is important for epithelial–mesenchymal transition and increased cell motility [28]. Quantitative RT–PCR analysis confirmed that depletion of GLP significantly decreased dex-induced expression of *CDH-1* mRNA after 8 h of dex treatment, and G9a depletion produced a similar although not significant trend (Fig 5D), indicating that G9a and GLP act as coactivators for this gene. Likewise, 24 h of dex treatment significantly increased E-cadherin protein expression at the plasma membrane (Fig 7A). However, G9a or GLP depletion largely prevented dex enhancement of E-cadherin expression, as indicated by quantification of the staining with ImageJ software (Fig 7A) and by immunoblot analysis (Appendix Fig S9C). Since E-cadherin inhibits cell migration (Appendix Fig S9D), we analyzed the effect of G9a/GLP depletion and dex on cell migration by the transwell migration assay. There was a significant decrease in migration in cells incubated with dex for 24 h compared to ethanol-treated cells (Fig 7B). However, depletion of G9a or GLP by siRNA significantly prevented repression of cell migration by dex (Fig 7B). In order to determine the impact of G9a methylation on this phenotype, we used the stable cell line where G9a expression (wild type or K185 mutant) is doxycycline inducible (Fig 7C). Dex treatment decreased migration of A549 cells as previously demonstrated, and similar dex inhibition of migration was observed in cells overexpressing wild-type G9a (Fig 7D). In contrast, overexpression of G9a K185R significantly prevented the dex-induced decrease in migration and in fact caused increased cell migration after dex treatment, suggesting that the overexpressed mutant version of G9a has a dominant-negative effect, suppressing the activity of endogenous G9a and interfering with the dex-induced decrease in migration. Consistent with these results, analyses of the E-cadherin expression by Western blot (Fig 7C) or immunofluorescence (Appendix Fig S9E) showed that there is little or no dex-induced increase in E-cadherin gene expression after overexpression of G9a K185R. These findings further demonstrate that methylation of G9a and subsequent recruitment of HP1 γ are involved in the regulation of cell migration, an important function in normal cell biology, EMT, and cancer metastasis in many systems. In addition, in another experimental model the estrogen-dependent proliferation of MCF-7 breast cancer cells was dependent on G9a and

HP1 γ (Appendix Fig S9F). Since HP1 γ is critical for the coactivator activity of G9a, this implicates the coactivator activity of G9a in estrogen-dependent proliferation of breast cancer cells.

Discussion

PTMs provide a switch that regulates G9a and GLP coactivator function

A growing list of transcriptional coregulators has been associated with both gene activation and gene repression [1,3,5], and indeed, TFs that recruit these coregulators also activate or repress different subsets of their direct target genes (i.e., those genes that are regulated by the TFs and coregulators and are associated with regulatory sites where the TFs/coregulators bind). Thus far, very little is known about the factors that dictate whether TFs and coregulators act positively or negatively on each of their direct target genes. A relevant observation is that TFs and coregulators act in a gene-specific manner; for example, different direct target genes of the same TF have distinct mechanisms of transcriptional activation, as indicated by the fact that they require different sets of transcriptional coregulators [4,5,29]. These observations lead to our working hypothesis that each gene has a unique regulatory environment that specifies which coregulators are required and is determined by several factors, including but perhaps not limited to: the specific DNA sequence to which the TF binds, which can alter the conformation of the TF [30,31]; the DNA sequence surrounding the TF binding site, which dictates which other TFs may bind with their associated coregulators; the status of various cellular signaling pathways and the presence or absence of their effector proteins (some of which make PTMs which may regulate DNA binding and activity of various TFs and coregulators) on regulatory sites associated with specific genes; and the local chromatin conformation which may also dictate which coregulators are required for the appropriate chromatin remodeling events needed to achieve gene regulation.

Here, using a model system of glucocorticoid-regulated gene transcription, we investigated the mechanism that controls transcriptional activation by two specific coregulators, G9a and GLP. G9a and GLP are two important, ubiquitous, and essential coregulators that have been implicated in many mammalian physiological processes. We demonstrated here that GLP acts in a gene-specific manner as a coregulator for GR: GLP facilitates glucocorticoid activation of some GR target genes and glucocorticoid repression of others, while a third subset of GR target genes is regulated by the hormone independently of GLP, as was already described for G9a [4]. Furthermore, there is substantial overlap in the dex-induced genes that are negatively affected by depletion of G9a or GLP, but a few GR target genes were regulated by GLP and not G9a, showing that these two proteins support the regulation of highly similar but not identical gene sets (Fig 5). We show here that two specific PTMs shared by G9a and GLP provide a molecular switch that regulates the ability of G9a and GLP to function as coactivators (Fig 7E). It is interesting to speculate that regulation of the coactivator function of G9a and GLP may have an effect on the decision as to whether G9a and GLP function as coactivator or corepressor on a given gene to which they are recruited. However, further work is required to address this issue.

Figure 7. G9a and GLP mediate glucocorticoid repression of cell migration.

- A** E-cadherin expression was analyzed by immunofluorescence. A549 cells transfected with non-specific siRNA (siNS) or SMART-pool siRNA targeting G9a (siG9a) or GLP (siGLP) were treated with 100 nM dex or ethanol for 24 h. The nuclei were counterstained with DAPI (blue). Representative images are shown. E-cadherin expression (green) per cell quantified by image analysis for at least 1,500 cells per experiments is shown as the mean $\pm$ SEM of four independent experiments. *P*-value was determined using a paired *t*-test. $*P \leq 0.05$. Scale bar represents 10 μ m.
- B** A549 cell migration was analyzed using transwell migration assays for the same cells as described in (A). Migratory cells on the bottom of the polycarbonate membrane were stained. Representative images are shown (left panel). Then, dye extracted from the cells was quantified at OD 560 nm. Relative migration index is shown as the mean $\pm$ SEM of four independent experiments (right top panel). The ratio of migration for cells treated with dex versus ethanol (Eth) from these four experiments is shown on the right bottom panel. *P*-value was determined using a paired *t*-test. $*P \leq 0.05$, $**P \leq 0.01$. Scale bar represents 100 μ m.
- C** A549 rTA cell lines containing a stably integrated doxycycline-regulated G9a WT or K185R transgene were treated or not with 10 ng/ml of doxycycline for 24 h prior to and during 24 h of dex treatment. A fraction of the cells was analyzed by immunoblot using indicated antibodies.
- D** Using the same cells described in (C), cell migration was assessed using transwell migration assays as described in (B). Analyses are shown as the mean $\pm$ SEM of four independent experiments. *P*-value was determined using a paired *t*-test. $*P \leq 0.05$, $**P \leq 0.01$. Scale bar represents 100 μ m.
- E** Model for transcriptional regulation of G9a/GLP-dependent GR target genes by G9a and GLP PTMs. After stimulation with hormone (filled black circle), GR binds to GR binding regions (GBR) on DNA and recruits G9a and GLP. G9a facilitates recruitment of p300 and Carm1 coactivators, which acetylate histones H3 and H4 (Ac) and methylate histone H3 at R17 (Me), respectively. If G9a and GLP are methylated, they recruit phospho-S93-HP1 γ , which facilitates recruitment of RNA polymerase II (PolII), which is phosphorylated (P) on S5 of the C-terminal domain repeats to activate G9a/GLP-dependent GR target genes. Dex-induced, G9a/GLP-dependent GR target genes include *CDH1* (encoding E-cadherin), which is important for the decreased cell migration caused by dex. However, if G9a or GLP are phosphorylated by Aurora kinase B, HP1 γ recruitment by G9a or GLP is prevented, thereby inhibiting dex-induced expression of the G9a/GLP-dependent GR target genes (inset).

HP1 γ recruitment by G9a and GLP is regulated by PTMs and is required for G9a and GLP coactivator function

We demonstrated here that GLP is methylated on K205 and phosphorylated on T206 by Aurora kinase B in a sequence of amino acids with high homology to the similarly modified region of G9a. The formation of the G9a/GLP-HP1 γ complex in cells is regulated by

G9a/GLP methylation and phosphorylation, as indicated by co-immunoprecipitation of overexpressed proteins and by PLA using endogenous proteins in A549 cells (Figs 1–3). G9a or GLP binding to HP1 γ requires lysine methylation of K185 in G9a or K205 in GLP and is inhibited by threonine phosphorylation (T186 or T206); furthermore, both G9a and GLP can nucleate a ternary complex with HP1 γ and GR.

Comparison of dex-induced gene expression for the G9a/GLP-dependent and the G9a/GLP-independent GR target genes served as an internally controlled experimental system to demonstrate the gene-specific nature of the G9a/GLP coactivator pathway and the role of the G9a/GLP PTMs in controlling their coactivator function (Fig 7E). There was a consistent contrast in the roles of all components of the G9a/GLP coactivator pathway in mediating dex-induced expression of the G9a/GLP-dependent and G9a/GLP-independent genes. Depletion of G9a, GLP, and HP1 γ , and use of an Aurora kinase B inhibitor all had distinct effects on the G9a/GLP-dependent versus G9a/GLP-independent genes (Fig 5). Similarly, dex-induced occupancy by G9a, GLP, HP1 γ , S93-phosphorylated HP1 γ , and RNA polymerase II was consistently different on G9a/GLP-dependent versus G9a/GLP-independent GR target genes (Fig 6 and Appendix Fig S8). Thus, multiple experimental comparisons of the roles of multiple components of the G9a/GLP coactivator pathway in dex-induced expression of these two groups of GR target genes provide compelling, well-controlled evidence for the importance of the PTMs of G9a and GLP in controlling their coactivator function for a specific subset of GR target genes. Furthermore, the fact that the mechanisms of G9a coactivator and corepressor functions are distinct and utilize different domains of G9a and GLP, along with the selective recruitment of G9a, GLP, and HP1 γ only to GR target genes where they are required as coactivators, provides very strong evidence to validate our conclusion that G9a, GLP, and HP1 γ are acting directly as coactivators on these genes, rather than by some indirect mechanism in which G9a, GLP, and HP1 γ are acting as corepressors (e.g., repressing a gene that encodes a repressor of the GR target genes).

In addition, these findings demonstrate that the molecular mechanism of coactivator function of G9a and GLP involves recruitment of HP1 γ . Thus, HP1 γ functions as a coactivator on these genes after dex treatment, mediating the coactivator function of G9a and GLP (Fig 5), by facilitating the recruitment of RNA polymerase II (Figs 6 and 7E). While the HP1 family of proteins (α , β , and γ) are primarily known for their roles in gene repression, HP1 γ in particular has also been shown to function as a coactivator for regulation of specific genes [18]. Consistent with that, even though they interact with methylated G9a [16], HP1 α and HP1 β do not function as coactivators for regulation of G9a/GLP-dependent or G9a/GLP-independent GR target genes (Appendix Figs S6E and S7E and F), in contrast to HP1 γ (Figs 5E and 6, and Appendix Fig S7A).

In a previous report [4], we concluded that the methyltransferase activity of G9a was required for its corepressor activity but not for its coactivator activity, based on an experiment where we pretreated A549 cells with a G9a/GLP-specific methyltransferase inhibitor for 1 h prior to dex treatment. The data reported here show that self- or reciprocal methylation by G9a and GLP is required for coactivator function which obviously contradicts the previous conclusion. The explanation lies in the length of pretreatment with the G9a/GLP-specific methyltransferase inhibitor. The 24-h pretreatment with the inhibitor used in the current study is required to substantially reduce the methylation of G9a K185 and GLP K205 and thus inhibit G9a/GLP coactivator function. Thus, the 1-h inhibitor pretreatment used in the previous study [4] was sufficient to prevent new methylation of histone H3K9, which is required for G9a/GLP corepressor function; but the 1-h pretreatment was not sufficient to reduce the N-terminal methylation of

G9a and GLP and thus did not significantly affect the coactivator function.

G9a and GLP coactivator function regulates cell migration of a lung cancer cell line

The biological function of the two PTMs on G9a and GLP, and of the regulation of their interaction with HP1 γ , has not been previously addressed. Using the A549 lung cancer cell model, we demonstrated that G9a and GLP mediate glucocorticoid repression of cell migration by cooperating with GR to induce the expression of target genes such as *CDH1* (which encodes E-cadherin) that are involved in cell migration (Fig 7). Furthermore, methylation of G9a on K185 is directly involved in this process. Indeed, overexpressed G9a K185R acts as a dominant negative, preventing dex-induced expression of E-cadherin and the resulting dex repression of cell migration (Fig 7 and Appendix Fig S9). These findings directly implicate the methylation of G9a and the resulting coactivator function of G9a in cell migration and thus demonstrate a specific biological regulatory function for G9a/GLP PTMs in the GR signaling pathways. The fact that reduction in *CDH1* expression is a critical part of the mechanism of epithelial–mesenchymal transition, which is involved in many developmental processes as well as tumor progression [28], suggests that the coactivator function of G9a and GLP may play critical roles in these developmental and pathogenic processes.

Possible mechanisms regulating G9a and GLP PTMs, and their implications

Regulation of the methylation and phosphorylation status of G9a and GLP modulates glucocorticoid regulation of the specific subset of GR target genes (among all the genes regulated by GR) that require G9a and GLP as coactivators. In effect, this provides a mechanism for modulating the hormone response. Since G9a and GLP are controlled by this dual-PTM switch and also serve as coregulators for many different TFs, it seems likely that the same PTM switch controls positive gene regulation by G9a and GLP much more broadly than just with steroid hormone receptors. Furthermore, since the same methylation/phosphorylation switch regulates binding of HP1 proteins to histone H3 (at methylated lysine 9), we speculate that a similar switch mechanism will be found to control positive versus negative gene regulation by other coregulators and TFs, controlling many others biological functions.

Based on current knowledge, there are many potential pathways to regulate the addition or removal of these two PTMs on G9a and GLP. Methylation could be regulated by controlling the intramolecular or intermolecular interaction of the N-terminal methylation site with the C-terminal regions of G9a or GLP containing the methyltransferase activity. Indeed, *in vitro* methylation experiments with G9a and GLP fragments indicate that *trans*-methylation as well as intramolecular auto-methylation is possible (Fig 1), suggesting similar mechanisms in cells since G9a and GLP heterodimerize. In addition, there are many different potential enzymes to test for G9a and GLP demethylation. JMJD1A, LSD1/KDM1, PHF8, KMD4A, and KMD7A can all demethylate H3K9 [32–35], which has almost the same local amino acid sequence context (ARKS) as the G9a and GLP methylation sites (ARKT), suggesting that these enzymes may also demethylate G9a and GLP.

In addition, the protein level and activity of Aurora kinase B are regulated in many ways. Transcription of the Aurora kinase B gene is regulated by the cell cycle [36,37] and by transcription factors such as c-Myc, p53, and ETS-1 [38,39]. Aurora kinase B activity is regulated by multiple protein–protein interactions, and by phosphorylation and dephosphorylation [36]. Stability of the protein [37] and mRNA [40] is also regulated. G9a has been shown to regulate proliferation and differentiation of skeletal muscle cells, regulating the cell cycle by two different mechanisms, serving as a corepressor for some genes and as a coactivator for other genes [41], suggesting a possible complex interaction with the regulation of methylation and phosphorylation of G9a and/or GLP in this context. It will be important to explore the many possible regulatory mechanisms for the G9a and GLP PTMs, including the identity and regulation of G9a/GLP demethylases, and which of the many Aurora kinase B regulatory mechanisms identified in the context of the cell cycle may apply to its function as a modulator of G9a and GLP coactivator activity. In addition, since G9a [10,42] and Aurora kinase B [37,38,43] are both over-expressed in many different types of cancer, it is important to ask whether the gene targets that require G9a as a coactivator, as a corepressor, or both are involved in the transformed phenotype.

Materials and Methods

Plasmids

The following plasmids were described previously: luciferase reporter plasmid MMTV-LUC (which contains glucocorticoid responsive elements), along with mammalian protein expression vectors for hG9a and hG9a fragments, hGLP, hGR, and mGrip1 [11,12,44]; and bacterial expression vector for GST-hG9a N (1–280) [4]. PCR-amplified DNA fragments encoding hG9a ΔN (735–1,210), hGLP ΔN (814–1,279), and hGLP N (31–357) were cloned into the EcoRI-BamHI, BamHI, or EcoRI-XhoI sites, respectively, of the vector pGEX-4T1. PCR-amplified cDNA fragment encoding hG9a and hGLP were cloned into the EcoRI site of the lentiviral vector of FUW.FTRT.GFP provided by Dr. Wange Lu (USC). For lentiviral production, the packaging vector psPAX2 and the envelope plasmid pMD2.G were used. G9a and GLP point mutants were generated with the QuikChange site-directed mutagenesis kit (Stratagene) using pSG5.HA-hG9a, pSG5.HA-hGLP, FUW.FTRT.GFP-hG9a, pGEX.4T1-hG9a (1–280), or pGEX.4T1-hGLP (31–357) as templates. The pcDNA-FLAG-Aurora-B-WT plasmid encoding human Aurora kinase B was provided by Dr. Masaaki Tatsuka (University of Hiroshima).

Cell culture

Cos-7, CV-1, MCF-7, and A549 cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. For ZM443979 (Tocris) Aurora kinase B inhibitor, UNC0638, UNC0642, and UNC0646 (Sigma) G9a/GLP catalytic activity inhibitors, cells were treated with the indicated concentration of the compound or with the equivalent volume of DMSO for the indicated amount of time.

For lentivirus particle production, 293T cells were plated in 100-mm dishes and transiently transfected by Lipofectamine 3000

(Invitrogen) according to the manufacturer's protocol with the transducing vector (FUW.FTRT.GFP-HA-G9a wild type or K185R mutant, or FUW.FTRT.GFP-HA-GLP wild type or K205R mutant), the packaging vector psPAX2, and the envelope plasmid pMD2.G. The medium was changed the next day, and viruses were harvested by collecting the medium at 48 and 72 h post-transfection. Virus-containing medium from two harvests was pooled, passed through a 0.45-μm filter, and stored at –80°C. For lentiviral transduction, A549 cells were seeded a day before to reach 80% of confluency at the day of infection. Medium containing virus was added to cells along with polybrene (Millipore) at the final concentration of 6 μg/ml; 24 h after infection, virus-containing medium was replaced with culture medium containing puromycin (1 μg/ml) for selection of infected cells. The resistant cell populations were used for the indicated experiments.

Protein depletion by siRNA

SMART-pool siRNAs used for depletion of G9a, GLP, HP1γ, HP1α, HP1β, CDH1, and Aurora kinase B and ON-TARGETplus Non-targeting siRNA#2 used as control non-specific siRNA (siNS) (Dharmacon) were transfected into A549 cells using Lipofectamine siRNAi max (Invitrogen) according to the manufacturer's protocol.

Lentivirus production and delivery of anti-GLP shRNA followed by microarray analysis

All the procedures were performed in parallel with the anti-G9a shRNA analysis, using four biological replicates from four independent experiments performed on different days, as previously described for G9a [4]. Global gene expression analysis was performed with Illumina Human-Ref8v3 microarrays, using total RNA samples from four biological replicates from independent experiments performed on different days. Each experiment included non-infected A549 cells or A549 cells infected with lentivirus encoding shNS or shGLP, treated with 100 nM dex or equivalent volume of vehicle ethanol for 24 h. Data analysis methods were described previously [4]. To define hormone-regulated genes, the untreated control gene set (pooled data of uninfected cells and cells infected with the virus encoding shNS) was compared with the control gene set that was hormone-treated; a *q*-value cutoff of 0.01 was applied along with a hormonal regulation fold change cutoff of 1.5 to facilitate subsequent experimental target gene validation and reduce the number of potential false positives. To define GLP-regulated genes, the control gene set that was hormone-treated was compared with the shGLP gene set that was hormone-treated, and a *q*-value cutoff of 0.05 with no fold change cutoff was applied. Forward primer used for PCR to create shGLP is as follows: 5'-CTTGTGGAAAGGACGAAACACCGAAGTTCGAGGAGCTAGAAATCATATTTCAAGAGATATCATCTCTAGCTTCTCGAACTTCTTTTCTGCAG-3' (underlining indicates shRNA targeting sequence). The complete microarray data have been deposited in GEO with accession number GSE94646.

Immunoprecipitation and immunoblot

Cos-7 or A549 cells were seeded on 10-cm dishes the day before transfection. Cells were transiently transfected (where indicated) using Lipofectamine 2000 (Invitrogen) with 5 μg each of the

indicated plasmids according to the manufacturer's protocol. At 48 h after transfection, cells were treated (or not) with dex for the indicated time period, and cell extracts were prepared in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.25% deoxycholate) supplemented with protease inhibitor tablets (Roche Molecular Biochemicals) and phosphatase inhibitors (1 mM NaF, 1 mM Na₃VO₄, and 1 mM β -glycerophosphate). Protein extracts were incubated with 1 μ g of the indicated primary antibodies overnight at 4°C with shaking. Protein A/G Plus Agarose (Santa Cruz sc-2003) beads were added, and the mixture was incubated 2 h at 4°C. The immunoprecipitates were separated on SDS-PAGE. Immunoblotting was conducted with primary antibodies against HA epitope (3F10 Roche Applied Science); G9a (G6919), FLAG (F1804), β -actin (A5441), or GAPDH (G9545) from Sigma; Aurora kinase B (ab2254), HP1 γ (ab10480), HP1 γ (ab56978), phospho-S93-HP1 γ (ab45270), or pan-methyllysine (ab23366) from Abcam; GR (sc-8992) from Santa Cruz; GLP (09-078), or pan phospho-threonine (AB1607) from Millipore; phospho-S211-GR (#4161), H3K9me3 (#13969), H3S10ph (#53348), histone H3 (#4499) from Cell Signaling; or E-cadherin (610182) from BD Transduction Laboratories. Secondary antibodies from Santa Cruz Biotechnology (anti-rat) and Promega (anti-rabbit and anti-mouse) were used for chemiluminescence detection using Super Signal West Dura (Thermo Scientific) for proteins with low expression levels and ECL prime detection reagent (Amersham) for all other proteins according to the manufacturers' instructions. In immunoprecipitation experiments, 3% of the input of each sample was analyzed by immunoblot using the antibodies listed.

Methyltransferase assays

Bacterially produced GST fusion proteins (2 μ g) of N-terminal fragments of G9a or GLP (GST-hG9a N or GST-hGLP N), mutant (GST-hG9a N K185R or GST-hGLP N K205R or GST-hG9a N T186A), or GST alone were incubated 90 min at 30°C with GST-hG9a Δ N or GST-hGLP Δ N in the presence (or not) of 1 mM of unlabeled SAM (New England Biolabs, B9003S). Methylated products were analyzed by standard SDS gel electrophoresis followed by immunoblot. The radioactive methylation assay were performed in the same experimental conditions in the presence of 1 μ Ci/ml of S-adenosyl-L[methyl-³H]methionine (55–85 Ci/mmol; PerkinElmer; NET155H250UC). Methylation reactions were separated on SDS-PAGE. Following electrophoresis, gels were incubated in Amplify fluorographic reagent (Amersham Biosciences) according to the manufacturer's instructions and visualized by fluorography.

Luciferase assays

CV-1 cells were plated in hormone-free medium with 5% charcoal-stripped serum in 24-well plates the day before transfection. Cells were transfected using Lipofectamine 2000 (Invitrogen) with the indicated plasmids according to the manufacturer's protocol. After transfection, the cells were grown in hormone-free medium for 48 h in the presence or absence of 100 nM dex. Cell lysis and luciferase assays on cell extracts were performed with Promega luciferase assay kit. An aliquot of the cell lysate was reserved for immunoblot analysis of input samples. The results were normalized as indicated and presented as the mean $\pm$ SEM of at least four independent experiments.

Chromatin immunoprecipitation

ChIP experiments were performed according to previously described protocols [4] with antibodies against GR (Santa Cruz sc-8992X), HP1 γ (Abcam ab10480), HP1 α (Cell signaling #2616), HP1 β (Cell signaling #8676), phospho-Rpb1 CTD (Ser5) (Cell signaling #13523), H3K9me3 (Cell signaling #13969), H3S10ph (Cell signaling #53348), and HA epitope (3F10 Roche Applied Science). Results are expressed relative to the signal obtained from input chromatin. Primer sequences are indicated (Table 1).

Real-time RT-qPCR

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription reaction was performed using iScript (Bio-Rad) according to specifications with 0.8 μ g of total RNA as template. Quantitative PCR amplification of the resulting cDNA was performed on a Roche LightCycler 480 using SYBR green I master mix (Roche). mRNA levels were normalized to the level of β -actin mRNA. Primer sequences are specified (Table 2).

Proximity ligation assay

The experiments were performed following the manufacturer's instructions as previously described [21,45]. Cells were grown on coverslips in 12-well plates, fixed in methanol for 2 min, and then washed twice in PBS. Firstly, the samples were saturated using the blocking solution, and then different pairs of primary antibodies (HP1 γ (Abcam ab10480) and GR (Santa Cruz sc-393232) in order to analyze HP1 γ -GR, G9a (Sigma G6919) and HP1 γ (Abcam ab56978) in order to analyze HP1 γ -G9a interaction, and HA-Tag (6E2) (Cell signaling #2367) and HP1 γ (Abcam ab10480) in order to analyze HA-HP1 γ interaction) were incubated with the fixed cells for 1 h at 37°C. After washes, the PLA minus and plus probes (containing the secondary antibodies conjugated with complementary oligonucleotides) were added and incubated 1 h at 37°C. After the ligation of oligonucleotides into a circular template, the addition of nucleotides and DNA polymerase allows a rolling-circle amplification reaction during an incubation of 100 min at 37°C. The amplification solution also contains fluorescently labeled oligonucleotides that hybridize to the amplification product. Afterward, the samples were mounted with Duolink II Mounting Medium containing DAPI in order to counterstain nuclei and then analyzed on Zeiss Imager.Z1 fluorescence microscope. For each sample, interactions were counted for 1,000 cells using ImageJ software [46].

Immunofluorescence

Cells were grown on coverslips in 12-well plates. Cells were then fixed in cold methanol for 2 min, washed twice in PBS, and incubated in 1 $\times$ PBS gelatin for 30 min. Then, the cells were incubated with E-cadherin antibody (610182) from BD Transduction Laboratories in Dako diluent (S0809) for 1 h at 37°C. After PBS washes, the cells were incubated for 1 h at 37°C with the mouse secondary antibodies coupled with Alexa Fluor 488 from Invitrogen (1:3,000) in Dako antibody diluent, then washed in PBS and mounted on glass slides in mounting solution (Dako). Slides were analyzed on Zeiss Imager.Z1 fluorescence microscope.

Table 1. ChIP primer list.

Primer name	Sense	Antisense
ENaC α -2.5 kb	5' AACTCCAGTCTCCCTTGAGC 3'	5' CCATGCTGCCTTAAGCTAGTG 3'
ENaC α GBR (-1.3 kb)	5' CACCTTCAGTGCCTGCTTC 3'	5' AGGCCAGGAATGTGTAATCG 3'
ENaC α TSS	5' TCAACTGGAAGGAACCAAGTC 3'	5' CTCGAGCTGTGCTGATTCT 3'
ENaC α +2.1 kb	5' CAACGAAATGACCTGGCTTT 3'	5' GGCCCTTCGTATATTCCAT 3'
ENaC α +5.7 kb	5' GACCTTTGGGAGAGTGAAGG 3'	5' CCACACACAAACCTGTGAC 3'
ENaC α +11 kb	5' CCGGAAATTAAGAGGAGCTG 3'	5' TACAGGTCAAAGAGCGTAGC 3'
CDH16 -1.5 kb	5' GCCAAGTGCCATACCTTCCTT 3'	5' CTCCTGCCATTCAATAAGCTG 3'
CDH16 GBR (-0.36 kb)	5' TTGAGCTGAGCACTGAAGCATG 3'	5' TGCAGCCACACCTTTTCACAC 3'
CDH16 TSS	5' TGGCTTTCCAAAGTCAATGAG 3'	5' GGCACTTGAGCAGGTAGGAG 3'
CDH16 +2.5 kb	5' ATCTCCGAGTCTGTATGTG 3'	5' TGAAGCCTCAAGGAAGAGGA 3'
CDH16 +5 kb	5' AGTGGGTGGGGTAAGGTCTC 3'	5' CAGGGCTCAGGAGCTGATAC 3'
CDH1 GBR (+21 kb)	5' CCTGCTCATCTTCTCCCA 3'	5' TGCACCAAGAACGCTTTATG 3'
HSD11B2 GBR (-7.5 kb)	5' TGTAAGTGGTGGCACTTGGA 3'	5' TTCCAAACACCTTGTCCTCCAA 3'
HSD11B2 TSS	5' GGGACTGGACACTCAACAGG 3'	5' GGTGGAGAAGTCTCCACTCT 3'
PPL GBR (-7.7 kb)	5' CAGCTTCACCCCTGTTTTGTA 3'	5' GGCCAGCACAATTTTCCACT 3'
FKBP5 GBR (+86 kb)	5' TGTGCCAGCCACATTCAGAACA 3'	5' GTAACCACATCAAGCGAGCTG 3'
FKBP5 TSS	5' TCCCATCTAGCTCTGGTCTCA 3'	3' GGGACTGCTTCTCACCATGT 3'
CITED2 GBR (-0.93 kb)	5' AGTTTGCGTTTGACAGTCTT 3'	5' AAGGTGGATCTGGGACGAG 3'
FOXO1 GBR (-0.2 kb)	5' AGATTTGGGGGAACGAAGCC 3'	5' GATGGCCCCGGAAGTTAAG 3'
H3K9me3 positive region	TCTTGAGCTTGCTTTCAT	TTCAATGACCTCAGCAGCAG
H3K9me3 negative region	CAGCTAATCAGCCTCCTTG	GCCTCAAGAAGCTGGACATC

Table 2. qPCR primer list.

Gene name	Sense	Antisense
CDH16	5' TCGGCAGTGGGCATCCTTGTA 3'	5' GCACGCTGTCTGCTGGTTGAT 3'
ENaC α	5' AACGGTCTGTCCCTGATGCT 3'	5' TTGGTGAGTCGCCATAATC 3'
HSD11B2	5' GACCTGACCAACCAGGAGA 3'	5' CCGCATCAGCACTACTTCA 3'
PPL	5' CAGGAGATCCTCCAATTCCA 3'	5' CTGGGAAGCTCTTCCCTCT 3'
CDH1	5' TTCCCAACTCCTCTCTG 3'	5' AAACCTTGCTTCTTTGTC 3'
FKBP5	5' AGGCTGCAAGACTGCAGATC 3'	5' CTGCCCATTGCTTTATTGG 3'
CITED2	5' GCCAGGTTTAAACACTCCCA 3'	5' CTGGTTTGCTCCGTTTCATCT 3'
FOXO1	5' ACAGTTTTCCAAATGGCTG 3'	5' CATCCCTTCTCCAAGATCA 3'
β -actin	5' CCACACTGTGCCATCTACG 3'	5' AGGATCTTCATGAGTAGTCAGTCAG 3'
HP1 α	5' GATGTCATCGGCACTGTTG 3'	5' GCACAATACTTGGGAACCTGA 3'
HP1 β	5' TTTGGTTTGCTCTCTCTCC 3'	5' AACACATGGGAGCCAGAAGA 3'
HP1 γ	5' AAGAGGCAGAGCCTGAAGAA 3'	5' TCTGTAAATCCCTTCCACTTCA 3'
G9a	5' ATGGGTGAAGCCGTCTCGGA 3'	5' ATCTTGGGTGCCTCCATGCG 3'
GLP	5' GATAGCGGAAATGGGGTTT 3'	5' GTAGTCCTCAAGGGCTGTGC 3'

Cell migration

A549 cells suspended in serum-free medium were plated in the upper part of a 24-well, 8- μ m pore, cell Transwell migration chamber (Cell Biolabs Inc, San Diego, CA, USA) according to the manufacturer's protocol. Medium with 10% FBS was placed in the

lower wells. 0.4×10^6 cells were incubated for migration at 37°C with 5% CO₂ for 24 h. Then, cells were fixed and stained with Cell Staining Solution (Cell Biolabs). After washing, images of migrated cells on the opposite side of the membrane were captured with an inverted microscope. Migratory cells were dissociated from the membrane using Extraction Solution (Cell Biolabs).

Optical density of the dye was measured at 560 nm in a 96-well microtiter plate.

Proliferation

Twenty-four hours after transfection with the appropriate siRNA, MCF-7 cells were plated in triplicate in 96-well plates at a density of 2,500 cells per well. One plate was harvested and analyzed each day of the time course. At each time point, cells were treated with MTS (Promega G3581) and incubated 1 h at 37°C. Absorbance was monitored at 490 nm with a 96-well plate reader.

Expanded View for this article is available online.

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Author contributions

CP and MRS conceived the project and wrote the manuscript. CP designed and performed the experiments. YH contributed to some co-immunoprecipitation experiments under the supervision of CP. DB performed the microarray experiments and D-YW analyzed the data. DSG established the A549 cell lines with doxycycline-inducible expression of G9a and GLP.

Conflict of interest

The authors declare that they have no conflict of interest.

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