A Role for Widely Interspaced Zinc Finger (WIZ) in Retention of the G9a Methyltransferase on Chromatin*

Jeremy M. Simon‡, Joel S. Parker§, Feng Liu¶, Scott B. Rothbart∥, Slimane Ait-Si-Ali**, Brian D. Strahl‡‡, Jian Jin***, Ian J. Davis****, Amber L. Mosley††, and Samantha G. Pattenden‡‡‡

From the ‡Carolina Institute for Developmental Disabilities, Department of Cell Biology and Physiology, and the Department of Genetics, Curriculum in Bioinformatics and Computational Biology, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, the §Department of Genetics and the Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, the ¶Department for Integrative Chemical Biology and Drug Discovery, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, Chapel Hill, North Carolina 27599, the ∥Center for Epigenetics, Van Andel Research Institute, Grand Rapids, Michigan 49503, the **Laboratoire Epigénétique et Destin Cellulaire, UMR7216, CNRS, Université Paris Diderot, Sorbonne Paris Cité, 75013 Paris, France, the ‡‡Lineberger Comprehensive Cancer Center, the Curriculum in Genetics and Molecular Biology, and the Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, the ‡‡‡Department of Structural and Chemical Biology, the Department of Oncological Sciences, and the Department of Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, New York, New York 10029, the ‡‡§Department of Genetics, the Lineberger Comprehensive Cancer Center, the Department of Pediatrics, and the Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, and the |||Department of Biochemistry and Molecular Biology and the Center for Computational Biology and Bioinformatics, Indiana University School of Medicine, Indianapolis, Indiana 46202.

Background: G9a-GLP lysine methyltransferases mono- and di-methylate histone H3 lysine 9 (H3K9me2). Results: Widely interspaced zinc finger (WIZ) regulates H3K9me2 levels through a mechanism that involves retention of G9a on chromatin.

Conclusion: The G9a-GLP-WIZ complex has unique functions when bound to chromatin that are independent of the H3K9me2 mark.

Significance: Combining pharmacologic and genetic manipulations is essential to any translational hypotheses related to G9a function.

G9a and GLP lysine methyltransferases form a heterodimeric complex that is responsible for the majority of histone H3 lysine 9 mono- and di-methylation (H3K9me1/me2). Widely interspaced zinc finger (WIZ) associates with the G9a-GLP protein complex, but its role in mediating lysine methylation is poorly defined. Here, we show that WIZ regulates global H3K9me2 levels by facilitating the interaction of G9a with chromatin. Disrupting the association of G9a-GLP with chromatin by depleting WIZ resulted in altered gene expression and protein-protein interactions that were distinguishable from that of small molecule-based inhibition of G9a/GLP, supporting discrete functions of the G9a-GLP-WIZ chromatin complex in addition to H3K9me2 methylation.

The posttranslational modification of the N-terminal lysines of histone proteins is associated with diverse biological consequences including changes in transcription and chromatin architecture. Lysine methylation is commonly performed by the SET (Su(var)3-9-Enhancer of zeste-Trithorax) domain-containing proteins (1–5). Two important members of this family are the G9a (EHMT2) and GLP2 (EHMT1) lysine methyltransferases. These enzymes are implicated in a variety of disease and biological processes; (6) they demonstrate both co-activator and co-repressor functions (6–8) and interact with other SET domain-containing proteins including SETDB1, Su(var)9H1, and the PRC2 complex (9, 10). G9a and GLP show similar substrate specificities (11–13); they methylate histones H1 (11, 12, 14), H3K27 (11, 15) and H3K56 (16), as well as a number of non-histone proteins (17–21), but are best characterized as the major lysine methyltransferases involved in mono- and di-methylation of H3K9 (11, 22–25).

Transcriptional silencing and the H3K9 mono- and di-methylation functions of G9a and GLP are dependent on heterodimerization through their respective SET domains (25). Studies in mouse G9a and Glp knock-out cells demonstrate that loss of either G9a or GLP results in degradation of the remaining protein partner (13, 25). Reconstitution of these cells with a heteromeric complex consisting of either a G9a or a GLP catalytic mutant suggests that the catalytic activity

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§ The abbreviations used are: GLP, G9a-like protein; WIZ, widely interspaced zinc finger; DMSO, dimethyl sulfoxide; qPCR, quantitative PCR; IP, immunoprecipitation; CTCF, CCCTC-binding factor; SAINT, significance analysis of interactome; CHX, cycloheximide; TSS, transcription start site.
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siRNA Knockdown and Compound Treatment—Cells were treated with the indicated media supplemented with 1 μM UNC0638 (30) or the volume equivalent of DMSO for 48 or 96 h, as indicated. All siRNAs were purchased from GE Healthcare Dharmacon, and transfections were performed as per the manufacturer’s instructions with DharmaFECT 4 transfection reagent. siRNAs used in this study were as follows: control (scrambled) siRNA (Dharmacon non-targeting 001810-04), G9a siRNA (Dharmacon 006937-05), and WIZ siRNA custom (ON-TARGETplus, Dharmacon) with sense sequence 5’-CAGCAGAGGUAAGGCAAUUU-3’ and antisense sequence 5’-UUGCCUUGACUCUCUGUUU-3’.

Quantitative RT-PCR—RNA was extracted using the RNaseasy Plus mini kit (Qiagen), and RNA was quantified on a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific). The reverse transcription reaction was performed with 500 ng of RNA using Superscript III First Strand Synthesis Supermix (Invitrogen 1172-050) according to the manufacturer instructions. The resulting cDNA was diluted 1:10, and 2 μl of the mixture was used for each PCR reaction. Quantitative PCR was performed using Roche FastStart Universal SYBR Green Master Mix (Roche Applied Science) in a 384-well plate format on an ABI 2900HT instrument. Oligo sequences are available upon request.

Immunoprecipitation—Whole cell protein extract was prepared by lysing cells in Cytobuster™ protein extraction reagent (EMD Millipore 71009) supplemented with 25 units of Benzonase nuclease (EMD Millipore 101654). Subcellular fractionation was performed using the Pierce Subcellular Fractionation Kit (Thermo Fisher Scientific 78840) according to the manufacturer’s instructions. FLAG-HA-G9a was immunoprecipitated from the samples described with Anti-FLAG® M2 affinity gel (Sigma A2220) overnight at 4 °C and washed three times in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20). Bound proteins were eluted by boiling in 1× SDS-PAGE loading buffer and subjected to Western blot analysis.

G9a Protein Stability Assay—FLAG-HA-G9a cells were plated on 6-well plates at a density of 5 × 10⁵ cells/well. After 24 h, cells were transfected with siRNA as described previously or treated with 1 μM UNC0638 (or volume equivalent of DMSO) and incubated for 48 h. Cells were then treated with 1 μM MG132 (or volume equivalent of DMSO) for 12 h followed by the addition of 25 μg/ml (Fig. 3A) or 50 μg/ml (Fig. 3B) cycloheximide (or volume equivalent of ethanol) for 0, 4, 6, 9, or 13.5 h. Cells were lysed in Cytobuster™ protein extraction reagent (EMD Millipore 71009) supplemented with 25 units of Benzonase nuclease (EMD Millipore 101654). The protein extract concentration was determined using Bradford reagent (Bio-Rad 500-0006) and a standard curve generated by known quantities of bovine serum albumin. Ten μg of lysate was run on SDS-PAGE and subjected to Western blotting.

Western Blotting and Densitometry—Western blotting was performed using a Typhoon imager (GE Healthcare) as described elsewhere (31) or using a LI-COR Odyssey as described elsewhere (32). Densitometry was performed using ImageJ software or LI-COR Image Studio software.

Experimental Procedures

Cell Culture—HEK293T cells were grown in DMEM supplemented with 10% FBS. FLAG-HA-G9a HeLa cells (9) were grown in MEM supplemented with 5% FBS and 5% horse serum. Stable pools of WIZ shRNA knockdown and control shRNA knockdown cells were created as follows. FLAG-HA-G9a HeLa cells (9) were transduced with lentivirus containing WIZ shRNA knockdown cells were created as follows. FLAG-HA-G9a HeLa cells (9) were transduced with lentivirus containing WIZ shRNA knockdown and control shRNA insert that does not target any known genes from any species (Sigma Mission pLKO.1-puro non-target shRNA, TRCN0000253784) or a control shRNA that contains an scramble sequence. Transduced cells were selected with 5 μg/ml puromycin, and the resulting stable pools were grown in MEM supplemented with 5% FBS, 5% horse serum, and 5 μg/ml puromycin.

WIZ Cloning—WIZ cDNA (Thermo Fisher Scientific MH51768-101549168) was PCR-ampliﬁed with o-WIZclone-1 (forward, 5’-GGGTTCTAGAGATGGTGGCCATTTGGG-3’) and o-WIZclone-2 (reverse, 5’-GCACCGCCG-GGGAGCTCTGCGCGCGCTG-3’) and cloned into pcDNA3.1/myc-His B (Invitrogen, V855-20) with XbaI and SacII.

of GLP, but not G9a, is dispensable for H3K9 methylation (13).

Although the interaction between G9a and GLP has been studied extensively, the interaction between these proteins and a third complex member, widely interspaced zinc finger (WIZ) protein, is less well understood. The mouse Wiz gene products were identified as two alternatively spliced isoforms, WizS and WizL, which were abundantly expressed in the brain compared with other tissue types (26). WIZ has C₂H₂-type zinc finger motifs, originally characterized in the Xenopus TFIIIA (27) and Drosophila Krüppel transcription factors (28). Typical C₂H₂-type zinc finger motifs are separated by seven amino acids. The WIZ zinc fingers are widely spaced, being separated by distances ranging from 16 to 258 amino acids in mouse (26) and from 16 to 263 amino acids in the longest human splice variant. Mutational analysis has demonstrated that WIZ interacts with the C-terminal SET domain of G9a or GLP through its C-terminal zinc ﬁnger (29). WIZ has been shown to bridge the interaction between G9a-GLP and the transcriptional co-repressors C-terminal binding protein 1 (CtBP1) and CtBP2, possibly to help recruit G9a-GLP to speciﬁc genomic loci (29), and is a non-histone methylation target for G9a (18).

WIZ has also been implicated in G9a and GLP protein stabilization (29). We found that WIZ knockdown leads to an H3K9me2 loss that is not attributable to the degradation of G9a or GLP protein; rather, WIZ is important for the retention of G9a on chromatin. Using pharmacological inhibition of G9a and gene-silencing methods to regulate WIZ, we compared G9a localization with evidence of enzymatic activity. Although the loss of G9a chromatin localization mediated by WIZ and small molecule inhibition of G9a and GLP activity (30) both resulted in a loss of H3K9me2, these treatments variably affected gene expression and G9a protein-interacting partners.
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**FIGURE 1.** Treatment with UNC0638 and knockdown of WIZ results in loss of H3K9me2.  
A, RT-qPCR of G9a, GLP, and WIZ mRNA expression in HEK293T cells treated with UNC0638 or vehicle control for 48 h, normalized to B2M expression. Error bars represent the standard deviation of three biological replicates. B, Western blots of whole cell extract from HEK293T cells treated with 1, 2, and 4 μM UNC0638 or vehicle control for 48 h. C, densitometry of Western blot bands represented by B. Error bars represent the standard deviation of three biological replicates. The y axis shows the G9a and H3K9me2 signals. The y axis shows the fold change in densitometry signal after 1 μM UNC0638 treatment relative to DMSO. p values are indicated. D, RT-qPCR showing mRNA levels in HEK293T cells treated with control or WIZ siRNA for 48 h. E, densitometry of Western blot bands represented in B. Error bars represent the standard deviation of three biological replicates. The y axis shows the fold change in densitometry signal of WIZ siRNA knockdown relative to control; p values are indicated. F, RT-qPCR showing mRNA levels in HeLa FLAG-HA-G9a cells stably transfected with control or WIZ shRNA, normalized to B2M mRNA levels. Error bars represent the standard deviation of four biological replicates. p values are indicated. NS, no significant difference. G, Western blots of whole cell extract and FLAG IP from HeLa FLAG-HA-G9a cells stably transfected with control or WIZ shRNA. J, densitometry of Western blot bands represented in H. Error bars represent the standard deviation of three biological replicates. The y axis shows the input and G9a-FLAG IP signals for the indicated antibody. The y axis shows the fold change in WIZ shRNA densitometry signal relative to control input signal. p values are indicated. NS, not significant.

**Chromatin Immunoprecipitation**—Chromatin immunoprecipitation was performed using the ChIP-IT High Sensitivity kit (Active Motif 53040) according to the manufacturer’s instructions, except that the input samples were purified by phenol:chloroform extraction. The following antibodies were used: anti-H3K9me2 (Abcam ab1220), anti-H3K9me3 (Abcam ab8898), anti-G9a (Abcam ab40542), and Rabbit IgG (Jackson ImmunoResearch 011-000-003). ChIP samples were quantitated using the Qubit 2.0 fluorometer (Invitrogen). A portion of each sample was confirmed by qPCR as described above. The fold change over background was calculated using the ΔΔCt method (33), where immunoprecipitation (IP)/input for the ChIP antibody signal was compared with the IP/input value for IgG (background). After a confirmation of the ChIP signal over background, the remainder of the sample was submitted for high-throughput sequencing. Oligo sequences are available upon request.

**High-throughput Sequencing and Data Analysis**—Libraries were prepared for high-throughput using the Illumina TruSeq kit as per the manufacturer’s recommendations. Single-end 50-bp reads were sequenced on an Illumina HiSeq. Reads were checked to be of sufficient quality, filtered to remove those with a significant adapter contribution using TagDust (34), and aligned to the reference human genome (hg19) using Bowtie (35). Regions of significant ChIP enrichment relative to input control (“peaks”) were identified using MACS2, assuming fragments were 250 bp in length. Heat maps were generated in MATLAB by plotting the normalized ChIP-seq signal around the midpoints (± 2 kb) of the union set of G9a peaks. Changes in the ChIP signal were illustrated by taking the arithmetic difference between treated and untreated samples. ChIP-seq data are available from NCBI Gene Expression Omnibus (accession number GSE67317). Genomic distributions were computed using CEAS (36). The CCCTC-binding factor (CTCF) and H3K4me3 ChIP-seq data from 293T cells were mined from BAM files provided by ENCODE (37). To find regions with significantly altered ChIP-seq enrichment, the number of overlapping aligned reads for all 500-bp non-overlapping windows across the genome was first tabulated for all samples. Only those windows with an average number of aligned reads exceeding 10 were retained. A binomial test was then applied to compare the G9a ChIP-seq signal between control
siRNA-treated and WIZ siRNA-treated cells at each of these windows; p values were corrected for multiple testing using the q value. Windows with q < 1 × 10⁻⁷ were retained and used for subsequent analyses.

Gene Expression Microarrays—RNA was extracted using the RNeasy Plus mini kit (Qiagen) and quantified on the Qubit fluorometer (Invitrogen). RNA quality was verified on an Agilent bioanalyzer before hybridization to human 8 × 60 K arrays (Agilent Technologies 28004). After scanning, microarray images were processed using Agilent Feature Extraction software according to the manufacturer-supplied protocols. The resulting background-subtracted and local regression (LOESS)-normalized log ratio data were collected and filtered to remove data generated from poor quality or low intensity spots. Probes with >30% missing data were removed from further evaluation. Dye swap replicates were inverted to match the log ratio direction of non-dye swap replicates. The remaining missing data were then imputed using weighted k-nearest neighbors (KNNimpute) (38). Testing for differential expression was performed using the statistical analysis of microarray (SAM) (39) one-class option for a comparison with DMSO or scrambled siRNA control and the two-class unpaired option when comparing UNC0638 with WIZ siRNA knockdown. A conservative false discovery rate threshold of 0.5% was set to identify differentially expressed genes in both experiments. The differential expression results were visualized using hierarchical cluster analysis based on average linkage clustering and a Pearson correlation distance metric. All statistical analyses were carried out using the R package (v3.1.1). Expression data are available from NCBI Gene Expression Omnibus (accession number GSE70914).

G9a Purification for Proteomics—G9a was purified from the HeLa FLAG-HA-G9a cell line (9) grown in a volume of 5 liters (~8 ml cell pellet), treated with either 1 μM UNC0638 or volume equivalent of DMSO for 48 h. Cells were centrifuged, washed once in PBS, and then frozen at ~80 °C prior to purification. Purification was performed as follows. All buffers were supplemented with 1× protease inhibitor mixture (Roche 05056489001). Cell pellets were thawed on ice, resuspended in 40 ml of nuclei prep buffer (50% glycerol, 0.5% Nonidet P-40, 1 mm PMSF, 10 mm Tris-HCl, pH 7.4, 10 mm NaCl, and 3 mm MgCl₂), and incubated on ice for 10 min or until the nuclei were visible with trypan blue stain. Nuclei were pelleted, washed once with reticulocyte standard buffer (RSB: 10 mm Tris-HCl, pH 7.4, 10 mm NaCl, and 3 mm MgCl₂), resuspended in 24 ml of nuclei lysis buffer (0.2% Triton X-100, 10 mm HEPES, pH 7.9, 1.5 mm MgCl₂, 0.56 mm NaCl, and 1 mm KCl) and incubated for 10 min on ice. The nuclei were then centrifuged over a 0.8-ml chromatography spin column (Bio-Rad 732-1550), and anti-FLAG M2 affinity gel (Sigma A2095) was equilibrated in 0.42 ml NaCl nuclei lysis buffer (~100 μl of beads) was incubated with the clarified lysate overnight at 4 °C. Beads were washed with 50 ml of wash buffer (0.2% Triton X-100, 10 mm HEPES, pH 7.9, 1.5 mm MgCl₂, 0.3 mm NaCl, and 10 mm KCl) and transferred to a 0.8-ml chromatography spin column (Pierce 89868). The protein was eluted by rotation at room temperature in 200 μl of elution buffer (50 mm Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 0.15 mm NaCl, and 0.2 mg/ml 2× FLAG peptide) three times for a total of ~600 μl of eluate. Eluates were diluted to 1.5 ml final volume in wash buffer and transferred to a chromatography spin column (Bio-Rad 732-6008). Anti-FLAG-agarose (Sigma A2095) was equilibrated in wash buffer (~100 μl beads) and incubated with the eluates overnight at 4 °C. Beads were washed with 30 ml of wash buffer and then transferred to a 0.8-ml chromatography spin column (Pierce 89868). Proteins were eluted in 200 μl of elution buffer (50 mm Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 0.15 mm NaCl, and 1.0 mg/ml HA peptide) four times for a total of ~800 μl of eluate. A portion of the eluates was visualized by silver staining, and the remaining sample was submitted for mass spectrometry analysis.

FIGURE 2. A combination of enzymatic inhibition and reduction in WIZ protein levels has an additive effect on G9a chromatin localization. A, RT-qPCR showing G9a, GLP, and WIZ mRNA levels in HEK293T cells treated with control or WIZ siRNA in combination with vehicle (DMSO) or 1 μM UNC0638 for 48 h. Error bars represent the standard deviation of three biological replicates. B, Western blots of fractionated lysates from HEK293T cells treated with control or WIZ siRNA in combination with vehicle (DMSO) or 1 μM UNC0638 for 48 h. The fractions are total (input), chromatin, and soluble.
Proteomic Analysis—Multidimensional protein identification technology (MudPIT) was carried out as described previously (40, 41). In brief, proteins from each elution were precipitated with trichloroacetic acid, and the resulting pellets were washed with 100% cold acetone. The dried protein pellets were then resuspended in 8M urea and reduced and alkylated with tris(2-carboxyethyl)phosphine and chloroacetamide as described previously (42). The proteins were then subjected to proteolytic digestion with LysC overnight at 37 °C with shaking followed by digestion with Trypsin Gold (Promega) after diluting the sample to 2 m urea. The reaction was quenched with formic acid and then pressure-loaded onto a microcapillary column pack with both strong cation exchange resin and reverse phase resin as described previously (42). A nine-step MudPIT run was performed for each sample on an LTQ-Velos Pro that was in-line with a Proxeon Easy nanoLC (41). The resulting RAW data files were processed by protein database matching using SEQUEST within Proteome Discoverer 1.4 (Thermo Fisher Scientific). Database matching was performed using a Homo sapiens FASTA database downloaded from UniProt.
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Results

Enzymatic Inhibition of G9a or Knockdown of WIZ Expression Leads to a Global Reduction in H3K9 Methylation Levels—We initially compared H3K9me2 levels following chemical inhibition of G9a catalytic activity and reduction in G9a protein levels due to WIZ knockdown. Inhibition of G9a catalytic activity in HEK293T cells with a small molecule probe, UNC0638 (30), did not result in a change in G9a, GLP, or WIZ mRNA levels (Fig. 1A) but did lead to a global reduction in H3K9me2 levels and an increase in G9a protein (Fig. 1, B and C). Treatment of HEK293T cells with a siRNA against WIZ reduced WIZ mRNA 5-fold (p < 0.0001) but did not affect G9a or GLP mRNA levels (Fig. 1D). H3K9me2 (p = 0.03) and WIZ (p = 0.001) protein levels decreased compared with the control siRNA (Fig. 1, E and F). There was also a slight (1.25-fold) decrease in G9a levels, which agrees with a previous study that linked WIZ with G9a protein stability (29). To determine whether WIZ knockdown affects long-term G9a protein stability, we stably expressed WIZ shRNA or control in a HeLa cell line that expresses a double tagged version of G9a (9). These cells demonstrated no change in G9a or GLP mRNA levels (p = 0.1311), whereas WIZ mRNA levels were reduced 2-fold (p = 0.035) (Fig. 1G). Even after multiple passages of this cell line, the overall change in G9a protein levels remained very small (1.25-fold, p = 0.02), whereas H3K9me2 levels were reduced 2.5-fold (p = 0.01) and WIZ protein levels were 2.5-fold lower (input, p = 0.02) (Fig. 1, H and I). These data suggest that reduction in H3K9me2 associated with WIZ silencing was not due to progressive loss of G9a protein.

WIZ Is Important for G9a Interaction with Chromatin—Because other zinc finger proteins have been shown to regulate lysine methyltransferase activity (46, 47), we investigated the possibility that WIZ is important for G9a retention on chromatin. To assess G9a chromatin interaction, we performed subcellular fractionation of HEK293T cells that were treated with UNC0638, WIZ siRNA, or combinations of the two to determine whether these treatments had an additive effect. Neither treatment nor the combination of the two had an effect on G9a or GLP mRNA levels, whereas WIZ mRNA levels were reduced to a similar extent in both the siRNA knockdown and the combination treatment (Fig. 2A). H3K9me2 levels were visibly reduced after treatment with UNC0638 in combination with control siRNA (Fig. 2B, compare lanes 5 and 6, 4.8-fold) or WIZ siRNA (Fig. 2B, compare lanes 5 and 8, 8.6-fold) and were slightly reduced after treatment with DMSO combined with WIZ siRNA (Fig. 2B, compare lanes 5 and 7, 1.6-fold). Consistent with the changes in H3K9me2 levels, G9a and GLP association with the chromatin fraction was altered after treatment with UNC0638 or WIZ siRNA. G9a levels decreased slightly after the addition of UNC0638 (Fig. 2B, compare lanes 5 and 6, 1.9-fold), WIZ siRNA (Fig. 2B, compare lanes 5 and 7, 1.8-fold),
or a combination of both treatments (Fig. 2B, compare lanes 5 and 8, 6.2-fold). Similar to G9a, GLP levels decreased in the chromatin fraction after the addition of UNC0638 (Fig. 2B, compare lanes 5 and 6, 1.9-fold), WIZ siRNA (Fig. 2B, compare lanes 5 and 7, 1.7-fold), or a combination of both treatments (Fig. 2B, compare lanes 5 and 8, 9.1-fold). The dramatic reduction in G9a and GLP protein levels in the chromatin fraction after the combination treatments (Fig. 2B, lane 8) indicates that enzymatic inhibition and reduction in WIZ protein levels have an additive effect, suggesting that WIZ may help stabilize G9a interaction with chromatin.

Because we observed increased G9a in the soluble fraction after UNC0638 treatment not attributable to an increase in G9a mRNA (Figs. 1A and 2A), we examined G9a protein stability in HeLa FLAG-HA-G9a cells. We inhibited de novo protein synthesis with cycloheximide (CHX) in combination with MG132 proteasome inhibitor, UNC0638, or WIZ siRNA knockdown (Fig. 3). Although treatment with UNC0638 for 48 h resulted in higher levels of G9a, none of the treatments demonstrated diminished G9a protein turnover including up to 13.5 h of CHX treatment (Fig. 3A). As a control, the levels of a control protein, COXIV, were reduced after 6 h of CHX treatment (Fig. 3B). Extending the experiment beyond 13.5 h was not possible (Fig. 3A) due to cell toxicity. Thus, we cannot rule out the possibility that UNC0638 increases long-term G9a protein stability.

To further examine G9a association with chromatin, we performed subcellular fractionation of HeLa cells expressing FLAG-HA-tagged G9a followed by FLAG IP of G9a. G9a association with GLP, WIZ, H3K9me3, or H3 did not change following UNC0638 treatment (Fig. 4, A and B), but H3K9me2 levels in three biological replicate experiments were lower in the input (p = 0.030) and IP (p = 0.001) samples compared with the H3 input signal (Fig. 4C). Although G9a continued to interact with GLP when WIZ was knocked down, its interaction with both K9 di-methyl (2.2-fold) and tri-methyl (2.3-fold) H3 and total H3 (2.2-fold) was noticeably reduced. These data indicate that a reduction in WIZ protein levels decreases the association of G9a with histones in the chromatin fraction (Fig. 4D). We then examined G9a and WIZ localization to either the nuclear or chromatin fractions (equivalent to Fig. 4, A and C, Input). Treatment with UNC0638 resulted in an increase in G9a protein levels in the nuclear compartment as noted earlier (Fig. 2B), but G9a levels remained unchanged in the chromatin fraction, and no alteration in WIZ protein levels was observed (Fig. 4D). In contrast, WIZ shRNA knockdown significantly reduced G9a levels in the chromatin fraction (Fig. 4F, 2-fold).

To confirm that this effect was WIZ-specific, we transfected a plasmid expressing the short human WIZ splice variant cDNA into the stable WIZ silenced HeLa cells (Fig. 1E) followed by subcellular fractionation (Fig. 5). The short isoform (WIZS) was selected for reconstitution for several reasons. First, it contains five zinc fingers that are highly conserved in mouse and...
human WIZ, whereas the additional zinc fingers present in the long isoform (WIZL) are less conserved (26, 48). Second, in mice, WizL and WizS are expressed at different times during development, with WizS being highly expressed in the developing cerebellum and WizL expression restricted to the mature cerebellum (26). Despite the disruption of WizL in C57BL/6 mice due to the insertion of an ETn retrotransposon, there is no obvious phenotype in the cerebellum, suggesting a nonessential role for WizL (48). Third, G9a and GLP interact with the C-terminal zinc finger of WIZ, which is present in the protein product for both splice variants (29). Note that the WIZ siRNA recognizes the 3'-end of the WIZ transcript, and therefore both isoforms were knocked down after treatment (Fig. 5A). Because WIZ mRNA was reduced about 2-fold in the shRNA stable cell line (Fig. 1E), and the pWIZ vector was overexpressed compared with endogenous WIZ (data not shown), we did not mutate the region targeted by WIZ shRNA in the pWIZ vector.

In the WIZ knockdown pool, G9a protein levels were reduced in the chromatin fraction compared with the control knockdown pool (p = 0.024, Fig. 5, B and C). Transfection of the pWIZ vector resulted in an increase in G9a protein levels in the chromatin fraction to levels comparable with those of the control knockdown pool (p = 0.028, Fig. 5, B and C). Thus, the protein product of the WIZS isoform is sufficient to restore G9a and WIZ protein levels to chromatin.

To determine whether G9a chromatin occupancy was affected by enzymatic inhibition or reduced levels of WIZ, we performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). HEK293T cells were treated with 1 μM UNC0638 or DMSO for 48 h (Fig. 6A) or 96 h

FIGURE 7. UCSC Genome Browser snapshots of specific genomic regions. A–C, changes in signal are highlighted in gray. Occupancy for G9a and H3K9me2 in HEK293T cells is shown in WIZ siRNA knockdown (siWIZ), control siRNA knockdown (siNS), and UNC0638-treated or DMSO-treated cells. A blue arrow represents transcribed regions, and the scale is shown in kb in the top right corner.

FIGURE 8. Genomic distribution of G9a. A, the percent distribution of genomic regions with G9a ChIP signal is plotted relative to the genomic coverage of those features. B, the percent distribution of regions where G9a signal is lost following WIZ siRNA treatment is plotted. C, line plot showing the average ChIP signal in HEK293 cells for H3K4me3 (red line) and CTCF (black line) centered around regions that lose G9a signal after WIZ siRNA treatment (blue line). Scale is shown in kb. Left, x axis represents the average ChIP-seq signal for H3K4me3 and CTCF (data from ENCODE). Right, x axis represents the average ChIP-seq signal for G9a (WIZ siRNA — control siRNA signal).
(Fig. 6B) or control siRNA or WIZ siRNA for 72 h (Fig. 6C) followed by ChIP with antibodies against G9a or H3K9me2 (30) (Figs. 6–8).

We first normalized the ChIP-seq enrichment computed over the union set of G9a binding sites to account for differences in the sequencing depth. We then assessed the difference in the G9a and H3K9me2 ChIP-seq signals between UNC0638-treated (Fig. 6, A and B) or WIZ siRNA (Fig. 6C)-treated and untreated cells by subtracting the signal from control cells. A reduction in the H3K9me2 signal was observed for all treated samples compared with controls (Fig. 6, A–C and E). Following UNC0638 treatment, the G9a signal was elevated (Fig. 6, A, B, and D), which might be explained by the increase in the G9a protein levels (Figs. 2B, 3, and 4A). This elevation in the G9a signal was general and subtle, with no one site in the treated samples having a statistically significant increase over controls. The signal difference decreased and became more diffuse after 96 h. In contrast, WIZ siRNA treatment resulted in a loss of G9a signal (Fig. 6, C and D). Examples of the changes at specific regions are highlighted in Fig. 7. These data suggest that the decreased G9a chromatin binding associated with a reduction in WIZ is responsible for the loss of H3K9me2.

Assessment of the genomic distribution of regions with G9a occupancy revealed that the majority of G9a signal was localized to introns or distal intergenic regions (Fig. 8A). UNC0638 treatment resulted in a slight increase in G9a occupancy at distal intergenic regions at 48 h (2.8%) and 96 h (4%), with no other large changes in occupancy distribution noted. Treatment with WIZ siRNA had little effect on overall G9a distribution, with the remaining G9a signal increasing slightly at promoters (1.6%) and decreasing slightly at distal intergenic regions (2.7%). Because WIZ siRNA knockdown resulted in an overall loss of G9a signal, we examined the genomic distribution of regions with reduced G9a occupancy compared with control siRNA knockdown (Fig. 8B). We identified 14,116 regions where G9a occupancy was significantly reduced following WIZ knockdown (binomial test, $q < 1 \times 10^{-5}$). Many of these differential regions were distal from genes (~83% of these sites were greater than 10 kilobases (kb) from a transcription start site (TSS), and the median distance to the nearest TSS was ~30 kb. There were, however, 2389 sites within 10 kb of a TSS. Despite the small proportion of sites (~17%) in the vicinity of a TSS, we noted that the loss of G9a was slightly skewed toward promoters and coding exons following WIZ siRNA treatment relative to the genomic coverage of those features. By associating the sites of G9a signal loss with ENCODE ChIP data for HEK293 cells (37), we observed signal enrichment with H3K4me3 and the CTCF (Fig. 8C), both of which are associated with active transcription.

The Effects on Gene Expression by WIZ Knockdown Do Not Completely Overlap with Those by Enzymatic Inhibition of G9a—Because WIZ silencing and G9a inhibition both led to reduced H3K9me2 levels, we asked whether gene expression would be similarly affected. G9a can act as both a co-activator and co-repressor of transcription (49–53). As the ability of G9a to affect transcription is not fully dependent on its catalytic activity, we predicted that treatment with UNC0638 would

![FIGURE 9. RT-qPCR data from samples used for gene expression microarray analysis.](http://www.jbc.org/)
with the heterodimer (13, 25), it is not surprising that G9a knockdown affects gene expression differently than either enzymatic inhibition (UNC0638 treatment) or loss of chromatin association (WIZ knockdown). Indeed, this observation is also true for genes with divergent expression changes following WIZ knockdown versus UNC0638 treatment (Fig. 10B).

For differentially expressed genes (Fig. 10B, 118 genes at <0.5% false discovery rate), enzymatic inhibition resulted in derepression of gene expression, whereas WIZ knockdown led to transcriptional repression. As noted earlier, only ~17% of the regions of G9a loss were within 10 kb of a TSS. Of these sites, 42% (or 1015) were near a gene that was represented on our microarray. Although only 32 of those regions were near a gene with a significant (q < 0.05) change in gene expression (Fig. 10C), all but one of those genes were repressed following WIZ knockdown. In contrast, UNC0638 treatment showed less overall change in the gene expression profiles in the same regions. The fact that loss of G9a ChIP signal after WIZ knockdown is associated with signals associated with active transcription, but are not generally seen directly at transcription start sites (Fig. 8, B and C), could indicate that G9a exerts co-transcriptional influence from a distance.

Taken together, these data suggest that binding of G9a to chromatin is important for its role as a co-activator, whereas enzymatic activity is associated with co-repressor function. These observations are consistent with the promoter and exon-skewed loss of G9a following siRNA WIZ knockdown (Fig. 8, B and C) and with prior studies, indicating that G9a catalytic activity is dispensable for co-activator function (49, 50, 52–54).}

**Discussion**

We demonstrated that WIZ is important for G9a interaction with chromatin. Stable knockdown of WIZ reduced H3K9me2 levels and resulted in a very slight reduction in G9a protein levels, which remained stable over time (Fig. 1, A and B). Differences could be due to the fact that there was only about a 40% reduction in WIZ protein levels and resulted in a very slight reduction in G9a protein levels and resulted in a very slight reduction in G9a protein levels, which remained stable over time (Fig. 1, A and B). This suggests that WIZ-mediated effect was specific, because reconstitution of exoge-
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![Diagram](image)

**FIGURE 11. Silver-stained gels showing purified samples submitted for mass spectrometry analysis.** Tandem affinity purification of G9a was performed with FLAG followed by HA. HA elution samples were submitted for mass spectrometry analysis. The FLAG elution (FLAG Elute) and HA purification unbound (HA UB) samples are shown for reference. **A,** purification from HeLa FLAG-HA-G9a cells treated with DMSO or 1 μM UNC0638 for 48 h. **B,** purification from HeLa FLAG-HA-G9a cells with either control shRNA or WIZ shRNA stable knockdown.

WIZ nously expressed WIZ into WIZ knockdown cells increased G9a chromatin-associated protein (Fig. 5, B and C), and genome-wide, as WIZ knockdown resulted in a dramatic loss of G9a occupancy on chromatin (Fig. 6).

The WIZ protein contains C$_2$H$_2$-type zinc finger motifs that have an unusually widely spaced configuration (26). In *Petunia*, the EPF family of proteins has two widely separated C$_2$H$_2$ zinc fingers that recognize and bind to specific DNA sequences (55–57). *Drosophila* proteins with widely spaced zinc finger motifs include the chromatin-associated suppressor of variegation 3–7 (58–60) and the homeotic protein Teashirt, which binds sequence-specific gene-regulatory sites in *vivo* (61) and behaves as a transcriptional repressor when transfected into human cells (62). The murine orthologues of Teashirt also act as transcriptional repressors and are involved in developmental roles similar to fly Teashirt (63, 64). Thus, despite an unusual configuration, proteins with widely spaced zinc finger motifs are compatible with DNA binding and transcriptional regulation. This conclusion was further supported by findings published while our manuscript was being prepared for submission (65).

**Co-transcriptional Activity of G9a—**Although both loss of WIZ and small molecule inhibition of G9a reduced the H3K9me2 levels, they appeared to do so by distinct mechanisms. UNC0638 binds in the substrate-binding site of G9a and is noncompetitive with the cofactor S-adenosyl-L-methionine (30). Yet, although UNC0638 treatment effectively decreased cellular H3K9me2 levels, it did not generally displace G9a from chromatin. In contrast, silencing the G9a complex member WIZ resulted in the loss of both H3K9me2 and G9a occupancy on chromatin (Figs. 6 and 7).

The power of a combinatorial approach to the study of G9a signaling was highlighted by differential gene expression analysis, which suggested that G9a catalytic activity was associated with the co-repressor function, whereas G9a occupancy on chromatin was more closely related to its function as a co-activator (Fig. 10B). The G9a catalytic domain is sufficient for transcriptional activation of several genes (49, 50, 52–54), and G9a has a number of non-histone substrates, including itself, which would be affected by enzymatic inhibition but potentially not a knockdown of WIZ (17–21). Our results confirmed that catalytic activity was dispensable for the regulation of a subset of genes on our microarray (Fig. 10B). A comparison of either UNC0638 treatment or WIZ siRNA knockdown to a G9a siRNA knockdown would not have revealed this stark contrast, because the loss of G9a produced a gene expression pattern that overlapped with both treatment conditions (Fig 10B).

Our ChIP-seq data showed that G9a loss occurred mainly at distal intergenic and intronic regions of the genome (Fig. 8B); however, the signal was slightly skewed toward promoters and coding exons. The sites of G9a loss also overlapped with sites of H3K4me3 and CTCF binding (Fig. 8C). These data were consistent with our gene expression analysis, which associated G9a occupancy on chromatin with its co-activator activity at a subset of genes (Fig. 10B).

Despite the strong association between G9a occupancy and transcriptional activity, we noted that the overall median distance of sites of G9a loss following WIZ siRNA knockdown to the nearest TSS was ~30 kb (Fig. 8B). In addition, the majority of sites occupied by G9a were located in distal intergenic regions (Fig. 8A). These findings suggested that G9a co-transcriptional activity in HEK293T cells was exerted mostly at a significant distance from the site of transcription initiation. The H3K9me2 mark is unique compared with some other histone modifications because of its broad regional deposition (72). It has been proposed that G9a target specificity is determined by multiple interactions with chromatin or sequence-specific DNA-binding molecules (6, 8). It would be interesting to determine whether G9a binding mediates chromatin looping to allow its interaction with multiple sites in the genome from a localized domain.

**G9a Protein-interacting Partners—**The loss of H3K9me2 by two distinct mechanisms allowed for comparison of the
genomic and proteomic differences resulting from either enzymatic inhibition or relocalization of G9a. Proteomic data suggested that the interaction of G9a with its non-histone targets was affected by enzymatic inhibition to a greater extent than reduction of WIZ and chromatin targeting (Fig. 12). As expected, GLP and WIZ were identified as significantly interacting proteins (SAINT/H11350 > 0.9). Additional known G9a-associated proteins include: chromodomain on Y-like 1 (CDYL1) (73, 74), which binds methylated H3K9; histone deacetylase 1 (HDAC1) and HDAC2 (75); mesoderm induction early response 1 (MIER1) (76), which represses transcription through recruitment of HDAC1; and chromobox homologs CBX1 (or heterochromatin protein 1 (HP1α)) and CBX3 (HP1γ) (77–80), readers of methylated H3K9. Mass spectrometry analysis by others has shown that many of these proteins interact to form complexes. CDYL1 bridges the REST repressor and G9a to regulate neuronal gene expression. In addition to G9a and GLP, CDYL1 co-purified with WIZ, HDAC1 and HDAC2, and MIER1 (81). HDAC2 was shown by mass spectrometry to associate with G9a, GLP, WIZ, MIER1 and MIER2, and the reciprocal purification with G9a bait yielded both HDAC2 and MIER1 (82).

We noted that enzymatic inhibition of G9a had more effect on its protein-interacting partners compared with WIZ knockdown (Fig. 12B). These data could be the result of an inefficient WIZ knockdown (WIZ protein levels were reduced by ~40%), or they could indicate that G9a displaced from chromatin maintains catalytic activity toward non-histone substrates. In support of the latter interpretation, proteins with the greatest abundance of changes in UNC0638-treated cells compared with controls were non-histone substrates or closely associated with non-histone substrates of G9a. Automethylation of G9a has been shown to be important for its interaction with CBX3 (17, 19, 67). Other non-histone substrate protein interactions that are reduced following treatment include CDYL1, HDAC1, GLP, and WIZ (18, 21). Knockdown of WIZ did not result in a change in association of G9a with CDYL1 or CDYL2, again implying that G9a enzymatic activity is important for this interaction. Other associations that were affected by enzymatic inhibition, but not WIZ knockdown, include CBX1 and ZNF644. These data suggest that methylation of non-histone substrates by G9a could be important for maintaining interactions with various protein complexes.

Overall, our findings underscore the potential of combining small molecule probes with gene silencing to dissect molecular pathways. Understanding the mechanistic basis for differences between pharmacologic and genetic manipulations is essential to any translational hypotheses related to G9a function (83–85).

Author Contributions—S. G. P. conceived and coordinated the study, performed experiments other than those listed below, and wrote the paper. I. J. D. and B. D. S. critically revised the paper. J. M. S. prepared sequencing libraries and analyzed the data shown in Figs. 6–8 and 10C. J. S. P. analyzed the data shown in Fig. 10, and F. L. and J. J. synthesized UNC0638. S. B. R. performed and analyzed experiments shown in Fig. 2B. A. L. M. performed the mass spectrometry and proteomic analysis shown in Fig. 12. All authors reviewed the results and approved the final version of the manuscript.

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Gene Regulation:
A Role for Widely Interspaced Zinc Finger (WIZ) in Retention of the G9a Methyltransferase on Chromatin

Jeremy M. Simon, Joel S. Parker, Feng Liu, Scott B. Rothbard, Slimane Ait-Si-Ali, Brian D. Strahl, Jian Jin, Ian J. Davis, Amber L. Mosley and Samantha G. Pattenden

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