Zinc Finger Protein Wiz Links G9a/GLP Histone Methyltransferases to the Co-repressor Molecule CtBP*

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G9a is a SET-domain mammalian histone methyltransferase responsible for mono- and dimethylation of lysine 9 in histone H3 (H3K9) at euchromatic regions. Recently we reported that G9a forms a stoichiometric heteromeric complex with another SET-domain-containing molecule, GLP/Eu-HMTase1. Although G9a and GLP can independently methylate H3K9 in vitro, G9a/GLP heteromeric formation seems to be essential for their function as an euchromatic H3K9 methyltransferase in vivo. To further elucidate how G9a/GLP-mediated histone methylation and transcriptional regulation are controlled, we purified and characterized G9a complexes from mouse embryonic stem cells. We identified a novel G9a/GLP-associating zinc finger molecule named Wiz that can interact with G9a and GLP independently but is more stable in the G9a/GLP heteromeric complexes. Interestingly, Wiz small inhibitory RNA knockdown not only Wiz but also G9a. GLP deficiency also decreases G9a levels, suggesting that the Wiz/G9a/GLP tricomplex may protect G9a from degradation and that Wiz plays a major role in G9a/GLP heterodimer formation. Furthermore, amino acid sequence analysis of Wiz predicted two potential CtBP binding sites, and indeed CtBP binding to Wiz and association of CtBP with the Wiz/G9a/GLP complex was observed. These data indicate that Wiz not only contributes to the stability of G9a but also links the G9a/GLP heteromeric complex to the CtBP co-repressor machinery.

In eukaryotes, DNA is wrapped around core histones to form nucleosome particles and condensed chromatin structures with various nuclear molecules. Therefore, regulation of chromatin structure and dynamics is a very critical step for genomic functions. Covalent histone modifications play critical roles in regulating these processes (1). Among these modifications, histone lysine methylation has an enormous impact on various chromatin-associated functions including transcriptional regulation, heterochromatin formation, DNA repair, and recombination (1, 2). Like phosphorylation regulates protein function through controlling protein-protein interaction (3), histone lysine methylation also controls protein (histone)-protein interaction. It has been shown that each methylated lysine residue of H3 and H4 is utilized differentially and recruits different functional molecules involved in different chromatin-associating processes (2, 4–16).

The function of H3K9 methylation was initially defined based upon the first described histone lysine methyltransferases (HMTases), mouse Suv39h1 and its Drosophila and yeast counterparts Su(var)3–9 and Cdr4 (17–19). These HMTases are members of SET-domain-containing molecules and specifically methylate H3K9, which plays a crucial role in heterochromatin formation and heterochromatic gene silencing (19, 20). H3K9 methylation has also been shown to control DNA methylation, typically in fungus Neurospora crassa and plant Arabidopsis thaliana (21, 22). Methylated H3K9 allows the binding of the chromodomain of heterochromatin protein 1 (HP1), a step that is crucial for most of the chromatin functions regulated by H3K9 methylation (4, 6, 19). In transcriptional regulation within euchromatin, H3K9 methylation is generally associated with transcriptional silencing (1); however, recent reports suggest that H3K9 methylation is also associated with transcriptional activation (elongation) (23, 24). Therefore, the roles of H3K9 methylation on transcriptional regulation remain controversial.

G9a is also a SET-domain-containing molecule and a major mammalian histone methyltransferase responsible for mono- and dimethylation of H3K9 at euchromatic regions (25, 26). Recently, we described that G9a forms a stoichiometric heteromeric complex with another SET-domain-containing molecule, GLP/Eu-HMTase1. Although G9a and GLP can independently methylate H3K9 in vitro, G9a/GLP heteromeric formation seems to be essential for exerting their function as an euchromatic H3K9 methyltransferase (27). It has been reported that both G9a and GLP are components of several transcriptional repression complexes, such as those involving E2F6, CtBP1, and CDP/cut (28–30). We have also revealed that G9a exists as ~1-megadalton complexes in mouse embryonic stem

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2 The abbreviations used are: HMTase, histone lysine methyltransferase; ES, embryonic stem; CtBP, C-terminal binding protein; Wiz, widely interspaced zinc finger motifs; DAPI, 4’,6-diamino-2-phenylindole; GFP, green fluorescent protein; TRF, telomeric repeat binding factor; EGFP, enhanced green fluorescent protein; siRNA, small inhibitory RNA; CID, ctbp interaction domain; ZF, zinc finger motif.
(ES) cells. To elucidate how G9a/GLP HMTases regulate H3K9 methylation, how this transcriptional regulation is controlled, and the significance of the G9a/GLP heteromeric complex, we isolated the G9a complex from mouse ES cells and identified and characterized one of the novel G9a-associating molecules, Wiz.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse ES cells were maintained in 10% fetal calf serum and LIF (500 units/ml)-containing medium.

**Generation of Stable Cell Lines**—G9a<sup>−/−</sup> ES cells (clone #22-10, (25)) were co-transfected with a cDNA encoding human G9a (GenBank<sup>TM</sup> accession number NM_006709) tagged with His-FLAG epitopes at the carboxyl terminus in the pCAGGS expression plasmid and a vector conferring Hygromycin B resistancy (pGK-hygroB) by the use of Lipofectamine 2000 reagent (Invitrogen). Resistant cells were selected in ES cell medium containing 150 μg/ml hygromycin B and checked for the expression of human G9a by anti-G9a or anti-FLAG antibody by Western blotting. One of the positive clones (termed as HF7) was used for G9a complex purification.

**G9a Complex Purification**—The G9a complexes were purified from a nuclear extract prepared from HF7 cells. As a control, mock purification was performed from a nuclear extract prepared from the G9a<sup>−/−</sup> ES cells expressing mouse G9a-L without tags (clone #15-3). After removing the cytoplasmic fraction with buffer A (10 mM HEPES-KOH at pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride), nuclear pellets were suspended and lysed with buffer D (20 mM HEPES-KOH at pH 7.9, 1.5 mM MgCl<sub>2</sub>, 420 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, and 20% glycerol) for 30 min on ice. The insoluble nuclear fraction was removed by centrifugation.

The nuclear extract was first applied to nickel-nitriilotriacetic acid-agarose beads (Qiagen) and bound materials were eluted with buffer D containing 0.2 M imidazole. The eluates were further incubated with anti-FLAG mouse monoclonal antibody (M2)-conjugated agarose beads (Sigma) and then eluted with FLAG peptide (Sigma). The purified proteins were resolved by 4–20% gradient SDS-PAGE and silver-stained. The polypeptides specific to HF7 cells were excised and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Aliquots of the purified G9a complexes were also loaded onto a 10-ml 10–30% glycerol gradient in buffer D, centrifuged at 32,000 rpm for 1 h, and fractionated. Each fraction was concentrated with trichloroacetic acid, resolved by SDS-PAGE, and visualized by silver staining or Western blotting.

**Immunofluorescence Analysis**—By the use of TransIT-LT1 (Mirus), pEGFP-C2-Wiz was transfected to NIH3T3 cells. After 2 days in culture, cells were collected, cytospun, and fixed with 4% paraformaldehyde for 10 min. Then the cells were permeabilized with 0.1% Triton X-100 for 10 min and incubated with anti-G9a (#8620) at 37 °C for 40 min. Anti-mouse IgG conjugated with Zenon Alexa Fluor 568 ( Molecular Probes) was used for detection. The nuclei were counterstained with DAPI, observed under fluorescence microscopy, and analyzed with AxioVision software (Zeiss).

**Immunoprecipitation and Western Blot Analysis**—For immunoprecipitation of endogenous G9a, GLP, and Wiz, ES cells were harvested with phosphate-buffered saline containing trypsin (0.05%) and EDTA (0.2 mM). Nuclear extracts were prepared as described under “G9a Complex Purification.” Nuclear extracts from 10<sup>7</sup> ES cells were incubated with 2 μg of antibodies overnight and immune complexes were collected with 20 μl of protein G slurry (1:1 ratio) for 1 h. The immune complexes were washed twice with 300 μl of buffer D. For the stringent immunoprecipitation experiments, RIPA buffer (phosphate-buffered saline containing 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor; Nakalai) was used for nuclear extraction and following immunoprecipitation steps.

For transient G9a, GLP, Wiz, and CtBP interaction analyses, HEK 293T cells were transfected using TransIT-LT1, with combinations of FLAG-tagged cDNAs driven by the pcDNA3 vector (Invitrogen) or the pM vector (Clontech) and the corresponding cDNAs subcloned into the pEGFP-C vectors (Clontech). After 2 days in culture, whole cell extracts were prepared with buffer D and used for immunoprecipitation as described above.

For Western blot analysis, immunoprecipitated molecules or total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, blocked with 5% milk, and probed with the specific antibodies described below.

**Antibodies**—For detection of mouse and human G9a, mouse monoclonal antibody #8620 (Perseus Proteomics Inc.) and hamster monoclonal antibody #14-1 (Medical & Biological Laboratories Co., Ltd.) were used, respectively. For detection of mouse and human GLP, mouse monoclonal antibody #422 (Perseus Proteomics Inc.) and hamster monoclonal antibody C7–5 (Medical & Biological Laboratories Co., Ltd.) were used, respectively. For detection of epitope-tagged protein, rabbit anti-GFP (Code No.598, MBL), anti-FLAG M2 (code number F3165, Sigma) and anti-GAL4-DNA-binding domain (RKSC1, Santa Cruz Biotechnology) were used. For specificity control in two sequential immunodepletions analyses, anti-TRF1 was used (31). For the negative control of immunoprecipitation experiments, anti-Myc (9E10) antibody was used. To control for protein loading, anti-tubulin (CP06, Oncogene) was used. For CtBP detection, mouse monoclonal antibodies against CtBP1 and CtBP2 (BD Biosciences) were used.

**Plasmid Constructions**—For the construction of FLAG-mWiz expression vector, mWiz cDNA (GenBank<sup>TM</sup> accession number AB255388) was inserted into XhoI site of the pcDNA3 vector, which has FLAG-tag sequence between HindIII/BamHI sites. For the construction of EGFP-mWiz expression vector, mWiz cDNA was inserted into BamHI/XbaI sites of pEGFP-C2 vector. For the construction of GAL4-mWiz expression vector, mWiz cDNA was inserted into BamHI/Sall sites of pm vector (Clontech). ZF6 version of Wiz was made by removal of cDNA coding amino acid positions from 853 to 956 (downstream of Pstl site). The ZF6 version of Wiz (amino acid positions from 851 to 956) was inserted by inserting mWiz cDNA corresponding to this region to Pstl/BamHI sites of pEGFP-C1 vector. For the construction of EGFP-tagged mCtBP1 and
Wiz Links G9a/GLP to CtBP

A

B

C

D

E

F

G

H
mCtBP2 expression vectors, mCtBP1 cDNA was inserted into EcoRI site of pEGFP-C2 vector and mCtBP2 cDNA was inserted into KpnI/XbaI sites of pEGFP-C3 vector. FLAG-mCtBP2 was constructed by inserting FLAG-tag sequence into KpnI site of pcDNA3.1-His-CtBP2 vector (kind gift from Dr. Shimotohno). All the mG9a, mGLP, and mSu39h1 expression vectors used were described previously (25–27).

Anti-Wiz Antibody Production—mWiz cDNA was subcloned into the bacterial expression vector pGEX-4T-3 (Amersham Biosciences). Recombinant fusion molecules (amino acid positions from 300 to 956 of the Wiz polypeptide) were used to immunize rabbits. Anti-Wiz antibody was affinity-purified using glutathione S-transferase-fused Wiz (amino acid positions from 300 to 514 of mWiz).

siRNA—Double-stranded RNA oligonucleotides (21 nucleotides) homologous to G9a, GLP, and Wiz were designed as follows: G9a, 5'-CCAUUG CUUUG ACAAAGUCAUG G (forward) and 5'-AUUGGU AGUUUG ACGACCAUGGA G (reverse); GLP, 5'-GCGUG GUCAGA GUAAG ACGCU A (forward) and 5'-AGUCUC AUACUA GUGACC AGCU G (reverse); Wiz #1, 5'-GGAGCA AGCCAA AAACCAAACCUA A (forward) and 5'-UUUGAG GGUUUG GGCUCU GCUCU U (reverse); Wiz #2, 5'-GAAUA AGGAA CGUGG AUCUU U (forward) and 5'-AGAUC CACGU UCCUU AUUUC C (reverse); Wiz #3, 5'-GCAGA ACAUC ACAUAA AUUUG A (forward) and 5'-AAAUU UUGUUG ACGUGG CUGCC G (reverse); Control siRNA, 5'-GGGAAG GCUCU UGAUG AUAAG G (forward) and 5'-UUUUCACUAA CAGGAG UCCUC U (reverse). Cells were treated with annealed siRNAs at a final concentration of 25 nm by the use of Oligofectamine (Invitrogen). As a control, unrelated nuclear protein siRNA was used.

Northern Blot Analysis—Eight micrograms of total RNAs were separated by 1% agarose-formaldehyde gel electrophoresis, transferred to a nylon membrane, and hybridized with 32P-labeled cDNA probes.

RESULTS

Purification of the G9a Complex—The G9a complex was purified from G9a−/− ES cells stably expressing human G9a tagged with carboxyl-terminal His-FLAG epitopes (clone HF7). Nuclear extracts from HF7 cells were first incubated with nickel-nitrilotriacetic acid-agarose, and the bound polypeptides were eluted with buffer containing 0.2 M imidazole. The G9a complexes were further purified with agarose beads conjugated with anti-FLAG antibody. Control mock purification was performed in parallel using G9a+ ES cells stably expressing mouse G9a-L without tags (clone 15-3). The second affinity purification step with anti-FLAG antibody resulted in the identification of several polypeptides that associated with G9a in the HF7 but not the 15-3 extracts (Fig. 1A). It is of note that G9a+ ES cell phenotypes such as Mage-a genes expression were rescued in HF7 cells (data not shown).

The components of the G9a complex were identified by mass spectrometry. As reported in previous studies (27–30), GLP was present in the complex. In addition to GLP, we identified a protein named Wiz (widely interspaced zinc finger motifs), previously reported as a factor containing five Kruppel (C2H2)-type zinc finger motifs in a widely interspaced manner (32) (Fig. 1B). Although brain specificity has been attributed to Wiz, our Northern blot analysis showed that Wiz is ubiquitously expressed in various mouse tissues (Fig. 1C), suggesting a more general function of Wiz.

To confirm the interaction between G9a and Wiz, we performed co-immunoprecipitation experiments. HEK 293T cells were transfected with vectors expressing EGFP-Wiz and/or FLAG-tagged G9a molecules (FLAG-G9a). Lysates prepared from the transfected cells were incubated with anti-FLAG antibody and the immunoprecipitates subjected to Western blots analyses. Use of anti-FLAG antibodies clearly showed that EGFP-Wiz co-immunoprecipitated with FLAG-tagged G9a (Fig. 1D, lane 4). Similarly, when we immunoprecipitated EGFP-Wiz with anti-GFP, FLAG-tagged G9a was co-precipitated (lane 8). Since in previous studies we have shown that nuclear-targeted EGFP alone does not bind to FLAG-tagged G9a (27), we conclude that G9a can stably associate with Wiz in cells.

Wiz Is a Nuclear Protein That Co-localizes with G9a—Next, to examine the sub-cellular localization of Wiz, NIH3T3 cells were transiently transfected with a EGFP-Wiz expression vector and stained with anti-G9a antibody. As shown in Fig. 1E, Wiz (EGFP-Wiz) was detected only in the nucleus but largely excluded from nucleoli (arrowhead) and, importantly, co-localized with G9a. These data further support the association of Wiz with G9a.

FIGURE 1. Novel G9a-associating zinc finger protein, Wiz. A, purification of G9a complex from mouse ES cells. The G9a-associated polypeptides were detected by silver staining. The identities of the associated polypeptides are indicated at the right. B, schematic representation of Wiz. Wiz possesses six widely interspaced Kruppel (C2H2)-type zinc finger motifs (ZF1–6) with intervals of 79–177 amino acids (a.a.). Alignment of the amino acid sequences of zinc finger motif in Wiz. ZF6 was previously unidentified zinc finger that has longer inter-spacing compared with others. Bold letters indicate conserved cysteines and histidines in the zinc finger motifs (lower). C, expression profiles of Wiz transcripts in mouse tissues. Eight micrograms of total RNA were probed with radiolabeled Wiz cDNA (top). 28 S ribosomal RNA was visualized by ethidium bromide staining as a loading control (bottom). D, interaction between G9a and Wiz. HEK 293 cells were transiently transfected with the expression vector for EGFP-Wiz alone or together with FLAG-G9a. Transfectants were lysed and immunoprecipitated with anti-FLAG antibody, and immunoprecipitates (IP) were analyzed by Western blotting (WB) with anti-FLAG or anti-GFP antibody (left panel). Furthermore, HEK 293 cells were transfected with the expression vector for FLG-G9a alone or together with EGFP-Wiz. Transfectants were lysed and immunoprecipitated with anti-GFP antibody, and immunoprecipitates were analyzed by Western blotting with anti-FLAG or anti-GFP antibody (right panel). E, EGFP-Wiz was introduced into NIH3T3 cells, and the cells were stained with G9a antibody. Nuclei were stained with DAPI. Both G9a and Wiz were detected exclusively in nuclei but did not accumulate at DAPI-dense heterochromatin and were excluded from nucleoli (arrowhead). F, generation of Wiz antibody. Western blot analysis showed that generated anti-Wiz, but not preimmune serum, specifically detected two sizes of molecules (about 120 and 130 kDa) in TT2 mouse ES cells and also 120-kDa molecules (arrowed) expressed from mWiz cDNA. This anti-Wiz also detected two isoforms of human Wiz (about 125 and 135 kDa, Janel HeLa and HEK 293). Using this antibody, immunoprecipitation (IP) was performed with nuclear extracts from mouse ES cells (TT2). The immunoprecipitation experiment indicated that G9a, GLP, and Wiz form complex in vivo (right panel). Antibodies used for immunoprecipitation are specified at the top of the figure. G, the G9a-containing complexes were separated on a 10–30% glycerol gradient by centrifugation. Input and fractions (the top to bottom) were resolved by SDS-PAGE and visualized by silver staining (data not shown) and Western blot (WB) with indicated antibodies. M, marker. H, G9a/GLP/Wiz predominantly forms triple complexes. Two sequential immunodepletions using anti-GLP antibody led to a drastic reduction of G9a, GLP, and Wiz proteins from nuclear extracts. Anti-TRF1 antibody was used for specificity control. IP, immunoprecipitation.
Endogenous Wiz Is in the G9a/GLP Complex—To confirm that this association exists between endogenously expressed G9a/GLP and Wiz, we generated specific antibody against Wiz. Western blot analysis revealed that our anti-Wiz antibody specifically detected two distinct bands in TT2 mouse ES cells (about 120 and 130 kDa, arrows) and human cell lines (about 125 kDa and 135 kDa in HeLa and HEK 293), as well as the 120-kDa protein (arrowhead) expressed from mWiz cDNA. Furthermore, addition of Wiz siRNA decreased the intensity of signals detected by this antibody in human cells (Fig. 4 and not shown). Therefore, we concluded that our antibody recognizes both mouse and human Wiz proteins. We then performed co-immunoprecipitation experiments using anti-G9a, anti-GLP, and anti-Wiz antibodies. We clearly showed that anti-G9a or anti-GLP antibodies co-immunoprecipitated not only GLP and G9a but also Wiz in TT2 mouse ES cells; in turn, anti-Wiz antibody co-immunoprecipitated G9a and GLP molecules (Fig. 1F, right panel). Furthermore, this G9a/GLP/Wiz interaction was observed in several mouse and human cell lines (Fig. 5C and supplemental Fig. S1). These results indicate that association of endogenous Wiz and the G9a/GLP complex exists widely.

In addition, we determined the homogeneity of the G9a complex described in the legend to Fig. 1A. The purified materials were separated on a 10–30% glycerol gradient by ultracentrifugation and each fraction was analyzed by silver staining (data not shown) and Western blot (Fig. 1G). G9a appears in at least three different forms (fractions 2–4, 5–9, and 12), and both GLP and Wiz co-precipitate with all forms. We next performed sequential immunodepletion analysis of Wiz with anti-GLP antibody (Fig. 1H). Two consecutive immunoprecipitations with anti-GLP resulted in an efficient depletion of not only G9a...
but also Wiz. From these observations, we conclude that Wiz is a major component of the G9a complex, at least in mouse ES cells.

Both G9a and GLP Can Interact with Wiz—To determine the domain that is responsible for the interaction between G9a and Wiz, we constructed several deletion mutants of G9a and performed co-immunoprecipitation assays with Wiz (Fig. 2A and data not shown). Interestingly, Wiz was unable to interact with the G9a mutant missing a SET-domain (G9aΔSET) (Fig. 2A, lanes 3 and 6). Since the SET-domains of G9a and GLP are highly homologous, this suggested that Wiz might also interact with GLP. Co-expression analysis of FLAG-Wiz with EGFP-GLP or EGFP-GLPΔSET in HEK 293T cells revealed that EGFP-GLP, but not EGFP-GLPΔSET, clearly co-immunoprecipitated with FLAG-Wiz (Fig. 2A, lanes 9 and 12). However, another SET-domain-containing molecule, Suv39h1, failed to bind Wiz (lane 15). Therefore, this SET-domain-dependent Wiz interaction appears specific to G9a and GLP.

ZF6 of Wiz Is Essential for the Interaction with Both G9a and GLP—We also analyzed which domain of Wiz is important for G9a and GLP association. As shown in Fig. 2B, the last zinc finger motif, ZF6, which has a larger interval between C2 and H2 (Fig. 1B, bottom) and not recognized as the zinc finger motif in the original report (32), was required for the interaction between both G9a and GLP (Fig. 2B, lanes 3, 6, 9, and 12).

To further evaluate whether the SET-domain of G9a or GLP and the ZF6 of Wiz are directly involved in their interactions, we performed co-immunoprecipitation assays using such domain-only molecules. As shown in Fig. 2C, EGFP-tagged Wiz ZF6 specifically interacted with the FLAG-tagged SET-domains of G9a or GLP, whereas EGFP alone did not associate with either of them (Fig. 2C, lanes 3, 6, 9, and 12). Finally, we introduced two types of point mutations into ZF6 that should impair zinc ion binding (33), designated C903A and H923A, and examined whether these ZF6 mutants can interact with G9a and GLP. As shown in Fig. 2D, both point mutations in ZF6 resulted in the loss of association with G9a and GLP (Fig. 2D, lanes 6, 9, 15, and 18). Together, these biochemical data strongly suggest that the SET-domains of G9a and GLP and the ZF6 of Wiz are necessary and sufficient for their interactions.

Wiz Binding Is Stabilized in the G9a/GLP Heteromeric Complex—Since Wiz bound not only G9a but also GLP in the transient transfection experiments, we next examined Wiz complex formation in G9a and GLP knock-out ES cells (Fig. 3A and B). In the wild-type TT2 ES cells, Wiz could be efficiently co-immunoprecipitated with anti-G9a or anti-GLP antibody (Fig. 3A). Interestingly, the association of G9a and Wiz were also detected but reduced in GLP−/− ES cells, whereas association of GLP and Wiz were mostly preserved in G9a−/− ES cells (Fig. 3A, lanes 6 and 10).

We then performed more stringent immunoprecipitation assays, using 0.1% SDS and 0.5% deoxycholate-containing RIPA buffer. In contrast to the stable G9a/GLP/Wiz complex in wild-type ES cells, the association of G9a or GLP and Wiz was mostly disrupted in G9a and GLP knock-out ES cells (Fig. 3B, lane 5 versus lane 6 and lane 9 versus lane 10). Based on these results, we conclude that Wiz binding is more stable in the G9a/GLP heteromeric complex.

Wiz Links G9a/GLP to CtBP—To further elucidate the functional significance of the association of Wiz with the G9a/GLP heteromeric complex, we performed knockdown of Wiz in HeLa cells. As shown in Fig. 4, Wiz proteins were effectively down-regulated in the cells transfected with Wiz-specific siRNAs (#1 and #2), whereas the cells transfected with

FIGURE 3. Wiz requires both G9a and GLP for stable complex formation. A, wild-type (TT2), G9a−/−, or GLP−/− ES cells were immunoprecipitated either with anti-G9a or anti-GLP antibodies. Input and precipitates were resolved by SDS-PAGE and immunoblotted with indicated antibodies. B, immunoprecipitations were performed using RIPA buffer instead of buffer D (Fig. 3A). In this condition, Wiz was mostly unable to associate with GLP in G9a−/− ES cells (left panel) and with G9a in GLP−/− ES cells (right panel). IP, immunoprecipitation.

FIGURE 4. Wiz contributes to G9a protein stability. Either Control, G9a, GLP, or Wiz siRNA were transfected in HeLa cells and harvested 3 days after transfection for Western blot (WB) analysis (left panel). Western blot analysis with anti-G9a indicated that Wiz siRNA transfected cells showed reduction of G9a protein level. As a loading control, each sample was immunoblotted with anti-α-tubulin. To exclude the nonspecific effect of Wiz siRNA, total RNA were prepared from each sample to perform G9a Northern blotting (right panel, top). Transfection of GLP or Wiz siRNA did not change G9a mRNA level compared with control.

Wiz Contributes to G9a Protein Stability—To further elucidate the functional significance of the association of Wiz with the G9a/GLP heteromeric complex, we performed knockdown of Wiz in HeLa cells. As shown in Fig. 4, Wiz proteins were effectively down-regulated in the cells transfected with Wiz-specific siRNAs (#1 and #2), whereas the cells transfected with
G9a or GLP siRNA showed no obvious difference in Wiz protein level compared with the Control siRNA-transfected cells. In agreement with our previous results in GLP/H11002/ES cells (27), knockdown of GLP by GLP siRNA resulted in reduction of G9a protein levels. Unexpectedly, the Wiz siRNA transfected cells also showed reduction of G9a protein levels. To exclude the possibility of a nonspecific effect of Wiz siRNA on G9a expression, we performed Northern blot analysis and confirmed that the expression level of G9a RNA in the Wiz siRNA transfected cells was comparable with that in the Control siRNA-transfected cells (Fig. 4, right panel, top). From these observations, we conclude that not only GLP, but also Wiz, contributes to the stability of G9a.

Wiz Forms a Complex with CtBP1 and CtBP2—Previously, several zinc finger proteins (such as Blimp-1, NRSF/REST, and Gfi1) have been reported to interact with G9a, and these proteins are thought to recruit G9a to their specific target gene loci (34–36). However, Wiz does not possess the typical equally spaced, tandemly arrayed zinc fingers, which are usually important for DNA recognition (33). Therefore, we looked for alternative function(s) for Wiz, other than recruitment of G9a/GLP to specific gene loci. One of the more likely possibilities is that Wiz may connect the G9a/GLP complex to other functional molecule(s). We searched for protein-binding motifs in Wiz and found two PXDLS-like putative CtBP-binding stretches (335PLNLT339 and 519PLNLS523) located near ZF3 and ZF4 (Fig. 5B, left panel). CtBP family proteins are generally known as transcriptional co-repressors and play important roles during development and oncogenesis (37). We tested whether Wiz can associate with CtBPs by transient transfection and co-immunoprecipitation experiments. As shown in Fig. 5B, right panel, most of the CtBP2 binding was disrupted when CID2 was mutated (lane 9) and totally lost when both CIDs were mutated (lane 12). Therefore, we conclude that Wiz can bind to CtBPs and that this interaction of Wiz is most likely mediated through these divergent CtBP-binding motifs (38, 39).

FIGURE 5. Association of CtBPs with Wiz and the G9a/GLP/Wiz/CtBPs complex formation. A, EGFP-CtBP1 or CtBP2 were co-expressed in HEK 293T cells, either with FLAG-G9a, GLP, or Wiz, specified at the top of the figure. Immunoprecipitates were collected with control anti-Myc antibody or anti-FLAG antibody. FLAG-Wiz, but not FLAG-G9a nor FLAG-GLP, significantly precipitated both CtBP1 and CtBP2. B, Wiz contains two CtBP interaction domains (termed CID) that are located close to ZF3 and ZF4, respectively. For the CID mutation analysis, CID1, CID2, or both were mutated to the ASASA sequence (left panel). FLAG-CtBP2 was co-expressed with Wiz CID mutants, specified at the top of the figure. Immunoprecipitates were collected with control anti-Myc antibody or anti-FLAG antibody. This experiment indicated that Wiz contains two CID domains, and most of the association is carried through CID2 (right panel). C, nuclear extracts from HeLa cells were prepared for precipitation of endogenous Wiz, CtBP1, and CtBP2. Antibodies used for immunoprecipitation are specified at the top of the figure. All of the antibodies used precipitated partner proteins. D, nuclear extracts from mouse ES cells (TT2) were prepared for precipitation of endogenous G9a, GLP, Wiz, CtBP1, and CtBP2. Antibodies used for immunoprecipitation are specified at the top of the figure. Only CtBP2 was detected in the α-G9a/GLP/Wiz triple complexes. IP, immunoprecipitation.
Finally, we performed immunoprecipitation assays using HeLa and mouse ES cells, to examine endogenous Wiz-CtBP complex formation. In previous studies by Shi et al. (29), G9a and GLP were present in the complex affinity-purified with FLAG-hemagglutinin-tagged human CtBP1 from HeLa cells, but Wiz association was not reported. However, in our hands, anti-Wiz clearly co-immunoprecipitated not only G9a and GLP but also CtBP1 and CtBP2 (Fig. 5C, left panel). Vice versa, anti-CtBP1 and -CtBP2 co-immunoprecipitated G9a, GLP, and Wiz molecules (right panel). Also in mouse TT2 ES cells, association of CtBP2, but not CtBP1, to the G9a/GLP/Wiz molecules was shown by the anti-G9a, anti-GLP, and anti-Wiz co-immunoprecipitation experiments (Fig. 5D).

Collectively, we conclude that the G9a/GLP heteromeric complex associates with CtBP co-repressors through Wiz.

**DISCUSSION**

In this study, we purified the G9a complex from mouse ES cells and identified Wiz as a novel binding partner for both G9a and GLP. Furthermore, we showed that Wiz association to the G9a/GLP complex was not restricted to mouse ES cells but also seen in different human and mouse cells (Fig. 5C and supplemental Fig. S1). The sequential immunodepletion analysis with anti-GLP antibody revealed that most of the G9a/GLP complexes associate with Wiz (or that G9a and GLP mainly exist as G9a/GLP/Wiz complexes) in mouse ES cells (Fig. 1H).

**Potential Function(s) of Wiz for the G9a/GLP Heteromeric HMTase**—Previously we reported that G9a and GLP preferentially exist as a stoichiometric heteromeric complex in cells that express both of these molecules although they can also form a homomeric complex (27). This finding was partly explained by the evidence that G9a is more stable in the G9a/GLP heteromeric complex than when expressed alone. However, GLP content appeared not to be affected in the G9a-deficient ES cells. Our new findings regarding Wiz interaction may challenge this presumed predominance of G9a/GLP. First, we showed that Wiz can bind to G9a and GLP (via the SET-domains of G9a and most of the G9a or GLP complexes in the form of a G9a/GLP heterodimer because this triple-complex is the most stable and could explain the preferential existence of the G9a/GLP complex over other homomeric complexes. Furthermore, since ZF6 within Wiz is essential for its binding to G9a/GLP, ZF6 may recognize a pocket, created by dimerized SET-domains. Also, Wiz within Wiz is essential for its binding to G9a/GLP, ZF6 may recognize a pocket, created by dimerized SET-domains. Also, Wiz may be involved in targeting G9a/GLP HMTases to specific gene loci. Therefore Wiz may enhance the ability to bind Wiz, these data could support the following possibility. Wiz may recognize and bind to DNA (data not shown), Wiz may act as an adaptor molecule, recruiting diverse types of transcriptional repressors other than CtBP, to accomplish gene silencing. (3) Finally, since Wiz can bind to DNA (data not shown), Wiz may be involved in targeting G9a/GLP HMTases to specific gene loci. HDAC, histone deacetylase.

**FIGURE 6. Potential function(s) of Wiz in the G9a/GLP complex.** (1), Wiz stabilizes G9a/GLP heterodimer complex by binding to their SET domains. (2), Wiz may act as an adaptor molecule, recruiting diverse types of transcriptional repressors other than CtBP, to accomplish gene silencing. (3), Finally, since Wiz can bind to DNA (data not shown), Wiz may be involved in targeting G9a/GLP HMTases to specific gene loci. HDAC, histone deacetylase.
ing their HMTase function in vivo. To elucidate this question, further studies are required.

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