Multifaceted Histone H3 Methylation and Phosphorylation Readout by the Plant Homeodomain Finger of Human Nuclear Antigen Sp100C*

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The decoding of histone post-translational modifications by chromatin-binding modules (“readers”) constitutes one major mechanism of epigenetic regulation. Nuclear antigen Sp100 (SPECKLED, 100 kDa), a constitutive component of the promyelocytic leukemia nuclear bodies, plays key roles in intrinsic immunity and transcriptional repression. Sp100C, a splicing isoform specifically up-regulated upon interferon stimulation, harbors a unique tandem plant homeodomain (PHD) finger and bromodomain at its C terminus. Combining structural, quantitative binding, and cellular co-localization studies, we characterized Sp100C PHD finger as an unmethylated histone H3 Lys4 reader that tolerates histone H3 Thr3 phosphorylation (H3T3ph), histone H3 Lys9 trimethylation (H3K9me3), and histone H3 Ser10 phosphorylation (H3S10ph), hallmarks associated with the mitotic chromosome. In contrast, whereas H3K4me0 reader activity is conserved in Sp140, an Sp100C paralog, the multivalent tolerance of H3T3ph, H3K9me3, and H3S10ph was lost for Sp140. The complex structure determined at 2.1 Å revealed a highly coordinated lysine e-amine recognition sphere formed by an extended N-terminal motif for H3K4me0 readout. Interestingly, reader pocket rigidification by disulfide bond formation enhanced H3K4me0 binding by Sp100C. An additional complex structure solved at 2.7 Å revealed that H3T3ph is recognized by the arginine residue, Arg713, that is unique to the PHD finger of Sp100C. Consistent with a restrictive cellular role of Sp100C, these results establish a direct chromatin targeting function of Sp100C that may regulate transcriptional gene silencing and promyelocytic leukemia nuclear body-mediated intrinsic immunity in response to interferon stimulation.

In eukaryotic organisms, epigenetic mechanisms are commonly employed to establish particular chromatin states in response to intrinsic or extrinsic signals (1). Decoding histone post-translational modifications by chromatin-binding reader modules constitutes one major mechanism of epigenetic regulation (2). Thus, it is important to explore how the histone-binding reader modules regulate chromatin structure and gene activity in specific cellular processes.

The speckled protein (Sp) family members (Sp100, Sp140, Sp110, and Sp140L) constitute a class of multimodular nuclear proteins that play key roles in intrinsic immunity and transcriptional regulation (3). Among them, Sp100 is a nuclear autoantigen first identified in patients with the autoimmune disease primary biliary cirrhosis (4). Sp100 is a constitutive component of promyelocytic leukemia nuclear bodies and serves as an intrinsic immune response factor along with PML, DAXX, and ATRX in a small ubiquitin-like modifier (SUMO)2-dependent manner (5). Human Sp100 proteins are transcribed from a single gene and alternatively spliced into four isoforms designated Sp100A, Sp100B, Sp100C, and Sp100-HMG, all of which harbor a heterochromatin protein 1 (HP1)-interacting region at the N terminus (6). The longer isoforms B, C, and HMG share one additional C-terminal SAND domain (named after Sp100, AIRE-1, NucP41/75, DEAF-1) that is preferable for unmethylated CpG DNA binding. Notably, Sp100C acquires a unique, tandem plant homeodomain finger and bromodomain (PHD-Bromo) upon alternative splicing. In human fibroblasts, IFN-β treatment changes the differential splicing of the Sp100 transcripts in favor of the Sp100C isoform, which blocks the transcription of the herpes simplex virus type 1 (HSV-1) immediate early genes ICP0 and ICP4 (7, 8). Adenovirus infection induces relocalization of Sp100C from promyelocytic leukemia nuclear bodies to viral replication centers, probably in order to repress viral factor transcription at the chromatin level in a manner similar to epigenetic reader SPOC1 (survival time-associated PHD protein in ovarian cancer 1/PHF13) (6).

Although the PHD-Bromo domain of Sp100C (Sp100C_{PB}) is a chromatin-related cassette indicative of gene regulation, no direct evidence has been reported regarding its histone binding activity. Sp140, a lymphocyte-specific Sp100C homolog that harbors a similar PHD-Bromo cassette, is a risk factor in chronic lymphocytic leukemia and may serve as a transcriptional co-activator (9–12). However, histone-binding activity

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2 The abbreviations used are: SUMO, small ubiquitin-like modifier; PHD, plant homeodomain; Bromo, bromodomain; PB, PHD-Bromo; ITC, isothermal titration calorimetry; EGFP, enhanced GFP; Kac, acetylated lysine.
of the Sp140 PHD finger was not detected in a previous study (13). Therefore, it remains an intriguing question whether the PHD-Bromo cassettes of Sp100C and Sp140 are indeed histone readers or not.

Here we characterized the PHD-Bromo cassettes of Sp100C and Sp140 as unmethylated histone H3 Lys4 (H3K4me0)3 readers. The co-crystal structures of Sp100C_pp bound to H3 peptides revealed that the PHD finger is responsible for H3K4me0 binding, whereas its C-terminal bromodomain is critical to ensure the structural integrity of the PHD finger mainly through hydrophobic contacts. Key recognition residues were determined by structure-guided mutagenesis studies. Moreover, we observed a multivalent tolerance of H3T3 phosphorylation (H3T3ph), H3K9 trimethylation (H3K9me3), and H3S10 phosphorylation (H3S10ph) by Sp100C_pb but not Sp140C_pb. This, in addition to SAND-mediated DNA recognition and HP1-mediated H3K9me3 binding, these results suggest that Sp100C_pp, as a histone-binding cassette, expands the chromatin targeting capability of Sp100 to prompt intrinsic immunity and gene silencing in response to interferon stimulation.

**Experimental Procedures**

*Molecular Cloning and Recombinant Protein Preparation—* The full-length gene of human Sp100C was PCR-amplified from human cDNA and cloned into the pEGFP-C1 vector (Clontech) for the transfection assay. The human Sp140 gene was a gift from Dr. Jiahui Han (Xiamen University). The PHD-Bromo domains encompassing residues 696–878 or 701–878 of Sp100C and 684–867 or 687–867 of Sp140 were subcloned into a pSUMOH10 vector (modified based on pET28b) containing an N-terminal His10-SUMO tag. All of the mutant Sp100C and Sp140 were generated by two-step overlap PCR and verified by sequencing. Wild type Sp100C_pb(696–878) was overexpressed in the Escherichia coli BL21 (DE3) strain (Novagen). After overnight induction with 0.2 mM isopropyl β-D-thiogalactoside at 16 °C in LB medium supplemented with 0.1 mM ZnCl₂, cells were harvested by centrifugation and suspended in buffer containing 20 mM Tris-HCl, pH 7.5, and 500 mM NaCl and then disrupted by an EmulsiFlex-C3 homogenizer (Avestin). The lysate was further cleared by centrifugation, and the supernatant was loaded onto a HisTrap affinity concentrator (Avestin). The peak fractions were pooled and concentrated to 3350 and 0.2M sodium citrate at a protein concentration of 10 mg ml⁻¹. The H3T3ph-bound complex crystals can be obtained using the following reservoir conditions: 20% PEG 4000, 0.1 M sodium citrate/citric acid, pH 5.5, and 10% 2-propanol. Crystals of the H3(1–15)T3ph-bound complex were obtained under the following reservoir conditions: 20% PEG 3350 and 0.2 M sodium citrate at a protein concentration of 10 mg ml⁻¹. The H3T3ph-bound complex crystals can be obtained using either wild type or a surface triple mutant (C754S/L772Q/C793S, all mapped out of the PHD finger of Sp100C) Sp100C_pb based on the crystallization conditions detailed above. Because the triple mutant crystal diffracted better, the data set collected using the triple mutant-H3T3ph complex crystal was used for final structure determination.

For data collection, crystals were flash-frozen in liquid nitrogen under cryoprotectant conditions consisting of the reservoir solution with 10% supplemented glycerol. Diffraction data were collected at beamline BL17U1 at the Shanghai Synchrotron Radiation Facility at 1.282 or 0.9793 Å. All data were indexed, integrated, and merged using the HKL2000 software package (14). Detailed data collection statistics are summarized in Table 1. The phase was determined using the zinc single-wavelength anomalous dispersion method with PHENIX (15). Model building was performed using COOT (16), and the structural refinement was performed using PHENIX. The H3(1–15)T3ph complex was solved by molecular replacement using the MOLREP program (17). Structural refinement statistics are summarized in Table 1. The structural figures were created using PyMOL (18) or Chimera (19). The electrostatic potential surfaces were calculated with the APBS tools integrated in Chimera.

**Isothermal Titration Calorimetry (ITC) Measurements—** All of the calorimetric experiments of the wild type or mutant Sp100C/Sp140 PHD-Bromo proteins were conducted at 20 °C with a MicroCal iTC200 instrument in 20 mM Heps Na, pH 7.5, and 100 mM NaCl buffer. The protein concentration was determined by the UV280 nm absorbance. The concentrations of histone peptides were quantified by either UV280 nm absorbance of an added C-terminal tyrosine residue (εTyr280 = 1,280 M⁻¹ cm⁻¹) or by weighing in large quantities. Acquired calorimetric titration curves were analyzed with Origin version 7.0 software (OriginLab) using the “One Set of Binding Sites” fitting model.

**Oxidation and Reduction Treatment—** The Sp100C triple mutant (C754S/L772Q/C793S) was used to avoid unwanted disulfide bond formation. 0.2 mM CuCl₂ was added to triple mutant Sp100C solution at 2 mg ml⁻¹ containing 20 mM Heps-Na, pH 7.5, 100 mM NaCl. After incubation at 4 °C for 30 min, the precipitants were removed by centrifugation, and the resultant supernatants were subjected to gel filtration over a Superdex G75 column (GE Healthcare) to remove the oxidizing reagent. The monomer peak fractions were pooled and subjected to an ITC assay. For the ITC assay of the re-reduced
Sp100C, 5 mM β-mercaptoethanol was added to both the protein and peptide samples and incubated for 1 h before titration.

**Co-localization Analysis**—HeLa cells were transfected with plasmids encoding the wild type, PB-truncated, or mutant EGFP-Sp100C using the Lipofectamine 2000 reagent (Invitrogen). 24 h after transfection, HeLa cells were fixed with 4% (w/v) paraformaldehyde in PBS for 15 min, permeabilized with 0.5% Triton X-100 at room temperature for 10 min, and washed with PBS. Cells were incubated with primary antibodies against histone H3K4me0 (1:1000; Millipore, 05-1341), H3K4me3 (1:2000; Millipore, 07-473), or H3T3ph (1:1500; Cell Signaling Technology, catalog no. 13576) diluted in 3% BSA overnight at 4 °C, washed with PBS, and incubated with Alexa Fluor 647-conjugated goat anti-mouse (1:1000; Cell Signaling Technology, catalog no. 4410) or donkey anti-rabbit (1:400; Life Technologies, Inc., A-31573) secondary antibodies for 1 h at room temperature. Nuclei were counterstained with DAPI Fluoromount-G (Southern Biotech, 0100-20). Immunofluorescent images were taken as z-stacks with a Zeiss710 confocal laser scanning microscope system and deconvoluted with AutoQuant X3 software (Media Cybernetics). Settings for the deconvolution procedure were held constant for all of the samples to allow comparison within any given experiment. Projected images were generated by ZEN 2 lite software (Carl Zeiss), and quantification was performed with Imaris (Bitplane). Statistical analyses and unpaired t test were performed with GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA).

For mitotic spreading, HeLa cells were treated with colchicine for 3.5 h, suspended (8 × 10^5 cells/ml) in hypotonic buffer, and transferred into a Cytospin chamber for centrifugation (Cytospin Cytocentrifuge, Thermo Scientific). The immunolabeling procedures were the same as described above except that cold 100% methanol was used for permeabilization at −20 °C.

**Results**

Sp100CPB Is an Unmethylated H3K4 Reader, an Activity Conserved in Sp140PB—Human Sp100C and its paralog Sp140 share similar domain architectures, with a characteristic PHD-Bromo cassette located at the C terminus (Fig. 1A). Despite the fact that H3 binding activity was not observed in Sp140 in a previous study using the PHD finger alone (13), sequence alignment with TRIM24 and TRIM33 suggests that the PHD fingers in Sp100C and Sp140 are unmethylated histone H3K4 readers, given the presence of candidate H3K4me0-binding acidic residues near the N terminus (Fig. 1B, triangle).

To experimentally investigate this, we overexpressed and purified Sp100CPB (amino acids 696–878) and then performed ITC assays using a set of unmethylated or methylated histone H3 N-terminal peptides. We determined a dissociation con...
Histone H3 Recognition by Sp100C

The bromodomain normally functions as a histone acetyllysine reader. To explore whether Sp100C<sub>PB</sub> recognizes a combinatorial H3 “K4me0-Kac” modification pattern engaging the PHD-Bromo cassette, an activity that has been observed for both TRIM24 and TRIM33, we synthesized Lys14-, Lys18-, and Lys23-acetylated peptides in the frame of H3(1–25) and Lys27-acetylated peptide of H3(1–34) and then subjected them to ITC titrations. As shown in Fig. 2C, additional acetylation(s) displayed minimal effects on H3K4me0 binding, in contrast with TRIM33 and TRIM24, for which the binding affinities were boosted 3- and 24-fold, respectively. ITC titrations using other common acetylated H3 and H4 peptides, including H3K5ac, H4K5ac, H4K8ac, H4K12ac, and H4K16ac, did not reveal any clear binding either (Fig. 2D). Sequence alignment showed that an invariant asparagine residue critical for Kac recognition was changed to tyrosine (Fig. 1B, star), suggesting loss of canonical Kac binding activity of the Sp100C bromodomain (20, 21).

Overall Structure of Sp100CPB Bound to H3K4me0—To explore the molecular basis for H3 recognition by Sp100C<sub>PB</sub>, we crystallized Sp100CPB bound to the H3(1–15)K9me3 peptide and solved the complex structure at 2.1 Å by single wavelength anomalous dispersion at the zinc edge (Table 1). In the complex structure, H3(1–8) could be traced around the canonical histone-binding surface of the PHD finger (Fig. 3A). Despite the presence of H3K9me3 in the H3 peptide used for crystallization, the density of the trimethylated Lys9 side chain is invisible due to its flexibility and lack of direct protein contacts, consistent with the observed trimethylated Lys9 tolerance (with a slight enhancement) (Fig. 3A).

The PHD finger and bromodomain of Sp100C form a compact structural unit stabilized by extensive hydrophobic interactions and two hydrogen bonding pairs (Glu<sup>774</sup>-Arg<sup>753</sup> and His<sup>727</sup>-Lys<sup>819</sup>) at the PHD-Bromo interface (Fig. 3, B and C). We determined a buried solvent-accessible surface area of 889.5 Å<sup>2</sup>. Although the Sp100C bromodomain does not directly participate in histone H3(1–8) binding (Fig. 3B), it may contribute to such recognition by ensuring the proper folding of a functional PHD finger. This may explain why histone H3 binding activity was not observed for the Sp140 PHD finger alone on NMR-based titration studies (13). Conservation analysis (Fig. 3D) suggests that the H3 binding surface is well conserved among Sp100C, Sp140, TRIM24, and TRIM33, indicative of a common H3K4me0 binding activity. By contrast, the bromodomain pocket is less conserved, consistent with the loss of a Kac

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**FIGURE 2.** Histone H3 recognition by the PHD-Bromo cassettes of human Sp100C and Sp140. ITC fitting curves of Sp100CPB (A) and Sp140PB (B) using Lys<sup>4</sup>- or Lys<sup>9</sup>-methylated histone H3 peptides. H3K4me0 denotes the unmethylated state of H3K4. C and D, ITC fitting curves of Sp100CPB using Kac histone H3 or H4 peptides.

**FIGURE 3.** Overall structure of Sp100CPB bound to H3K4me0.
**Histone H3 Recognition by Sp100C**

**TABLE 1**

Data collection and refinement statistics

|                          | Sp100C<sub>Sp</sub> (C754S/L772Q/C793S)-H3(1–15)K9me3 | Sp100C<sub>Sp</sub>-H3(1–15)K9me0 | Sp100C<sub>Sp</sub>-H3(1–15)K9me3 |
|--------------------------|-----------------------------------------------------|----------------------------------|----------------------------------|
| **Data collection**      |                                                     |                                  |                                  |
| Wavelength (Å)           | 1.282                                               | 0.9793                           | 0.9793                           |
| Space group              | P2,2,2                                              | P2,2,2                           | C2                               |
| Cell dimensions (Å)      | 52.7, 102.4, 44.7                                   | 52.8, 103.0, 45.0                | 83.8, 99.2, 53.7                 |
| a, b, c (degrees)        | 90, 90, 90                                          | 90, 90, 90                       | 90, 93.2, 90                     |
| Resolution (Å)           | 50-2.3 (2.34-2.30)<sup>a</sup>                      | 50-2.1 (2.14-2.10)               | 50-2.7 (2.75-2.70)               |
| R<sub>merge</sub> (%)    | 11.9 (60.4)                                         | 11.6 (66.5)                      | 13.6 (72.5)                      |
| I/σI                     | 21.9 (3.8)                                          | 22.3 (3.7)                       | 19.1 (3.3)                       |
| Completeness (%)         | 99.0 (100)                                          | 99.9 (100)                       | 99.9 (100)                       |
| Redundancy               | 10.5 (10.6)                                         | 7.1 (7.2)                        | 5.6 (5.7)                        |
| **Refinement (F > 0)**   |                                                     |                                  |                                  |
| Resolution (Å)           | 34.2–2.1                                            | 32.1–2.7                         |                                  |
| No. of reflections (test set) | 14,906 (713)                                   | 12,032 (1238)                    |                                  |
| R<sub>work</sub>/R<sub>free</sub> (%) | 16.7/21.9                                           | 22.4/27.6                        |                                  |
| No. of atoms             | Protein/peptide                                     | 1444/64                          | 2849/166                         |
| Zinc ion                 | 2                                                   | 4                                |                                  |
| Water                    | 155                                                 | 24                               |                                  |
| B-factors (Å<sup>2</sup>)| Protein/peptide                                     | 33.4/31.5                        | 58.9/62.5                        |
| Zinc ion                 | 21.6                                                | 38.9                             |                                  |
| Water                    | 41.7                                                | 49.0                             |                                  |
| Root mean square deviations| Bond lengths (Å)                                   | 0.006                            | 0.003                            |
| Bond angles (degrees)    | 0.82                                                | 0.64                             |                                  |

<sup>a</sup> SAD, single-wavelength anomalous dispersion.

<sup>b</sup> Values in parentheses are for the highest resolution shell.

**binding activity of the Sp100C bromodomain.** The exact molecular function of the Sp100C bromodomain remains to be explored.

**Details of the H3 Tail Binding Pocket**—The H3 binding surface is largely negatively charged, which is best for basic H3(1–8) peptide recognition (Fig. 4A). Upon complex formation, about 43% of H3(1–8) was buried, with a buried solvent-accessible surface area of 582 Å². Specific recognition of histone H3(1–8) by the Sp100 PHD finger is characterized by extensive hydrogen bonding and electrostatic interactions (Fig. 4, A and C). In addition to an induced anti-parallel β-sheet formation involving H3 Arg<sup>2</sup>–Lys<sup>4</sup> and Sp100C Arg<sup>713</sup>–Cys<sup>716</sup>, H3 sequence specificity is determined by the following interactions: 1) anchoring of the H3 N-terminal amine through hydrogen bonding to Asp<sup>706</sup>, Lys<sup>737</sup>, and Ala<sup>725</sup> (Fig. 4B); 2) H3R2 side chain recognition by Asp<sup>718</sup>, Asn<sup>701</sup>, and Cys<sup>717</sup> (Fig. 4B); 3) highly coordinated recognition of unmethylated H3K4 by Asp<sup>696</sup>, Asn<sup>701</sup>, and Asp<sup>718</sup> (Fig. 4B); 4) anchoring of H3T6 by four pairs of hydrogen bonds involving residues Asn<sup>703</sup> and Gly<sup>712</sup> (Fig. 4D); 5) amide-aromatic stacking of histone H3 Ala<sup>7</sup>–Arg<sup>6</sup> (A7-R8) step with Sp100C Trp<sup>711</sup> aromatic ring (Fig. 4E, top).

Notably, an intimate encapsulation of H3K4me0 by the Sp100C PHD finger (Fig. 4, A and B) was observed, which explains the pronounced affinity drop (~13-fold) following the monemethylation of H3K4 due to the introduced steric clash. In addition, Trp<sup>711</sup>, that stacks against the H3 Ala<sup>7</sup>–Arg<sup>6</sup> step, is unique to Sp100C. In TRIM33, Trp<sup>711</sup> corresponds to the side-chain free residue Gly<sup>889</sup>, which causes H3R8 to flip down and thus enable a cation–π interaction between H3K9me3 and Trp<sup>889</sup> (Ile<sup>704</sup> in Sp100C) (Fig. 4E, bottom). This explains why H3K9me3 is able to prompt the TRIM33-H3 interaction (22). By contrast, the bulky aromatic ring of Trp<sup>711</sup> holds up the H3 Ala<sup>7</sup>–Arg<sup>6</sup> step and prevents H3K9me3 from contacting Sp100C (Fig. 4E, top), which explains the observed H3K9me3 tolerance.

**Mutagenesis and H3K4me0 Co-localization Analysis**—Mutagenesis and ITC binding assays were performed to evaluate the importance of key H3K4me0 recognition residues of the Sp100C PHD finger. As expected, alanine substitution of Asp<sup>696</sup>, Asn<sup>701</sup>, Asp<sup>718</sup>, and Asp<sup>718</sup> led to a binding loss of 7–330-fold (Fig. 5A), stressing their importance in the H3 Arg<sup>2</sup> and Lys<sup>4</sup> readout. In particular, N701A displayed the most significant binding reduction, probably due to the loss of both H3 Arg<sup>2</sup> recognition by Asn<sup>701</sup> side chain and the collapse of the H3K4 ε-amino coordination sphere formed by the extended N-terminal motif of 696–703 (Fig. 4B). Consistently, deletion of residues 696–700 caused a 25-fold binding loss (Fig. 5A), highlighting the important role of an extended N-terminal motif of the PHD finger for histone H3 recognition (23).

To further validate the H3K4me0 binding by Sp100C in the cellular context, we next performed immunofluorescence assays exploring their co-localization in HeLa cells transiently transfected with wild type (WT) or mutant EGFP-tagged full-length Sp100C constructs. As shown in Fig. 5B, all EGFP-tagged Sp100C displayed a punctate pattern in the nucleus. By contrast, histone H3K4me0 showed a rather diffused distribution pattern. Upon close inspection of the Sp100C foci (Fig. 5B, top row), we observed partial but clear co-localization between WT Sp100C and H3K4me0 as reflected by tracks of merged yellow signals. By contrast, the D696A, N701A, N703A, and D718A mutants displayed largely compromised co-localization signals. Consistent with the fact that trimethylation of H3K4 disrupts Sp100C<sub>Sp</sub> binding, we detected no co-localization signals between WT Sp100C and H3K4me3 in a control immunofluorescence assay.

Quantification analysis (Fig. 5C) revealed a co-localization Pearson coefficient of 0.36 between WT Sp100C and H3K4me0. The Pearson coefficients drop to ~0.08, ~0.15, ~0.10, and ~0.14 for D696A, N701A, N703A, and D718A, respectively.
Consistent with the most pronounced H3K4me0 binding loss of N701A in ITC titration, the Pearson coefficient of N701A (−0.15) is closest to that of the Sp100C WT-H3K4me3 control (−0.17). Collectively, the negative correlations observed for the Sp100C PHD finger mutants underscore the importance of an H3K4me0 binding activity for proper Sp100C chromatin targeting. Moreover, the medium correlation of Sp100C with H3K4me0 suggests histone H3K4me0 recognition accounts for a particular subset of Sp100C cellular functions.

Pocket Rigidification by Disulfide Bond Formation Promotes Histone H3 Binding—A close inspection of the H3K4me0-binding pocket of Sp100C<sub>PB</sub> revealed two cysteine residues, Cys<sup>698</sup> and Cys<sup>716</sup>, positioned in a pair so as to demarcate the H3K4 and H3 Arg<sup>2</sup> binding pockets (Fig. 6A, left), a feature not observed for the TRIM24 or BPTF PHD finger (Fig. 6A, middle and right). The sulfur atoms of Cys<sup>698</sup> and Cys<sup>716</sup> are 3.7 Å apart in the complex structure, reflecting a reduced state. The close spatial proximity of Cys<sup>698</sup> and Cys<sup>716</sup> led us to speculate that disulfide bond formation between this cysteine pair might be able to rigidify the H3K4me0 reader pocket and thereby regulate H3 binding.

To experimentally test this, we generated an oxidized Sp100C<sub>PB</sub> by Cu<sup>2+</sup>-catalyzed disulfide bond formation (24). To avoid unwanted intermolecular cross-linking, we used a surface triple mutant Sp100C<sub>PB</sub> (C754S/L772Q/C793S, originally introduced to facilitate crystallization), in which two surface cysteine residues were mutated to serine. ITC titrations suggested that the triple mutant Sp100C<sub>PB</sub> binds to the H3(1–15)K4me0 peptide well with a calculated $K_D$ of 3.4 μM. Interestingly, disulfide bond formation enhanced Sp100C<sub>PB</sub>-H3 interaction by 2-fold with a calculated $K_D$ of 1.7 μM. Importantly, re-reducing the oxidized Sp100C<sub>PB</sub> using β-mercaptoethanol brought the affinity back to 3.9 μM, a value comparable with the level before Cu<sup>2+</sup> treatment (Fig. 6B). Thermodynamic parameter analysis revealed that the observed binding enhancement was most driven by reduced entropy change from ~17 to ~9 cal/mol/degrees (Fig. 6C). This suggests that the H3K4 binding pocket is rigidified by disulfide bound formation in
its oxidized form, thus being entropically more favorable for the Sp100C-PHB-H3 interaction compared with the reduced state.

Sp100C Tolerates and Recognizes H3T3 Phosphorylation—H3T3ph is a hallmark of chromosome segregation and contributes to asymmetric histone inheritance (25) as well as heritable epigenetic silencing (26). There exists a basic residue, Arg713, in the vicinity of H3T3 (Fig. 4B), which suggests that the Sp100C PHD finger might recognize H3T3ph, a mechanism recently reported in the case of an engineered ADD domain of Dnmt3a (27). Using the H3(1–15)T3ph peptide, we determined a binding \( K_{D} \) of 3.4 \( \mu \)M with Sp100CPB by ITC (Fig. 7A), confirming the H3T3ph tolerance of Sp100CPB. Introduction of an acidic glutamate at position 713 by mimicking Sp140 (Fig. 7B) abolished H3T3ph binding without significantly affecting H3K4me0 recognition by Sp100CPB (Fig. 7, A and C). In the case of Sp140, H3T3ph displayed an effect of “binary switch” (28), in which the interaction of Sp140PHB with the H3(1–15) tail was decreased from 3.0 to 44 \( \mu \)M upon H3T3 phosphorylation (Fig. 7D). Interestingly, an Sp100C-mimicking E701R mutation of Sp140PHB rescued H3T3ph binding with a \( K_{D} \) of 4.3 \( \mu \)M. Moreover, glutamine mutation of Sp100C-PHB Arg713 and Sp140PHB Glu701 significantly compromised H3T3ph peptide binding (Fig. 7, A and D). Collectively, these results underscore the importance of a basic arginine residue of the Sp100C PHD finger in H3T3ph-specific recognition.

We next solved the crystal structure of Sp100C-PHB bound to the H3(1–15)T3ph peptide at 2.7 Å (Table 1). Based on the \( F_{o} - F_{c} \) omit map, we were able to trace residues 1–10 of the histone peptide with H3T3ph protruding toward Arg713 of Sp100CPB (Fig. 7E). The new H3T3ph complex can be well superimposed with the H3K4me0-bound complex. We calculated a root mean square deviation of 0.483 Å over the 153 Ca atoms. In particular, H3T3ph is stabilized by direct hydrogen...
bonding and ion pair interactions between Arg713 and H3T3ph, followed by adaptive conformational adjustments of Phe715 and Arg713 side chains (Fig. 7E). In sum, the above findings establish that Sp100C PB is an effective reader of histone H3T3ph in vitro.

We also extended our binding assay to other phosphorylated H3 peptides, including H3T6ph, H3S10ph, and H3K9me3S10ph. Whereas H3T6ph reduced Sp100C PB-H3(1–15) binding by 30-fold, we found that H3S10ph and H3K9me3S10ph were tolerated with KD values of 2.4 and 2.8 μM, respectively (Fig. 7F).

The observed “binary switch” of H3S6ph is probably due to steric clash introduced at the H3S6 side chain (Fig. 4D). Different from the H3T3ph tolerance through Arg713 recognition, the mechanism underlying H3S10ph and H3K9me3S10ph tolerance probably results from the lack of direct contacts between Sp100C PB and the phosphate group (Fig. 7E).

**FIGURE 5. Mutagenesis and co-localization analysis.** A, ITC fitting curves of WT and mutant Sp100C PB titrated with the unmethylated H3(1–15)K4 peptide. B, immunofluorescence of HeLa cells reacted with antibodies against H3K4me0 or H3K4me3 (red) and EGFP-tagged WT or mutant Sp100C (green). White boxes, representative Sp100C foci magnified in the top row. Scale bar, 5 μm. C, quantification of the EGFP signal co-localized with H3K4me0 or H3K4me3 (counts are based on 10 cells). ****, p < 0.0001 by unpaired t test with Welch's correction, compared with the EGFP-tagged WT unmethylated group.

**Sp100C Targets the Inner Centromeric Region via Its PHD Finger and Regulates Cell Cycle Progression**—To further validate the H3T3ph recognition by Sp100C in the cellular context, we next performed an immunofluorescence assay of mitotic chromosomes with an antibody against H3T3ph in HeLa cells transiently transfected with different EGFP-tagged Sp100C constructs. As shown in Fig. 8A, the H3T3 signal was strongest at the inner centromere regions of spread mitotic chromosomes. The WT, R713E, or PB-truncated Sp100C appeared in a speckled pattern over mitotic cells. Consistent with this, WT but not R713E or PB-truncated Sp100C partially co-localizes with the H3T3ph-enriched regions (Fig. 8, see arrows in A and quantification in B), suggesting a tolerance of the inner centromere localization of WT Sp100C. With the R713E mutation, Sp100C was detected at the outer region of the centromere, where the H3T3ph signal is minimal, suggesting a “binary
**FIGURE 6.** Pocket rigidification by disulfide bond formation regulates histone H3 binding. A, comparison of the residues demarcating H3 Arg\(^2\) and Lys\(^4\) pockets in the complex structures of Sp100C\(_{PHD}\)-H3K4me0, TRIM24\(_{PHD}\)-H3K4me0, and BPTF\(_{PHD}\)-H3K4me3. The Cys\(^698\)-Cys\(^716\) pair is indicated with a pink circle. B, ITC fitting curves of the Sp100C\(_{PHD}\) surface triple mutant (C754S/L772Q/C793S) (Sp100C\(_{mu3}\)) titrated with unmethylated H3(1–15) peptide. The untreated, Cu\(^{2+}\)/H11001-oxidized (Sp100C\(_{mu3O}\)), and β-mercaptoethanol re-reduced (Sp100C\(_{mu3R}\)) forms of Sp100C\(_{mu}\) proteins were used for ITC titration. C, summary of thermodynamic parameters of the ITC assays shown in Fig. 3B. Parameters of the oxidized Sp100C\(_{mu3}\)-H3K4me0 binding assay are colored pink.

**FIGURE 7.** H3T3ph tolerance by the PHD finger of Sp100C in contrast with that of Sp140. A, ITC fitting curves of Sp100C\(_{PHD}\) or its mutants with the T3 phosphorylated H3(1–15) peptide. B, sequence alignment of the PHD N terminus between Sp100C and Sp140. The residues that determine H3T3ph tolerance are denoted with a red star. C, ITC fitting curves of WT and R713E mutant Sp100C\(_{PHD}\) titrated with unmodified H3(1–15) peptide. Note that the recognition of unmodified H3(1–15) is only minimally affected by R713E mutation. D, ITC fitting curves of Sp140\(_{PHD}\) or its mutants with the T3 phosphorylated H3(1–15) peptide. E, N-terminal structure of Sp100C\(_{PHD}\) bound to H3(1–10)T3ph peptide. The PHD finger is shown with pink ribbons, and histone H3 peptide is depicted with yellow sticks. Blue mesh, \(F_{c}-F_{o}\) omit map around the H3 peptide contoured at the 2.5σ level. Note the newly formed hydrogen bonds (cyan dashes) between Arg\(^{713}\) and H3T3ph. For comparison, the H3T3ph complex structure (pink ribbon and yellow stick) is superimposed over the H3K4me3-bound complex (gray). Note the conformational changes of Arg\(^{713}\) and Phe\(^{715}\) side chains (indicated by the red arrowheads). F, ITC fitting curves of Sp100C\(_{PHD}\) titrated with H3(1–15)T6ph, H3(1–15)S10ph, or H3(1–15)K9me3S10ph peptide.
Histone H3 Recognition by Sp100C

**Unique Features of Unmethylated H3K4 Readout by the Sp100C PHD Finger**—PHD fingers have been identified in many chromatin-associated factors that have typical histone binding activities (23, 32). Despite this, it was reported in a previous study that the PHD finger of Sp140 did not harbor the histone binding activity (13), which raised concerns as to whether the PHD fingers of Sp100 family members were indeed histone readers or not. Here we demonstrate that PHD fingers of both Sp100C and Sp140 display unmethylated histone H3K4 reader activity with single digit micromolar level binding affinity.

Our structural studies revealed several unique features of the Sp100C PHD finger related to unmethylated H3K4 binding. First, an extended N-terminal loop forms a most coordinated H3 lysine 4 ε-amine recognition sphere that endows Sp100C with relatively stringent methylation sensitivity. As revealed in the complex structure, the N-terminal loop encompassing Asp⁶⁹⁶ (the −9 position; the first zinc-coordinating Cys is defined as position 0) to Asn⁷⁰³ (the −2 position) provides approximately five pairs of hydrogen bonding interactions around the H3K4 ε-amine, thus enabling robust binding (Fig. 9A). By contrast, 1–4 pairs of hydrogen bonding interactions were observed in the Dnmt3a, TRIM33, AIRE-1, TRIM24, and BHC80 PHD fingers (Fig. 9, B–F). The encapsulation of the tip of the H3K4 side chain makes the Sp100C PB-H3 interaction sensitive to H3K4 methylation. In support of this, we observed ~13-fold binding reduction upon H3K4 monomethylation, suggesting that Sp100C PB is preferentially an unmethylated H3K4 reader. Second, we observed that reader pocket rigidity by the disulfide bond formation between Cys⁶⁹⁸ and Cys⁷¹⁶ prompted Sp100C PB-H3 interaction (Fig. 6, A and B). Because viral infection often induces oxidative stress (33), such as in the process of HSV-1 infection (34) that can be repressed by Sp100C expression (7, 8), the observed redox responsiveness of Sp100C might serve as a fine-tuned antiviral mechanism by prompting Sp100C chromatin targeting. Interestingly, the above mentioned disulfide bond-forming cysteine pair is conserved in Sp100C, Sp140, and Sp140L, suggesting a similar redox-regulated histone readout mechanism.

Finally, the Sp100C PHD finger and bromodomain form an integral functional unit through extensive hydrophobic contacts. Such a domain organization is similar to the cases of TRIM24 and TRIM33 but different from that of BPTF and other independently folded PHD fingers (22, 35–37). The requirement of an adjacent bromodomain for functional PHD finger folding may explain why the Sp140 PHD finger alone did not display clear histone H3K4me0 binding activity in the previous report (13).

**Sp100C**<sub>pb</sub> Is a Multifaceted Histone H3 Methylation and Phosphorylation Sensor—In addition to H3K4me0 readout, our structural and binding studies established that the PHD finger of Sp100C tolerates adjacent histone modifications, such as H3T3ph, H3K9me3, H3S10ph, and H3K9me3S10ph. Among them, phosphorylated H3T3 is specifically recognized by an arginine residue localized to the ε1 strand of Sp100C PHD finger. Interestingly, this arginine residue (Arg<sup>713</sup>) is unique to Sp100C among the reported histone H3K4me0 readers, including Sp140 (23, 38). In most PHD fingers, non-basic polar residues (e.g. Glu or Gln) are located at a position corresponding to the stress response (30), genome integrity (31), and transcriptional repression (29). Therefore, Sp100C might serve as a fine-tuned antiviral mechanism by prompting Sp100C chromatin targeting.

**Discussion**

Sp100 family members are constitutive components of promyelocytic leukemia nuclear bodies that play important roles in the stress response (30), genome integrity (31), and transcriptional repression (29). The Sp100C splicing isoform harbors a unique PHD-Bromo cassette at its C terminus and is specifically up-regulated upon interferon stimulation (7). Utilizing structural, quantitative binding, and cellular co-localization studies, we have demonstrated that the Sp100C PHD finger is a multifaceted histone reader that recognizes H3K4me0, being sensitive to H3K4 methylation, whereas it tolerates H3T3ph, H3K9me3, H3S10ph, and H3K9me3S10ph. It is noteworthy that these histone modifications either display strong correlations with gene repression or mark mitotic chromosomes for proper cell cycle progression, consistent with a restrictive cellular function of Sp100C. In contrast, whereas Sp140 displayed similar H3K4me0 reader activity, it can be switched off by either H3T3ph or H3K9me3, suggesting divergent cellular functions between the two paralogs.
FIGURE 9. Comparison of histone H3K4me0 hydrogen-bonding spheres among H3K4me0-binding PHD fingers of Sp100C (A), Dnmt3a (B), TRIM33 (C), AIRE (D), TRIM24 (E), and BHC80 (F). PHD fingers and histone H3 peptide are represented as blue and yellow ribbons. Key recognition residues are depicted as sticks. Key coordinating residues are numbered by defining the first zinc-coordinating cysteine as 0. Corresponding binding $K_D$ values are shown below each structure. Coordinates are taken from Protein Data Bank entries 4QBQ for Dnmt3a$_{ADD}$-H3, 3U5N for TRIM33$_{PB}$-H3, 2KFT for AIRE-1$_{PHD}$-H3, 3O37 for TRIM24$_{PB}$-H3, and 2PUY for BHC80$_{PHD}$-H3, respectively.

FIGURE 10. A working model on multivalent chromatin targeting by Sp100C. Promyelocytic leukemia nuclear bodies contain PML, Sp100, DAXX, and ATRX, and are important host restriction factors in intrinsic immunity. Interferon stimulation specifically induces the expression of the Sp100C splicing isoform. Sp100C contains an N-terminal homodimerization HSR domain and an HP1-interacting motif that are common to all Sp100 isoforms. HP1 is a specific H3K9me3 reader; thereby, Sp100C could target to the H3K9me3-enriched heterochromatin region in an HP1-dependent manner. The C-terminal SAND domain is common among Sp100B, Sp100C, and Sp100HMG and harbors unmethylated CpG-DNA binding activity. The PHD-Bromo cassette is unique to Sp100C. In addition to a role in stabilizing the adjacent PHD finger, the exact ligand binding function of the bromodomain remains a question mark. Our work reported here suggests that in addition to HP1-mediated heterochromatin targeting and SAND-mediated CpG-DNA binding, the PHD-Bromo cassette could further extend the chromatin targeting capability of Sp100C by recognition/tolerance of H3 "K4me0," "K4me0-T3ph," "K4me0-K9me3," "K4me0-S10ph," and "K4me0-K9me3-S10ph." Presumably, this multivalent chromatin targeting activity will be beneficial to support a role of Sp100C as a restriction factor in regulating cell cycle, viral replication, transcription, and latent infection.
Histone H3 Recognition by Sp100C

Arg\textsuperscript{713}, which usually leads to an “off” switch upon phosphorylation of H3T3, as supported by our binding studies (Fig. 7, A and D). In mitosis, H3T3ph is a hallmark of the inner centromere and is essential for functional spindle formation (39). Our chromosome spreading and co-localization studies confirmed the H3T3ph tolerance of Sp100C and revealed its centromeric localization in mitotic phase. Collectively, these data suggest a role of Sp100C in regulating chromosome segregation and cell cycle progression.

We also observed that Sp100C\textsubscript{PB} tolerates H3K9me3 and H3K9me3S10ph, two other hallmarks associated with the mitotic chromosome (40, 41). Unlike the TRIM33 PHD finger that recognizes H3K9me3 with a tryptophan residue through cation–π interactions (22), Sp100C\textsubscript{PB} lacks such a residue at the same position but gains another tryptophan, Trp\textsuperscript{711}, to stack against histone H3 Ala\textsuperscript{7}–Arg\textsuperscript{8} (Fig. 4E). In the complex structure of Sp100C\textsubscript{PB} bound to the H3(1–15)K9me3 peptide, residues after H3R8 are exposed to solvent and are thus too flexible to be modeled (Fig. 3A). Therefore, both H3K9me3 and H3K9me3S10ph are tolerable by Sp100C\textsubscript{PB} due to lack of contacts. Interestingly, the Sp100C binding partner HP1 is a classical H3K9me3 reader but is sensitive to adjacent H3S10ph, which functions to reject HP1 from H3K9me3-enriched heterochromatic regions (42). Therefore, our work suggests an important role of the PHD finger in mediating Sp100C chromatin targeting even when its binding partner HP1 is switched off by H3S10ph.

In conclusion, our studies demonstrate that, in addition to HP1-mediated heterochromatic targeting and SAND-mediated Cpg-DNA binding, the PHD-Bromo cassette expands the chromatin targeting capability of Sp100C by its readout of histone H3 “K4me0,” “K4me0–T3ph,” “K4me0–K9me3,” and “K4me0–K9me3–S10ph,” which probably plays important roles in regulating normal chromosome segregation as well as having an impact on viral replication, transcription, and even certain latent infection processes (Fig. 10).

Author Contributions—H. L. initiated and conceived the study. X. Z. designed and performed the experiments under the guidance of H. L. D. Z., X. X., and Z. H. provided technical support. H. L. and X. Z. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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