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Research Article

Chaetocin disrupts the SUV39H1–HP1 interaction independent of SUV39H1 methyltransferase activity

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Chemical tools to control the activities and interactions of chromatin components have broad impact on our understanding of cellular and disease processes. It is important to accurately identify their molecular effects to inform clinical efforts and interpretations of scientific studies. Chaetocin is a widely used chemical that decreases H3K9 methylation in cells. It is frequently attributed as a specific inhibitor of the histone methyltransferase activities of SUV39H1/SU(VAR)3–9, although prior observations showed chaetocin likely inhibits methyltransferase activity through covalent mechanisms involving its epipolythiodixopiperazine disulfide 'warhead' functionality. The continued use of chaetocin in scientific studies may derive from the net effect of reduced H3K9 methylation, irrespective of a direct or indirect mechanism. However, there may be other molecular impacts of chaetocin on SUV39H1 besides inhibition of H3K9 methylation levels that could confound the interpretation of past and future experimental studies. Here, we test a new hypothesis that chaetocin may have an additional downstream impact aside from inhibition of methyltransferase activity. Using a combination of truncation mutants, a yeast two-hybrid system, and direct *in vitro* binding assays, we show that the human SUV39H1 chromodomain (CD) and HP1 chromoshadow domain (CSD) directly interact. Chaetocin inhibits this binding interaction through its disulfide functionality with some specificity by covalently binding with the CD of SUV39H1, whereas the histone H3–HP1 interaction is not inhibited. Given the key role of HP1 dimers in driving a feedback cascade to recruit SUV39H1 and to establish and stabilize constitutive heterochromatin, this additional molecular consequence of chaetocin should be broadly considered.

Introduction

H3K9 methylation is a conserved modification across higher eukaryotes and is important in gene silencing and maintenance of constitutive heterochromatin. Misregulation of its methyltransferases has been associated with cancers and neurodegenerative diseases [1,2]. Thus, along with other histone modifying enzymes, histone methyltransferases are promising 'druggable' targets. Tools to control their activity are also of considerable interest for basic research.

In 2005, the small molecule, chaetocin, was identified as a specific inhibitor of the H3K9 methyltransferase activity of SUV39H1 (human) and its homolog SU(VAR)3–9 (Drosophila melanogaster) [3]. However, biochemical assays later showed that the epipolythiodixopiperazine (ETP) disulfide 'warhead' functionality of chaetocin may be acting to inhibit enzyme activity [4] by covalently reacting with methyltransferase proteins [5,6]. Despite questions regarding its mechanism and specificity of action, chaetocin has remained in active use in the biological sciences as it generally reduces H3K9 methylation levels. Crucially, the general assumptions in experimental studies remain that the primary molecular impact of chaetocin is reduction in H3K9 methylation and that these reductions result from inhibition of methyltransferase activity, whether direct or indirect. Chaetocin also continues to be marketed by most chemical vendors as an inhibitor of methyltransferase activity (Table 1). However, the reactive ETP functionality of chaetocin hints that there may be additional concurrent

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Table 1 Commercial suppliers of chaetocin

	Supplier	Supplier-provided Description	Country
1	Absource Diagnostics	Histone methyltransferase inhibitor	Germany
2	AdooQ Bioscience	Histone methyltransferase SUV39H1 inhibitor	U.S.A.
3	APExBIO	SUV39H1 Inhibitor	U.S.A.
4	Benchchem	NA	U.S.A.
5	BioVision Inc. (Part of Abcam)	A selective histone lysine methyltransferase (HKMT) inhibitor	U.S.A.
6	bioWORLD	Histone methyltransferase SUV39H1 inhibitor.	U.S.A.
7	BPS Bioscience	Inhibitor of histone methyltransferases	U.S.A.
8	Cambridge Bioscience	NA	U.K.
9	Cayman Chemical	Inhibits the histone methyltransferase (HMT) SU(VAR)3–9	U.S.A.
10	ChemShuttle	Histone Methyltransferase	U.S.A.
11	Creative Biolabs	Specific inhibitor of the lysine-specific methyltransferase SU(VAR)3–9	U.S.A.
12	Enzo Life Sciences, Inc.	Methyltransferase inhibitor	U.S.A.
13	Fermentek	A fungal metabolite which inhibits G9a histone methyltransferase	Israel
14	Focus Biomolecules	Selective inhibitor of lysine specific histone methyltransferase	U.S.A.
15	Hello Bio	Potent histone methyltransferase SUV39H1 inhibitor	U.S.A.
16	MedChemExpress	Specific inhibitor of the HMT SU(VAR)3–9	U.S.A.
17	Millipore Sigma	HMTase Inhibitor II	Germany
18	R&D Systems (Bio-techne)	Histone methyltransferase SUV39H1 inhibitor	U.S.A.
19	Santa Cruz Biotechnology	Alternate Names: Methyltransferase inhibitor	U.S.A.
20	Scientific Laboratory Supplies	Specific inhibitor of lysine-specific histone methyltransferase	U.K.
21	Selleck Chemicals	Histone methyltransferase inhibitor	Japan
22	STEMCELL Technologies	Epigenetic modifier; Inhibits histone methyltransferases SU(VAR)3–9	U.S.A.
23	StressMarq Biosciences Inc.	Methyltransferase inhibitor	Canada
24	Tebu Bio	HMT inhibitor	France
25	Tocris Bioscience (Bio-techne)	Histone methyltransferase SUV39H1 inhibitor	U.K.
26	TOKU-E	Chaetocin is an inhibitor of lysine-specific methyltransferase SU(VAR)3–9 both *in vitro* and *in vivo*	Japan
27	Toronto Research Chemicals	Nonspecific inhibitor of histone lysine methyltransferases	Canada

mechanisms and distinct molecular effects mediated by chaetocin beyond disrupting SUV39H1 enzymatic activity and reducing H3K9 methylation levels. Revealing any new and distinct effects would be important in properly interpreting and designing experiments and in the accuracy of biological conclusions drawn using chaetocin.

Here, we hypothesize that chaetocin may act through a distinct mechanism not previously considered and that does not involve inhibition of methyltransferase activity. It is known that SUV39H1, through its chromodomain (CD), directly interacts with the chromoshadow domain (CSD) of heterochromatin protein 1 (HP1). This interaction facilitates HP1 recruitment to heterochromatin. Because HP1 dimerizes, it then also recruits additional molecules of SUV39H1 to heterochromatin in a feedback loop that enhances H3K9 methylation and heterochromatin stability [7–9]. Unknown and inadvertent inhibition of this important protein–protein

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interaction by chaetocin could lead to distinct and previously unconsidered mechanistic interpretations in the cell biology literature.

We pursue two approaches to test this hypothesis. First, taking advantage of the natural lack of constitutive heterochromatin (characterized by H3K9 methylation) in *Saccharomyces cerevisiae*, we create a yeast two-hybrid reporter to monitor the binding interaction between SUV39H1 and HP1. We observe that the reporter gene activates only when SUV39H1 (bait) and HP1 (prey) are expressed together. Using truncation mutants lacking the methyltransferase SET domain of SUV39H1, we show this binding interaction occurs through the SUV39H1 CD and the HP1 CSD, and not through the known interaction of the HP1 CD with H3K9 methylation, which is absent from *S. cerevisiae*. We then show that chaetocin inhibits this interaction. Furthermore, this inhibition is specific to SUV39H1 and HP1, as chaetocin does not affect other two-hybrid protein pairs. Second, we develop a pull-down assay to directly verify these results in highly controlled *in vitro* conditions and describe an optimized protocol to produce SUV39H1 which is typically refractory to recombinant expression. We show chaetocin induces a dose-dependent decrease in binding between the SUV39H1 CD and HP1 CSD. Furthermore, deactivation of the ETP functionality of chaetocin by a reducing agent abrogates this inhibition. Our results provide direct evidence that chaetocin inhibits the interaction of SUV39H1 and HP1, that this inhibition is independent of methyltransferase activity, and that it likely acts through its disulfide moiety. This molecular interaction is critical in heterochromatin formation and stability, and the ability of chaetocin to disrupt it should be broadly integrated into the mechanistic interpretations of new and prior results using the chemical.

Results

ZF–SUV39H1 and HP1–VP16 activate a yeast two-hybrid GFP reporter

Full length SUV39H1 is known to be challenging to recombinantly express and purify [5]. Therefore, our first aim is to develop a rapid and facile cell-based assay to assess the interaction between SUV39H1 and HP1 and the effect of chaetocin. *Saccharomyces cerevisiae* naturally lacks methylation of H3K9, H3K27, and DNA, and lacks the associated methyltransferases (i.e. SUV39H1, G9a, EZH2, DNMT1/3/3A). It therefore serves as an orthogonal experimental host to assess binding interactions between human components of constitutive heterochromatin by minimizing potential interactions with host proteins or host methylated chromatin. In this two-hybrid system, the bait comprises human SUV39H1 fused to a three-finger zinc finger (ZF) array that binds to two operators in the promoter of a green fluorescent protein (GFP) reporter gene [10] (Figure 1); the prey comprises human HP1α, HP1β, or HP1γ fused to the transcriptional transactivator virus proein 16 (VP16). ZF–SUV39H1 is under the transcriptional control of an anhydrotetracycline (ATc)-regulated promoter, and HP1–

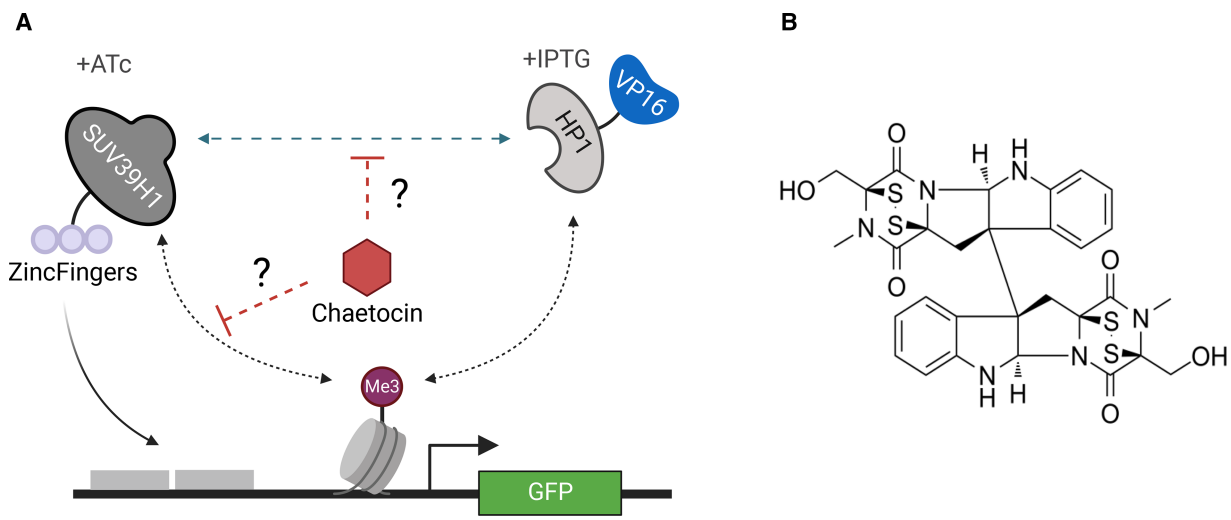

Figure 1. Yeast two-hybrid system and chemical structure of chaetocin.

(**A**) Schematic showing the yeast two-hybrid system and two possibilities for chaetocin activity: inhibition of SUV39H1 methyltransferase activity or inhibition of the interaction between SUV39H1 and HP1. (**B**) Chemical structure of chaetocin.

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VP16 is under the transcriptional control of an IPTG-regulated promoter. As expected, the expression of HP1–VP16 itself does not activate the reporter; only when both fusion proteins are expressed does the reporter activate (Figure 2). Similar activation is observed for all three isoforms of HP1.

SUV39H1 and HP1 interact directly, and methyltransferase activity is dispensable for reporter activation

SUV39H1 consists of a methyltransferase (SET) domain at its C-terminus and a chromodomain at its N-terminus. HP1 consists of an N-terminal CD and a C-terminal CSD. There are two potential ways in which HP1–VP16 can be recruited to the reporter (Figure 1): through its CD binding to H3K9 methylation; or through its CSD binding to the CD of SUV39H1. We use truncation and point mutants of SUV39H1 and HP1γ to deconvolute these two possibilities (Figure 3). Importantly, HP1γ–VP16 successfully activates the reporter even when the C-terminal SET domain of SUV39H1 contains an inactivating mutation (H324K) or is completely absent (ΔC). Likewise, the CD domain of HP1γ is dispensable for this interaction. This indicates that the two-hybrid assay is capturing the binding interaction between the SUV39H1 CD and HP1γ CSD and does not require the enzymatic activity of SUV39H1 nor H3K9 methylation.

Chaetocin inhibits the interaction between SUV39H1 and HP1

With the two-hybrid system established and showing that SUV39H1 and HP1 directly interact, we next ask whether chaetocin can inhibit this interaction (Figure 4A). When ZF–SUV39H1 is expressed at a moderate level (0.01 μg/ml ATc inducer), chaetocin inhibits GFP activation in a dose-dependent manner, with substantial inhibition even at 1 μM, near the reported IC50 of ∼0.8 μM. Higher expression levels of ZF–SUV39H1 (0.05 μg/ml ATc) abrogate the ability of chaetocin to inhibit this interaction even at 15 μM chaetocin, suggesting ZF–SUV39H1 may consume chaetocin irreversibly, consistent with a disulfide-based modification of SUV39H1. Similar results are observed for all three isoforms of HP1.

It is possible that chaetocin is inhibiting the ZF array from binding the DNA operators or inhibiting the transactivating activity of VP16. However, when the ZF array and VP16 are directly fused together, chaetocin does not inhibit the activation of the reporter, indicating chaetocin is not affecting the DNA binding or transactivating properties of the system (Figure 4B).

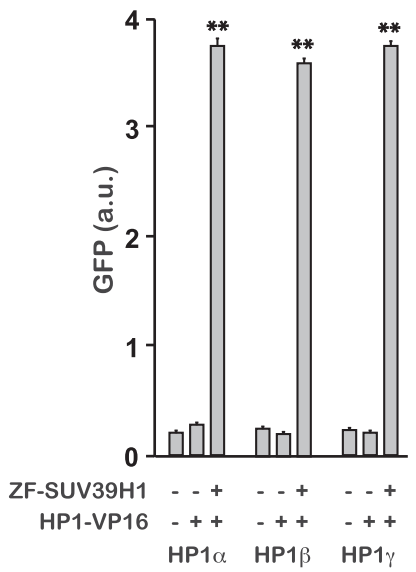

Figure 2. ZF–SUV39H1 and HP1–VP16 activate a yeast two-hybrid GFP reporter.

Flow cytometry shows GFP reporter activation only when both ZF–SUV39H1 and HP1–VP16 fusion proteins are expressed. Similar activation is observed for all three isoforms of HP1. Median values of three biological replicates are displayed along with standard deviations. Statistics: one-way ANOVA with Tukey–Kramer post hoc for each set of cultures containing the same HP1 isoform, ** $P < 0.01$ for pairwise comparisons.

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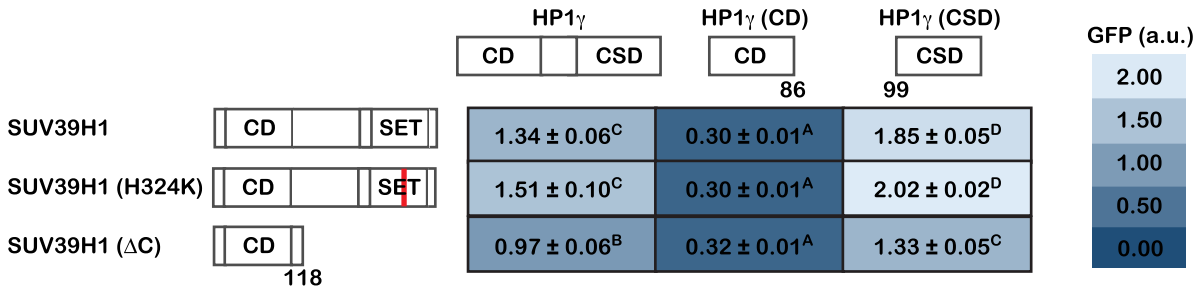

Figure 3. SUV39H1 and HP1 interact directly, and methyltransferase activity is dispensable for reporter activation.
Flow cytometry data of truncation mutants show that the two-hybrid interaction between SUV39H1 and HP1γ relies on the direct interaction of the SUV39H1 CD and HP1γ CSD. The bait displayed on the left are fused to a zinc finger array. The prey displayed at the top are fused to VP16. Median values of three biological replicates are displayed along with standard deviations. Statistics: one-way ANOVA with Tukey–Kramer post hoc, $P < 0.01$ for pairwise comparisons, with conditions sharing the same superscript letters not statistically significant from each other.

Chaetocin may also non-specifically inhibit protein–protein interactions. As we previously showed that cryptochrome circadian regulator (CRY2) and calcium- and integrin-binding protein 1 (CIB1) interact upon blue light stimulation in a similar two-hybrid assay [11], we can use this system as a convenient test of chaetocin's effect on general protein–protein interactions. Using this protein pair, ZF–CRY2 and CIB1–VP16 drive reporter activation under Dark, Low Light and High Light, 3 or 15 μM chaetocin does not inhibit this interaction (Figure 4C).

Chaetocin disrupts the direct interaction between SUV39H1 and HP1 independent of methyltransferase activity and through disulfide functionality

The two-hybrid system provides a facile approach to assess protein–protein interactions, and it provides new evidence of chaetocin inhibiting the interaction between SUV39H1 and HP1. However, even in *S. cerevisiae* which lacks constitutive heterochromatin this approach may suffer from potential unknown pleiotropic effects or indirect mechanisms of inhibition including through undetectable levels of histone methylation for example. Furthermore, the requirement of transporting chaetocin into the yeast cell abrogates the ability to quantitatively assess the inhibitory concentrations of chaetocin for the SUV39H1 and HP1 interaction. Thus, this evidence from the two-hybrid system motivates the development of a recombinant *in vitro* assay.

However, full length SUV39H1(1–412 aa) is known to be refractory to recombinant production and purification; therefore, commercial SUV39H1 comprises only amino acids 82–412. This may be due to its high cysteine composition (5.34% compared with an average of 2.26% in mammalian proteins). In particular, the pre-SET domain alone contains 14.5% cysteines, and the N-terminal aa1–40 contains 12.5% cysteines and is the domain that binds with HP1 [12]. A positive correlation exists between the number of cysteines in a sequence and the disordered nature of flanking sequences, affecting recombinant protein express [13]. We address this challenge through a series of optimization experiments to reveal conditions for ample and high-quality SUV39H1 protein (Supplementary Figure S1). Through these experiments, we find that the key conditions facilitating expression and purification of SUV39H1 are: (1) fusion of a maltose binding protein (MBP) tag to the N-terminus to promote solubility; (2) using an optimized concentration of a reducing agent in wash, elution, and storage buffers to reduce the aggregation caused by the cysteine residues; (3) lowering the temperature and shaking speed during bacterial culture to minimize inclusion body formation; and (4) lysing and purifying cells freshly after their induction. These optimizations enable the production of purified SUV39H1, SUV39H1 mutants, and EGFP–HP1 as shown via SDS–PAGE (Supplementary Figure S1).

The capability to produce ample SUV39H1 unlocks the ability to answer several detailed questions about the activity of chaetocin. For example, using the H324K and ΔC mutants, we can ask whether chaetocin directly inhibits the interaction between SUV39H1 and HP1. To do this, we establish a pull-down assay (Figure 5A). Here, SUV39H1–3xCMYC fusion proteins are bound to anti-CMYC magnetic beads and then incubated with enhanced green fluorescent protein (EGFP) fused to the C-terminus of HP1. After washes, any EGFP–HP1 protein bound to SUV39H1 is eluted, and the EGFP fluorescence is quantified by a fluorescence plate reader.

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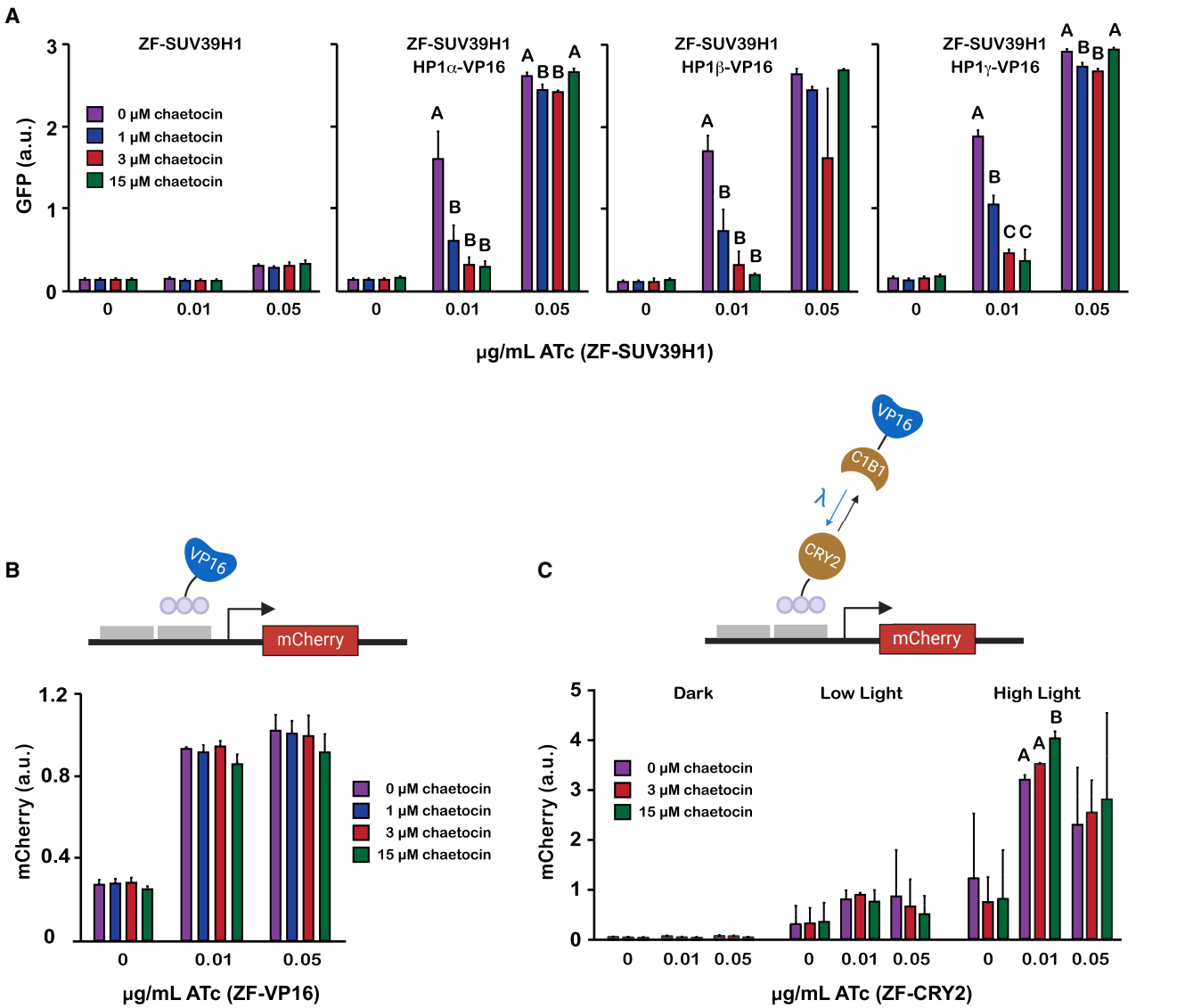

Figure 4. Chaetocin inhibits the interaction between SUV39H1 and HP1.

(**A**) Flow cytometry results show that chaetocin inhibits activation of the reporter in a dose-dependent manner with similar inhibition observed for all three HP1 isoforms. In contrast, chaetocin does not inhibit activation of the reporter by (**B**) a ZF–VP16 fusion protein nor (**C**) an optogenetically activatable ZF–CRY2 and CIB1–VP16 protein pair for both low and high expression levels. Median values of three biological replicates are displayed along with standard deviations. Statistics: one-way ANOVA with Tukey–Kramer post hoc is performed for each experimental group having the same ATc concentration, $P < 0.01$ for pairwise comparisons, and conditions sharing the same superscript letters not statistically significant from each other.

Two initial experiments optimize the conditions for the system. First, quantifying the fluorescence of just the purified proteins reveal that the autofluorescence of SUV39H1 is minimal while EGFP–HP1 provides a strong dynamic range based on its concentration (Figure 5B). Second, using TEV protease to remove the MBP tag from the SUV39H1(H324K) and SUV39H1(ΔC) mutants improved the accessibility of their CD domains and showed two-fold greater binding to EGFP–HP1 (Figure 5C).

To ask if chaetocin directly inhibits the interaction between SUV39H1 and HP1, 0.1, 1, 3, and 15 μM chaetocin are added in the assay with EGFP–HP1 and either SUV39H1(H324K) (Figure 5D) or SUV39H1(ΔC) (Figure 5E). In both cases, a dose-dependent decrease in the fluorescence signal shows that chaetocin directly inhibits the interaction of SUV39H1 and HP1, and that the methyltransferase domain and activity are dispensable for this effect. Chaetocin may disrupt the interaction of SUV39H1–3xCMYC with the anti-CMYC

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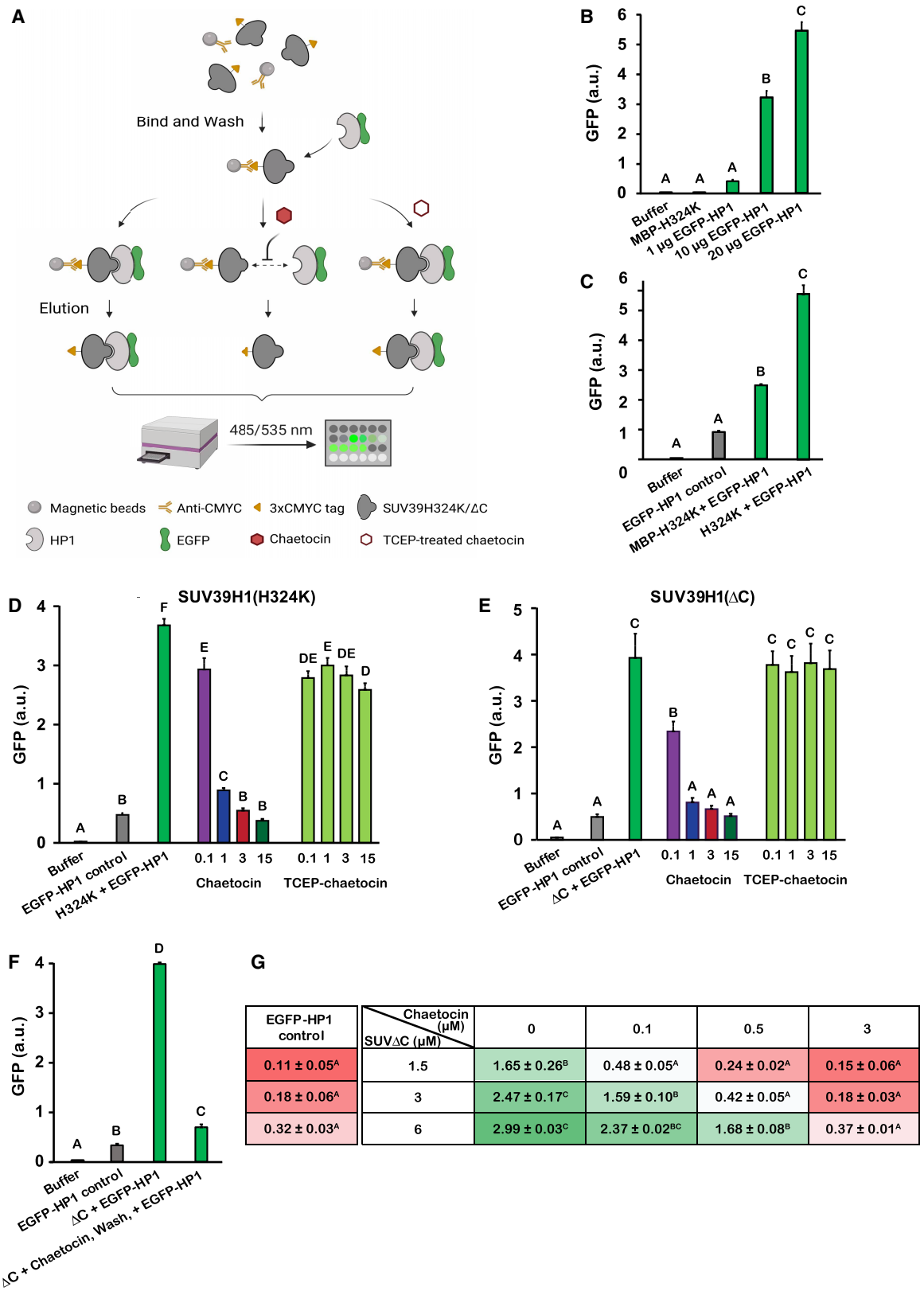

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EGFP-HP1 control	Chaetocin (µM) / SUVΔC (µM)	0	0.1	0.5	3
0.11 ± 0.05^{A}	1.5	1.65 ± 0.26^{B}	0.48 ± 0.05^{A}	0.24 ± 0.02^{A}	0.15 ± 0.06^{A}
0.18 ± 0.06^{A}	3	2.47 ± 0.17^{C}	1.59 ± 0.10^{B}	0.42 ± 0.05^{A}	0.18 ± 0.03^{A}
0.32 ± 0.03^{A}	6	2.99 ± 0.03^{C}	2.37 ± 0.02^{BC}	1.68 ± 0.08^{B}	0.37 ± 0.01^{A}

Figure 5. Chaetocin disrupts the direct interaction between SUV39H1 and HP1 independent of methyltransferase activity and through disulfide functionality. Part 1 of 2

(**A**) Schematic showing three types of experimental conditions: without chaetocin, with chaetocin, with TCEP-treated

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Figure 5. Chaetocin disrupts the direct interaction between SUV39H1 and HP1 independent of methyltransferase activity and through disulfide functionality. Part 2 of 2

chaetocin. (**B**) A linear range of detection for EGFP–HP1 protein, directly quantified, is established. Autofluorescence from SUV39H1 (MBP–H324K) is negligible. (**C–G**) Fluorescence detection of elutions from the *in vitro* binding assay. (**C**) When MBP is cleaved from SUV39H1(H324K), there is a two-fold greater binding to EGFP–HP1. (**D** and **E**) Chaetocin, but not TCEP-treated chaetocin, disrupts the direct interaction between SUV39H1 and HP1. (**F**) When SUV39H1 is first incubated with chaetocin, followed by free chaetocin being washed out, the binding between SUV39H1–HP1 is abrogated. (**G**) A titration matrix shows that chaetocin irreversibly inhibits the SUV39H1 CD from binding HP1 in a dose dependent manner. Median values of three biological replicates are displayed along with standard deviations. Statistics: one-way ANOVA with Tukey–Kramer post hoc, $P < 0.01$. For Figure 5G, ANOVA-TK is performed for each experimental group having the same SUV39H1(ΔC) concentration, $P < 0.01$ for pairwise comparison. Conditions sharing the same superscript letters not statistically significant from each other.

magnetic beads. Controlling for this possibility, SDS–PAGE confirms that SUV39H1(ΔC) is in the elution while EGFP–HP1 is in the supernatant of assay samples treated with 3 μM chaetocin (Supplementary Figure S2), indicating that chaetocin is not disrupting the SUV39H1–3xCMYC binding to the anti-CMYC beads at an inhibitory concentration of chaetocin.

We next ask whether this disruption acts through the disulfide functionality of chaetocin. To break the disulfide bonds of the ETP group in chaetocin, 50 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) is incubated with chaetocin for 30 min before addition to the pull-down assay samples. TCEP-treated chaetocin samples yield similar fluorescence levels to samples without chaetocin (Figure 5D,E), suggesting that chaetocin disrupts the SUV39H1 and HP1 interaction through its disulfide group. Importantly, this includes samples with only 0.1 μM chaetocin, below its reported IC_{50} value, suggesting disruption of the disulfide functionality abrogates any potential reversible inhibition of the interaction by chaetocin. SDS–PAGE verifies that both SUV39H1(ΔC) and EGFP–HP1 are in the elution (Supplementary Figure S2).

We also perform several additional experiments to further test the hypothesis that chaetocin is irreversibly reacting with SUV39H1 and disrupting its ability to bind HP1, and not acting in a reversible or even catalytic manner to inhibit this protein–protein interaction (PPI). In one set of experiments, SUV39H1–3xCMYC(ΔC) is bound to anti-CMYC magnetic beads and 3 μM chaetocin is added. The beads are washed to remove free chaetocin, and then EGFP–HP1 is mixed with the beads. Fluorescence quantification shows that EGFP–HP1 does not bind with chaetocin-treated SUV39H1(ΔC) even when chaetocin has been washed out (Figure 5F). A titration matrix assay further verifies this result (Figure 5G). 0, 0.1, 0.5, 3 μM chaetocin are added to 1.5, 3, or 6 μM SUV39H1(ΔC) before 80 nM of EGFP–HP1 is added. When 0.1 μM chaetocin is added, it reduces EGFP–HP1 binding by 76%, 38% and 23% for 1.5, 3, 6 μM SUV39H1(ΔC), respectively. An amount of 3 μM chaetocin completely prevents all EGFP–HP1 binding for all 3 concentrations of SUV39H1(ΔC) (Figure 5G). The results indicate that chaetocin disrupts binding in a dose and concentration dependent manner. We also ask further if this inhibition is specific to SUV39H1 or if it generally disrupts interactions of proteins, for example, with HP1. We therefore repeat the same assay with commercial full-length histone H3 and EGFP–HP1. It is known that the HP1 CSD interacts with the GTVAL motif of histone H3 [14], and we observe this binding as well (Supplementary Figure S3). Importantly, when chaetocin is incubated with histone H3, chaetocin does not inhibit the H3–HP1 interaction (Supplementary Figure S3).

Discussion

Our results put forward an inhibitory mechanism in which chaetocin disrupts the interaction between SUV39H1 and HP1. Prior work focused primarily on methyltransferase inhibition. However, HP1 is a conserved protein and holds diverse roles in heterochromatin assembly and transcriptional silencing [15]. This has important and broad implications for the interpretation of past and future literature using chaetocin. For example, while both inhibition of methyltransferase activity and HP1 recruitment could lead to a similar net effect of reduced H3K9 methylation, these two mechanisms are different and could yield different interpretations of how heterochromatin is formed, the kinetics of silencing and reactivation processes, and our understanding of HP1 recruitment and its role in phase condensate formation. Given the critical role of the SUV39H1–HP1 interaction in heterochromatin formation and stability, this new molecular effect of chaetocin should be broadly considered when interpreting and designing experiments using this chemical.

Specific molecular details may also inform future work. For example, our results show that chaetocin acts on the CD of SUV39H1 in a dose dependent way through its disulfide moiety to disrupt the SUV39H1 and HP1 interaction. In contrast chaetocin does not inhibit the interactions between histone H3 and the HP1 CSD nor between CRY2–CIB1 and the CMYC-antibody, and also does not inhibit the ability of ZF-VP16 to transactivate the two-hybrid reporter. These other proteins have relatively lower levels of cysteines compared with SUV39H1 [16]. A detailed analysis of the impact of chaetocin on the structures of Su(var)3–9 family proteins could inform our mechanistic understanding of how this disruption is occurring, but also reveal if there are potential functions of SUV39H1 that are preserved. In addition, a global analysis of the impact of chaetocin on cysteine-rich proteins could identify other cellular effects of the chemical. For example, chaetocin has already been shown to form covalent adducts with hypoxia inducible factor-1alpha (HIF-1α) [17] and components of the thioredoxin–thioredoxin reductase (Trx–TrxR) system [18].

HP1 is also known to self-dimerize, an important interaction for heterochromatin formation and stability. While previous work has discovered mutations at conserved residues abrogating this dimerization, these did not involve cysteines (IY165/168EE) [12]. Experiments testing whether chaetocin affects this self-dimerization, as well as with other molecular interactions with HP1 and SUV39H1, would further clarify the net effects of the chemical on heterochromatin.

There are also finer molecular details previously discussed regarding the specificity of chaetocin inhibition of methyltransferase activity. We also consider their relevance to the disruption of the SUV39H1–HP1 interaction here. In particular, prior discussions indicated chaetocin acts through ETP-based irreversible inhibition of SUV39H1 methyltransferase activity, but also suggested that the specificity of chaetocin could be maintained through short exposures of chaetocin at low concentrations below the originally reported IC_{50} of 0.8 μM [3]. In our work assessing the disruption of the SUV39H1–HP1 interaction, low concentrations of chaetocin 0.1 μM were able to disrupt their binding, but this disruption was abrogated by TCEP treatment suggesting that an irreversible mechanism through the ETP functionality was required.

Chaetocin has also been evaluated as a potential therapeutic agent to treat multiple cancers [19–22], chronic heart failure [23], and African Trypanosomiasis [24], as well as to improve the cloning of embryos by somatic cell nuclear transfer (SCNT) [25]. A more complete understanding of its mechanism of action may therefore benefit future therapeutic strategies as well as potentially inform dosages, timing of administration, and the anticipation of off-target effects. Ultimately, interfacing molecular and biochemical measurements *in vivo* may also be important to assess the therapeutic potential and specificity of chaetocin.

Methods

Yeast culture and strains

Methods for yeast culture and strain generation closely follow prior work [10], from which the following paragraph is adapted. The *S. cerevisiae* strain YPH500 (Strategene) was used as the background strain for the yeast two-hybrid system. Culturing and genetic transformation were done as previously described using URA3 (GFP reporter, pRS406 backbone), HIS3 (ZF–SUV39H1, pNH603), or LEU2 (HP1–VP16, pNH605) as selectable markers. The reporter plasmid was constructed by cloning two ZF43–8 binding sequences directly upstream of a minimal CYC1 promoter driving EGFP. ZF–SUV39H1 and HP1–VP16 fusion proteins were expressed from TetR and LacI-regulated GAL1 promoters. The host strain was generated by genomically integrating into the background strain an expression cassette that constitutively expresses TetR, LacI, and GEV (cloned into single-integrating plasmid pNH607 [HO]. Constitutive expression of the repressors in glucose-containing media ensures low basal levels of expression of the fusion proteins from the engineered GAL1 promoters, which can be relieved by the respective addition of the chemical inputs, ATc (0.5 μg/ml) and IPTG (20 mM), along with b-estradiol (5 μM) to the medium.

Flow cytometry assays

Methods for flow cytometry assays closely follow prior work [26], from which the following paragraph is adapted. Three single yeast colonies for each strain were picked after plasmid transformations and inoculated into 500 ml of SD-media (synthetic dropout media containing 2% glucose with defined amino acid mixtures) in Costar 96-well assay blocks (V-bottom; 2 ml max volume; Fisher Scientific). The cultures were grown at 30° C with 900 rpm shaking for 24–48 h. Cultures were then re-inoculated in SD-complete media to an OD_{600} of 0.05 to 0.1 and grown at 30°C with 900 rpm shaking for 12 h. Cells were treated with 10 mg/ml cycloheximide

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to inhibit protein synthesis and then assayed for yEGFP expression by flow cytometry. 10 000 events were acquired using a MACSQuant VYB flow cytometer with a 96-well plate sampler. Events were gated by forward scatter and side scatter and all values obtained were from three isogenic strains. Plots were generated based on side scatter versus FL1 fluorescence. Median fluorescence values were obtained from the flow cytometry histograms using FlowJo software.

Cloning, expression and purification of SUV39H1 mutants and EGFP–HP1

The *E. coli* codon-optimized gene coding for SUV39H1(H324K)-3xCMYC and SUV39H1((ΔC)-3xCMYC were obtained from Twist and subcloned into pMAL (Addgene 79008) vector separately with an N-terminal MBP and 6His tag. The EGFP–HP1 gene fragment was subcloned into a pET (Addgene 29711) vector with an N-terminal Sumo and 6His tag. Proteins were produced in BL21(DE3) cells and purified by FPLC IMAC cartridges. The eluted proteins were concentrated using Amicon Ultra filters (50 kDa MW cutoff) and stored at −80°C. Proteins were analyzed via SDS–PAGE (Supplementary Figure S1).

Fluorescence intensity assay

MBP–SUV39H1(H324K)-3xCMYC and MBP–SUV39H1(ΔC)–3xCMYC were digested by TEV protease to remove MBP and 6His tag at 4°C, overnight, separately (Supplementary Figure S1). H324K–3xCMYC and ΔC–3xCMYC were separately incubated with anti-CMYC beads in 1× TBS-T buffer (25 mM Tris, 0.15 M NaCl, 0.25% Tween-20 Detergent), after washing, 0, 0.1, 1, 3, 15 μM chaetocin (Cayman Chemical, Item No. 13156, CAS 28097-03-2, >95% purity) were added separately followed by addition of EGFP–HP1. Mixtures were incubated at room temperature for 30 min with mixing. For TCEP-treated chaetocin, 50 mM TCEP was added to chaetocin and incubated for 1 h before chaetocin was added. Supernatants were removed, and proteins were eluted with 50 mM NaOH. The fluorescence intensity of the elations were measured on a TECAN plate reader. EGFP–HP1 incubated with anti-CMYC beads were used as the background control. Same operation for binding assay with commercial histone H3 and EGFP–HP1. For wash assay, a wash step was operated to remove free chaetocin before EGFP–HP1 was added to the assay. For titration matrix assay, 25, 50, 100 μl anti-CMYC beads were set up to bind with 1.5, 3, 6 μM SUV39H1(ΔC) separately. 0, 0.1, 0.5, 3 μM chaetocin were added to each amount of SUV39H1(ΔC) followed by addition of 80 nM constant amount of EGFP–HP1.

SDS–PAGE assay

The elution and supernatant of pull-down samples were collected and mixed with reducing reagent and sample loading buffer. After incubating at 95°C for 5 min, the samples were loaded into a Bis-Tris gel for SDS–PAGE analysis. Gel staining followed the Imperial Protein Stain protocol (Fisher PI24615).

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Albert J. Keung: Conceptualization, Resources, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing — original draft, Project administration, Writing — review and editing. **Linna Han:** Data curation, Software, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing — original draft, Writing — review and editing. **Jessica B. Lee:** Data curation, Software, Formal analysis, Funding acquisition, Validation, Investigation, Visualization, Methodology,

Writing — original draft, Writing — review and editing. **Elaine W. Indermaur:** Data curation, Formal analysis, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing — review and editing. LH and JBL can be listed in either order on professional documents.

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Abbreviations
CD, chromodomain; CIB1, calcium- and integrin-binding protein 1; CMYC, cellular myelocytomatosis; CRY2, cryptochrome circadian regulator 2; CSD, chromoshadow domain; DNMT, DNA methyltransferase; EGFP, enhanced green fluorescent protein; ETP, epipolythiodixopiperazine; EZH2, enhancer of zeste homolog 2; HMT, histone methyltransferase; HP1, heterochromatin protein 1; IPTG, isopropyl β-D-1-thiogalactopyranoside; MBP, myelin basic protein; SDS–PAGE, Sodium dodecyl-sulfate polyacrylamide gel electrophoresis; TCEP, tris (2-carboxyethyl)phosphine; Trx–TrxR, thioredoxin–thioredoxin reductase; VP16, virus protein 16; ZF, zinc finger.

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