The Fission Yeast Jmj2 Reverses Histone H3 Lysine 4 Trimethylation

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Histone methylation regulates transcription, chromatin structure, and the epigenetic state of the cell. Recent studies identified the JmjC domain as a catalytic module for histone demethylation. Schizosaccharomyces pombe contains seven JmjC proteins, but it was unclear whether any of them functioned as histone demethylases. In this report, we show that the JmjC protein Jmj2, which is evolutionarily conserved from yeast to human, reversed trimethylated H3-Lys-4 to di- and mono- but not unmethylated products. Overexpression of Jmj2 but not a catalytically inactive mutant reduced H3-Lys-4 trimethylation levels in vivo and suppressed the toxicity caused by overexpression of the H3-Lys-4-me3-binding protein Yng1 in budding yeast. Genome-wide analysis showed that the loss of jmj2 was associated with an increase in the H3-Lys-4-me3 signal, which was enriched near the transcriptional start sites and the coding regions. At the mating-type locus, the loss of jmj2 or substitution of jmj2 with a catalytically inactive form is correlated with increased reporter gene transcription and H3-Lys-4-me3/2 levels, suggesting that Jmj2 and its demethylase activity may play a role in heterochromatin biology. Our findings identified a novel S. pombe histone demethylase with specificity toward di- and trimethylated histone H3-Lys-4 and a possible role in heterochromatin regulation.

Methylation occurs on both lysine and arginine residues, including H3-Lys-4-, Lys-9, Lys-27, Lys-6, H4-Lys-20, as well as Lys-9 located within the core of histone H3 (4, 5). Methylation at these sites has been linked to transcriptional activation and repression, as well as DNA damage response (5, 6), demonstrating a widespread role for histone methylation in various aspects of chromatin biology (3). An added level of complexity results from the fact that lysine residues can be mono-, di-, or trimethylated, which may represent distinct interaction surfaces for the recruitment of different factors that differentially impact the functional outcomes.

Histone trimethylation at H3-Lys-4 is linked to active transcription (7–11). H3-Lys-4 methylation is dynamically regulated by histone methylases and the recently identified mammalian demethylase LSD1 (12), which mediates demethylation of H3-Lys-4-me1/2, but not H3-Lys-4-me3, due to the inherent chemistry (12). Given the critical role of H3-Lys-4-me3 in active transcription, it is of significant interest to investigate whether there are demethylases that specifically reverse H3-Lys-4 trimethylation.

JmjC domain is a recently identified catalytic module for histone demethylation (13–18). Its chemistry allows for this type of enzymes to mediate demethylation of mono-, di-, and trimethylated lysine residues (reviewed in Refs. 19 and 20). Indeed, recent studies identified a subfamily of JmjC domain-containing proteins that mediate demethylation of trimethylated histone H3-Lys-9 and H3-Lys-36 (15–18). In Schizosaccharomyces pombe, there are seven JmjC domain proteins, but it was unclear whether any of them functioned as demethylases. In this report, we provide both in vitro and in vivo evidence that Jmj2 is an H3-Lys-4 trimethyl histone demethylase that catalyzes demethylation of H3-Lys-4-me3 to H3-Lys-4-me1/2 but had no effect on methylation at other lysine residues. Consistent with these observations, overexpression of Jmj2 resulted in a specific reduction of H3-Lys-4-me1/2, but not H3-Lys-4-me3, due to the inherent chemistry (12). Given the critical role of H3-Lys-4-me3 in active transcription, it is of significant interest to investigate whether there are demethylases that specifically reverse H3-Lys-4 trimethylation.

The abbreviations used are: IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; WT, wild type; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; SC, synthetic complete; RT, reverse transcription.
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**Experimental Procedures**

**Yeast Strains**—Deletion of *jmj2* and knocking in of the nmt1 promoter were performed by using a PCR-based module method (44). Generation of the point mutant H468A was also carried out by a PCR-based module method using a 70-bp oligonucleotide (44). Generation of the point mutant H468A was also carried out by a PCR-based module method using a 70-bp oligonucleotide (44).

**Expression Vectors**—*jmj2* cDNAs (wild type and H468A mutant) were amplified from *S. pombe* genomic DNA using *Platinum* PfX polymerase (Invitrogen) and introduced into the Invitrogen Gateway Entry System. After sequencing of the plasmids, the cDNAs were transferred to the FLAG-baculovirus vector.

**Protein Purification**—FLAG-*jmj2* and FLAG-A468H were purified from *S. cerevisiae* as described previously (18).

**In Vitro Demethylation Reactions**—Demethylation reactions were carried out with 2–10 μg of purified protein using either 1 μM peptide or 5 μg of calf thymus type II-A histones (Sigma) as substrates in a buffer containing 20 mM Tris-HCl, pH 7.3, 150 mM NaCl, 50 μM (NH₄)₂Fe(SO₄)₂, 1 mM α-ketoglutarate, and 2 mM ascorbic acid for 3–5 h at 30 °C. The reactions were incubated for 1 h at 37 °C and quenched by 125 mM glycine for 5 min at room temperature.

**MALDI-TOF Mass Spectrometry**—1 μl of demethylation reaction was analyzed by MALDI-TOF mass spectrometry on an Applied Biosystems Voyager DE-STR instrument as shown previously (18).

**Western Blot**—For total cell extracts, yeast cultures grown to *A*₆₀₀ = 0.5 in Edinburgh minimal medium supplemented with amino acids were harvested, cell extracts were prepared using a mini bead beater for cell disruption, and equal amounts of proteins were run in 15% polyacrylamide gels. Dilutions 1:1000 in phosphate-buffered saline, 1% bovine serum albumin of the following antibodies were used for Western blotting of histones: anti-monomethyl H3-Lys-4 (Millipore catalog number 07-450), anti-dimethyl H3-Lys-4 (Millipore catalog number 07-427), anti-trimethyl H3-Lys-4 (Millipore catalog number 07-423), anti-monomethyl H4-Lys-20 (Millipore catalog number 07-440), anti-dimethyl H4-Lys-20 (Millipore catalog number 07-031), anti-trimethyl H4-Lys-20 (Millipore catalog number 07-463), anti-trimethyl H3-Lys-36 (Millipore catalog number 07-549), anti-monomethyl H3-Lys-9 (Millipore catalog number 07-450), anti-dimethyl H3-Lys-9 (Millipore catalog number 07-441), anti-trimethyl H3-Lys-9 (Millipore catalog number 07-442), and anti-H3 (Millipore catalog number 05-928).

**Chromatin Immunoprecipitation (ChIP)—** *S. pombe* cells were fixed by 1% formaldehyde for 30 min at 30 °C and quenched by 125 mM glycine for 5 min at room temperature. ChIP experiments were carried out as described previously (45) by using antibodies against H3-Lys-4-methyl 2 (Millipore catalog number 07-030) and H3-Lys-4-methyl 3 (Millipore catalog number 07-423). Genome-wide mapping of H3-Lys-4-Me3 was performed according to the manufacturer's instructions (Nimblegen). ChIP analyses were carried out by real-time PCR (Roche Applied Science) (see Fig. 3) or radioactive PCR (see Fig. 5). The following primers were used for ChIP: *specc285.03*, 5′-AAGTA-GATAAAATCTTCTCATTGC-3′ and 5′-GGAGTTGATATT-TTACATGCC-3′; *cgs2*, 5′-AAACCAAGGTATCAATAGG-TACC-3′ and 5′-TTAGTATTGGATAGATGGG-3′; *cox18*, 5′-TTTACACTGATCATGCTGTCG-3′ and 5′-CCTCTCTGTTGACATACTCTCG-3′; and *Ura4*, 5′-GGAGG-GATGAAAAATCCCATGC-3′ and 5′-TTCGACACAGGATT-ACGACC-3′.

**Microra Group Design and Hybridization**—A whole-genome tilting array was implemented using a total of 191,581 distinct 55-mer tiles with 65-bp spacing, designed using the forward strand of the 2003 *S. pombe* genome assembly. No Tₘ, matching was performed. Each spot was replicated twice on the array, and random GC content probes were included for quality control purposes. The platform design can be found at NCBI GEO under accession number GPL4749.

**Analysis of Microarray Data**—Nimblegen-supplied data files were imported into the Bioconductor computing environment. After performing quantile normalization, genotype-dependent ChIP enrichments were estimated for each probe by fitting a fixed linear model accounting for array, dye, and genotype effects to the data using the LIMMA package (46). Moderated *t*-statistics and the log-odds score for differential expression or ChIP enrichment were computed by empirical Bayes shrinkage of the standard errors with the false discovery rate controlled to 0.05. Results were loaded into a relational data base for positional analyses into a Generic Genome Browser. Files containing per-probe signal estimates are available (see supplemental data), and all microarray data have been deposited under the provisional NCBI GEO series GSE8175.

**RT-PCR**—For RT-PCR, 5 μg of RNA was used in a RT reaction by using random N9 primer.

**Ura4** Reporter Assay—5-fold serial dilutions of exponentially growing liquid cell cultures were spotted on plates starting with 2 × 10⁵ cells. The strains were grown on rich medium (YEM), Edinburgh minimal medium supplemented with amino acids without uracil, and Edinburgh minimal medium supplemented with amino acids and uracil and 1 mg/ml 5-fluoroorotic acid. Cells were growing for 4 days at 32 °C.

**Charomycyes cerevisiae** ortholog YJR119c, but not the catalytically inactive mutants, suppressed the toxicity caused by overexpression of the H3-Lys-4-me3-binding protein YNG1, suggesting a role of H3-Lys-4 histone demethylases in transcriptional regulation. Finally, we show that Jmj2 demethylase activity is necessary for regulating heterochromatin at the mating-type locus, suggesting a potentially important role of this enzyme in heterochromatin biology. Our findings provided unequivocal evidence demonstrating that the evolutionarily conserved Jmj2 functions as a histone H3-Lys-4 tridemethylase and may play important roles in both euchromatic and heterochromatic transcription.
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| Jmj2 + peptide | Peptide alone |
|----------------|--------------|
| **H3 K4me3**   | 2622 2650    |
| **H3 K4me2**   | 2622 2636    |
| **H3 K4me1**   | 2608 2622    |

| Jmj2 + peptide | Peptide alone |
|----------------|--------------|
| **H3 K9me3**   |              |
| **H3 K9me2**   |              |
| **H3 K9me1**   |              |

| Jmj2 + peptide | Peptide alone |
|----------------|--------------|
| **H3 K36me3**  |              |
| **H3 K36me2**  |              |
| **H3 K36me1**  |              |

| Jmj2 + peptide | Peptide alone |
|----------------|--------------|
| **H4 K20me3**  |              |
| **H4 K20me2**  |              |
| **H4 K20me1**  |              |
RESULTS

Jmj2 is a Histone H3-Lys-4-me3-specific Demethylase—The fission yeast S. pombe has seven JmjC domain-containing proteins (supplemental Fig. 1). Among them, Epe1 has been shown to counteract H3-Lys-9 methylation and heterochromatin spreading (21, 22). Although Epe1 is an ortholog of the human histone demethylase JHDM1 and requires the integrity of the JmjC domain to antagonize heterochromatin spreading, it does not appear to have the demethylase activity when assayed on histone substrates. Although it lacks some of the key residues required for histone demethylation (14), whether Epe1 has demethylase activity on other protein substrates remains to be determined. Another jmjC containing protein, Msc1, has been shown to be involved in the regulation of the histone acetylation (23), underlining the evolutionary conservation of these proteins as chromatin regulators.

We took a candidate approach and, in this study, focused on one of the members of this family, Jmj2 (SPAC1002.05c). Jmj2 contains the JmjN and JmjC domains, which have been shown to be important for histone demethylation (24). It also contains an AT rich interacting domain (ARID)/BRIGHT domain, which is a DNA binding motif (25, 26), and a C5HC2 zinc finger whose function is unknown (supplemental Fig. 1). To determine whether Jmj2 was a histone demethylase, we isolated a full-length jmj2 cDNA from S. pombe genomic DNA by PCR, subcloned it into a Gateway entry vector, and subsequently transferred it to N-terminal FLAG tag baculovirus expression vector for expression and purification to near homogeneity (supplemental Fig. 2A). We used a MALDI-TOF mass spectrometric approach to determine whether Jmj2 had histone demethylase activity, using as substrates peptides corresponding to the methylated histone N-terminal tails. As shown in Fig. 1, Jmj2 mediated demethylation at H3-Lys-4 but not at H3-Lys-9, H3-Lys-36, or H4-Lys-20. Jmj2 appeared to be capable of demethylating mono-, di-, and trimethylated H3-Lys-4 in the peptide-based assay. However, when assayed on bulk histones, Jmj2 acted predominantly as an H3-Lys-4-me3/2-specific demethylase, with no detectable effect on H3-Lys-4-me1/2 (Fig. 2A). Like other JmjC domain demethylases, Jmj2 requires Fe(II) and α-ketoglutarate as cofactors for the reactions (14, 18). Omission of α-ketoglutarate, as well as the point mutation of one of the conserved histidine residues important for iron chelation (H468A) (18, 19, 24, 27), resulted in a robust inhibition of the demethylation reaction mediated by Jmj2 (supplemental Fig. 2, B and C).

Regulation of H3-Lys-4 Methylation by Jmj2 in Vivo—We investigated the ability of Jmj2 to regulate H3-Lys-4 methylation as a demethylase in vivo. We generated S. pombe strains in which the wild type Jmj2 or the catalytically inactive Jmj2 mutant (H468A) were under the control of the thiamine-repressible promoter nmt1, which in the absence of thiamine allows for high levels of expression of the gene of interest (28). As shown in Fig. 2B, overexpression of Jmj2 resulted in a significant decrease in the H3-Lys-4-me3 level, as well as an appreciable reduction of H3-Lys-4-me2, but had no significant effect on H3-Lys-4-me1/2. This is consistent with the in vitro data on bulk histones (Fig. 2A), suggesting that Jmj2 is predominantly an H3-Lys-4-me2/3-specific demethylase. Importantly, overexpression of the catalytically inactive Jmj2 (H468A) had no effect on either H3-Lys-4-me3 or H3-Lys-4-me2 (Fig. 2B, compare lane 4 with lane 2, top and middle panel), indicating that the changes in H3-Lys-4-me2/3 levels upon overexpression of Jmj2 were likely to be due to its histone demethylase activity. However, deletion of Jmj2 had no discernable effects on global H3-Lys-4 methylation (Fig. 2B, lane 2, top panel). Yeast cells deleted of Jmj2 were viable, suggesting that Jmj2 is not an essential gene. The lack of change of the H3-Lys-4-me3 level could be due to either the presence of additional H3-Lys-4 trimethyl demethylases or the possibility that Jmj2 regulates only a small percentage of the genome; therefore, its ablation would not be expected to cause a global increase of H3-Lys-4-me levels. As a further control, mono-, di-, and trimethylation of H4-Lys-20 and trimethylation of H3-Lys-36 were shown to be unaffected by either the overexpression or the loss of Jmj2 (Fig. 2C), supporting the idea that Jmj2 is an H3-Lys-4-me-specific demethylase.

ChIP-on-Chip Identifies Genomic Regions That Are Regulated by Jmj2—Since deletion of the Jmj2 gene does not have a significant impact on the global H3-Lys-4 methylation levels, we next determined the effect of Jmj2 deletion by surveying the entire genome using ChIP-chip analysis. We noticed misregulation of H3-Lys-4 trimethylation where H3-Lys-4-me3 was significantly increased at diverse genomic loci in jmj2Δ (Fig. 3A). Specifically, average gene analysis, which includes all the open reading frames in the genome, showed that, in jmj2Δ, H3-Lys-4-me3 was maximally enriched relative to wild type

FIGURE 1. Identification of Jmj2 enzymatic activity by MALDI-TOF mass spectrometry. Spectra from MALDI-TOF analysis of methylated histone peptides after incubation either with 2.5 mg of purified full-length Jmj2 protein (Jmj2 + peptide) or without protein (peptide alone) are shown. The shaded spectra correspond to H3-Lys-4-me3, H3-Lys-4-me2, and H3-Lys-4-me1/2 peptides demethylated by Jmj2 protein. The molecular weights of H3-Lys-4 peaks are indicated, and the peaks corresponding to a demethylation product are marked with a star.

FIGURE 2. In vitro and in vivo demethylase activity of Jmj2 on histone substrates. A, Jmj2 demethylates bulk histones. Bulk histones were incubated without protein (left lane) or with 5 mg of purified FLAG-Jmj2 (right lane) and blotted with specific antibodies anti-H3-Lys-4-me3 (top panel), H3-Lys-4-me2 (middle top), H3-Lys-4-me1/2 (middle bottom), or H3 (bottom). B and C, in vivo demethylase activity of Jmj2. Whole cell lysates were prepared from exponentially growing cultures of: lane 1, WT strain; lane 2, Jmj2-overexpressing strain; lane 3, jmj2Δ strain; and lane 4, H468H point-mutant-overexpressing strain. Whole cell lysates were probed with the following antibodies: anti-H3-Lys-4-me3, -H3-Lys-4-me2 and -H3-Lys-4-me1/2 (B) or, as control, anti-H4-Lys-20-me1, -H4-Lys-20-me2, -H4-Lys-20-me3, and -H3-Lys-36-me3 (C).
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**A**

Overview of ChrT

WT-H3K4me3

jmj2Δ-H3K4me3

contrast

**B**

Mean Enrichment

WT H3K4me3

jmj2Δ H3K4me3

WT H3K4me2

Gene Position

**C**

SPCC285.03

Wt-H3K4me2

Wt-H3K4me3

jmj2Δ-H3K4me3

Cgs2

Wt-H3K4me2

Wt-H3K4me3

jmj2Δ-H3K4me3

Cox18

Wt-H3K4me2

Wt-H3K4me3

jmj2Δ-H3K4me3
cells around the transcriptional start site, as well as in the 5’ half of the coding region and throughout the promoter (Fig. 3B). Significantly, this analysis also showed substantial decreases in H3-Lys-4-me3 levels in other genomic regions in jmj2Δ cells (Fig. 3A), including the 3’ end of coding sequences (Fig. 3B). To confirm the ChIP-chip findings, we carried out independent chromatin IP experiments. As shown in Fig. 3C, both H3-Lys-4-me3 and H3-Lys-4-me2 were enriched relative to WT near the transcriptional start site at a number of gene loci upon deletion of jmj2. These included the genes spcc285.03, cgs2, and cox18 (right panels). This provided further in vivo evidence that jmj2 functions as a demethylase that reverses H3-Lys-4 di- and trimethylation.

**Jmj2 and Yjr119c Antagonize Yng1, Which binds H3-Lys-4-me3 and Is Important for H3-Lys-4-me-directed Transcription**—In budding yeast, the catalytic subunit of transcription factor II H (TFIHF), Kin28, phosphorylates serine 5 of C-terminal domain of polymerase II (CTD) to recruit Set1 containing COMPASS complex (29). Therefore, serine 5 phosphorylation of CTD and H3-Lys-4-me3 are enriched at the 5’ end of genes (29, 30). Recently, it was shown that Nua3 histone acetyl transferase (HAT) complex preferentially binds to H3-Lys-4-me3 through the plant homeo domain (PHD) finger of Yng1 and promotes H3-Lys-14 acetylation and transcription of a subset of genes (31, 32). Yng1 overexpression results in a high toxicity that is strongly dependent on H3-Lys-4 methylation (32). It is possible that removal of Lys-4 methylation by overexpression of an H3-Lys-4 histone demethylase might suppress this toxicity. Therefore, Yng1 overexpressing cells were used to isolate a budding yeast-specific H3-K4 histone demethylase in vivo. High copy plasmids expressing each JmjC domain protein were introduced in cells expressing Yng1 from GAL1 promoter, and the resulting transformants were tested for the ability to grow on galactose medium. Confirming the previously published data, Yng1 overexpression strongly inhibited the cell growth, and the toxicity was completely abolished by deletion of the H3-Lys-4 methyltransferase SET1 (Fig. 4A). Interestingly, overexpression of Yjr119c, the ortholog of Jmj2 in budding yeast, also conferred resistance to Yng1 overexpression, suggesting that Yjr119c may function as a trimethyl-specific H3-Lys-4 histone demethylase in budding yeast (Fig. 4B). Importantly, four other JmjC domain proteins, Jhd1, Rph1, Gis1, and Ecm5, did not rescue the Yng1 toxicity (data not shown). We next examined the effect of Yjr119c on H3-Lys-4 methylation by Western blot analysis. As shown in Fig. 4D, H3-Lys-4-me3 was significantly reduced in Yjr119c-overexpressing cells but not in cells overexpressing a catalytic mutant yjr119c (H427A), which has a mutation in the Fe(II) binding site. Interestingly, overexpression of the mutant Yjr119c did not rescue the Yng1 toxicity, although the mutant protein was expressed at levels comparable with wild type Yjr119c (Fig. 4, B and D), indicating that Yjr119c rescues the Yng1 toxicity by directly regulating H3-Lys-4 methylation in vivo. Importantly, overexpression of Jmj2, but not the catalytically inactive mutant H468A, also rescued the Yng1 toxicity in the budding yeast (Fig. 4C). Taken together, these findings suggest that Jmj2 and Yjr119c are likely to play an important role in regulating transcription via demethylation of H3-Lys-4-me2/3.

**Jmj2 Demethylase Activity Is Required for Regulation of Heterochromatin at the Mating-type Region**—Fission yeast heterochromatin is characterized by H3-Lys-9 hypermethylation and H3-Lys-4 hypomethylation (33), raising the possibility that H3-Lys-4 demethylases may play a role in the establishment and/or maintenance of the heterochromatic regions. The ChIP-chip experiments did not reveal a significant misregulation of H3-Lys-4-me3 at the centromeres or subtelomeres, where H3-Lys-4-me3 is present at a very low level in both WT and jmj2Δ. The strain that we used for ChIP-chip does not contain the K-region, which is required for the efficient establishment of the silenced chromatin state at the mating-type region (34), precluding our analysis of any potential effects of Jmj2 in this heterochromatin region. To determine whether the heterochromatin in this region is affected by jmj2 deletion, we used a reporter strain that contains the ura4+ gene inserted in the mating-type region, adjacent to mat3M (Fig. 5A). In this strain, due to the position effect variegation, ura4+ remains silenced, and the cells are unable to grow in the absence of uracil. As shown in Fig. 5B, jmj2Δ and the catalytically inactive point mutant H468A strains were able to grow slightly better in medium lacking uracil, as compared with cells with WT or overexpressed levels of Jmj2. This improved growth was most likely due to the ability to express the inserted ura4+ gene since the mutant cells are sensitive to FOA (Fig. 5B). Chromatin IP identified significantly higher levels of H3-Lys-4 di- and trimethylation of the ura4+ reporter gene in both the jmj2Δ and H468A strains (Fig. 5C). This increase of H3-Lys-4 methylation was also correlated with a higher level of the ura4+ mRNA detected by reverse transcription-PCR (Fig. 5D). These experiments suggest that Jmj2 might be involved in regulating H3-Lys-4 methylation and heterochromatin at the mating-type

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**FIGURE 3. Genome-wide analysis of H3-Lys-4-me3 changes upon jmj2 deletion.** A, a general distribution profile of H3-Lys-4-me3 in the WT and jmj2Δ strains. A schematic diagram of chromosome I is shown, as well as the sequential zooming-in of 50 and 6 kb of a representative euchromatic region in the genomic tiling array. The top part of the panels shows a schematic representation of the open reading frames. The tracks represent enrichment on H3-Lys-4-me3 in the WT strain (top track) and jmj2Δ strain (middle track) and the contrast between both signals (bottom track). The color code is as follows: a darker bar color means more significant enrichment value than a lighter bar color. B, an average gene profile of H3-Lys-4 methylation in the WT and jmj2Δ strains. Values from ChIP-on-chip experiments and all the open reading frames in the genome were used in an average gene analysis of H3-Lys-4-me3 enrichment in the WT and jmj2Δ strains as well as H3-Lys-4-me2 in the WT strain. Positive values of the x axis represent gene position as a percentage of total gene length, whereas negative values correspond to the number of base pairs upstream of the gene start. The y axis represents the enrichment mean values, C, changes in H3-Lys-4 methylation of individual genes. The left panels show H3-Lys-4 methylation levels of individual genes determined by ChIP-on-chip analysis, including H3-Lys-4-me2 in the WT strain (top track), H3-Lys-4-me3 in the WT strain (middle track), and H3-Lys-4-me3 in the jmj2Δ strain (bottom track). The right panels show the enrichment of H3-Lys-4-me2 and H3-Lys-4-me3 in the same genes, analyzed by conventional ChIP and real-time PCR in both WT strain (wt) and jmj2Δ strain (ko). On the top of each panel, there is a schematic representation of the gene and the location of the region analyzed. WCE, whole cell extract.
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**A**

| WT      | SC-Glucose | SC-Galactose |
|---------|------------|-------------|
| Vector  | pGAL-YNG1  | pGAL-YNG1   |
| set1Δ   | pRS424     | pRS424-yr119c |

**B**

| Vector | SC-Glucose | SC-Galactose |
|--------|------------|-------------|
| pGAL   | pGAL-YNG1  | pGAL-YNG1+H468A |

**C**

| Vector | SC-Glucose | SC-Galactose |
|--------|------------|-------------|
| pGAL   | pGAL      | pGAL-YNG1   |

**D**

| Vector | SC-Glucose | SC-Galactose |
|--------|------------|-------------|
| pGAL-YNG1 | H3         | H3K4me2    |
|         | H3K4me3   | H3K36me3   |
|         | H3K4me2-yr119c | H3K4me3   |

**E**

| Vector | SC-Glucose | SC-Galactose |
|--------|------------|-------------|
| pGAL   | Rpb3      | Rpb3        |
|        | HA        | HA          |

**FIGURE 4. Overexpression of Jmj2 and Yjr119c rescue the Yng1 toxicity in budding yeast.** A, the Yng1 toxicity is dependent on H3-Lys-4 methylation. pRS316 and pGAL-YNG1 were introduced into wild type and set1Δ mutant. The resulting transformants were grown in synthetic complete (SC) medium containing 2% glucose, and then 10-fold serial dilutions of yeast cells were plated on SC medium lacking uracil and tryptophan containing either 2% glucose or 2% galactose as a carbon source. SC plates containing glucose were photographed after 48 h, and plates containing galactose were photographed after 72 h. B and C, overexpressions of Yjr119c and Jmj2 confer resistance to Yng1 overexpression. The indicated plasmids were introduced into a wild type strain containing either pRS316 or pGAL-YNG1. Cells were spotted onto SC medium lacking uracil and tryptophan (B) or uracil and leucine (C) containing either 2% glucose or 2% galactose as a carbon source. SC plates containing glucose were photographed after 48 h, and plates containing galactose were photographed after 72 h. D, Yjr119c is a histone demethylase for H3-Lys-4-Me3 in budding yeast. The indicated plasmids were transformed into wild type or set1Δ mutant strain, and the transformants were grown in medium containing 2% glucose as a carbon source. Equal amounts of chromatin fractions from the indicated cells were analyzed by Western blot analysis with anti-H3, anti-H3-Lys-4-me3, anti-H3-Lys-4-me2, anti-H3-Lys-36-me3, anti-hemagglutinin (HA), or anti-Rpb3 antibody. E, expression of Jmj2 in budding yeast. The indicated plasmids were transformed into wild type and set1Δ mutant strain, and the transformants were grown in medium containing 2% galactose as a carbon source. Equal amounts of total cell extracts from the indicated cells were analyzed by Western blot analysis with anti-hemagglutinin or anti-Rpb3 antibody.

**DISCUSSION**

In mammals, there are at least eight histone H3-Lys-4 methyltransferases (3). Prior to this study, LSD1 was the only H3-Lys-4 demethylase (12). LSD1 is chemically restricted to demethylation of mono- and dimethylated H3-Lys-4, raising the question of whether H3-Lys-4 trimethylation was enzymatically reversible. The finding of Jmj2 demonstrates that the H3-Lys-4 trimethyl state is also enzymatically reversible. In fact, this function appears to be conserved from *S. cerevisiae* to human, indicating important roles for these enzymes in chromatin and gene regulation (35, 37–43).

When histone peptides were used as substrates, Jmj2 demethylated all three methylation states at H3-Lys-4 in vitro (Fig. 2). However, its activity became restricted to H3-Lys-4-Me3 and H3-Lys-4-Me2 when bulk histone proteins were used as substrates in vitro (Fig. 3). Consistently, analysis of endogenous histone H3-Lys-4 methylation states upon overexpression of Jmj2 also showed restricted activity of Jmj2 toward tri- and dimethylated H3-Lys-4 (Fig. 4). Thus, it is most likely that the major histone substrates for Jmj2 are H3-Lys-4-Me2/3. It is interesting to note that deletion of *jmj2* or replacement of the wild type *jmj2* with a catalytically inactive *jmj2* had no effect on the global histone H3-Lys-4 methylation states in vivo (Fig. 4A). This suggests possible functional redundancy and the existence of additional H3-Lys-4 demethylases in *S. pombe*. Supporting this view, *S. pombe* has two other Jmj2-related JmjC domain proteins, Msc1 and Lid2, that share sequence and structural similarities to the human JARID1 family of proteins, composed of four members, JARID1A (RBP2), JARID1B (PLU-1), JARID1C (SMCX), and JARID1D (SMCY) (reviewed in Refs. 19 and 20), all of which have recently been shown to be H3-Lys-4 demethylases (35). Collectively, we have identified Jmj2 as a histone H3-Lys-4 tri- and dimethyl-specific demethylase, and this finding suggests that Msc1 and Lid2 may be H3-Lys-4 demethylases as well.

H3-Lys-4 trimethylation has been linked to active transcription (7–11). Consistent with this view, genome-wide ChIP-chip analysis identified H3-Lys-4 trimethylation to be enriched around transcriptional start sites (Fig. 3B). Importantly, deletion of *jmj2* resulted in an increase in H3-Lys-4 trimethylation at these genomic loci, consistent with the idea that Jmj2 is an H3-Lys-4-Me3-specific demethylase. Interestingly, *jmj2* deletion showed lower than wild type levels of H3-Lys-4 trimethylation toward the 3′ end of genes, which may contribute to our failure to detect significant global H3-Lys-4 methylation changes in the *jmj2* deletion mutant by Western blot using modification-specific antibodies (Fig. 2B). Despite our efforts, we were unsuccessful at ChIP-chipping or ChIP-pinging Jmj2. Although we consider it likely that Jmj2 directly demethylates histones at the target genomic loci, the lack of the ChIP result formally leaves open the question of whether the effect is direct or indirect.

In budding yeast, H3-Lys-4 methylation recruits NuA3 HAT complex to promote H3-Lys-14 acetylation and transcription of genes including *YML062C* and *YGR157W* (31). Yng1, a component of NuA3, binds to H3-Lys-4 methylation through its PHD finger, and genome-wide analysis showed that Yng1 is localized at the 5′ end of genes enriched with H3-Lys-4-Me3 (31, 36). Consistent with this observation, we found that H3-Lys-14 acetylation together with H3-Lys-4-Me3 was increased in *jmj2* deletion mutant (data not shown).
Furthermore, we showed that both Jmj2 and Yjr119c rescue the Yng1 toxicity by directly antagonizing H3-Lys-4-me3 in vivo (Fig. 4). These findings suggest a negative role of these histone demethylases in transcriptional regulation. De-methylation of H3-Lys-4 by Jmj2 or Yjr119c might inhibit transcription by disrupting the Yng1-H3-Lys-4-me3 interaction, resulting in the loss of H3-Lys-14 acetylation at the 5’ end of genes. Although the findings support the idea that Jmj2 and Yji119c regulate transcription via their demethylase activity, much still remains to be understood. For instance, it is unclear why we did not observe transcriptional changes of those genes where deletion of jmj2 resulted in an increase in H3-Lys-4-me3 (Fig. 3) (data not shown). Lastly, the biological function of Jmj2 is only beginning to be understood. Our current data suggest that Jmj2 likely plays a role in heterochromatin biology by regulating H3-Lys-4 methylation and transcription at the mating-type locus. Although ChIP-chip failed to identify H3-Lys-4-me3 alteration at the centromeric and subtelomeric regions, it is possible that a lack of an effect reflects functional redundancy. Thus, whether Jmj2 plays a role at other heterochromatin regions still remains to be determined.

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