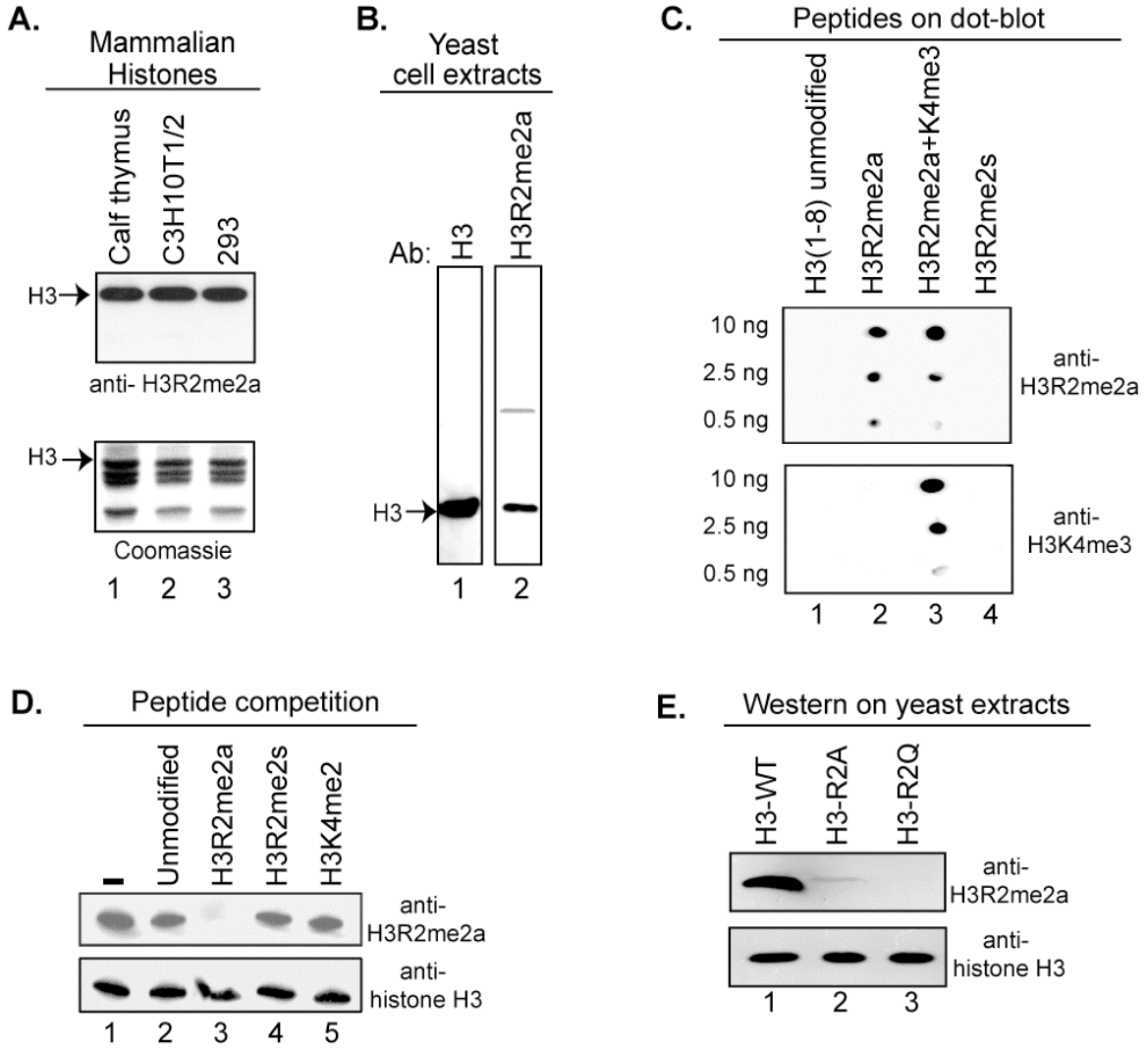

Figure S1. H3R2 is asymmetrically dimethylated in mammals and yeast.

(A) Histones purified from calf thymus, mouse fibroblasts (C3H10T1/2) and human embryonic kidney cells (293) were used in Western blot analysis with the anti-H3R2me2a antibody. Equal loading of histones was monitored by coomassie stain. (B) Whole yeast extracts prepared from the BY4741 strain were analyzed by Western blotting using the anti-H3 antibody (lane 1) or the anti-H3R2me2a antibody (lane 2). (C)

Dot-blot analysis using an unmodified H3 peptide (aa 1-8, lane 1), an asymmetrically methylated H3R2 peptide (aa 1-8, lane 2), an asymmetrically methylated at H3R2 plus trimethylated at H3K4 peptide (aa 1-8, lane 3), or a symmetrically dimethylated H3R2 peptide (aa 1-8, lane 4). The peptides were spotted on a PVDF membrane at the indicated concentrations and then probed with an anti-H3R2me2a antibody (top panel) or an anti-H3K4me3 antibody (bottom panel). (D) Peptide competition experiment was performed by western blot analysis using 0.5 μg of purified yeast histones and antiH3R2me2a antibody in the presence of 1 μg/ml of unmodified H3 peptide (aa 1–8, lane 2), asymmetrically dimethylated H3R2 peptide (aa 1–8, lane 3), symmetrically dimethylated H3 peptide (aa 1–8, lane 4), or dimethylated H3K4 peptide (aa 1-8, lane 5). Lane 1 represents a no peptide control. An anti-H3 antibody was used to show equal loading of yeast histones (bottom panel). (E) Whole yeast extracts prepared from wild type H3 (lane 1), H3R2A mutant (lane 2), or H3R2Q mutant (lane 3) strains were subjected to western blot analysis using an anti-H3R2me2a antibody (top panel) or an anti-H3 antibody (bottom panel).

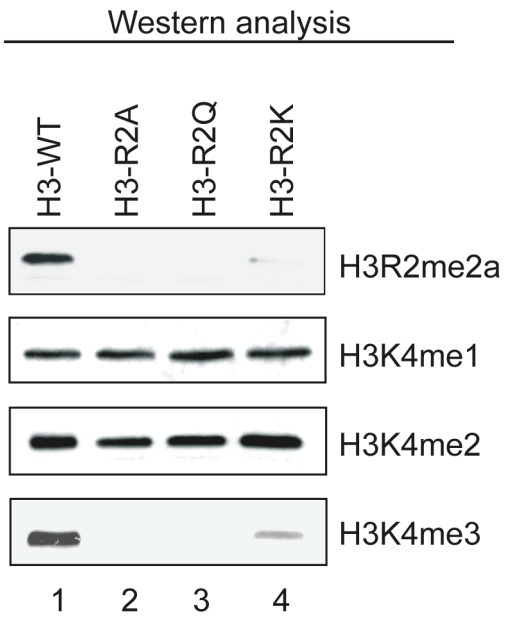

Figure S2. Mutation of H3R2 abolishes tri-methylation at H3K4me3.

Whole yeast extracts prepared from the H3R2K (lane 4), H3R2Q (lane 3), H3R2A (lane 2) and isogenic WT (lane 1) strains were analyzed by Western blotting using antibodies to H3R2me2a, H3K4me1, H3K4me2, and H3K4me3.

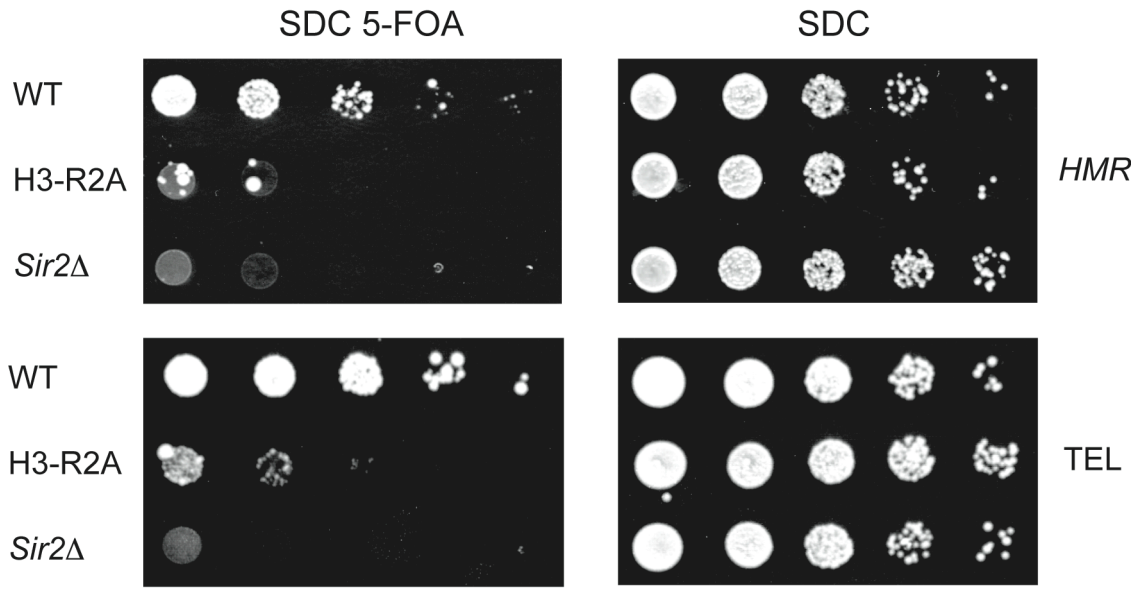

Figure S3. H3R2 is required for heterochromatic silencing.

Cells from the H3-R2A, *sir2Δ,* and isogenic wild-type (WT) yeast strains were grown to mid-log phase. Approximately 1.2 x 10^4 cells were serially diluted 10-fold, and spotted to minimal complete medium (SDC, right panels), and SDC+5-fluoroorotic acid (5-FOA, left panels) plates. Heterochromatic silencing of a *URA3* reporter, integrated at the two heterochromatic sites, *HMR* and telomere-07L (TEL), was measured by growth of colonies on media containing 1g/liter 5-FOA. Plates were photographed after 72 h incubation at 30 ºC.

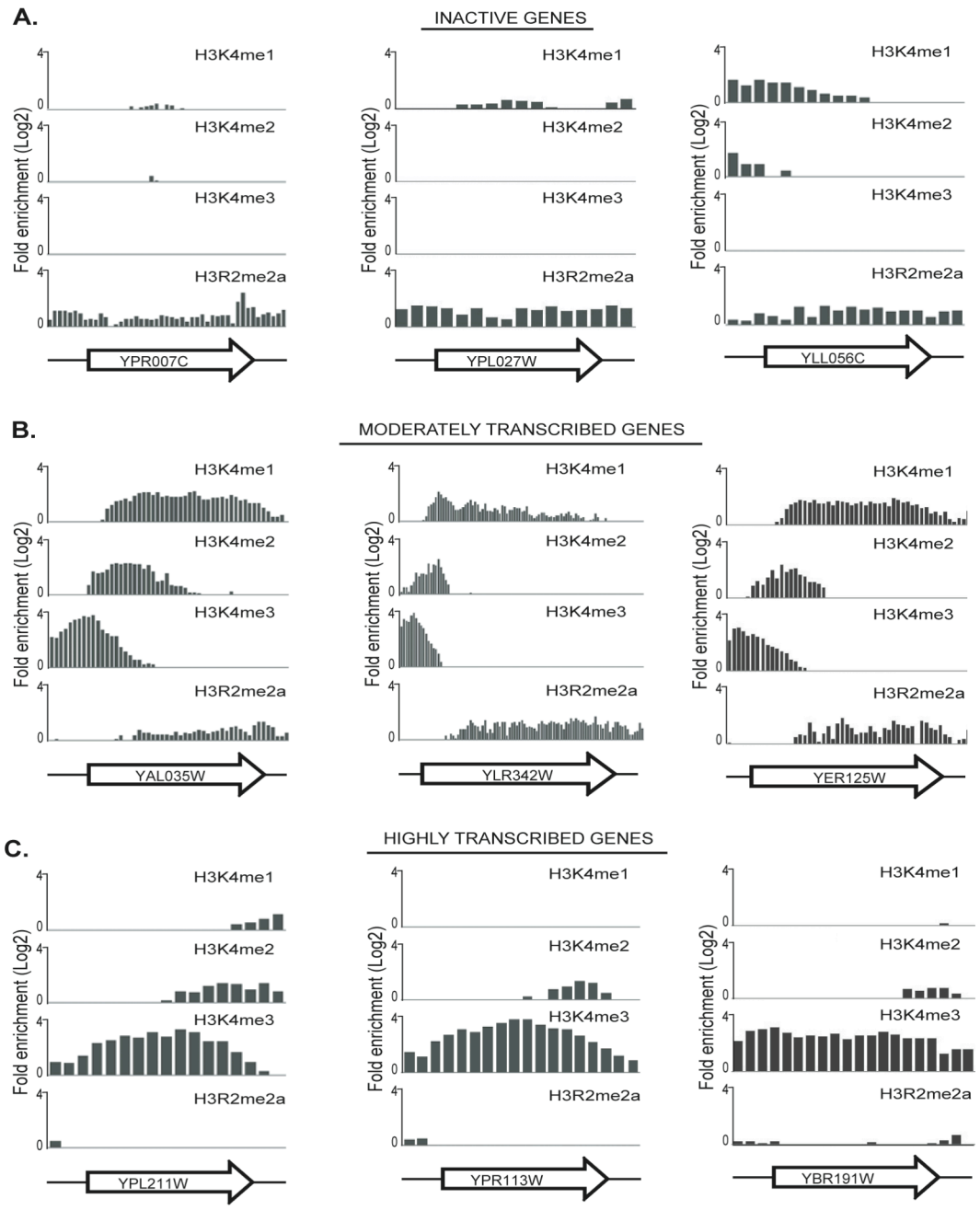

Figure S4. Distribution of H3K4me and H3R2me2a across yeast genes.

ChIP-on-chip analysis was performed using antibodies to H3K4me1, H3K4me2, H3K4me3, and H3R2me2a. The distribution of these modifications across genes is compared at three differentially expressed states. The name of each gene (arrow) is shown below the graphs.

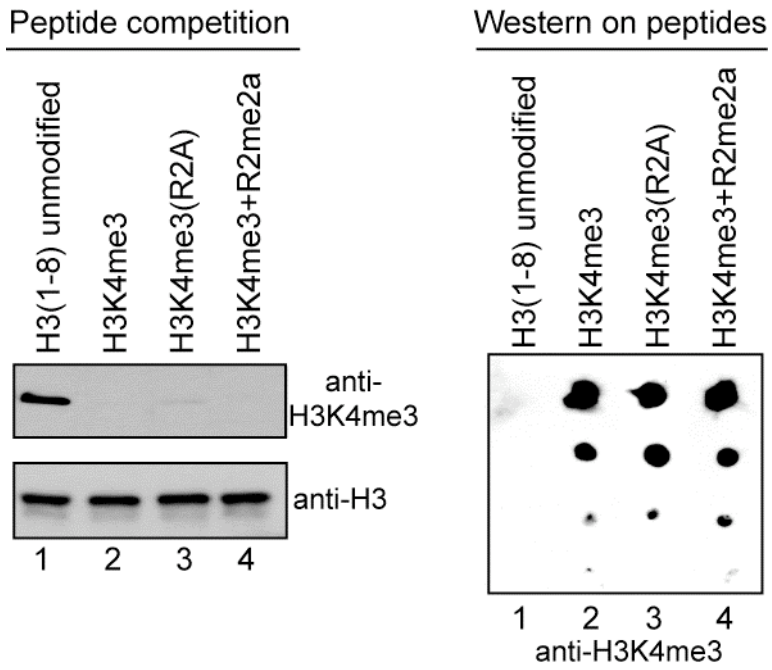

Figure S5. The anti-H3K4me3 antibody recognizes its epitope when the adjacent arginine 2 is either mutated or methylated.

Peptide competition experiments on total yeast extracts (left panel) and dot-blot analysis of peptides (right panel) were performed as in Figure S1C and S1D using an anti-H3K4me3 antibody in the presence of 1 μg/ml of unmodified H3 peptide (aa 1–8, lane 1), trimethylated H3K4 peptide (aa 1-8, lane 2), trimethylated H3K4 peptide containing an R2A mutation (aa 1–8, lane 3), or trimethylated H3K4 peptide that was concomitantly asymmetrically dimethylated at H3R2 (aa 1–8, lane 4).

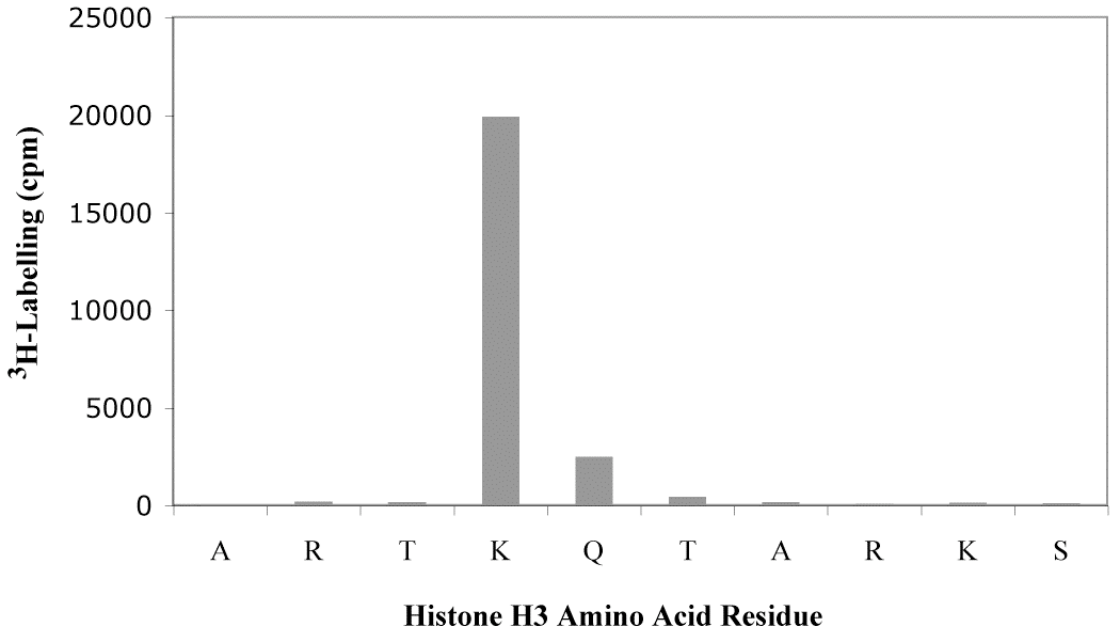

Figure S6. Set1p methyltransferase activity is specific for lysine 4 of histone H3.

The unmodified H3 N-terminal peptide (H3 1-17) was methylated with yeast purified Set1p complex in the presence of ^{3}H-S-Adenosyl methionine and subjected to Edman degradation. Fractions corresponding to each amino acid were collected and monitored by liquid scintillation counting. The amino acid sequence of H3 is indicated below the fractions.

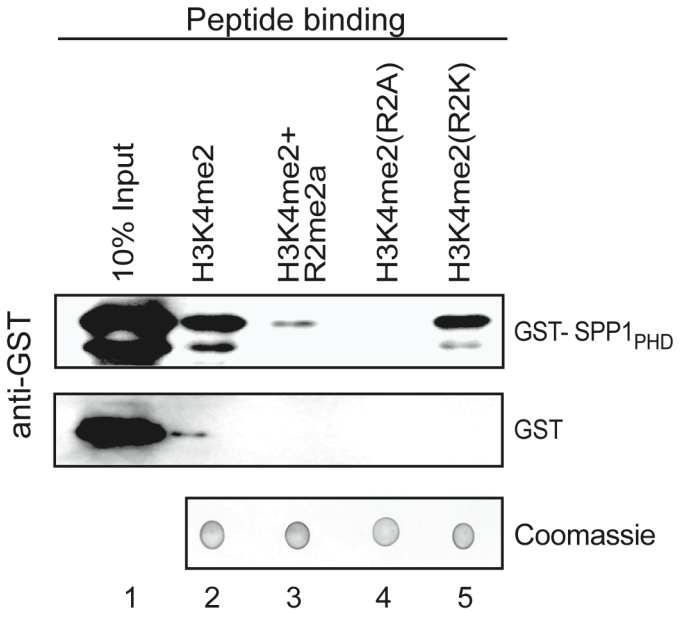

Figure S7. Methylation or mutation at H3R2 inhibits Spp1 binding to methylated-H3K4.

In vitro binding assays were performed using synthetic histone H3 N-terminal peptides (aa 1-21) and either recombinant GST-Spp1PHD (top panel) or GST alone (middle panel). The bound proteins were monitored by western analysis using a GST antibody. Peptide coupling was controlled by dot-blotting followed by coomassie staining (bottom panel).

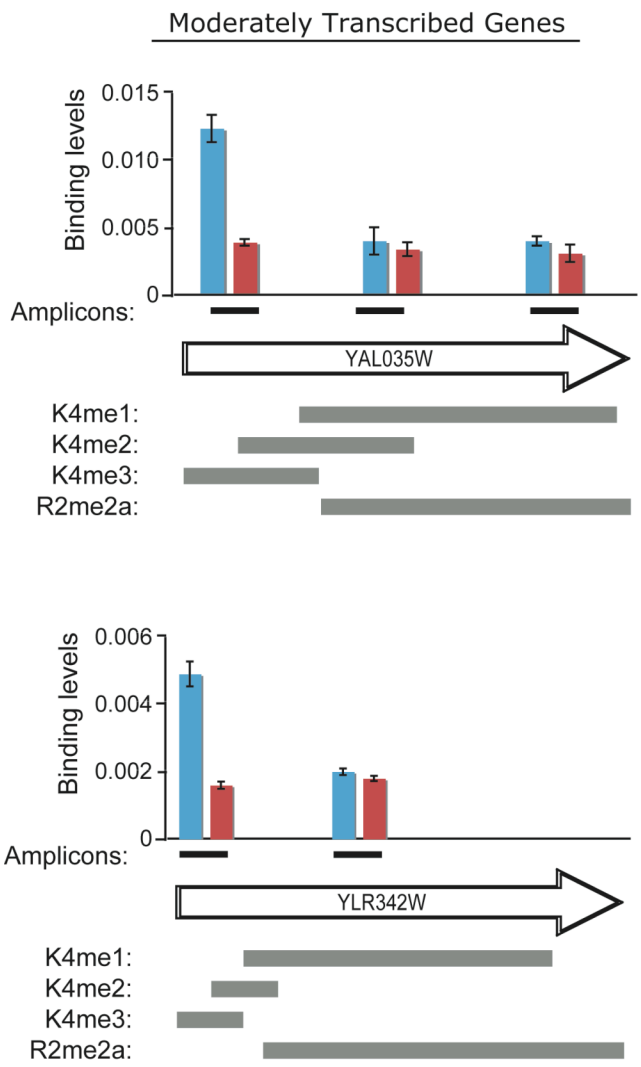

Figure S8. Spp1 occupancy is blocked by H3R2me2a.

In vivo binding analysis of Spp1 was performed using ChIP assays followed by quantitative PCR. Chromatin from yeast cells expressing a tagged (Myc-Spp1) or an untagged (Control) form of Spp1 was immunoprecipitated with an anti-Myc antibody. The analysed amplicons within each gene (arrow) are indicated by black lines. Standard errors were calculated for duplicate experiments. The grey bars below each plot show the distribution of mono-, di-, tri-H3K4 and H3R2me2a within each gene.

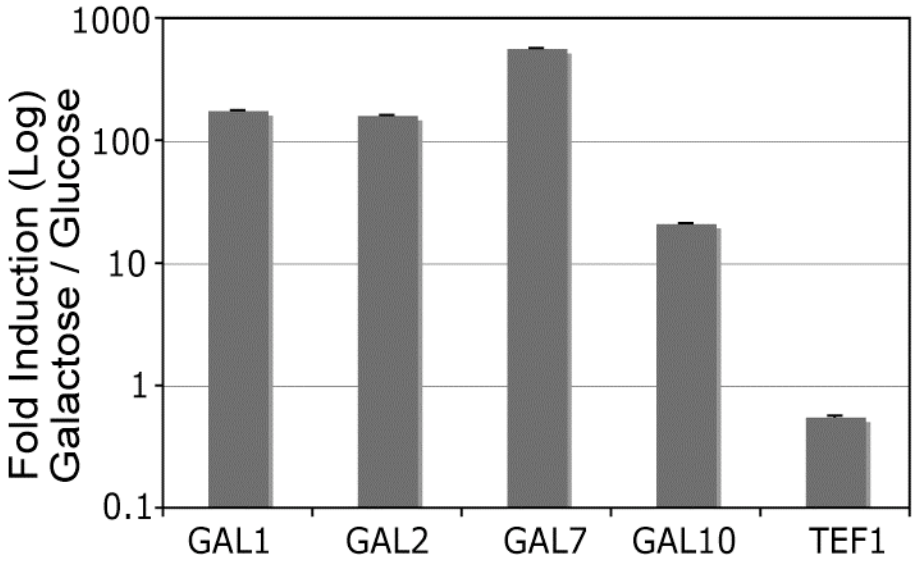

Figure S9. Transcriptional activation of galactose genes.

RT-PCR analysis was performed on yeast cells (BY4741) that were grown to mid-log phase in media containing glucose (repressive condition) and cells that were shifted to media containing galactose (activating condition) for 24 hours, using primers specific to the indicated genes. The expression level of each gene was normalized to the RNA levels of *RTG2*. Values show the fold induction when shifting cells from glucose to galactose. Standard errors were calculated for duplicate experiments.

Primer name	Primer sequence (5' to 3')
Gal1 Forward	AAACTTGCACCGGAAAGGTTTGCCAGT
Gal1 Reverse	CAGGGCCCATATTCGCTTTAACAACAGC
Gal2 Forward	GCTGTGCCTTTGGTGGTATT
Gal2 Reverse	ACCGAGACGACAATCGAAAG
Gal7 Forward	CCTTTGAATGCGACTGGTGATGAATTGAGTAAT
Gal7 Reverse	CAGCTTGTTCCGAAGTTAAATCTCTTTGAGGCT
Gal10 Forward	GGTGGTGCTGGATACATTGGTTCACACAC
Gal10 Reverse	CCTCATAGAAGGGAATGTGATGCTTGGTCAA
Rtg2 Forward	CGAGACTGAGGTCGTGTCGAGAAACTT
Rtg2 Reverse	GAACCTATGTCGACGATTCCACACAAG
Chromosome-V Forward	TAAGAGGTGATGGTGATAGGCGT
Chromosome-V Reverse	CCCTCGGGTCAAACACTACAC
Tef1 Forward	CGGTGAATTCGAAGCCGGTATCTCT
Tef1 Reverse	GCGTGTTCTCTGGTTTGACCATCCTTA
TEL06R Forward	GGCAAGGGTAAAAACCAGTGAGGCC
TEL06R Reverse	GAGTTCGGATCACTACACACGGAAATGG
HML Forward	TCCAGATTCCTGTTCCTTCC
HML Reverse	TTCCAGACGCTATCCTGTGA
HMR Forward	TAGAGTGTGGTCGTGGCGGAGGTTG
HMR Reverse	ACAAACATTGAGAACAAGAGCAAGA
rDNA Forward	GGACGGTGGCCATGGAA
rDNA Reverse	CATTCGGCCGGTGAGTTG
YAL035W-A Forward	AAAGAAGACGTGCCGCTTTA
YAL035W-A Reverse	CCAGACCGGCAACTTTGA
YAL035W-B Forward	AACGTTCAAGGTGGTGAAGC
YAL035W-B Reverse	CACCAATCTGTTGGGTGATG
YAL035W-C Forward	GCTGTGAAAACCGACCCTAC
YAL035W-C Reverse	TTGGACAGGTTGATGGTTGA
YLR340W-A Forward	CCATGGTTAGAAGAGCCATCA
YLR340W-A Reverse	TTCGAAGTCTGGCAAGTCG
YLR340W-B Forward	AACGGTCAAGTGTTCCCATC
YLR340W-B Reverse	AGCGGAAACGAAGTGAGAAA
YLR342W-A Forward	TGGTGGTCAGTATACCGCTTC
YLR342W-A Reverse	CGACGAATTTGGTTCTCCAT
YLR342W-B Forward	TGTGAATGGTCATTCGTTCC
YLR342W-B Reverse	GAGCACCAGCCCATTTTCT
YPR112C-A Forward	TTGATGACGAGATTCGCAAG
YPR112C-A Reverse	TGATGTGACTTGTGAGCTTGG
YPR112C-B Forward	CCAAAGGATTTGCATACGTTC
YPR112C-B Reverse	TCAACATAGGCGTTCACTGC
YPR112C-C Forward	TCAATGGGGTTTGGTTTTGT
YPR112C-C Reverse	TGCAGCTATCACGGCATTAG
YPL017C-A Forward	AAGCACGATGGAGGATTTTG
YPL017C-A Reverse	CAACCAATTACCAGGACATCG
YPL017C-A Forward	CGTTGAATCTCAAAGCGAGA
YPL017C-A Reverse	GGCAGAAGCCAACTCATTGT

Table S1. Sequence of primers used in both ChIP and RT-PCR assays.

Supplementary Methods

Yeast strains and plasmids

Wild-type (BY4741, Open Biosystems): *MAT*a, *ura3*Δ*0*, *leu2*Δ*0*, *his3*Δ*1*, *met15*Δ*0*; JHY6: *MAT*a, *ura3-52*, *lys2-801*, *ade2-101*, *trp*1–289, *his3*Δ*1*, *leu2*–3,112, Δ*hhf2-hht2*, Δ*hhf1-hht1*, pMS333[URA3-*HHT2-HHF2*]; UCC1369: *MAT*a, *ade2::hisG*, *his3*Δ*200*, *leu2*Δ*0*, *lys2*Δ*0*, *met15*Δ*0*, *trp1*Δ*63*, *ura3*Δ*0*, *adh4::URA3-TEL-VIIL*, *ADE2-TEL-VR*, *hhf2-hht2::MET15*, *hhf1-hht1::LEU2*, pMP9; UCC1188: *MAT*α, *leu2*Δ*1*, *lys2-801, trp1, ura3*, *RDN1::URA3*, *hhf2-hht2::HIS3*, *hhf1-hht1::LEU2*, pMP9; UCC7262: *MATa*, *ade2*, *his3*, *leu2*, *lys2*, *ura3*, *ADE2-TEL-VR*, *hhf2-hht2::MET15*, *hhf1-hht1::LEU2*, *hmra::URA3*, pMP9; UCC7266: *MATa*, *ade2*, *his3*, *leu2*, *lys2*, *ura3*, *ADE2-TEL-VR*, *hhf2-hht2::MET15*, *hhf1-hht1::LEU2*, *hml::URA3*, pMP9. C13 ABYS-86: *Mat*α *ura3 leu2-3 his3-112 pra1-1 prb1-1 prc1-1 cps1-3*. Mutations of H3R2 and H3K4 were introduced into the *HHT2* gene in plasmid pMR206 (*TRP1-HHT2-HHF2*) using the QuickChange (Stratagene) site directed mutagenesis kit and verified by sequencing. Mutant and wild-type control plasmids were introduced into strain JHY6 to make the strains JHY6-H3WT, JHY6-H3R2A, JHY6-H3R2Q, and JHY6H3K4A. Derivatives of pMR206 were also used to replace plasmid pMP9 in strains UCC1369, UCC1188, UCC7262, and UCC7266 to make the following strains: UCC1369-H3WT, UCC1369-H3R2A, UCC1369-H3R2Q, UCC1188-H3WT, UCC1188-H3R2A, UCC1188-H3R2Q, UCC7262-H3WT, UCC7262-H3R2A, UCC7262-H3R2Q, UCC7266-H3WT, UCC7266-H3R2A, and UCC7266-H3R2Q**.** The strain JHY6-H3WT was also used in PCR-mediated

disruption to create strains JHY6-*set1C1068A* (*set1C1068A::HIS3*) and JHY6-*Sir2Δ* (*sir2::KAN)*. The strain JHY6 was a gift from S. Berger, strain C13 ABYS-86 was a gift from D. H. Wolf and the strains UCC1369, UCC1188, UCC7262, and UCC7266 were gifts from Daniel Gottschling.

Western blotting

Yeast cells were grown to an A_{600} OD of 0.8 in a 30°C shaker. Total yeast extracts were prepared by first resuspending cell pellets in 10X volume of SDS loading buffer (3% 2-mercaptoethanol, 3% SDS, 0.1% Bromophenol Blue, 10% glycerol) and then the samples were alternately boiled and chilled three times to rupture cell membranes. Core calf thymus histones were obtained from Roche Applied Sciences. Acid extraction was used to prepare histones from H3K293 and C3H10T1/2 cells which were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (FBS) in a 5% CO2 incubator at 37°C. Western blotting was performed using standard procedures. The nitrocellulose membranes (Whatman, Inc.) were blocked overnight in BSA buffer (Tris-buffered saline, 0.01% Tween 20, and 5% bovine serum albumin) at 4°C. The antibodies used included anti-H3 (ab1791, Abcam plc), anti-H3R2me2a (ab8046, Abcam plc), anti-H3K4me1 (ab8895, Abcam plc), anti-H3K4me2 (ab7766, Abcam plc), anti-H3K4me3 (ab8580, Abcam plc), anti-H3K36me3 (ab9050, Abcam plc), and anti-H3K79me3 (ab2621, Abcam plc).

Antibody characterization

Dot-blotting was performed as previously described[1]. Briefly, 2 ul of peptide dilutions were spotted onto PVDF membranes (RPN2020F, Amersham) and let to air-dry. The

membranes were then blocked for 2 hrs with BSA buffer (Tris-buffered saline, 0.01% Tween 20, and 5% bovine serum albumin) at room temperature prior to performing a standard immunoblot analysis. For peptide competitions, 500 ng of isolated yeast histones, prepared as described below, were separated on a 15% SDS-PAGE. A standard western blotting procedure was followed except that the antibodies were incubated with the peptides (0.5ug/ml) for 30 min at room temperature before probing the membranes. The antibodies used included anti-H3 (ab1791, Abcam Plc), anti-H3R2me2a (ab8046, Abcam Plc), and anti-H3K4me3 (ab8580, Abcam Plc).

The specificity of the anti-H3R2me2a (ab8046, Abcam Plc) antibody is further validated by the fact that its reactivity towards mammalian histones is sensitive to the deletion of CARM1/PRMT4[2]. This shows that the antibody is specific to methylated H3R2 since deletion of the enzyme (CARM1) that catalyzes this modification *in vitro*[3] affects the antibody.

Yeast histone/chromatin preparation

Cells were grown in rich media (YPD) to A_{600} OD of 0.7; Washed once on PBS and resuspended in Spheroplasting buffer (0.6M sorbitol, 20mM Potassium Phosphate Ph=7, 5mg of zymolase 20T per gram of cell). After 30 min incubation, at room temperature with gentle shaking, cells were centrifuged at 3500 rpm for 10 min and washed once with (0.6M sorbitol, 20mM Potassium Phosphate Ph=7). The cell pellet was then resuspended in 4ml Ficoll buffer (18% Ficoll, 20mM PIPES pH=6.3, 0.5 mM CaCl2, 1Mm DTT, 1Mm EDTA) per gram of cell, disrupted in a manual douncer by 5-6 strokes and centrifuged at 6000rpm for 10 min. The supernatant was discarded. Pelleted cells were resuspended in 10 ml Extraction buffer (10mM HEPES pH=7.5, 1mM EDTA, 0.5 M

NaCl, 0.5% NP-40), incubated on ice for 20 min and centrifuged at 14000 rpm for 10 min. The supernatant was discarded. The washes with Extraction buffer were repeated until the pellet started to loose opacity. Then, the pellet was resuspended in 2ml Tris-HCl pH=8, 10% glycerol and dialysed O/N against the same buffer. Finally the sample was aliquoted and stored at –80°C.

Chromatin Immunoprecipitation (ChIP)

Wild-type (BY4741), JHY6-H3WT, and JHY6-H3R2A strains were grown to an A_{600} OD of ~0.9 in a 30°C shaker. For the galactose induction experiments BY4741 cells were cultured overnight in complete medium (YPD) containing 2% glucose. The next morning the cells were split into two YPD samples, one containing 2% glucose and the other containing 2% galactose, and grown at 30°C for another 24 hours. Cell pellets from both conditions collected at A_{600} OD of ~0.9 were used for ChIP experiments and reverse-transcription PCR (see below). Formaldehyde cross-linking and chromatin immunoprecipitations were performed as described before[4] with the following exceptions: the immunocomplexes were eluted from the Sepharose beads (17-5280-01, Amersham) using a total of 200 ul elution buffer (100 mM Sodium bicarbonate, 1% SDS) and RNAse (11119915001, Roche) treatment was performed during reversing of the cross-links at 65°C for 5 hours. After reversing the cross-links, each individual ChIP sample was purified using the Qiaquick PCR purification kit (QIAGEN) and DNA was eluted from the columns with 50 ul of EB buffer. The following antibodies were used for immunoprecipitation: 3 ul/IP of anti-H3 (ab1791, Abcam Plc), 3 ul/IP of anti-H3K4me3

(ab8580, Abcam Plc), 3 ul/IP of anti-H3K4me2 (ab7766, Abcam plc), 3 ul/IP of anti-H3K4me1 (ab8895, Abcam plc), 3 ul/IP of anti-H3R2me2a (ab8046, Abcam Plc), 25 ul/IP of anti-Rap1 (sc-6663, Santa Cruz Biotechnology), 25 ul/IP of anti-Sir2 (sc-6667, Santa Cruz Biotechnology), and 2 ul/IP of rabbit anti-mouse IgG (5180-2104, Biogenesis, used as a negative control).

ChIP-on-chip Data Analysis

Primer positions were mapped using exonerate[5], and this information, along with an S. cerevisiae gff annotation file, was read into an R data structure using scripts from the Bioconductor package "tilingArray"[6]. Raw NimbleGen output files were used for the analysis of the data. Two channels were used for each array; for each channel, the bi-weight mean for all data points was calculated and used to scale the data. The corrected data was then used to calculate a ratio of immunoprecipitated to control DNA for each spot. Finally, a ratio of these data to similarly corrected histone H3 data was used to normalise the data to nucleosome occupancy levels. Composite average profiles were created in a similar manner as described previously[7]. The ends of ORFs were defined at fixed points corresponding to the positions of translational start and stop sites. The length of the ORF was then sub-divided into 40 regions of equal length, and the middle of each probe was assigned according to its nearest relative bin position. Probes 800 bp before start sites and 800 bp after stop sites were similarly assigned following sub-divisions into 20 bins for both regions.

Real-time PCR

Real time PCR analysis was performed on an ABI PRISM 7000 sequence detection system using SYBR Green (Applied Biosystems) as described previously[8]. Briefly, standard curves for each primer set were calculated from amplification of wild-type genomic DNA diluted in 1:10, 1:100. 1:1000, 1:10000, and 1:100000. After each run, a dissociation curve was performed to ensure that no primer dimmers contaminated the quantification and that the product had the expected melting temperature. Each PCR reaction was performed in duplicate and the analysis was repeated twice from independent ChIP experiments. A signal intensity value for each sample was calculated from the average of the 2 experiments. Relative fluorescent intensities for the ChIP experiments were calculated as follows: $[(\text{Ab signal}^{X}/\text{Ab signal}^{Y})-(\text{IgG signal}^{X}/\text{IgG signal}^{Y})] / [(\text{H3 signal}^{X}/\text{H3 signal}^{Y})-(\text{IgG signal}^{X}/\text{IgG signal}^{Y})]$, where Ab is the antibody of interest, IgG is the negative control antibody, H3 is the histone H3 antibody, X is the locus of interest and Y is the intergenic region on Chromosome-V which was used as an internal background control. The sequences of the primers used for PCR analysis can be found in Supplementary Table 1.

Quantitative reverse-transcription PCR

Total yeast RNA was prepared from 3 x 10^{7} cells of each indicated growth condition using the RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol. To ensure complete removal of contaminating DNA from the RNA preparations, the TURBO DNA-free kit (Ambion) was used. First strand cDNA synthesis was achieved using SuperScript II reverse transcriptase (cat.18080, Invitrogen) with a primer cocktail, containing 50 uM oligo(dT) (Ambion) and 50 ng random hexamers (Invitrogen), as

described in the manufacturer's instructions. The cDNA samples were then used as templates for real-time PCR (see above).

Array Design

The S. cerevisiae genome tiling array contained a total of 379,521 50-mer oligonucleotides, positioned at every 14 basepairs (bp) throughout the yeast genome representing both DNA strands. The design included random GC probes as controls.

Peptide synthesis

Peptides corresponding to amino acids 1-17 of histone H3 were synthesized in house using the Fmoc strategy on a solid-phase peptide synthesizer (Intavis, Germany). Peptides were synthesized on amide resin as 20-mers containing the first 17 amino acids of the histone H3 N-terminal tail followed by a double glycine spacer and a biotinylated lysine residue on the C-terminus. Unmodified amino acids as well as methylated arginine and lysine derivatives used for the synthesis were purchased from Novagen or Bachem. Following synthesis, peptides were cleaved from the resin using TFA, precipitated using ether, and air-dried. The identity and quality of the peptides was checked by mass spectrometry. Histone H3 N-terminal peptides corresponding to amino acids 1-8 and 121 were obtained from Almac Sciences (Edinburgh, Scotland). Peptides were synthesized with at least 90% purity and were resuspended in 10mM Hepes pH 7.5 containing 0.005% Igepal (Sigma).

In vitro methyltransferase and peptide pull-down assays

Set1-complex purification and methyltransferase assay was performed as described[9]. PtA-Set1 was expressed and the Set1-complex was purified from the C13 ABYS-86 strain. Approximately 1μg of Set1-complex was incubated in 50mM Tris-HCl pH 8.5, 20mM KCl, 10mM $MgCl_2$, 10mM β-mercaptoethanol, 0.05mM DTT, 250mM sucrose, 0.2% dodecyl-β-D-maltoside buffer, with 2 μl of ^{3}H-SAM (^{3}H-S-adenosyl-L-methionine, TRK865, Amersham Pharmacia) and ~2μg of peptides corresponding to aa1-17 of histone H3 as substrate, for 1h at 30 $^{\circ}$C. The reactions were resolved in 17% Tricine gels, transferred by Western blot onto PVDF membrane and exposed to KodaK Biomax MS autoradiogram films for 12h. Peptides in the reaction were also visualized by Coomasie staining of the PVDF membrane, cut off and subjected to Edman degradation (PNAC facility, University of Cambridge, www.bioc.cam.ac.uk/pnac/proteinsequencing.html) followed by quantification in a scintillation counter (Beckman Coulter, LS6500). The molar concentration of each peptide was determined according to the amount of the first alanine present in each peptide. The radioactive counts released from each peptide were then normalized to the molar concentration of each peptide.

The Spp1 PHD-domain, covering amino acids 20-76, was amplified from genomic yeast DNA and cloned into vector pGEX-5X-1. Binding assays using 3 ug GST-Spp1^{PHD} and 20 ug of N-terminal H3 peptides were performed as previously described[10] with minor modifications. The binding was performed at 4°C overnight in PDB-150 buffer (50 mM TRIS [pH8.0], 150 mM NaCl, 10 uM $ZnCl_2$, 5 mM EDTA, 0.5% Igepal, protease inhibitor cocktail [Roche Applied Sciences]), followed by washing in PDB-150.

Supplementary References

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