Genetic screen for suppressors of increased silencing in rpd3 mutants in Saccharomyces cerevisiae identifies a potential role for H3K4 methylation

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Abstract

Several studies have identified the paradoxical phenotype of increased heterochromatic gene silencing at specific loci that results from deletion or mutation of the histone deacetylase (HDAC) gene RPD3. To further understand this phenomenon, we conducted a genetic screen for suppressors of this extended silencing phenotype at the HMR locus in Saccharomyces cerevisiae. Most of the mutations that suppressed extended HMR silencing in rpd3 mutants without completely abolishing silencing were identified in the histone H3 lysine 4 methylation (H3K4me) pathway, specifically in SET1, BRE1, and BRE2. These second-site mutations retained normal HMR silencing, therefore, appear to be specific for the rpd3S extended silencing phenotype. As an initial assessment of the role of H3K4 methylation in extended silencing, we rule out some of the known mechanisms of Set1p/H3K4me mediated gene repression by SET1, BRE1, and BRE2. While first discovered in the 1960s (Allfrey et al. 1964), histone post-translational modifications became a major focus of chromatin research with the identification of the first histone modifying enzymes and acetyltransferase (Brownell et al. 1995) and the RPD3 ortholog histone deacetylases (HDACs; Taunton et al. 1996). Since then, an explosion of discoveries has been made identifying epigenetic writers, erasers, and readers, with demonstration of their roles in numerous chromatin processes including gene transcription and silencing, and DNA replication, repair, and recombination (Allis and Jenuwein 2016).

Keywords: silencing; RPD3; BRE1; BRE2; SET1; COMPASS; histone modifications; chromatin; Saccharomyces cerevisiae

Introduction

While first discovered in the 1960s (Allfrey et al. 1964), histone post-translational modifications became a major focus of chromatin research with the identification of the first histone modifying writers and erasers, the tetrahymena GCN5 ortholog acetyltransferase (Brownell et al. 1996) and the RPD3 ortholog histone deacetylases (HDACs; Taunton et al. 1996). Since then, an explosion of discoveries has been made identifying epigenetic writers, erasers, and readers, with demonstration of their roles in numerous chromatin processes including gene transcription and silencing, and DNA replication, repair, and recombination (Allis and Jenuwein 2016).

While early results suggested binary correlation of histone acetylation with active gene expression and deacetylation with repression (Hebbs et al. 1988; Johnson et al. 1990; Park and Szostak 1990), several studies in Saccharomyces cerevisiae have shown that loss of function of the HDAC RPD3 paradoxically leads to suppression of defective silencing or an increase in heterochromatic silencing at all three SIR (silent information regulator) dependent loci in yeast (Sussel et al. 1995; Rundlett et al. 1996; Vannier et al. 1996; Smith et al. 1999; Sun and Hampsey 1999). Increased silencing in rpd3 mutants has also been observed in metazoans at telomeres in Drosophila (De Rubertis et al. 1996).

Previous work from our lab showed that mutation of S. cerevisiae RPD3 led to the spread of silencing beyond the normal tDNA heterochromatin barrier element at the HMR locus resulting in silencing of downstream reporter genes (Jambunathan et al. 2005), and more recent studies have shown a possibly related restoration of defective yeast SIR-mediated silencing by second-site point mutations in RPD3 (Thurtle-Schmidt et al. 2016). Both this study and our unpublished results suggest that this increased silencing is due to loss of function of the RPD3L versus the RPD3S complex. While some explanations put forth for this increased heterochromatic silencing include boundary/barrier activity of Rpd3p (Ehrentraut et al. 2010) or redistribution of Sir proteins in rpd3 mutants (Zhou et al. 2009), it is not entirely clear how loss of HDAC activity results in an increase of heterochromatic silencing at specific loci.

To address this question in more detail and identify additional potential effectors of this rpd3 phenotype, we sought to isolate suppressor mutants that reverse the increased spreading of silencing at HMR in rpd3 mutant strains without completely abolishing silencing as would occur with second-site mutations in the SIR genes. MATα strains containing the rpd3Δ::KanMX allele and
an HMR-ADE2 silencing reporter gene were subjected to transpo-
sion mutagenesis, and isolates that reversed silencing of the ec-
topic ADE2 gene but retained a mating phenotype indicative of
normal HMRs silencing were characterized. Of the
mutants identified, most were in the histone H3 lysine 4 (H3K4)
methylation pathway, with multiple independent hits in SET1,
BRE1, and BRE2. We also show results that known mediators of
H3K4 repression are not involved and that the RNA Polymerase
III complex remains actively bound at the HMR-tDNA in rpd3Δ
strains.

Materials and methods

Selected transposon mutant strains isolated in the screen that
were further characterized in this study are listed in Table 1. All
strains described in the figures and their genotypes are listed in
Table 2. Oligonucleotides used with brief descriptions are listed in
Table 3.

Parent HMR-ADE2 rpd3 reporter strains DDY3133 and DDY2973
(Table 2) were constructed by crossing DDY814 (Jambunathan et al.
2005) with rpd3Δ::KanMX strains. When grown in media contain-
ing suboptimal levels of exogenous adenine, yeast colonies deficient in
adenine biosynthesis accumulate a red pigment derived from the
Ade2p substrate (Roman 1956). HMR-ADE2 rpd3Δ strains grow as
red colonies on agar minimal media (Yeast nitrogen base, U.S.
Biologica lY2025) containing 45 μg/ml adenine (15% of the normal
300 μg/ml level used) due to increased spreading of silencing that
represses the ectopic ADE2 allele at HMR. This sufficient but sub-
optimal level of adenine is critical to the colony color assay, as
lower levels (less than 15 μg/ml) of adenine activate the ADE2 pro-
mitter to overcome silencing and higher levels (over 90 μg/ml) feed-
back inhibit the pathway, both leading to white colony growth.
Colonies typically were grown for 3 days at 30°C, then plates were
held at 4°C for 3-4 days to obtain optimum pigmentation color prior
to photographing on a dissecting microscope. Slow-growing
rpd3 hist1 hsl2 mutants were grown for 5 days at 25°C to obtain op-
timum pigmentation.

The yeast transposon mutagenized library was obtained from
Mike Snyder (Stanford University), and mutagenesis was per-
formed as described (Burns et al. 1994; Ross-Macdonald et al.
1997, 1999a, 1999b). Transformations were plated on minimal
media lacking leucine and containing 15% adenine (minus leu-
15% ade), and rare white Leu+ colonies were picked and
restreaked on minus leu-15% ade plates to verify the stability of
the phenotype. Identification of mutagenized genes was per-
formed using the vectorette PCR method as described (Ross-
Macdonald et al. 1999b). Primary isolates were verified as single
transposon insertions by backcrossing with DDY814 to verify 2:2
segregation of the LEU2 marked transposon insertion, and
Table 1 List of primary transposon insertion mutant strains
characterized in this paper

| Isolate | Gene | Location | Insertion site | Strain number |
|---------|------|----------|---------------|---------------|
| L8      | BRE2 | Chr XII  | 176,534       | DDY5690       |
| L11     | NGG1 | Chr IV   | 814,931       | DDY5624       |
| L13     | SET1 | Chr VIII | 348,435       | DDY5625       |
| M8      | BRE2 | Chr XII  | 176,241       | DDY5626       |
| A4      | BRE1 | Chr IV   | 324,598       | DDY5642       |
| A5      | SET1 | Chr VIII | 347,931       | DDY5691       |
| A8      | BRE2 | Chr XII  | 175,842       | DDY5692       |
| A35     | SET1 | Chr VIII | 348,615       | DDY5643       |
| L31     | SIR4 | Chr IV   | 920,020       | DDY5640       |
| M4      | SIR4 | Chr IV   | 920,651       | DDY5641       |

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formed using the vectorette PCR method as described (Ross-
Macdonald et al. 1999b). Primary isolates were verified as single
transposon insertions by backcrossing with DDY814 to verify 2:2
segregation of the LEU2 marked transposon insertion, and
complete cosegregation of Leu+ Kan+ markers with white and
Leu– Kan+ with red colony phenotypes.

Direct deletion of SET1, BRE1, and BRE2 was performed by stan-
dard yeast genetics procedures by amplifying the LEU2 gene from
plasmid pRS405 (Sikorski and Hieter 1989) with primers contain-
ing homology to the immediate upstream and downstream
regions of each gene (listed and described in Table 3). After multi-
ple attempts at directly deleting these genes in the rpd3Δ back-
ground yielded only one deletion strain, PCR products were
transformed into strain DDY2973 containing URA3-marked plas-
mid pDD1340 expressing wild-type RPD3. We speculated that our
haploid rpd3Δ strains were mitotic recombination deficient, which
has been demonstrated for homozygous rpd3Δ diploid
yeast (Dora et al. 1999). We estimated an approximately 10-fold
increase in recombination efficiency in RPD3 plasmid-containing
transformations. Leu+ transformants were selected on minus
leu-15% ade plates, and white colonies were restreaked to 5-FOA
(5-fluoroorotic acid) plates to isolate Leu+ colonies that lost the
URA3-marked RPD3 plasmid. These 5-FOAΔ rpd3Δ isolates were
confirmed for proper SET1, BRE1, or BRE2 gene deletion by PCR on
each end (confirmation oligos listed in Table 3), then restreaked
on minus leu-15% ade plates to reconfirm the white colony phe-
notype. Plasmid pDD1340 was constructed by PCR amplification
of RPD3 from ~240 bp upstream of the ORF to ~350bp down-
stream with oligos DDO-2155 and DDO-2156 and Q5 polymerase
(New England Biolabs), using an RPD3-containing genomic library
plasmid (with a 12 kilobase insert) as template. The PCR product
was digested with Xho I and Not I then cloned into URA3 vector
pRS416 (Sikorski and Hieter 1989) also digested with Xho I and Not
I. Plasmid isolates were confirmed by transformation into
strain DDY2973 and complementation of the red colony phenotype
back to white.

Mating assays were performed as previously described (Donze
and Kamakaka 2001; Jambunathan et al. 2005). To generate the strains
for the mating assay in Figure 5, strain DDY1344 (Jambunathan et al.
2005) was mated to DDY3828, sporulated, and tetrads dissected. Since the HMR-barrier locus is not marked and both bre2Δ and Tn rpd3 alleles were marked with LEU2, gen-
types were verified by both LEU2 segregation patterns and PCR. HMRΔS-barrier loci were confirmed by PCR with oligonucleotides
DDO-681 and DDO-951, and bre2α::LEU2 alleles verified by PCR
with DDO-928+DDO-199. Double rpd3 bre2 strains were identified by 2:2 segregation of leucine prototrophy and bre2Δ confirmed
by PCR. Deletion of only RPD3 was confirmed as Leu+ isolates that
were negative for the bre2 deletion.

Northern blot analysis of the 19 base pair marked HMR-tRNA
was performed as described (Braglia 2007). Chromatin immuno-
precipitation of FLAG-tagged BRF1 was also performed as previ-
ously described (Rusché et al. 2002; Simms et al. 2008).

All yeast strains and plasmids described in this study are
available on request. The authors affirm that all data necessary
for confirming the conclusions of this article are represented fully
within the article, its tables and figures and the cited literature.

Screen design

To identify suppressors that reverse rpd3Δ mediated increased sil-
cencing through the tDNA barrier at the HMR domain, we
employed a colony color assay that indicates spreading of silenc-
ing beyond the tDNA at the

| Isolate | Gene | Location | Insertion site | Strain number |
|---------|------|----------|---------------|---------------|
| L8      | BRE2 | Chr XII  | 176,534       | DDY5690       |
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| L31     | SIR4 | Chr IV   | 920,020       | DDY5640       |
| M4      | SIR4 | Chr IV   | 920,651       | DDY5641       |
and white colony growth (DDY814). We previously demonstrated that rpd3 mutants result in spread of silencing beyond the boundary, resulting in repression of ADE2 and growth as red colonies on 15% ade media (Jambunathan et al. 2005; Simms et al. 2008). Strains D DY3133 and D DY2973 (Table 2) were mutagenized using LEU2-marked transposon mutagenesis and plated on minus leu-15% ade glucose minimal agar. White Leu+ colonies were identified as containing transposon insertions in genes that are potential suppressors of extended rpd3A silencing.

Leu+ transposon insertion isolates were verified by several tests before identification of the transposon insertions, which led to only a fraction of the primary isolates being pursued. Initially, each white colony was restreaked on minus leu-15% ade plates to verify the maintenance of phenotype, and multiple isolates initially appearing as white reverted back to red growth and were not pursued. Isolates that displayed a consistent white to very light pink colony phenotype were tested for mating against a MATa tester strain (DDY20, as complete loss of silencing (e.g., transposon insertions into one of the SIR genes) results in a

Table 2 Saccharomyces cerevisiae strains used and generated in this study

| Name | Genotype | Source |
|------|----------|--------|
| D DY20 | MATa his4 | J. Rine |
| D DY277 | MATa his3 leu2 lys2A trp1 ura3 HMRA1+ + tDNA Barrier at a2 | Donze Lab |
| D DY282 | MATa his3 leu2 lys2A trp1 ura3 HMRA1 | Donze Lab |
| D DY814 | MATa ade2 his3 leu2 trp1 ura3 HMR-ADE2 | Donze Lab |
| D DY465 | MATa his3 leu2 lys2A trp1 ura3 HMR RNAA | Donze Lab |
| D DY466 | MATa his3 leu2 lys2A trp1 ura3 HMR RNAA | Donze Lab |
| D DY1344 | MATa his3 leu2 lys2A trp1 ura3 HMR-ADE2 trp1 HMR RNAA | Donze Lab |
| D DY2973 | MATa ade2 his3 leu2 trp1 ura3 HMR-ADE2 rpd3A::KanMX | Donze Lab |
| D DY3133 | MATa ade2 his3 leu2 lys2A trp1 ura3 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | Donze Lab |
| D DY3396 | MATa his3 leu2 trp1 ura3 sir2::TRP1 rpd3A::KanMX HMR RNAA +19 | Donze Lab |
| D DY3398 | MATa his3 leu2 lys2A trp1 ura3 sir2::TRP1 HMR RNAA +19 | Donze Lab |
| D DY3400 | MATa his3 leu2 trp1 ura3 rpd3A::KanMX HMR RNAA +19 | Donze Lab |
| D DY3401 | MATa his3 leu2 lys2A trp1 ura3 HMR RNAA +19 | Donze Lab |
| D DY3402 | MATa his3 leu2 trp1 ura3 rpd3A::KanMX HMR RNAA +19 | Donze Lab |
| D DY3403 | MATa his3 leu2 trp1 ura3 rpd3A::KanMX HMR RNAA +19 | Donze Lab |
| D DY3684 | MATa ade2 his3 leu2 trp1 ura3 HMR RNAA +19 HMR-ADE2 BRP1-3XFLAG KanMX | Donze Lab |
| D DY3688 | MATa ade2 his3 trp1 ura3 HMR RNAA +19 HMR-ADE2 BRP1-3XFLAG KanMX rpd3A::URA3 | Donze Lab |
| D DY3828 | MATa ade2 his3 leu2 trp1 ura3 bre2A::LEU2 | Donze Lab |
| D DY5605 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | Donze Lab |
| D DY5609 | MATa ade2 his3 leu2 lys2A trp1 ura3 VII-L-URA3-TEL prr1A::TRP1 HMR-ADE2 rpd3A::KanMX | Donze Lab |
| D DY5657 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 rpd3A::Kan hst1A::LEU2 | This study |
| D DY5675 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 rpd3A::Kan hst1A::LEU2 bre2A::TRP1 | This study |
| D DY5625 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | This study |
| D DY5626 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | This study |
| D DY5632 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | This study |
| D DY5635 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | This study |
| D DY5636 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 rpd3A::KanMX | This study |
| D DY5639 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | This study |
| D DY5640 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | This study |
| D DY5641 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | This study |
| D DY5642 | MATa ade2 his3 leu2 trp1 ura3 HMR-ADE2 rpd3A::KanMX Tn:LEU2 bre2 | This study |
| D DY5664 | MATa ade2 his3 leu2 trp1 ura3 HMR-ADE2 rpd3A::KanMX Tn:LEU2 bre2 | This study |
| D DY5665 | MATa ade2 his3 leu2 trp1 ura3 HMR-ADE2 rpd3A::KanMX | This study |
| D DY5667 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | This study |
| D DY5676 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMR-ADE2 VII-L-URA3-TEL prr1A::TRP1 rpd3A::KanMX | This study |
| D DY5681 | MATa his3 leu2 trp1 ura3 HMRA1 + tDNA Barrier at a2 Tn:LEU2 rpd3 | This study |
| D DY5683 | MATa ade2 his3 leu2 lys2A trp1 ura3 HMRA1 + tDNA Barrier at a2 Tn:LEU2 rpd3 bre2A::LEU2 | This study |
| D DY5689 | MATa ade2 his3 leu2 trp1 ura3 HMR-ADE2 rpd3A::KanMX bre2A::LEU2 | This study |
| D DY5700 | MATa ade2 his3 leu2 trp1 ura3 HMR-ADE2 rpd3A::KanMX bre2A::LEU2 | This study |
| D DY5701 | MATa ade2 his3 leu2 trp1 ura3 HMR-ADE2 rpd3A::KanMX bre2A::LEU2 | This study |

All strains are isogenic to S. cerevisiae W303-1A with the genotype ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100. Lysine auxotrophic W303 derivatives have a complete deletion of the LYS2 ORF (lys2A).
naming phenotype. Out of 71 primary white colony isolates, 18 were nonmating. Two randomly selected nonmating isolates were found to have independent transposon insertions within the SIR4 gene (DDY5640 and DDY5641). Transposon insertion sites were determined by the anchor bubble ligation-vectorette PCR method (Ross-Macdonald et al. 1999b), and insertion coordinates in Table 1 correspond to the current update of the Saccharomyces Genome Database (Cherry et al. 2012). Multiple isolates did not yield a PCR product and the insertion site could not be identified.

**Results**

**Transposon mutagenesis screen for suppressors of rpd3Δ extended silencing yields hits predominately in the histone H3K4 methylation pathway**

Table 1 lists the identified Leu+ transposon mutagenized strains that lost the rpd3Δ extended silencing phenotype and grew as white colonies and retained a normal mating phenotype (therefore, did not lose normal SIR-mediated silencing), plus two randomly selected nonmating isolates. Interestingly, most of the identified mutants were in the histone H3K4 methylation pathway, including multiple hits in each BRE1, BRE2, and SET1. An additional insertion in NGG1, which codes for a component of the SAGA coactivator complex resulted in a very light pink and variegated phenotype, suggesting a partial suppression of increased rpd3Δ mediated silencing. However, this insertion also resulted in a slow growth phenotype, and it is not clear whether slow growth affects the accumulation of the pigment in this reporter assay. Figure 2 shows restreaked colonies grown on minus leu-15% ade plates derived from selected primary isolated mutants.

As described above, two randomly selected nonmating isolates were found to have transposon insertions within the SIR4 gene. Identification of white colony isolates containing insertions within the SIR genes was expected, as they completely abolish HMR silencing. Additional expected insertions were identified in adenine biosynthesis pathway genes, including ADE3, ADE4, ADE5,7, and ADE6, as second adenine pathway mutations are known to suppress the ade2 red colony phenotype (Roman 1956). To verify single transposon insertions and cosegregation of the white colony phenotype, selected primary isolates were backcrossed to strain DDY814 which contains the HMR-ADE2 marker gene. For each of the NGG1, SET1, BRE1, and BRE2 insertions, tetrad analysis verified that the LEU2 marked transposon segregated 2:2 verifying single insertions (unpublished data).

Individual haploid strains derived from these backcrosses were consistent with the suppression phenotype observed in the primary isolates: all haploid segregants containing only the

Table 3 Oligonucleotides used in this study

| Oligo #  | Sequence | Description                          |
|----------|----------|--------------------------------------|
| DDO-045  | CGCCAGGTTTTTCCAGCTGAC              | M13 -47, anchor bubble PCR and sequencing |
| DDO-046  | GAGGAGAGACGCTGCTGTAAGGAGG         | Anchor bubble adaptor reverse          |
| DDO-047  | GACTCTCCCCCTCGAATCAGGAGGAAGAT    | Anchor bubble adaptor forward          |
| DDO-048  | CGAATCGTACGGCTGTAAGGAGAATCGCT    | Anchor bubble PCR                      |
| DDO-198  | GCAGCTCTCAGATGATTATCCTGC         | pRS universal RC, upstream             |
| DDO-199  | CGCCAGCAGATGGCTAAGGAAGAAGG       | pRS universal RC, downstream           |
| DDO-2155 | TCAAGGAGGGTCTCCAGAGATATCAACTCAGAGCTATAG    | RPD3 clone XOHI upstream               |
| DDO-2156 | TCAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | RPD3 clone NOTI downstream             |
| DDO-2167 | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | SET1 pRS delete top                   |
| DDO-2168 | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | SET1 pRS delete bottom               |
| DDO-2169 | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | SET1 upstream check 326 BP           |
| DDO-2170 | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | SET1 downstream check 312 BP         |
| DDO-925  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | BRE1 pRS KO upstream                  |
| DDO-926  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | BRE1 pRS KO downstream                |
| DDO-2183 | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | BRE2 pRS KO upstream                 |
| DDO-928  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | BRE2 upstream check 425 BP           |
| DDO-929  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | BRE2 KO downstream check 390 BP      |
| DDO-681  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | HMR a2                                |
| DDO-951  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | HMR at tDNA                             |
| DDO-59   | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | Chromosome III tRNA Brf1 ChIP-A top    |
| DDO-60   | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | Chromosome III tRNA Brf1 ChIP-A bottom |
| DDO-482  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | HMR I silencer Brf1 ChIP-B top        |
| DDO-483  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | HMR I silencer Brf1 ChIP-B bottom     |
| DDO-484  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | HMR DNA Brf1 ChIP-C top               |
| DDO-485  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | HMR DNA Brf1 ChIP-C bottom            |
| DDO-1027 | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | ADE2 promoter Brf1 ChIP-D top         |
| DDO-1028 | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | ADE2 promoter Brf1 ChIP-D bottom      |
| DDO-767  | GTAATGATTTAAGGGCGCCAGCTATTCCCACCAGGCTG | +19 tRNA specific Northern probe      |
**Figure 1** Schematic diagram of screen to identify suppressors of increased silencing in rpd3 mutants. (A) MATα strains with a defective native ade2-1 allele and containing functional ADE2 integrated downstream of the HMR-tDNA barrier express ADE2 and grow as white colonies. (B) Deletion of RPD3 results in increased extended silencing through the barrier that represses ADE2 expression and results in red colonies on minimal media containing suboptimal amounts of adenine. (C) LEU2-marked transposon mutagenesis of rpd3Δ strains identified rare Leu+ white colonies on media lacking leucine and containing suboptimal adenine. (D) White colonies were screened in a mating assay to identify and exclude unwanted nonmating isolates that have completely lost silencing (e.g., those with transposon insertions in the SIR genes).

**Figure 2** Representative primary Leu+ isolates from mutagenesis of HMR-ADE2 rpd3Δ strains were restreaked multiple times for single colonies to verify stable propagation of the white colony phenotype. A single isolate containing the insertion in NGG1 gave rise to a variegated light pink phenotype. Additionally, isolates DDY5625 (set1) and DDY5692 (bre2) gave rise to rare very light pink colonies upon multiple restreaks of white colonies. DDY5640 and DDY5641 are two randomly selected Leu+ nonmating white colony isolates, and both contained different transposon insertions in the SIR4 gene consistent with a complete loss of silencing.

rpd3Δ::KanMX allele grew as red colonies on 15% ade media, and those with both rpd3Δ::KanMX and Tn LEU2 insertions in SET1, BRE1, or BRE2 grew as white colonies (Figure 3A) and retained their mating phenotype (examples shown in Figure 3B). These results confirm that loss of the H3K4 methylation pathway suppresses the increased silencing phenotype of rpd3 mutants.
without completely abolishing silencing at the HMR locus. One strain containing a transposon insertion into SIR4 was included in the assay as a nonmating control (DDY5641, Figure 3). As an additional verification of suppression, we directly deleted SET1, BRE1, or BRE2 in the parent strain DDY2973. White colonies from these knockout transformations were verified for the gene deletions by PCR on both ends of the LEU2 marker gene and were streaked on minus leu-15% ade plates. Figure 4 shows two independent isolates of each knockout, and each grew as white colonies on the indicator media. As a control, a randomly selected Leu+ isolate from bre1Δ transformations that was not integrated at BRE1 (likely recombined at the leu2-3,112 locus) maintained the red colony color of the parent strain.

As a final verification that mutations in the SET1/COMPASS pathway specifically suppress the increased rpd3Δ mediated silencing at HMR and was not simply affecting the ADE2 promoter of the reporter gene or the expression of other ADE pathway genes, we used a silencing-dependent mating assay that relies on the repression of a different promoter (schematically depicted in Figure 5A). Our original increased silencing rpd3Δ mutants were identified using this mating assay (Jambunathan et al. 2005) where the HMR-tDNA barrier is inserted between the HMR-a1 gene in a MATα background. Deletion of the HMR-I silencer and the barrier tDNA still allows repression of a1 by the HMR-E silencer alone in the parent MATα strain, as the cells exhibited a normal mating phenotype (Figure 5B, DDY282). In strain DDY277, blocking of silencing by the ectopically inserted tDNA barrier resulted in the expression of the a1 gene in the MATα background resulting in a nonmating phenotype. The increased spread of silencing in rpd3Δ mutants again repressed a1 and allowed mating (DDY5681), and this phenotype was reversed in a strain containing mutations in both rpd3 and bre2 which became nonmating (DDY5679).

The SET1 pathway has been demonstrated to repress target genes by promoting the recruitment of alternative HDACs, namely Hst3p, or a combination of Hst1p and Hos2p (Kim and Buratowski 2009; Jaiswal et al. 2017). We tested the potential role of these HDACs as possible mediators of extended silencing in rpd3Δ mutants by constructing deletions in these genes in our
the seven other copies of this tRNA Thr isoacceptor in HMR blot analysis to assay relative transcription levels of the rpd3 tagged Brf1p, which is part of the Pol III complex. Results in or around the tDNA (see Discussion and the nonmating phenotype. (Donze and Kamakaka 2001; Braglia et al. 2001; Bryk et al. 2002; Kim and Buratowski 2009).

Enhancement of Sir-protein mediated silencing by loss of RPD3 function is an intriguing paradox, made more interesting by the numerous studies that uncovered this phenomenon affecting all three Sir-protein repressed domains in S. cerevisiae as described in the introduction. To identify genes that might be misregulated in rpd3 mutants or otherwise involved in the extended silencing phenotype, we conducted a suppressor screen using transposon mutagenesis. Interestingly, most of the hits were in the histone H3K4 methylation pathway in SET1, BRE1, and BRE2. It is of note that we initially began this screen using a more cumbersome ultraviolet light induced mutagenesis procedure to isolate white colonies, followed by yeast plasmid library complementation of individual strains back to the red phenotype. Before switching to the transposon mutagenesis procedure, we isolated complementing plasmids containing BRE1 and BRE2 (R. A. Kleinschmidt, PhD dissertation, http://digitalcommons.lsu.edu.libezp.lib.lsu.edu/do/search/?q=etd-07052011-094524/), consistent with our findings reported here.

COMPASS (Complex Proteins Associated with Set1) is a conserved multiprotein complex recruited to chromatin to methylate H3K4 (and possibly other substrates) and contains both Set1p and Bre2p (Miller et al. 2001, Cenik and Shilatifard 2021). SET1 encodes the sole H3K4 methyltransferase activity in S. cerevisiae which is responsible for mono-, di- and trimethylation of this residue. BRE2 encodes a key structural factor within this complex that is also required for methylation (South et al. 2010; Hsu et al. 2018, Qu et al. 2018). Recruitment of COMPASS and subsequent H3K4 methylation requires prior histone H2B ubiquitylation mediated by the Bre1p E3-ubiquitin ligase complexed with the Rad6p ubiquitin-conjugating enzyme (Dover et al. 2002; Sun and Allis 2002). While initially thought to promote transcriptionally active chromatin structures as acetylation due to its prevalence near active promoters, H3K4 methylation was also found to be associated with repression of a subset of yeast genes (Briggs et al. 2001; Bryk et al. 2002; Kim and Buratowski 2009). Interestingly, although the mutualization procedure was calculated to be saturating, we did not find transposon insertions in RAD6, or in genes involved in Dot1p methylation of H3K79 which also requires H2B ubiquitylation (Briggs et al. 2002).

We also looked at several possible mechanisms for the formation and reversal of rpd3 mediated extended silencing at HMR by mutations that affect the SET1 pathway. Thurtle-Schmidt et al. (2016) also identified rpd3 mutations as suppressors of defective silencing in a catalytically inactive sir2N345A mutant. In that study, restored silencing was mediated by the Sir2 related Sirtuin HDAC Hst3p, as rpd3 hst3 mutants did not suppress the sir2 mutation. We deleted HST3 in our HMR-ADE2 rpd3 strains, but the resulting strains maintained the red colony phenotype (Figure 6), ruling out a role for H3K4me targeting of Hst3p in our system. One difference here (and possibly in other studies) might be that in the Thurtle-Schmidt study, the URA3 reporter gene used is located between the HMR-E and HMR-1 silencers, while our ADE2 reporter is downstream from the HMR-1 silencer. This might reflect an interesting potential difference in the mechanisms of normal versus rpd3A mediated silencing propagation between the silencers versus downstream of HMR-1 in rpd3 mutants. While
several studies have described that mutation of the SET pathway weakens SIR-mediated silencing in yeast, most used reporter genes at telomeres and the rDNA locus or between the HMR or HML silencers. The maintenance of mating in our primary SET pathway mutants and their progeny from crosses (Figure 3) suggests that normal HMR silencing of the a1 gene is not abolished in SET pathway mutants in the rpd3Δ background. Another role of the COMPASS complex is in the repression of middle sporulation genes which is mediated by another Sirtuin HDAC Hst1p in complex with Sum1p and Rfm1p (Jaiswal et al. 2017). However, deletion of HST1 in our HMR-ADE2 rpd3Δ strains again maintained the red colony phenotype (Figure 6). Another proposed mechanism of Set1p directed repression is through H3K4me recruitment of the SET3 complex (Kim and Buratowski 2009), which contains two HDACs, encoded by HST1 and HOS2.

Double deletion of these genes in our reporter strains again maintained the red colony phenotype (Figure 6). Another Northern blot analysis of a marked HMR-tDNA confirms its expression in rpd3Δ mutants. The HMR-tRNA transcript is detected using a complementary oligonucleotide probe specific to the 19 base pair extension (DDO-767, Table 3) to distinguish it from transcripts emanating from the other seven copies of this tRNAThr isoacceptor. Strains deleted for the HMR-tDNA show no signal, confirming the specificity of the assay. The blot was stripped and reprobed with a bulk tRNAThr 76 base oligonucleotide probe (complementary to the tRNAThr(AGU)C, SGD YNCC0014W final processed transcript) as a loading control. Strains used are (left to right) DDY3401, 3402, 3403, 3404, 3396, 3398, 465, and 466.

![Figure 6](image-url) Deletion of known effectors of H3K4me mediated gene repression do not affect extended rpd3Δ silencing. Strains were constructed in the rpd3Δ background to have deletions of HDAC genes HST3, HST1, or both HST1 and HOS2. Since hst1Δhos2Δ strains grew slowly, patches of ~5 mm diameter of each strain were made to obtain comparable growth to verify the red phenotype (lower panels).

![Figure 7](image-url) Binding of RNA Polymerase III transcription complex and HMR-tRNA expression are not affected by increased rpd3Δ silencing. (A) Chromatin immunoprecipitation was performed using anti-FLAG antibody in wild type and rpd3Δ strains containing FLAG-tagged Brf1p. PCR primer sets distal to (B and D) and overlapping the HMR-tDNA (C) were used to determine Pol III complex formation. Primer set A surrounds a tDNA distal to the HMR domain and was used as a positive control. A strain lacking the FLAG epitope on Brf1p was used as a negative control. (B) Northern blot analysis of a marked HMR-tDNA confirms its expression in rpd3Δ mutants. The HMR-tRNA transcript is detected using a complementary oligonucleotide probe specific to the 19 base pair extension (DDO-767, Table 3) to distinguish it from transcripts emanating from the other seven copies of this tRNAThr isoacceptor. Strains deleted for the HMR-tDNA show no signal, confirming the specificity of the assay. The blot was stripped and reprobed with a bulk tRNAThr 76 base oligonucleotide probe (complementary to the tRNAThr(AGU)C, SGD YNCC0014W final processed transcript) as a loading control. Strains used are (left to right) DDY3401, 3402, 3403, 3404, 3396, 3398, 465, and 466.

firing known to be influenced by SET1 through H3K37 methylation (Santos-Rosa et al. 2021).

Also, intriguing are our findings that while silencing spreads beyond the HMR-tDNA barrier in rpd3 mutants, the Pol III complex remains bound to the tDNA sequence and remains active (Figure 7). This contrasts with a previous study where we demonstrated that loss of function of the nonhistone proteins Nhp6a and Nhp6b leads to spreading of silencing past the tDNA barrier and loss of transcription of the HMR-tDNA (Braglia et al. 2007). We hypothesize that in the absence of RPD3 function, the tDNA is possibly somehow looped out to allow discontinuous spreading of heterochromatin around the actively transcribed tDNA. It will be interesting to test this hypothesis by chromatin conformation capture methods and to determine how loss of RPD3 HDAC function allows formation of such a chromatin structure to allow silencing to bypass the barrier and how this spreading is then suppressed by loss of H3K4 methylation. Since the Pol III complex remains intact, this would also require a detailed analysis of potential differences in H3K4 methylation marks on nucleosomes.
immediately adjacent to the HMR-tDNA in wild type versus rd3 mutants.

So how does loss of RPD3 function lead to increased silencing at HMR? One study suggests that RPD3 acts as a barrier protein by removing the acetylated lysine substrate for Sir2p and inhibiting the local formation of the OAA/ADP–ribose product of Sir2-mediated deacetylation which has been implicated in promoting silencing (Ehrentraut et al. 2010). However, an earlier study casts some doubt on this potential mechanism by demonstrating that Sir2-mediated silencing can propagate via deacetylation by a non-Sir2 protein in the absence of local OAA/ADP–ribose production (Chou et al. 2008). However, consistent with a direct role of Rd3p in barrier function we found in our bioinformatic analysis of published Rd3p ChIP-seq data (McKnight and Tsukiyama 2015) that weak but consistent peaks of Rd3p are present at numerous tDNAs in wild-type yeast, including at HMR. We are currently constructing yeast strains to confirm this potential association by conventional ChIP. Since SIR-mediated silencing in S. cerevisiae is modeled to propagate by sequential Sir2p deacetylation followed by Sir3p and Sir4p binding (Rusché et al. 2002), if Rd3p is indeed localized at tDNAs it could contribute to barrier function by simply removing the acetylated lysine docking sites for Sir2p activity over a range of nucleosomes near the HMR-tDNA. The subsequent loss of Rd3p function would then allow Sir2p to promote the propagation of silencing on both sides of the tDNA by attaching to and deacetylating the nucleosomes normally targeted by Rd3p. Why subsequent spreading of silencing in rd3 mutants is then dependent on the SET1 pathway remains to be determined. These studies are significant to gain a deeper understanding of the interplay and crosstalk of chromatin writers, erasers, and readers (Allis and Jenuwein 2016) but may also have relevance to current proposed applications of HDAC and other chromatin modification enzyme inhibitors in health care (West and Johnstone 2014; Ansari 2019; Bhat et al. 2021; Dang and Wei 2021). While therapeutic results have been seen using drugs that inhibit chromatin enzymes, the global effects of removing one chromatin-modifying activity on the myriad of others and all possible interactions and potential side-effects are not fully characterized. Other possibilities to consider are that chromatin-modifying enzymes have been shown to act on nonhistone targets (Toro et al. 2021; Bryk et al. 2021). Tumour function lead to increased silencing of mutants included Amanda Bowers and Abrar Hussein.

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**Conflicts of interest**
The authors declare that there is no conflict of interest.

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**Data availability**
All yeast strains and plasmids described in this study are available on request. The authors affirm that all data necessary for confirming the conclusions of this article are represented fully within the article, its tables and figures and the cited literature.

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