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Genomewide Screen for Negative Regulators of Sirtuin Activity in *Saccharomyces cerevisiae* Reveals 40 Loci and Links to Metabolism

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ABSTRACT

Sirtuins are conserved proteins implicated in myriad key processes including gene control, aging, cell survival, metabolism, and DNA repair. In *Saccharomyces cerevisiae*, the sirtuin Silent information regulator 2 (Sir2) promotes silent chromatin formation, suppresses recombination between repeats, and inhibits senescence. We performed a genomewide screen for factors that negatively regulate Sir activity at a reporter gene placed immediately outside a silenced region. After linkage analysis, assessment of Sir dependency, and knockout tag verification, 40 loci were identified, including 20 that have not been previously described to regulate Sir. In addition to chromatin-associated factors known to prevent ectopic silencing (Bdf1, SAS-I complex, Rpd3L complex, Ku), we identified the Rtt109 DNA repair-associated histone H3 lysine 56 acetyltransferase as an anti-silencing factor. Our findings indicate that Rtt109 functions independently of its proposed effectors, the Rtt101 cullin, Mms1, and Mms22, and demonstrate unexpected interplay between H3K56 and H4K16 acetylation. The screen also identified subunits of mediator (Soh1, Srb2, and Srb5) and mRNA metabolism factors (Kem1, Ssd1), thus raising the possibility that weak silencing affects some aspect of mRNA structure. Finally, several factors connected to metabolism were identified. These include the PAS-domain metabolic sensor kinase Psk2, the mitochondrial homocysteine detoxification enzyme Lap3, and the Fe-S cluster protein maturase Isa2. We speculate that PAS kinase may integrate metabolic signals to control sirtuin activity.

SIRTUINS are a conserved family of proteins found in all domains of life. In eukaryotic cells, they have been characterized as deacetylases, ADP-ribosylases, or both (Tanny *et al.* 1999; Imai *et al.* 2000; Landry *et al.* 2000). Work in a variety of systems has shown that they play roles in many key cellular processes including gene regulation, aging, cell survival, metabolic control, and DNA repair (Brachmann *et al.* 1995; Kaeberlein *et al.* 1999; Lin *et al.* 2000; Langley *et al.* 2002). Small molecule inhibitors and activators of sirtuin activity have received considerable attention recently as potential therapeutic agents for aging-associated diseases including Parkinson's disease and type II diabetes (Smith and Denu 2007). Thus, there is substantial general interest in understanding how this family of enzymes is regulated.

The founding member of the sirtuin family is the budding yeast *Saccharomyces cerevisiae* gene-silencing factor Silent information regulator 2 (Sir2), which is required for the formation of virtually all silent chromatin in budding yeast reviewed in Rusche *et al.* (2003). Sir2 acts in conjunction with the other Sir proteins to promote silencing. It forms a NAD-dependent histone deacetylase complex with Sir3 and Sir4 and is recruited to the silent mating-type cassettes through interactions with Sir1. Additionally, silencing at telomeres is mediated by the recruitment of the Sir proteins by Rap1 protein. Sir2 acts at the rDNA to promote silencing and to suppress recombination between rDNA repeats (Smith and Boeke 1997). Sir2 also inhibits the senescence of mother cells; it has been suggested that this is related to its anti-recombination activity at the rDNA (Sinclair and Guarente 1997).

The Sir complex appears to spread laterally from its nucleation points (Morris and Moazed 2007). This is thought to be accomplished through a cycle of histone-tail deacetylation by Sir2 that enables binding of additional copies of the Sir complex through the histone-tail binding sites in Sir3 and Sir4, whose binding to nucleosomes is inhibited by histone-tail acetylation. This mechanism of Sir protein spreading presents a potential problem in that ectopic spread of silencing activity to regions designated to be transcriptionally active would presumably be deleterious. Recent work has identified several mechanisms that prevent the local ecoptic spread of silent chromatin in budding yeast. A tRNA gene at the right (telomere-proximal) border of the *HMR***a** acts as a boundary element that blocks the lateral spread of silent chromatin (Donze *et al.* 1999). Subsequent work has identified similar boundary elements in other species (Noma *et al.* 2006; Scott *et al.* 2007). Substitution of histone H2A with the H2A.Z variant in euchromatin also antagonizes silencing, as do three

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distinct methylations in histone H3 (Van Leeuwen *et al.* 2002; Meneghini *et al.* 2003; Tompa and Madhani 2007; Venkatasubrahmanyam *et al.* 2007), and acetylation on lysine 16 on the H4 tail (Kimura *et al.* 2002; Suka *et al.* 2002). In at least one case, these factors act redundantly to block the global spread of silencing: we recently reported that H2A.Z substitution and the Set1 complex act together to prevent the global spread of silencing (Venkatasubrahmanyam *et al.* 2007).

A forward insertional mutagenesis screen aimed at identifying factors that prevent the spread of silencing from the *HMR***a** locus has been reported (Jambunathan *et al.* 2005). This work utilized a rearranged *HMR***a** locus containing a partial duplication in which the previously mentioned tRNA boundary element at *HMR***a** was moved to delete a segment of the *HMR***a**1–**a**2 region, an intact copy of the *HMR***a**1 gene was placed to the right of this element, and the *HMR*-I silencer was deleted. Using mating as an assay for the repression of the **a**1 gene, this screen identified the known anti-silencing factors *SAS4*, *SAS5*, and *RPD3*, as well as one protein not previously linked to silencing, the bromodomain protein *YTA7*.

To identify novel negative regulators of sirtuin activity in this context, we sought to extend this analysis to the whole-genome level using the yeast nonessential deletion collection. We crossed the collection to a strain containing a simple insertion of a sensitized *URA3* reporter gene just to the right of the tRNA boundary element that flanks the *HMR***a** locus.

MATERIALS AND METHODS

SGA screen: Colonies were transferred using a Virtek colony arrayer as described in Tong *et al.* (2001). The yeast *MAT***a** knockout library (http://www.openbiosystems.com/GeneExpression/Yeast/YKO/) was grown for 2 days on rich media (YPAD) and pinned onto lawns of the reporter (bait) strain, and then grown for another day at 30° to cross. Diploid progeny were selected on rich media plus G418 (120 mg/liter) and ClonNat (60 mg/liter) drugs and grown for 2 days. Diploids were transferred to presporulation media (GNA) and grown at 30° for 1 day, and then transferred to sporulation media (NGS) and incubated at 23° in an open-air humidified chamber for 5 days. Colonies were transferred to SGA –His –Arg +canavanine plates and grown for 2 days at 30° to select for *MAT***a** haploid progeny. Colonies were then transferred to SGA –His –Arg +canavanine +G148 plates and grown at 30° for 1 day to select for recombinant progeny. Colonies were then transferred to SGA –His –Arg +canavanine +G148 +Hygromycin B (180 mg/liter) plates and grown at 30° for 2 days, followed by 1 day of growth on SGA –His –Arg +canavanine +G148 +Hygromycin B +clonNat plates to select for *MAT***a** progeny containing a gene knockout allele and the reporter gene. These colonies were transferred to synthetic complete (SC) media +5-FOA (2 g/liter) plates and grown for 3–5 days prior to phenotypic scoring. Positively scored colonies were streaked to SC –Ura plates to verify maintenance of the *Candida albicans URA3* reporter gene and subsequently replica plated to 5-FOA media to confirm the resistance.

Strains: All strains used in this study are listed with their genotype in supplemental Table S1. The reporter strains used were generated by integrating the *C. albicans URA3* gene at position 295,754 of *S. cerevisiae* chromosome III by homologous recombination. The dominant drug markers *NatMX4* and *HphMX4* (Hygromycin B resistance) (Goldstein and McCusker 1999) were integrated at positions 299,553 and 290,254 of chromosome III, respectively. Strain genotypes of single knockouts recovered from the screen were confirmed by sequencing. Strains containing deletions of the *SIR3* gene were created by homologous recombination using either *HphMX4* or the *LYS2* gene from *C. albicans* and were confirmed by PCR for presence of the integrated deletion cassette and loss of the wild-type *SIR3* allele. Primers used for strain construction are listed in supplemental Table S2.

Chromatin immunoprecipitation: All chromatin immunoprecipitation (ChIP) experiments were performed according to the protocol used in Raisner *et al.* (2005). For each sample, we used 2.5 μl of the H3K56-ac antibody, which was generously donated by the lab of Michael Grunstein. We used 20 μl of the Sir3 antibody, which was used as serum and generated against a GST-tagged C-terminal fragment of the protein. We used 2.5 μl of the H4K16-ac antibody (Upstate no. 07-329) and 1 μl of the H3 antibody (Abcam no. Ab1791) per sample.

Plate assays: For all plate assays, cells were pregrown on rich (YPAD) media and then resuspended in water and plated in fivefold dilutions. The same dilutions were plated at the same time to rich media (SC), rich media (SC) containing 5-FOA (1 g/liter), and media lacking uracil (SC –Ura). Plates were incubated for 3 days at 30° prior to photography.

RESULTS

Genomewide screen to identify anti-silencing factors: To identify factors responsible for antagonizing the spread of Sir activity to proximal regions, we devised the following strategy to screen the entire yeast nonessential gene deletion library using a reporter-based assay. The reporter strain contains a promoter-truncated allele of the *C. albicans URA3* homolog, which is able to complement *S. cerevisiae ura3* mutants (Figure 1A). Several promoter-truncation alleles of varying length were integrated outside of *HMR*, 200 bp to the telomere-proximal side of its characterized boundary element, and tested for activity. The goal of performing these integrations was to obtain a low-expressing allele of *URA3* that was sensitized to spread of silencing events in mutants such as the *htz1*Δ mutant (Meneghini *et al.* 2003). Our criteria for selecting the reporter strain were threefold: (1) a reproducible 5-fluoroorotic acid resistance (FOA^R) phenotype in the *htz1*Δ mutant but not in wild-type cells to demonstrate sensitivity to the spread of silencing, (2) the smallest possible promoter fragment, and (3) a Ura+ phenotype when plated on –Ura media indicating that the reporter has sufficient expression of *C. albicans URA3* that allows for assaying on these media. The strain containing the allele with 70 bp of its endogenous promoter (Figure 1B) best fit these criteria and was chosen as the bait strain in our screen.

We adapted the SGA method developed by others (Tong *et al.* 2001) to cross our reporter strain with the available gene deletion library to generate a library of yeast colonies that were *MAT***a** haploids, bearing the

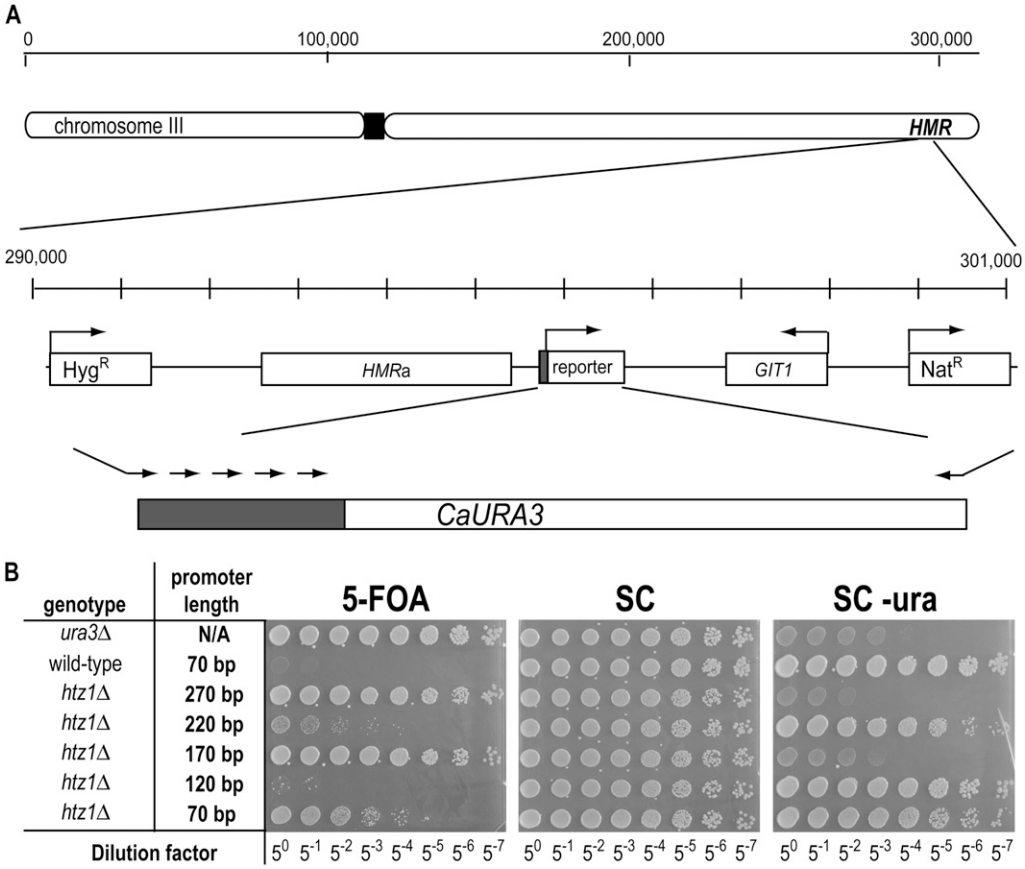


FIGURE 1.—Reporter strain for assaying spread of silencing activity from *HMR***a**. (A) Promoter-truncated alleles of the *Candida albicans URA3* gene were integrated adjacent to the right boundary element of the silenced *HMR***a** locus. Reporter alleles were integrated by homologous recombination of PCR-amplified genomic *C. albicans URA3* of varying length, containing 50 bp of homology upstream and downstream of the region 200 bp to the right of *HMR***a**. (B) Fivefold serial dilutions to test growth rate of 70-bp promoter bearing reporter construct on rich media (SC), 5-FOA, and media lacking uracil (SC – Ura).

reporter gene along with a single gene deletion. In addition, the parent strain contained dominant drug selection markers to track the segregation of the reporter allele, independent of potential silencing effects. These strains were arrayed on plates in 768-colony format in which each of 384 strains was arrayed in duplicate. They were then transferred to 5-FOA-containing plates for phenotypic testing. Because this automated method transfers a relatively large percentage of the colony to the new plate, and also because 5-FOA will select for spontaneous *C. albicans ura3* mutants that produce colony papillations, we were selective about scoring positives. Specifically, only colonies that were symmetric and lacked obvious papillations and were present as pairs (meaning that they had to arise from independent meioses) were chosen. These putative positive colonies were tested for *URA3* expression by assaying their ability to grow on synthetic media lacking uracil (SD −Ura) to screen for undesired Ura− mutants. Our original reporter strain contained a single dominant selectable drug marker, *NatMX4*, integrated 3800 bp telomere-proximal to the reporter at coordinate 299,553 of chromosome III (Figure 1A). Because of the large number of meioses that are potentially screened by this method, 5-FOA-resistant colonies arose. Upon further investigation, these were determined to be Ura− strains that likely arose from recombination events between the reporter and the drug marker. The first pass of the screen with this strain had a 5-FOA^R rate of ~5% (~220/4600). However, the majority of these strains were found to have undergone recombination between the reporter and the positive drug selection marker and were therefore Ura−. To avoid this problem, a second reporter strain was generated with an additional drug marker (*HphMX4*) integrated 5300 bp centromere proximal to the reporter at coordinate 290,254 of chromosome III. The probability of a double-recombination event leading to a drug-resistant strain lacking the reporter allele is considerably less likely than the single crossover event, reducing the likelihood of recovering 5-FOA-resistant colonies due to loss of the reporter gene. The screen was repeated accordingly and an additional 100 mutants that fit our criteria were scored as positives.

Secondary screenings to confirm phenotypic linkage: The two passes of the screen yielded 320 total candidates. Because this large-scale screening technique allowed for a variety of events that would lead to false detections, we imposed additional criteria:

1. The colonies needed to grow when streaked as singles on SD –Ura, while retaining the ability to yield FOA^R colonies when replica-plated, a hallmark of anti-silencing factors.

TABLE 1

Genes identified in this study that display an antisilencing function

Systematic name	Gene name	Cellular localization	GO terms	FOA plating efficiency	FOA plating efficiency of double mutant with *sir3*Δ	*SIR* dependent?
YNL134C	*ynl134c*	Cytoplasm/nucleus	Alcohol dehydrogenase (NADP+) activity	6.40*E*-05	2.56*E*-06	Yes
YML102W	*cac2*	Nucleus	Chromatin assembly, DNA repair, nucleosome assembly	3.20*E*-04	2.56*E*-06	Yes
YOR144C	*elg1*	Cytoplasm/nucleus	Telomere maintenance, DNA replication, DS-break repair	1.60*E*-03	2.56*E*-06	Yes
YMR106C	*yku80*	Nucleus	Chromatin assembly, chromatin silencing, telomere maintenance	3.20*E*-04	2.56*E*-06	Yes
YER088C	*dot6*	Cytoplasm/nucleus	Chromatin silencing at rDNA, chromatin silencing at telomere	8.00*E*-03	2.56*E*-06	Yes
YBR275C	*rif1*	Nucleus	Chromatin silencing, telomere maintenance	3.20*E*-04	2.56*E*-06	Yes
YGL127C	*soh1*	Nucleus	Mediator complex, telomere maintenance, DNA repair	1.60*E*-03	2.56*E*-06	Yes
YHR041C	*srb2*	Cytoplasm/nucleus	Mediator complex, telomere maintenance	3.20*E*-04	2.56*E*-06	Yes
YGR104C	*srb5*	Nucleus	Mediator complex, telomere maintenance	3.20*E*-04	2.56*E*-06	Yes
YDL070W	*bdf2*	Cytoplasm/nucleus	Bromodomain protein, redundant with Bdf1	1.60*E*-03	1.28*E*-05	Partial
YLR399C	*bdf1*	Nucleus	Swr1 complex, chromatin remodeling	6.40*E*-05	1.28*E*-05	Partial
YDR334W	*swr1*	Nucleus	Swr1 complex, chromatin remodeling, Swi2/Snf2-related ATPase	3.20*E*-04	2.56*E*-06	Yes
YOR213C	*sas5*	Cytoplasm/nucleus	H3/H4 histone acetyltransferase activity, chromatin silencing at telomere	4.00*E*-02	2.56*E*-06	Yes
YMR127C	*sas2*	Cytoplasm/nucleus	H3/H4 histone acetyltransferase activity, chromatin silencing at telomere	4.00*E*-02	2.56*E*-06	Yes
YDR181C	*sas4*	Cytoplasm/nucleus	H3/H4 histone acetyltransferase activity, chromatin silencing at telomere	4.00*E*-02	2.56*E*-06	Yes
YLL002W	*rtt109*	Nucleus	Histone acetyltransferase activity, negative regulation of transposition, DNA damage reponse	3.20*E*-04	2.56*E*-06	Yes
YJL115W	*asf1*	Nucleus	Chromatin assembly, chromatin silencing, histone exchange, histone acetylation	3.20*E*-04	2.56*E*-06	Yes
YKR048C	*nap1*	Cytoplasm	Histone binding, nucleosome assembly	6.40*E*-05	2.56*E*-06	Yes
YOL004W	*sin3*	Mitochondrion	Rpd3S, Rpd3L, histone deacetylase	4.00*E*-02	2.56*E*-06	Yes
YIL084C	*sds3*	Nucleus	Histone deacetylation, Rpd3L complex	4.00*E*-02	2.56*E*-06	Yes
YMR263W	*sap30*	Nucleus	Histone deacetylation, Rpd3L complex	8.00*E*-03	2.56*E*-06	Yes
YNL097C	*pho23*	Nucleus	Histone deacetylation, Rpd3L complex	8.00*E*-03	2.56*E*-06	Yes
YPL181W	*cti6/rxt1*	Nucleus	Histone deacetylation, Rpd3L complex	8.00*E*-03	2.56*E*-06	Yes
YBR095C	*rxt2*	Nucleus	Histone deacetylation, Rpd3L complex	8.00*E*-03	2.56*E*-06	Yes
YAL013W	*dep1/fun54*	NA	Histone deacetylation, Rpd3L complex	4.00*E*-02	2.56*E*-06	Yes
YER072W	*vtc1*	ER	Microautophagy, vacuole transport	8.00*E*-03	2.56*E*-06	Yes
YLR436C	*ecm30*	Cytoplasm	Cell wall organization and biogenesis, cytoplasm	6.40*E*-05	2.56*E*-06	Yes
YPR067W	*isa2*	Mitochondrion	Biotin biosynthesis, mitochondrion	3.20*E*-04	2.56*E*-06	Yes
YIL055C	*yil055c*	NA	NA	3.20*E*-04	2.56*E*-06	Yes
YNL239W	*lap3*	Cytoplasm/mitochondrion	Cysteine-type peptidase activity, nucleic acid binding, mitochondrion	3.20*E*-04	2.56*E*-06	Yes
YCL005W	*ldb16*	Lipid particles	Mitochondrion	3.20*E*-04	2.56*E*-06	Yes
YKL116C	*prr1*	Cytoplasm	Receptor signaling protein, serine/threonine kinase activity	3.20*E*-04	6.40*E*-05	Partial
YOL045W	*psk2*	Cytoplasm	PAS domain protein, serine/threonine kinase	3.20*E*-04	6.40*E*-05	Partial

(*continued*)

TABLE 1

(Continued)

Systematic name	Gene name	Cellular localization	GO terms	FOA plating efficiency	FOA plating efficiency of double mutant with *sir3*Δ	*SIR* dependent?
YLR079W	*sic1*	Cytoplasm/nucleus	CDK inhibitor	1.60*E*-03	2.56*E*-06	Yes
YGL173C	*kem1*	Cytoplasm	5′–3′ exoribonuclease activity, telomere maintenance	6.40*E*-05	2.56*E*-06	Yes
YIL122W	*pog1*	Nucleus	RNA Pol II transcription factor activity	3.20*E*-04	2.56*E*-06	Yes
YDR293C	*ssd1*	Cytoplasm	RNA binding, cell wall organization	1.60*E*-03	2.56*E*-06	Yes
YGL081W	*ygl081w*	NA	Unknown protein, FHA domain	3.20*E*-04	2.56*E*-06	Yes
YPR028W	*yop1*	ER	Vesicle-mediated transport, cell membrane	6.40*E*-05	2.56*E*-06	Yes
YML095C	*rad10*	Cytoplasm/nucleus	DS break repair, nucleotide-excision repair factor 1 complex	4.00*E*-02	1.60*E*-03	Partial
YGL163C	*rad54*	Cytoplasm/nucleus	Chromatin remodeling, DS break repair, telomere maintenance	2.00*E*-01	2.00*E*-01	No
YOR305W	*YOR305w*	Mitochondrion	Unknown protein, mitochondrion localization	2.00*E*-01	2.00*E*-01	No
YMR007W	*YMR007w*	NA	ORF, dubious	8.00*E*-03	1.60*E*-03	No
YBR078W	*ecm33*	NA	GPI-anchored protein, cell membrane, mitochondrion	3.20*E*-04	3.20*E*-04	No
YLR337C	*vrp1*	Punctate/actin	Actin binding, actin organization	1.60*E*-03	1.60*E*-03	No
YMR206W	*YMR206W*	NA	ORF, dubious	3.20*E*-04	3.20*E*-04	No

2. The phenotype was tightly linked to the knockout. For this, a minimum of 100 random spores were plated for each mutant and subsequently tested for linkage between the knockout, the reporter genotype, and 5-FOA resistance.
3. The identity of the knockout allele was verified by amplification and sequencing of the bar codes present in each deletion allele. This was necessary due to potential errors in the knockout collection and possible cross-contamination events arising in the course of the automated strain generation and scoring.

After removal of false positives, 46 nonredundant positively scoring mutants remained. The final results of both screens are summarized in Table 1. While we have confirmed the identity of the knockouts in all the strains, as well as the linkage of the knockout alleles with the FOA^R phenotype, we cannot of course rule out the possibility that a spontaneous mutation in a gene tightly linked to the marked gene deletion is responsible for a given phenotype.

Phenotypic characterization of anti-silencing mutants: To quantitatively assess the anti-silencing phenotypes of the mutants, we plated serial dilutions of each mutant on 5-FOA media (Figure 2). Each plate contained the parental strain (wild type containing reporter construct), a *ura3*Δ strain, and an *htz1*Δ harboring the reporter as controls. Mutants in known pathways or complexes, such as the SAS-I histone acetyl transferase genes *SAS2*, *SAS4*, and *SAS5*, were plated side by side (Figure 2A). Other mutants were grouped together on the basis of available gene ontology (GO) assignments curated at the Saccharomyces Genome Database (http://www.yeastgenome.org). Additionally, all of the strains were plated on SC media and media lacking uracil (SC – Ura) to control for plating efficiency and *URA3* expression. As shown in Figure 2, genes belonging to common functional categories tended to display similar phenotypic strength. For example, mutants in the Rpd3-L complex provide one example (Figure 2B). Phenotypic strength varied from a strong anti-silencing defect such as that of the *sas2*Δ mutant, in which nearly 10% of the colonies grow on 5-FOA (Figure 2A; compared to SC media), to weak ones such as the *nap1*Δ mutant (Figure 2E), which had an approximate relative plating efficiency of $1/5^5$. As a reference, the wild-type reporter strain was generally observed to have less than $1/5^8$ relative plating efficiency, while the *ura3*Δ control strain displayed ~100% efficiency. FOA plating efficiency for strains was determined by comparing the titer for which several colonies grew on FOA to the titer on SC plates for which a similar number of colonies was observed. The quantitative relative plating efficiencies for the mutants are listed in Table 1.

Sir dependence of increased repression of *C. albicans URA3* reporter in FOA+ mutants: The screen

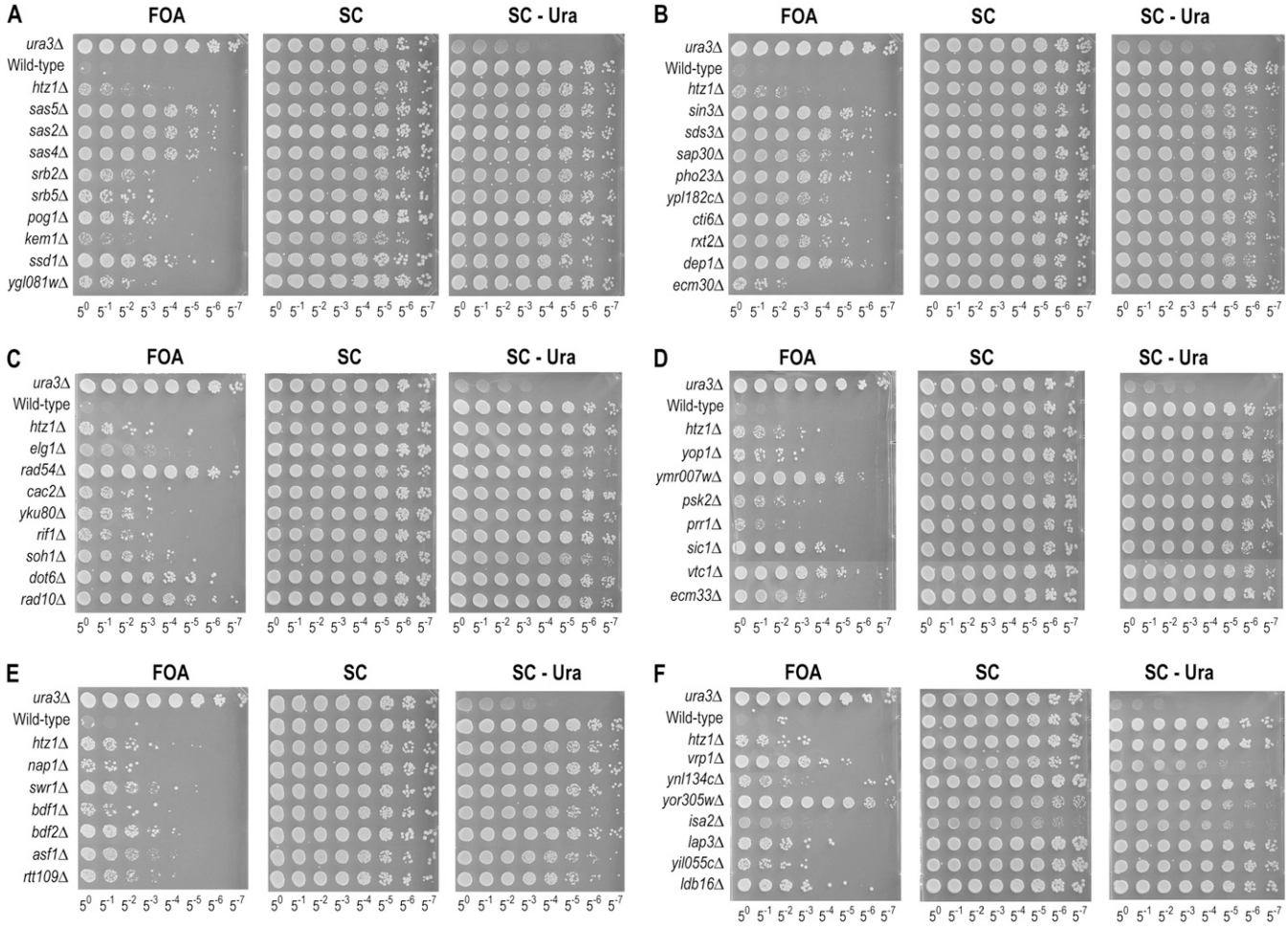


Figure 2.—Quantitative reporter plating assay for spread of silencing. Serial platings of mutant strains show increased growth on 5-FOA media, assembled into subcategories. Each plate contains a *ura3*Δ strain as a positive control, and a reporter strain bearing no knockouts (wild type) as a negative control, and an *htz1*Δ mutation in the reporter strain as an example of an ectopic silencing phenotype. (A) SAS-I complex and Mediator components. (B) Rpd3-L complex components. (C) Telomere maintenance genes. (D) Genes affecting protein phosphorylation and others. (E) Swr1 and Rtt109 complex components. (F) Metabolism-linked genes.

was designed to identify any mutant strain for which there was a large enough decrease in *URA3* expression to allow for increased growth on 5-FOA. Mutants were predicted to fall into two categories: genes that are essential for full *URA3* expression independently of silencing and genes that normally prevent a spread of silencing activity from *HMR***a**. Because we were interested only in the latter, we sought to identify mutants that have phenotypes dependent on Sir activity. To do this, we deleted *SIR3*, which encodes an essential subunit of the Sir complex. In a *sir3*Δ background, bona fide anti-silencing mutants should revert to a 5-FOA-sensitive phenotype in the absence of *SIR3*. Conversely, mutants in processes such as uracil biosynthesis, transcription *per se*, or drug resistance should not display a *sir3*Δ-suppressible phenotype.

For each mutant isolated in the screen, we deleted *SIR3* and tested for 5-FOA resistance. Deletion of *SIR3* by itself did not confer any fitness benefit compared to wild type (Figure 3A). As in Figure 2, each plate displayed in Figure 3 includes wild-type and *ura3*Δ strains as controls. Additionally, each plate assays a single paired example of a 5-FOA-resistant deletion strain next to its respective double mutant with *sir3*Δ as a reference. As shown in Figure 3, A–C, a large fraction (35/46) of the genes from the screen displayed 5-FOA phenotypes that were fully suppressed by deletion of *SIR3*. Phenotypes of mutants in *PRR1* and *RAD10* showed partial suppression of the phenotype, as evidenced by intermediate 5-FOA resistance (Figure 3, C and G, and supplemental Figure S1), whereas mutants in *BDF1* and *BDF2* had weak suppression (Figure 3E and supplemental Figure S1). Mutants in *PSK2* showed variable degrees of suppression (Figure 3C *vs.* supplemental Figure S1). Intermediate and weak suppression suggests dual roles for these genes in anti-silencing and transcription. Finally, the genes *RAD54*, *VRP1*, *ECM33 YOR206W*, *YMR007W*, and *YOR305W* (Figure 3, F and G) displayed phenotypes that were unaffected by deletion of *SIR3*, suggesting they affect expression

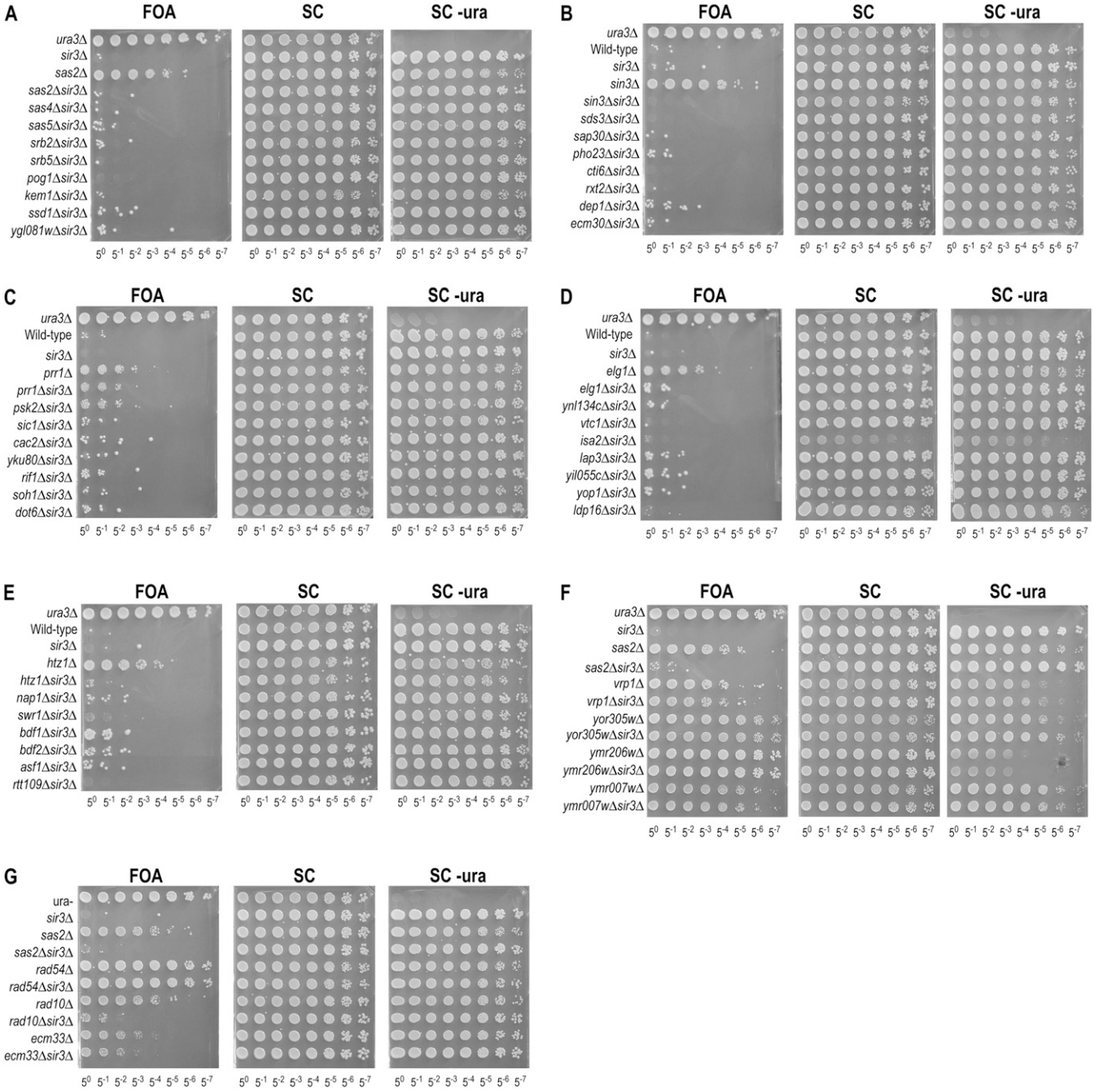


Figure 3.—Sir-suppressible and nonsuppressible anti-silencing phenotypes of mutant strains. Serial platings of mutant strains show increased growth on 5-FOA media, assembled into subcategories. Each plate contains *ura3*Δ as a positive control for 5-FOA resistance, and a reporter strain bearing no knockouts (wild type) as a control for 5-FOA sensitivity, and *sir3*Δ in the reporter strain as a negative control for Sir-independent effects. (A–D) Cases of robust suppression of 5-FOA phenotypes by *sir3*Δ. (E and G) Cases of weak suppression. (F and G) Cases of nonsuppression.

or genetic mutability of the *URA3* reporter gene. Quantitative estimations of the relative 5-FOA plating efficiencies for the double deletions are listed in Table 1.

Rtt109/Asf1 have a novel function to antagonize silencing of the reporter gene: Our screen identified Rtt109 and Asf1 as sirtuin-dependent anti-silencing factors. These proteins form a complex that acetylates the lysine 56 core residue of histone H3 (H3K56) (Driscoll *et al.* 2007; Han *et al.* 2007). The complex acetylates non-chromatin-associated H3 during DNA replication. Together with the Hst3 deacetylase that acts outside of S phase, this mechanism restricts H3K56 acetylation to S phase (Celic *et al.* 2006; Maas *et al.* 2006). For unknown reasons, cells defective in this modification are sensitive to DNA damage. On the basis

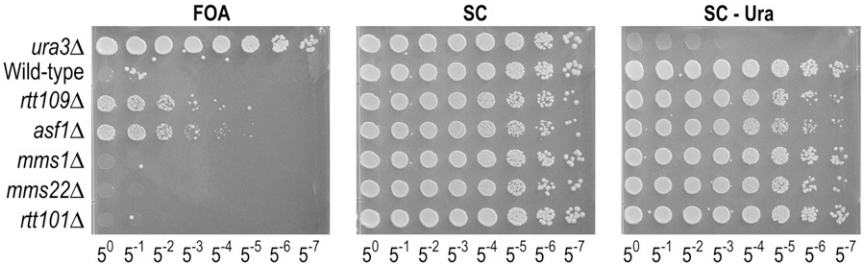
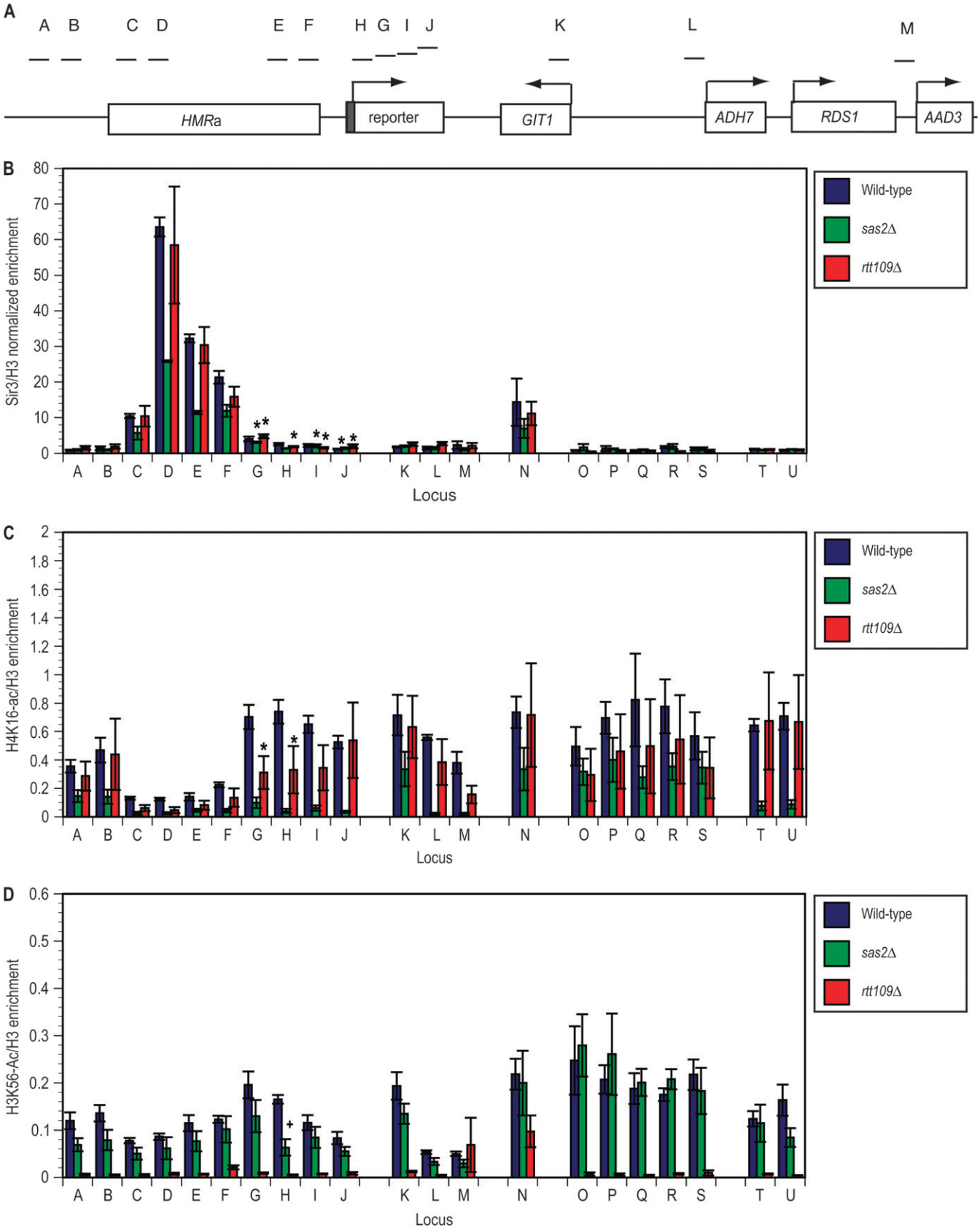


FIGURE 4.—Rtt109 and Asf1 have phenotypes distinct from the Rtt101 cullin complex. Indicated genotypes were analyzed for reporter gene expression as described in Figures 2 and 3.

of genetic interaction maps, it has been suggested that the role of this mechanism in DNA repair is facilitated by the proteins Asf1, Mms1, Mms22, Rtt101, and Rtt109 (COLLINS *et al.* 2007) . Interestingly however, only *RTT109* and *ASF1* share additional synthetic interactions with the Swr1 complex, which deposits H2A.Z (COLLINS *et al.* 2007). This is consistent with the results of the screen, which identified anti-silencing phenotypes only for *asf1*Δ and *rtt109*Δ mutants (Figure 2). However, it remained possible that mutants in *MMS1*, *MMS22*, and *RTT101* were false negatives. Therefore, we used homologous recombination to generate deletions of these genes in the reporter strain background and tested them for growth on 5-FOA media (Figure 4). We observed that *mms1*Δ, *mms22*Δ, and *rtt101*Δ did not display resistance to 5-FOA, in contrast to *asf1*Δ and *rtt109*Δ mutants.

Silencing outside of *HMR*a in the *rtt109*Δ mutant results in decreased H4K16 acetylation without a detectable increase in Sir3 binding: We investigated the phenotype of the *rtt109*Δ mutant further using ChIP, using the probes shown in Figure 5A. Probes A–F span the *HMR***a** silent cassette, probes G–J span the *C. albicans URA3* reporter gene, probes K–M correspond to the promoter regions of the three genes to the right (telomere-proximal) of the reporter gene, and probe N corresponds to the promoter of *HML*α-proximal gene *MRC1*. We also examined the promoter regions of several genes in euchromatic segments of chromosome III (probes O–S) and two probe sets (probes T and U) in the middle of the large ORF of the *BUD3* gene (used for normalization in Figure 5B). We first performed ChIP using polyclonal antibodies against Sir3 in the wild-type reporter strain, as well as in the mutant strains *rtt109*Δ and the previously characterized anti-silencing mutant *sas2*Δ as a control. Consistent with previous reports, Sir3 is dramatically enriched within *HMR***a**, while regions outside have very little or no observable Sir3 (Figure 5B). The data in all of our ChIP experiments were normalized to values obtained using ChIP performed with antibodies to histone H3 to control for nucleosome density. Additionally, for the Sir3 ChIP data, we normalized the data to the signal obtained from the *BUD3* ORF region, which shows no detectable Sir3 binding in wild-type cells. Notably, the promoter regions of the reporter construct (probe G) displayed less Sir3 association than the immediately adjacent *HMR***a** region, but more than that observed at control regions. Strikingly, when we examined Sir3 localization in the *rtt109*Δ and *sas2*Δ strains, no increased association of Sir3 within the reporter gene region was apparent, suggesting in this context that Rtt109 and Sas2 act downstream of Sir binding to antagonize silencing. Since a key function of the Sir complex is to deacetylate lysine 16 of histone H4 (H4K16), we examined the acetylation status of this residue (Figure 5C). We observed decreased acetylation on H4K16 using probes that cover the reporter gene in the *rtt109*Δ mutant (Figure 5C), which supports the idea that the increased activity of the Sir complex is responsible for the change in reporter gene expression in this mutant. Consistent with previous reports, we observed a dramatic depletion of this acetylation from *HMR***a** and we observed that its presence was dependent on the H4K16 HAT Sas2 at many, but not all, locations (Figure 5C). We also examined H3K56 acetylation by ChIP (Figure 5D). Although its removal has been suggested to be important for silencing (XU *et al.* 2007), we observed only a modest decrease of this modificiation within *HMR***a** compared to euchromatic sites and only a slight decrease at the reporter gene in the *sas2*Δ mutant.

FIGURE 5.—ChIP data for Sir3, H3K56 acetylation, and H4K16 acetylation. (A) Schematic of *HMR***a** and the surrounding region with locations of PCR amplicons used for quantitation. Primer set N corresponds to the promoter of the *HML*α-proximal gene *MRC1*, and primer sets O, P, Q, R, and S correspond to the promoters of the euchromatic genes *CDC10*, *CWH43*, *NFS1, DCC1*, and *RBK1*. Primer sets T and U are to the middle of the ORF region of *BUD3*. All data shown are averages of three independent ChIP experiments with standard error of the mean error bars. (B–D) *t*-Tests were applied to data for probes G–J only. (B) Sir3 ChIP data for wild-type, *sas2*Δ, and *rtt109*Δ strains. Data are normalized to H3 ChIP values for each locus, and all loci are normalized to the average of primer sets T and U. *t*-Tests for the null hypothesis that Sir3 levels did not change between wild type and the *sas2*Δ and *rtt109*Δ mutants were passed by loci denoted by *. (C) H4K16-ac ChIP values for wild type, *sas2*Δ, and *rtt109*Δ, normalized to H3 enrichment. *t*-Tests for the hypothesis that K16 acetylation levels decreased between the *rtt109*Δ mutant and wild type were passed by loci denoted by *. (D) H3K56-ac ChIP values for wild type, *sas2*Δ, and *rtt109*Δ, normalized to H3 enrichment. A *t*-test for the hypothesis that there is a significant difference in H3K56 acetylation between wild-type and *sas2*Δ cells was passed by the locus that is denoted by +.


A
A B C D E F H G I J K L M
HMRa
reporter
GIT1
ADH7
RDS1
AAD3
B
Sir3/H3 normalized enrichment
Locus
Wild-type
sas2Δ
rtt109Δ
C
H4K16-ac/H3 enrichment
D
H3K56-Ac/H3 enrichment

Importantly, the signal we observed in this region was dependent on *RTT109*.

DISCUSSION

Systematic screen for negative regulators of sirtuin activity: We present a strategy and results for identification of genes that negatively affect the Sir2-dependent repression of a reporter gene placed just outside a chromatin boundary element in *S. cerevisiae*. Our screen is similar in overall design to that reported by Jambunathan *et al.* (2005), which identified the bromodomain/AAA+ ATPase-encoding gene *YTA7* as a new negative regulator of silencing (Jambunathan *et al.* 2005). The major differences are that (1) we used a sensitized *C. albicans URA3* reporter gene and growth on FOA as output instead of a rearranged *HMR***a**1 locus and mating and (2) we used an SGA approach and the yeast deletion collection instead of mTn insertional mutagenesis. Although our screen did not identify *YTA7*, it did identify a number of previously described anti-silencing factors that were identified in the previous screen including *RPD3*, *SAS4*, and *SAS5*. As discussed above, given the potential for threshold and reporter-specific effects, false negatives are difficult to avoid in any genomewide reporter-based screen. Nonetheless, we identified 20 new loci that negatively regulate silencing outside of the tRNA boundary element. These assignments were based on individual mutants passing tests of linkage to the deletion collection marker, verification of the deletion barcodes, and dependency on *SIR2* for the increased-repression FOA phenotype.

Negative regulation of Sir2 by regulators of chromatin and DNA metabolism: In our effort to take an unbiased approach to identify novel negative regulators of silencing, we uncovered a disparate range of protein activities. Figure 6 describes the reported cellular locations, complexes, and functions of the 40 proteins identified in our screen that displayed *SIR*-dependent increased repression of the *C. albicans URA3* reporter gene on the basis of available annotation curated at the Saccharomyces Genome Database (http://www.yeastgenome.org). The majority of the chromatin modifying factors that we identified have been implicated previously in regulation of *SIR* activity. These include Bdf1 (a component of the Swr1 complex that deposits both H2A.Z and the TFIID complex), the SAS-I H4K16 acetyltransferase complex, the Rpd3-L HDAC complex, and a number of factors reported to control telomere length (Yku80, Rif1, Dot1, Pog1, Cac2, Elg1, and Mediator). The latter mutants may act by producing a longer telomere near *HMR***a**, which lies ~35 kb from the right telomere of chromosome III. Increased Sir complex nucleation might then lead to increased Sir-dependent repression of the reporter gene. Alternatively, some members of this group might act by distinct mechanisms as suggested previously for Yku (Maillet *et al.* 2001). The negative role

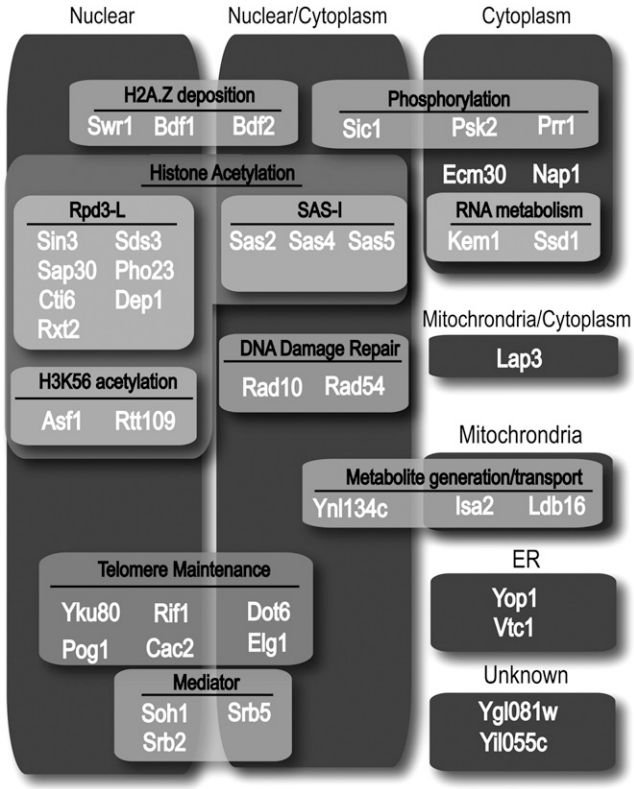


Figure 6.—Chart depicting functional and localization categories of Sir-dependent genes in this study. Genes are assigned functional categories on the basis of available functional or biochemical annotation (boxes with light shading), and those are overlaid on cellular localization annotations.

for Rpd3-L in silencing remains a mystery. Despite deacetylating histone tail residues in a similar fashion to the Sir HDAC complex, Rpd3-L appears to oppose the activity of the Sir HDAC complex (De Rubertis *et al.* 1996). One possibility is that Rpd3-L antagonizes direct acetylation of the Sir complex. Indeed, N-terminal acetylation of Sir3 has been reported to promote silencing (Wang *et al.* 2004).

Function of H3K56 acetylation: Another protein complex identified in our screen is the Rtt109 histone H3K56 acetyltransferase complex (Figure 6). H3K56 acetylation is cell-cycle regulated and peaks in S phase due to the downregulation of the Hst3 Sir2-related HDAC that removes the modification (Celic *et al.* 2006; Maas *et al.* 2006). Mutations of H3K56 have been shown to disrupt silencing (Xu *et al.* 2007). Specifically, mutation of this residue to a state that mimics either the acetylated (K > Q) or deacetylated (K > R) lysine resulted in decreased silencing of a subtelomeric silencing reporter gene as did a mutation (K > G) that removed the side chain altogether. This loss of silencing was not associated with a decrease in silencing-protein binding; however, there is an apparent increase in chromatin accessibility in that region (Xu *et al.* 2007). These results, however, do not clarify what state of K56 is favored

for complete silencing. Mutation of *SIR2* resulted in increased K56 acetylation in silenced regions, and it has been suggested that deacetylation of this residue by Sir2 is necessary for silencing (Xu *et al.* 2007). Our data indicate a fairly modest reduction in K56 acetylation at *HMR***a** in the wild-type strain relative to euchromatic sites (Figure 5D). Thus, it may be that acetylation antagonizes silencing at a post-Sir-binding step, but its complete removal is not essential for silencing. As described above, it has been suggested that Rtt101, a cullin homolog, cooperates with Mms1 and Mms22 to affect the function of H3K56 acetylation in genome stability. However, our results indicate that these factors are dispensable for the anti-silencing function of the Rtt109 complex, indicating a branch in the pathway.

In contrast, two other factors implicated in DNA repair, Rad10 and Elg1, affect silencing. These do not seem to act by increasing the mutability of the *C. albicans URA3* reporter gene since their effects were *SIR3* dependent. Elg1, which functions in an alternative RFC-like clamp loader complex, has been implicated previously in both genome stability and silencing (Ben-Aroya *et al.* 2003). Rad10 is a single-stranded DNA endonuclease involved in nucleotide excision repair (Prakash 1977). Precisely how these factors control silencing remains unknown, but they suggest an intriguing link between DNA repair factors and the regulation of sirtuin function. Such links are not without precedent. For example, Sir2 itself suppresses recombination between the rDNA repeats (Gottlieb and Esposito 1989).

Negative regulation of Sir2 by factors linked to metabolism: Given the established role of Sir2 and its orthologs in aging and metabolism in a variety of organisms, there is a great deal of interest in understanding how Sir2 activity is globally regulated. Current thinking focuses mostly on the fact that sirtuins require NAD for their decetylase and/or ADP-ribosylation activities, but other mechanisms of control have not been ruled out. Three proteins identified in our screen seem notable in this respect. First, we identify the PAS domain kinase Psk2 as a negative regulator of Sir2. This factor has been shown to phosphorylate a number of proteins involved in metabolism and translation (Rutter *et al.* 2002). Therefore, it is possible that Psk2 could regulate Sir2 in response to metabolic conditions. However, what the PAS domain binds to or senses and the biologically relevant outputs of this conserved kinase remain poorly understood.

It is intriguing to note that one of the factors identified in our screen, Ygl081w, contains a FHA domain. Such domains in other proteins have been shown to be a binding motif for phosphorylated proteins (Li *et al.* 2004). Likewise, our screen identified Lap3, a protein originally identified as a bleomycin hydrolase, but that is thought to function to limit the levels of the toxic metabolic side-product homocysteine in cells (Xu and Johnston 1994). One hypothetical possibility is that Lap3 limits silencing by hydrolyzing the *o*-acetyl-ADP-ribose product of the Sir2 deacetylase reaction, which has been shown *in vitro* to promote the assembly of the Sir complex. If Lap3 and Psk2 functions are related, perhaps the PAS domain of Psk2 binds *o*-acetyl-ADP-ribose. Finally, we note that Isa2, identified in our screen, is required for the maturation of Fe-S cluster proteins, again suggesting a link between Sir2 and metabolism. Further analysis of the genetic relationships of these genes should help to elucidate the pathways by which these genes control sirtuin outputs.

Conclusion: Our screen represents the most comprehensive survey to date for negative regulators of sirtuin activity in any system. Our survey identified 40 genes, including 20 genes not previously known to be involved in regulating Sir. The precise mechanisms by which they function in this context and their relationship to each other remain fertile ground for future investigation. Although we have emphasized potential regulation of Sir2 activity, any of the factors we describe could act by affecting steps in the expression of Sir proteins, ranging from synthesis to degradation of the corresponding mRNAs and proteins. Likewise, given that there are limited pools of Sir2 in the cell and competition for Sir2 association between the silent cassettes, telomeres, and the rDNA, it is possible that some of the mutations described here affect that Sir2 distribution. We note, however, that the rDNA-specific loss-of-silencing mutations described previously (Smith and Boeke 1997) were not identified in our screen with the exception of *cac1*. Regardless of the mechanism of action of the individual factors, we hope that our results will provide a useful resource for the field for future investigation of sirtuin regulation. In particular, the exploration of links between Sir2 and processes of DNA repair and metabolic control are likely to lead to advances in our understanding of Sir2 function and control. Given the central importance of this family of regulators in biology, understanding how they are controlled in *S. cerevisiae* may ultimately provide insights into the regulation of the sirtuins in humans, which have been suggested to play roles in common maladies ranging from aging to diabetes to cancer.

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