Antagonistic roles for the ubiquitin ligase Asr1 and the ubiquitin-specific protease Ubp3 in subtelomeric gene silencing

Tyler S. McCann[a], Yan Guo[b], W. Hayes McDonald[c,d], and William P. Tansey[a,1]

[a]Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232; [b]Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN 37232; [c]Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37025; and [d]The Mass Spectrometry Research Center, Vanderbilt University, Nashville, TN 37027

Ubiquitin, and components of the ubiquitin–proteasome system, feature extensively in the regulation of gene transcription. Although there are many examples of how ubiquitin controls the activity of transcriptional regulators and coregulators, there are few examples of core components of the transcriptional machinery that are directly controlled by ubiquitin-dependent processes. The budding yeast protein Asr1 is the prototypical member of the RPC (RING, PHD, CBD) family of ubiquitin-ligases, characterized by the presence of amino-terminal RING (really interesting new gene) and PHD (plant homeo domain) fingers and a carboxyl-terminal domain that directly binds the largest subunit of RNA polymerase II (pol II), Rpb1, in response to phosphorylation events tied to the initiation of transcription. Asr1-mediated oligo-ubiquitylation of pol II leads to ejection of two core subunits of the enzyme and is associated with inhibition of polymerase function. Here, we present evidence that Asr1-mediated ubiquitylation of pol II is required for silencing of subtelomeric gene transcription. We show that Asr1 associates with telomere-proximal chromatin and that disruption of the ubiquitin-ligase activity of Asr1—or mutation of ubiquitylation sites within Rpb1—induces transcription of silenced gene sequences. In addition, we report that Asr1 associates with the Ubp3 deubiquitylase and that Asr1 and Ubp3 play antagonistic roles in setting transcription levels from silenced genes. We suggest that control of pol II by nonproteolytic ubiquitylation provides a mechanism to enforce silencing by transient and reversible inhibition of pol II activity at subtelomeric chromatin.

ubiquitin | chromatin | gene silencing | transcription

The ubiquitin (Ub)–proteasome system features in a multitude of cellular processes, including the regulation of transcription (1). Over the past two decades, it has become clear that Ub-dependent processes impact multiple steps in gene expression, ranging from signaling histone modifications (2) to coordinating events of mRNA export from the nucleus (3). Many of the functions of Ub in transcription are directed against regulatory factors, and there are only a handful of examples in which core components of the transcriptional machinery are regulated by Ub-dependent transactions. The best understood example from this category is the Ub-mediated destruction of the largest subunit of RNA polymerase II (pol II) in response to DNA damage (4), which functions as a failsafe mechanism to remove stalled pol II complexes from damaged DNA segments. Beyond a damage response, however, it is unclear whether ubiquitylation controls the normal activity of pol II.

We previously reported that the *Saccharomyces cerevisiae* protein Asr1 is a RING (really interesting new gene) finger Ub-ligase that targets RNA polymerase II for nonproteolytic ubiquitylation (5). Asr1 defines the evolutionarily preserved RPC (RING, PHD, CBD) family of Ub-ligases, characterized by the presence of amino-terminal RING and PHD (plant homeo domain) fingers and a domain that binds to the carboxy-terminal domain (CTD) of the largest subunit of pol II, Rpb1 (Fig. S1A). Binding of the CTD-binding domain (CBD) of Asr1 to Rpb1 depends on

phosphorylation of serine residue 5 (Ser5) within the Rpb1 CTD repeats (5), a modification that occurs commensurate with the initiation of transcription. When bound to pol II, Asr1 oligo-ubiquitylates five lysine residues adjacent to the Rpb1 CTD, as well as unknown residues within Rpb2, the result of which is the ejection of two core subunits of the enzyme—Rpb4/Rpb7 (5). Interestingly, despite association of Asr1 with the 10 subunits of pol II required for transcriptional elongation (6), the form of pol II that copurifies with Asr1 is catalytically inactive (5), suggesting that Asr1 both modifies and inactivates RNA polymerase. Whether inactivation is linked to expulsion of Rpb4/7, or polymerase ubiquitylation per se, is unknown.

Despite the well-characterized biochemical actions of Asr1, a physiological role for this protein has remained enigmatic. Indeed, *asr1*-null cells are phenotypically normal (5), leaving open the question of what biological endpoint is mediated via interaction of Asr1 with pol II. Here, we demonstrate that the Ub-ligase activity of Asr1 is required for silencing of subtelomeric chromatin. We show that Asr1 associates with telomere-proximal genes and demonstrate that some of the prosilencing actions of Asr1 are mediated via ubiquitylation of Rpb1. We also demonstrate that Asr1 associates with the Ub-specific protease Ubp3 and recruits it to pol II and that Asr1 and Ubp3 act antagonistically to set expression levels of subtelomeric genes.

Significance

Ubiquitylation is integral to a myriad of cellular processes, including protein destruction, cell cycle control, and regulation of gene activity. Here, we show that ubiquitylation plays a role in inactivating the expression of genes that are located close to telomeres. We present evidence that the ubiquitin ligase Asr1 associates with subtelomeric DNA and ubiquitylates RNA polymerase II to prevent it from transcribing genes at these locations. We also show that Asr1 interacts with Ubp3, an enzyme that reverses ubiquitylation, and that the two play antagonistic roles in silencing subtelomeric genes. These findings show how ubiquitylation of a core component of the transcriptional machinery impacts gene activity and reveal a mechanism for controlling the expression of telomere-proximal genes.
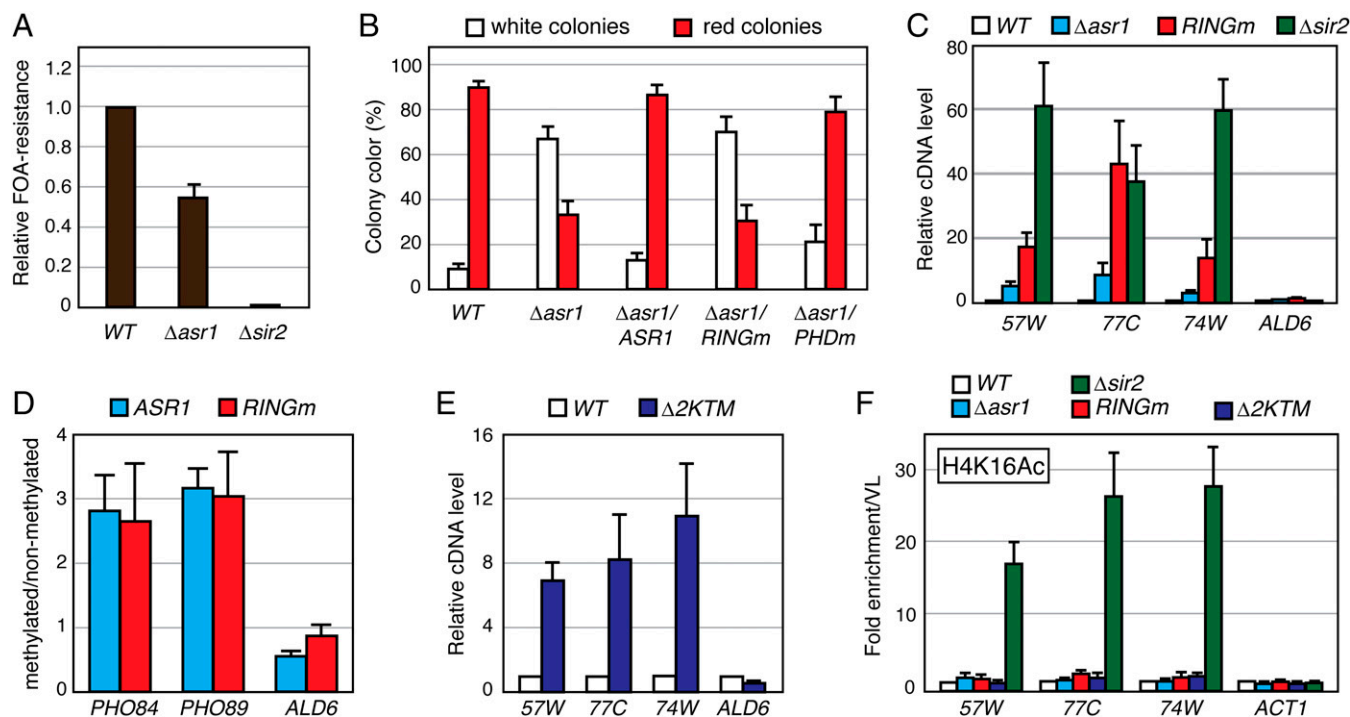
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Fig. 1. The ubiquitin ligase activity of Asr1 is required for subtelomeric gene silencing. (*A*) Asr1 is required for full silencing of a telomere-proximal *URA3* reporter. Equal amounts of yeast (*WT*, LPY4819; Δ*asr1*, LPY4819 ΔAsr1; Δ*sir2*, LPY4977) were plated on media with or without 5-FOA, and the relative number of FOA-resistant colonies was calculated. Error bars represent SEM (*n* = 3). (*B*) The Ub-ligase activity of Asr1 is required for silencing of a telomere-proximal *ADE2* reporter. Yeasts (*WT*, UTAT TM4; Δ*asr1*, UTAT TM5; Δ*asr1/ASR1*, UTAT TM6; Δ*asr1/asr1*[RINGm], UTAT TM7; Δ*asr1/ASR1*[PHDm], UTAT TM8) were plated on nonselective media, and the ratio of white and red colonies was calculated. Error bars represent SEM (*n* = 3). (*C*) The Ub-ligase activity of Asr1 is required for silencing telomere-proximal genes. Total RNA was extracted from yeast (*WT*, BY4741; Δ*asr1*, YTM5; *asr1*[RINGm], YTM27; Δ*sir2*, ΔSir2), and RT–QPCR was used to measure transcript levels from the indicated loci (*57W*, *YFR057W*; *77C*, *YNR077C*; *74W*, *YCL074W*; *ALD6*). Error bars represent SEM (*n* = 6–8). (*D*) Asr1 associates with subtelomeric chromatin. DNA was isolated from congenic strains expressing either an Asr1–DAM (YTM38) or Asr1[RINGm]–DAM (YTM39) fusion, or unfused DAM expressed from the *ASR1* promoter (YTM40). DNA was then either untreated, or cleaved with DpnII. QPCR was performed using primers flanking a DpnII site within each gene to determine the extent of cutting. Signals for each fusion strain were normalized to that from the strain expressing unfused DAM protein, and data were plotted as the ratio of methylated/nonmethylated DNA. Error bars represent SEM (*n* = 5). (*E*) Asr1-dependent ubiquitylation sites on Rpb1 are required for silencing. The experiment was performed as in *C*, except on RNA isolated from WT yeast (BY4742) or yeast expressing the Rpb1–Δ2KTM mutant (YTM29). Error bars represent SEM (*n* = 4–5). (*F*) Loss of silencing in Asr1-mutant cells is not accompanied by an increase in H4K16 acetylation. ChIP was performed on chromatin isolated from the indicated strains (*WT*, BY4741; Δ*asr1*, YTM5; *asr1*[RINGm], YTM27; *Rpb1*–Δ2KTM, YTM29; Δ*sir2*, ΔSir2), using an anti-H4K16 acetylation (H4K16Ac)-specific antibody. Coprecipitating DNAs were quantified by QPCR and normalized to the signal from a primer set that amplifies an intergenic portion on the left arm of chromosome V. Error bars represent SEM (*n* = 4).

Results and Discussion

The Ub-Ligase Activity of Asr1 Is Required for Subtelomeric Gene Silencing. We performed comparative transcriptomic analysis (RNA-seq) on RNA isolated from yeast expressing either WT Asr1 or a RING finger Asr1 mutant (Asr1[RINGm]) that lacks Ub-ligase activity (5). This analysis identified 27 genes that are induced, and 29 that are repressed, by a factor of two or more in *Asr1*[RINGm] mutant cells (Table S1). Genes in each category did not cluster according to ontology, but we noticed that 33% of genes in the induced set lie within 50 kb of a telomere. In contrast, for repressed genes, only 7% were telomere-proximal. The induction of subtelomeric gene expression in *Asr1*[RINGm] cells suggests that the Ub-ligase activity of Asr1 may be required for authentic patterns of subtelomeric silencing.

To determine whether Asr1 is involved in silencing, we monitored the effect of mutations in Asr1 on expression of one of two reporters, *URA3* (7) or *ADE2* (8), integrated at telomere-proximal DNA on the right arm of chromosome V. Deletion of the silent information regulator *SIR2* was included as a positive control. Silencing of *URA3* renders yeast resistant to 5-fluoroorotic acid (FOA); silencing of *ADE2* produces red colonies. In both assays, deletion of *ASR1*—or mutation of its RING finger—reduced the extent of silencing, as measured by a decrease in the number of FOA-resistant (Fig. 1*A*) and red (Fig. 1*B* and Fig. S1 *B* and *C*) colonies. For a separate assessment of the extent to which silencing

is perturbed in Asr1 mutant cells, we used reverse transcription quantitative PCR (RT–QPCR) to measure transcript levels from three telomere-proximal genes we have shown to be silenced (9) but were not detected in our RNA-seq analysis: *YFR057W*, *YNR077C*, and *YCL074W*. Here (Fig. 1*C*), deletion of *ASR1* resulted in a two- to fivefold increase in the expression of all three genes, whereas mutation of the Asr1 RING increased telomere-proximal gene expression by a factor of 15- to 40-fold. In contrast, expression of the euchromatic *ALD6* gene was unaffected by mutation of Asr1. Together, these data demonstrate that the Ub-ligase activity of Asr1 is required for subtelomeric gene silencing.

Next, we asked whether Asr1 associates with subtelomeric chromatin. For this purpose, we performed DNA adenine methyltransferase identification (DamID) (10). Here, Asr1 is expressed in yeast as a fusion to the *Escherichia coli* DNA adenine methyltransferase. If Asr1 binds chromatin, the DNA at that site will be ectopically methylated and resist cleavage by a methylation-sensitive restriction enzyme. Using this approach (Fig. 1*D*), we observed that both WT Asr1 and the Asr1[RINGm] mutant associate with the telomere-proximal *PHO84* and *PHO89* genes, two of the most strongly-induced genes in *Asr1*[RINGm] mutant cells (Table S1). The euchromatic *ALD6* gene, in contrast, was not appreciably methylated. This result suggests that Asr1 physically interacts with telomere-proximal chromatin and does so in a RING finger-independent manner.
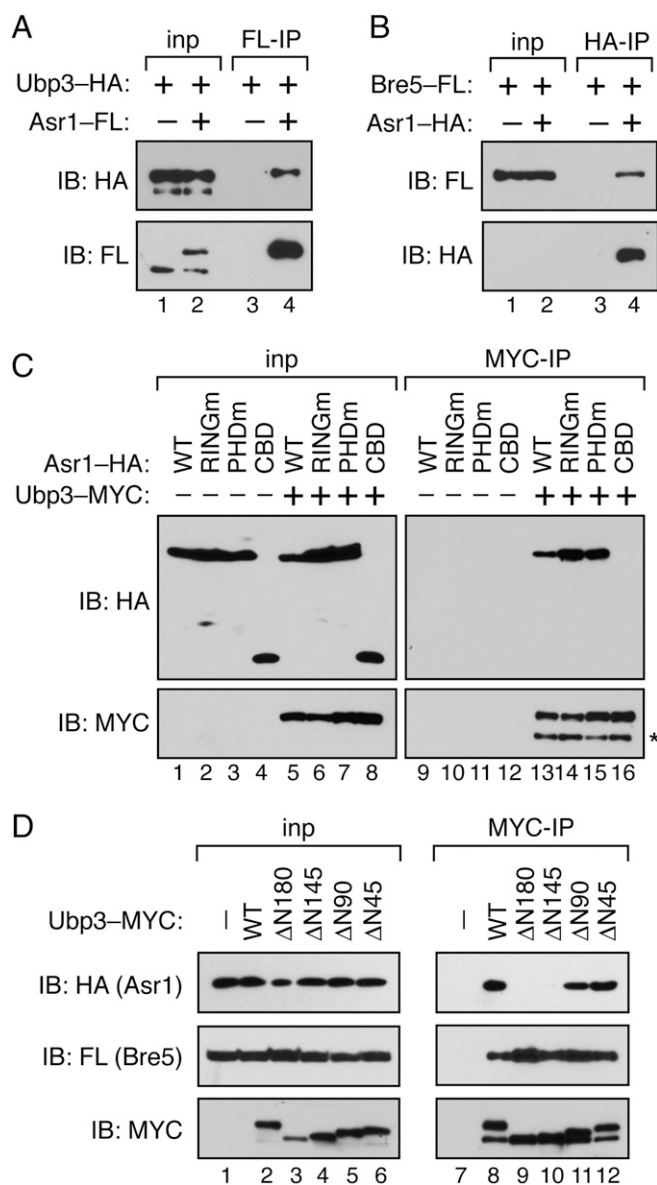
Fig. 2. Asr1 associates with the Ubp3/Bre5 deubiquitylase. (*A*) Asr1 coprecipitates with Ubp3. Extract was prepared from cells expressing HA-tagged Ubp3, either alone (YTM1) or in conjunction with FLAG (FL)-tagged Asr1 (YTM2). Immunoprecipitation (IP) was performed with an anti-FLAG antibody (F-IP), and products were subjected to immunoblotting (IB) with anti-HA and -FLAG antibodies. For Ubp3–HA, 1% of the input (inp) to the IP was also analyzed by IB; for Asr1–FLAG, 7.5% of the input was analyzed. (*B*) Asr1 coprecipitates with Bre5. Extract was prepared from cells expressing FLAG (FL)-tagged Bre5, either alone (YTM3) or in conjunction with HA-tagged Asr1 (YTM4). IP was performed with an anti-HA antibody (H-IP), and products were subjected to IB with anti-HA and -FLAG antibodies. For Bre5–FL, 1% of the input (inp) to the IP was analyzed by IB; for Asr1–HA, 7.5% of the input was analyzed. (*C*) The amino terminus of Asr1 is required for association with Ubp3. Extract was prepared from yeast cells expressing galactose-inducible HA-tagged Asr1 proteins, either alone (WT, YTM9; Asr1[RINGm], YTM10; Asr1[PHDm], YTM11; CBD, YTM12) or in the presence of a plasmid expressing MYC-tagged Ubp3 (WT, YTM13; Asr1[RINGm], YTM14; Asr1[PHDm], YTM15; CBD, YTM16). IP was performed with an anti-MYC antibody (MYC-IP), and products were subjected to IB with anti-HA and -MYC antibodies. The * indicates a low molecular weight MYC-reactive species that we assume is a degradation product of Ubp3 that forms during the IP. (*D*) The amino terminus of Ubp3 mediates interaction with Asr1. Extract was prepared from yeast cells expressing galactose-inducible HA-tagged Asr1 and FLAG-tagged Bre5, either alone (–, YTM9) or in the presence of a plasmid expressing MYC–tagged Ubp3 proteins (WT, YTM13; ΔN180, YTM17; ΔN145, YTM18; ΔN90,

To determine whether ubiquitylation of Rpb1 is relevant to the involvement of Asr1 in subtelomeric gene silencing, we asked whether silencing is perturbed in cells that express a mutant of Rpb1 in which sites of Asr1-mediated ubiquitylation are mutated (Δ2KTM) (5). Indeed, we observed increased expression of *YFR057W*, *YNR077C*, and *YCL074W*—but not *ALD6*—in the presence of the Δ2KTM Rpb1 mutant (Fig. 1*E*). Notably, induction of these genes was three- to fivefold lower than we observed in the presence of the Asr1[RINGm] mutant (Fig. 1*C*), revealing that loss of ubiquitylation of Rpb1 only partially contributes to the loss of gene silencing that we observed in the presence of inactive Asr1. We conclude that Asr1-mediated ubiquitylation of Rpb1 is involved in repression of telomere-proximal gene expression and that Asr1 ubiquitylates other substrates to silence gene activity at these sites.

Finally, we asked whether induction of subtelomeric gene activity in Asr1 mutant cells is accompanied by an increase in acetylation of histone H4 at lysine 16 (H4K16) at telomeres, as observed upon deletion of *SIR2* (11). Interestingly, however, despite levels of gene induction comparable with *SIR2* deletion (Fig. 1*C*), neither *ASR1* deletion nor the RING finger mutation nor the Δ2KTM mutation in Rpb1 elicited any significant change in the H4K16 acetylation state at *YFR057W*, *YNR077C*, and *YCL074W* (Fig. 1*F*). This result demonstrates that the mechanism through which silenced genes are activated in Asr1 mutant cells differs from those connected to loss of classic Sir2-dependent silencing processes.

Ubp3 Associates with RNA Polymerase II via Asr1. Compared with deletion of *ASR1*, mutation of the Asr1 RING finger has a much more substantial effect on activation of telomere-proximal genes (Fig. 1*C*). This differential effect could be due to a dominant-negative action by the Asr1[RINGm] protein, but it is also possible that Asr1 interacts with proteins that antagonize its role in repression of telomere-proximal genes. Previously, we reported that TAP-tagged Asr1 isolated from yeast cells associates with 10/12 core subunits of pol II, excluding Rpb4 and -7 (5). To identify additional proteins that associate with Asr1, we subjected an Asr1–TAP purification to further proteomic screening, comparing Asr1–TAP with a parallel purification of Rpb1–TAP (Table 1). Two proteins that were overrepresented in the Asr1–TAP purification were the Ub-specific protease Ubp3 and its essential cofactor Bre5. The selective enrichment of Ubp3 in the Asr1–TAP preparation is intriguing because Ubp3 is known to interact with pol II and to deubiquitylate Rpb1 (12), and because Ubp3 has been shown to function as an antisilencing factor in *S. cerevisiae* (13), albeit through an unknown mechanism. We confirmed that endogenous Asr1 associates with both Ubp3 (Fig. 2*A*) and Bre5 (Fig. 2*B*), demonstrating that Ubp3 and Bre5 are bona fide Asr1-interaction partners.

Next, we mapped the regions in Asr1 and Ubp3 that mediate their association (Fig. 2*C*). We found that interaction of Asr1 with Ubp3 does not depend on the integrity of the Asr1 RING or PHD fingers but does require the amino terminus of Asr1, because expression of the CBD alone is insufficient to support interaction with Ubp3. Within Ubp3, we showed that deletion of the amino-terminal 145 or 180 residues of Ubp3 (Fig. S2) disrupts interaction with Asr1 (Fig. 2*D*). Taking these data together, we conclude that the interaction between Asr1 and Ubp3 is likely mediated via amino-terminal sequences in both proteins.

Because Asr1 and Ubp3 are both pol II-interaction partners, we considered that association of Asr1 and Ubp3 results from the simultaneous interaction of each protein with RNA polymerase II. This possibility, however, is not correct because the CBD of Asr1—which suffices for interaction with Ser5-phosphorylated Rpb1—does not associate with Ubp3 (Fig. 2*C*). We therefore considered an

YTM19; ΔN45, YTM20). IP was performed with an anti-MYC antibody (MYC-IP), and products were subjected to IB with anti-HA, -FLAG, and -MYC antibodies.

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Table 1. Validated proteins associated with Asr1-TAP

Protein	Rpb1–TAP	Asr1–TAP	Asr1/Rpb1
Rpb1	2,418	70	0.03
Rpb2	2,066	58	0.03
Rpb3	1,007	26	0.03
Rpb4	526	4	0.01
Rpb5	377	10	0.03
Rpb6	202	3	0.01
Rpb7	684	0	0.00
Rpb8	88	2	0.02
Rpb9	120	4	0.03
Rpb10	338	2	0.01
Rpb11	256	5	0.02
Rpb12	34	0	0
Asr1	1	20	20
Ubp3	13	62	4.8
Bre5	6	17	2.8

The table lists the total spectrum counts for each protein detected in the Asr1–TAP or Rpb1–TAP preparations, and the ratio of counts between them (Asr1/Rpb1). Only validated Asr1-associated proteins are presented in this list.

alternative model, in which association of Ubp3 with pol II is indirect and mediated via its interaction with Asr1. Three observations support this concept. First, the amino-terminal 180 residues of Ubp3, when fused to the green fluorescent protein (GFP), suffice for interaction with both Asr1 and the pol II subunit Rpb3 (Fig. 3*A*). Second, deletion of *ASR1* blocks the ability of Ubp3 to interact with Rpb3 and S5-phosphorylated-Rpb1 (Fig. 3*B*, lane 9). And third, the ΔN180 mutant of Ubp3, which is defective for interaction with Asr1, is also defective for interaction with Rpb1 and Rpb3 (Fig. 3*B*, lane 12). The overlapping determinants within Ubp3 for Asr1 and pol II association, and the dependence of the Ubp3-pol II interaction on Asr1, reveal that interaction of Ubp3 with RNA polymerase II is mediated via Asr1.

Antagonistic Role of Asr1 and Ubp3 in Silencing. The opposing molecular functions of Asr1 and Ubp3 prompted us to ask whether these proteins play antagonistic roles in silencing. As controls, we asked whether other phenotypes associated with loss of *UBP3* are impacted by mutations in Asr1, focusing on (*i*) increased sensitivity to 6-azauracil (6-AU) (12), (*ii*) increased resistance to the DNA-methylating agent methyl methanesulfonate (MMS) (14), and (*iii*) increased sensitivity to rapamycin (15). For all three agents, we reproduced the effects of *UBP3* deletion but saw no synthetic interactions with an *ASR1* deletion or RING finger mutation (Fig. S3 *A–C*). We also found that the ΔN145 and ΔN180 mutants of Ubp3 had WT activity in these assays (Fig. S3 *D–F*), revealing that interaction with Asr1 is not connected to the known functions of Ubp3 in these phenotypes.

When we probed for silencing, however, we observed strong genetic interactions between *UBP3* and *ASR1*. In the *URA3* reporter assay, deletion of *UBP3* resulted in a striking increase in the number of FOA-resistant colonies (Fig. 4*A*), consistent with reports that loss of Ubp3 increases telomere-proximal silencing (13). Importantly, simultaneous deletion of *ASR1* reversed this increase, demonstrating that Asr1 opposes the function of Ubp3 in this context. Interestingly, the level of silencing of the *URA3* reporter in Δ*ubp3*Δ*asr1* cells is still higher than in Δ*asr1* cells, revealing that Ubp3 must have Asr1-independent functions that repress the silent chromatin state. In the *ADE2* reporter strain, the majority of colonies were red in WT cells (Fig. 4*B*), making it impossible to observe any increase in silencing, but here we also found the extent of silencing to be significantly reduced in Δ*ubp3*Δ*asr1* cells, compared with the Δ*ubp3* mutation alone. Direct analysis of transcript levels from the telomere-proximal *PHO12*, *PHO84*, and *PHO89* genes revealed a similar pattern of behavior, with the induction of

expression of all three genes observed in the presence of the Asr1^{RINGm} mutant blocked by deletion of *UBP3* (Fig. 4*C*). These data demonstrate that Asr1 and Ubp3 antagonistically control subtelomeric gene silencing, and that the association of these two proteins in this context is distinct from the function of Ubp3 in mediating sensitivity to 6-AU, MMS, and rapamycin.

Consistent with the idea that at least part of the antagonistic actions of Asr1 and Ubp3 is mediated via Rpb1 ubiquitylation, we found that deletion of *UBP3* partially reverses the decrease in the extent of ubiquitylation of S5-phosphorylated-Rpb1 observed in both Δ*asr1* and *asr1*^{RINGm} cells (Fig. 4*D*). The links between Ubp3 and Asr1 in this process are strengthened by the observation that overexpression of WT Ubp3, but not the Asr1 interaction-defective ΔN180 Ubp3 mutant (Fig. 4*E*), results in an almost complete loss of silencing in this context (Fig. 4*F*). Moreover, we found that
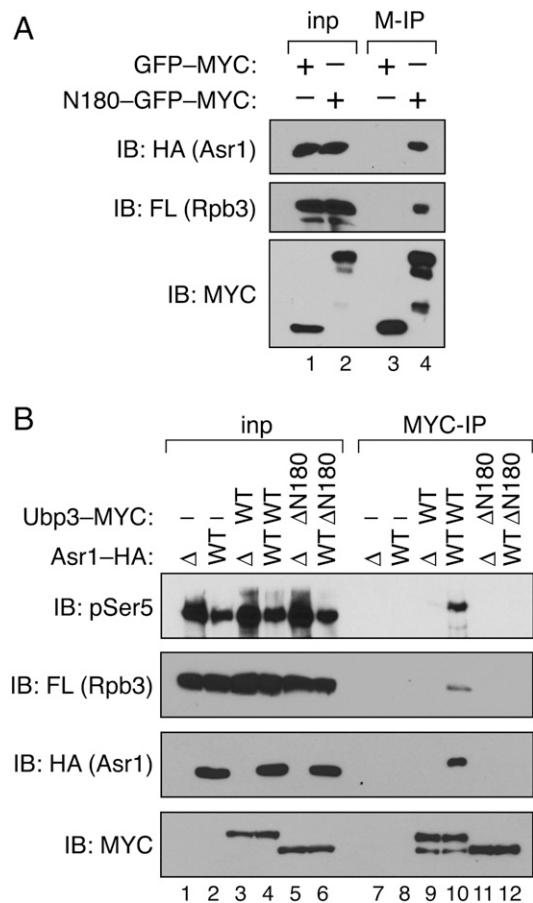

Fig. 3. Interaction of Ubp3 with RNA polymerase II is mediated via Asr1. (*A*) The amino terminus of Ubp3 suffices for interaction with Asr1 and pol II. Extract was prepared from cells expressing HA-tagged Asr1, FLAG-tagged Rpb3, and either MYC-tagged GFP alone (YTM43), or MYC-tagged GFP fused to the amino-terminal 180 residues of Ubp3 (YTM42). Immunoprecipitation (IP) was performed with an anti-MYC antibody (M-IP), and products were subjected to immunoblotting (IB) with anti-HA, -FLAG, and -MYC antibodies. For Rpb3–FLAG and Asr1–HA, 2.5% of the input was analyzed; for Ubp3–MYC, 0.1% of the input was analyzed. (*B*) Ubp3 requires Asr1 to interact with pol II. Extracts were prepared from yeast expressing FLAG-tagged Rpb3, and carrying combinations of (*i*) an *ASR1* gene deletion (Δ) or expression of WT HA-tagged Asr1, and (*ii*) WT MYC-tagged Ubp3 (WT) or the ΔN180 MYC-tagged Ubp3 mutant (lanes 1 and 7, YTM21; lanes 2 and 8, YTM22; lanes 3 and 9, YTM23; lanes 4 and 10, YTM24; lanes 5 and 11, YTM25; lanes 6 and 12, YTM26). IP was performed with an anti-MYC antibody (MYC-IP), and products were subjected to IB with anti-pSer5, -FLAG, -HA, and -MYC antibodies. For pSer5, Rpb3–FLAG, and Asr1–HA, 2.5% of the input was analyzed; for Ubp3–MYC, 0.1% of the input was analyzed.
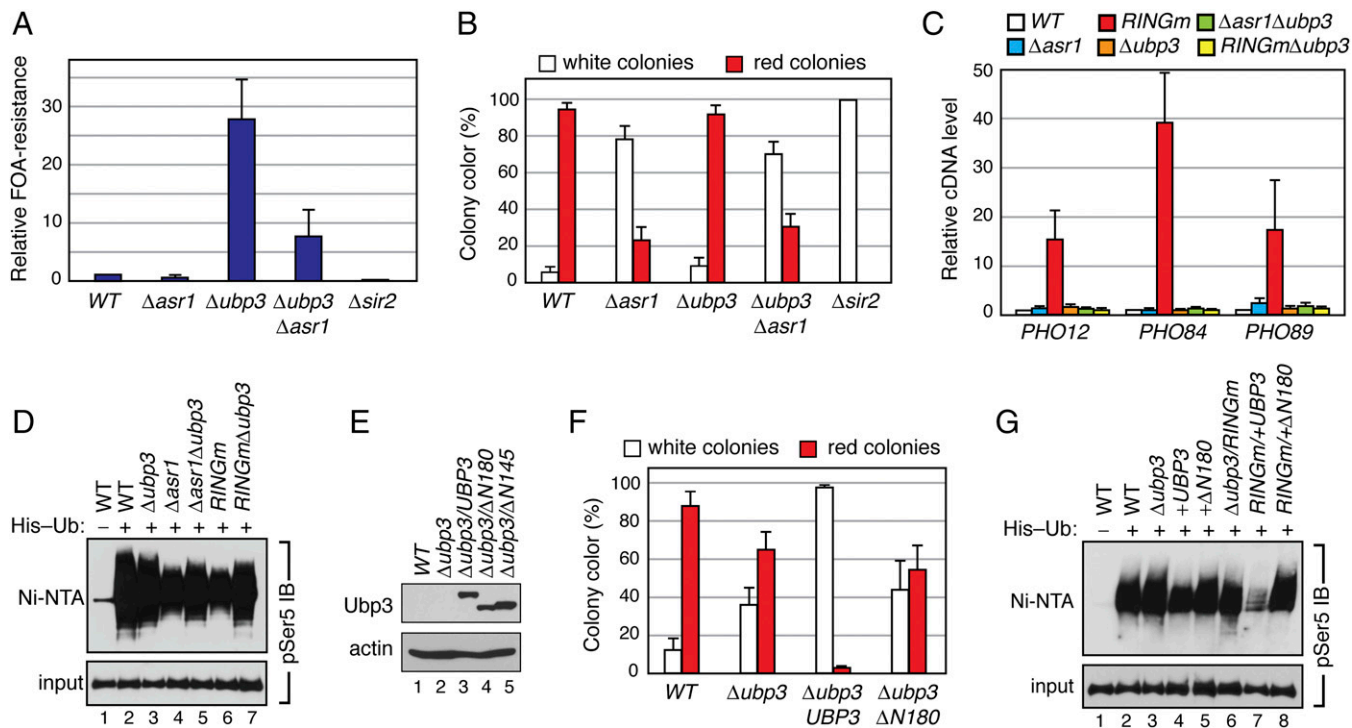
Fig. 4. Asr1 and Ubp3 act antagonistically in silencing of subtelomeric chromatin. (*A*) Deletion of *ASR1* attenuates the impact of *UBP3* deletion on silencing. Equal amounts of yeast (*WT*, LPY4819; Δ*asr1*, LPY4819 ΔAsr1; Δ*ubp3*, LPY4819 ΔUbp3; Δ*ubp3Δasr1*, LPY4819 ΔAsr1 ΔUbp3; Δ*sir2*, LPY4977) were plated on media with or without 5-FOA, and colonies were counted. Error bars represent SEM (*n* = 3). (*B*) Loss of *ASR1* is epistatic to loss of *UBP3*. Yeast (*WT*, YPH499UTAT; Δ*asr1*, UTAT TM1; Δ*ubp3*, UTAT TM2; Δ*asr1Δubp3*, UTAT TM3; Δ*sir2*, UTAT TM12) were plated on nonselective media (CSM), and the ratio of white and red colonies for each plate was calculated. Error bars represent SEM (*n* = 3). (*C*) Deletion of *UBP3* reverses the impact of the Asr1^{RING} mutation deletion on silencing. Total RNA was extracted from yeast (*WT*, BY4741; Δ*asr1*, YTM5; *asr1^{RINGm}*, YTM27; Δ*ubp3*, ΔUbp3; Δ*asr1Δubp3*, YTM6; *asr1^{RINGm}Δubp3*, YTM28), and RT–QPCR was used to measure transcript levels from the indicated loci. The expression level of each gene in WT (*WT*) congenic cells was set to one. Error bars represent SEM (*n* = 8). (*D*) Asr1 and Ubp3 oppositely impact ubiquitylation of serine 5-phosphorylated Rpb1. Denaturing extracts were prepared from a WT yeast strain (lane 1, YTM31), or yeast expressing polyhistidine-tagged ubiquitin (His–Ub) and carrying the indicated genotype (*WT*, YTM32; Δ*ubp3*, YTM35; Δ*asr1*, YTM33; Δ*asr1Δubp3*, YTM36; *asr1^{RINGm}*, YTM34; *asr1^{RINGm}Δubp3*, YTM37). Ubiquitylated proteins were recovered on Ni-NTA resin. Levels of serine 5-phosphorylated Rpb1 in the Ni-NTA–bound material and a sample of the input were detected by immunoblotting with a pSer5-specific antibody (pSer5 IB). (*E*) Steady-state levels of Ubp3 deletion mutants assayed in *F*. Ubp3 was MYC-tagged and visualized by an anti-MYC IB. An antibody against actin was used as a loading control. (*F*). The amino terminus of Ubp3 is required for its prosilencing function. Yeast cells (*WT*, UTAT TM4; Δ*ubp3*, UTAT TM9; Δ*ubp3UBP3*, UTAT TM10; Δ*ubp3ΔN180*, UTAT TM11) were plated to single colony density on leucine drop-out media, and the ratio of white and red colonies for each plate was calculated. Error bars represent SEM (*n* = 3). (*G*) The amino terminus of Ubp3 is required for its effects on ubiquitylation of serine 5-phosphorylated Rpb1. A His–Ub assay, as in *D*, was performed on a WT yeast strain (lane 1, YTM49) or yeast expressing polyhistidine-tagged ubiquitin (His–Ub) and carrying the indicated genotype (*WT*, YTM50; Δ*ubp3*, YTM51; +*UBP3*, YTM52; +Δ*N180*, YTM53; Δ*ubp3/RINGm*, YTM54; *RINGm/+UBP3*, YTM55; *RINGm/+N180Δ*, YTM56).

overexpression of Ubp3 reduces the extent of ubiquitylation of S5-phosphorylated Rpb1 (Fig. 4*G*), a phenomenon that is more prominent in the presence of the catalytically inactive Asr1^{RINGm} protein. Notably, in both cases, this action of Ubp3 is blocked by the ΔN180 mutation. Together, these data demonstrate that Asr1 and Ubp3 antagonistically control Rpb1 ubiquitylation and that the ability of Ubp3 to target S5-phosphorylated Rpb1 for deubiquitylation depends on its interaction with Asr1.

Many genes induced in response to the Asr1^{RING} mutation are located far from telomeres. To determine whether the connection between Asr1 and Ubp3 extends beyond telomere-proximal genes, we selected three robustly induced euchromatic genes from Table S1 and asked how their expression is changed in response to mutations in both proteins (Fig. S4). At *YBR056C–B* and *PRM7*, we observed modest induction in the Δ*asr1* and *asr1^{RINGm}* strains, but these levels were not impacted by additional disruption of *UBP3*. At *SPL2*, however, we observed induction in the presence of the Asr1^{RING} mutation, which was blocked upon subsequent deletion of *UBP3*. Interestingly, and unlike the other two loci, *SPL2* expression was also induced by deletion of *SIR2*. Thus, despite lying distal to a telomere, *SPL2* is under the control of the silent information regulators. The finding that the ostensibly

euchromatic *SPL2* gene is regulated by Asr1, Ubp3, and Sir2 in a manner similar to subtelomeric loci further strengthens the notion that Asr1 and Ubp3 act antagonistically to control the expression of silent chromatin.

Conclusions

Here, we have found that the ubiquitin-ligase activity of Asr1 is required for silencing of at least a subset of subtelomeric loci and have shown that part of this activity is mediated via ubiquitylation of Rpb1. We have also found that Asr1 recruits the deubiquitylating enzyme Ubp3 to pol II and that Asr1 and Ubp3 play antagonistic roles in controlling Rpb1 ubiquitylation and expression levels from subtelomeric chromatin. Based on our observations, we conclude that Asr1 and Ubp3 associate with one another, as demonstrated for other Ub-ligase/Ub-protease pairs such as BRCA1 and BAP1 (16). We suggest that Ubp3 is recruited to pol II via the CTD-binding ability of Asr1 and that the relevant enzymatic activities of each are directed against factors involved in silencing telomeric chromatin, one of which is the serine 5-phosphorylated Rpb1.

Our model for how Asr1/Ubp3 control transcription at telomere-proximal genes is as follows. We hypothesize that pol II that has initiated transcription at these sites is phosphorylated at serine 5

within the CTD. These phosphorylation events are then recognized by the CBD of Asr1, which leads to the recruitment of Asr1/Ubp3/Bre5. At this point, we propose that Asr1 ubiquitylates Rpb1—and likely other factors—the result of which is ejection of the Rpb4/7 heterodimer and termination of subtelomeric transcription. Although Rpb4/7 are dispensable for transcriptional elongation in vitro (6), this model is compatible with our observation that Asr1-associated pol II is catalytically inactive (5) and that transcriptional elongation by pol II in vivo can be stimulated by Rpb4/7 (17–19). After transcription is terminated, we suggest that corecruitment of Ubp3 with Asr1 allows rapid reversal of Rpb1 ubiquitylation, reassembly of the pol II complex, and restoration of polymerase function. In this way, nonproteolytic ubiquitylation of Rpb1 by Asr1 acts as a transient and reversible second tier mechanism for silencing telomere-proximal gene activity, over and above control via the silent information regulators.

Materials and Methods

Yeast Strains and Plasmids. Yeast strains and plasmids are listed in Tables S2 and S3, respectively.

RNA Sequencing and Analysis. RNA from strains BY4742 and YTM27 was isolated by hot acidic phenol extraction. Ribosomal RNA reduction was performed, and RNA was fragmented and converted to cDNA. Library preparation was performed using the NEBNext DNA Library Prep Master Mix Set for Illumina (NEB). Then, 50 million single-end reads were obtained for each sample on an Illumina HiSeq2500 Sequencer. RNA-seq data are deposited at GEO (accession no. GSE72740).

Tandem Affinity Purification and Proteomics. The tandem affinity purification (TAP) procedure was performed as described (5) using yeast strains Asr1–TAP

and Rpb1–TAP. After the final elution step, proteins were concentrated by TCA precipitation, and samples were subjected to trypsin digestions and analysis using an 8-step MudPIT as described (20, 21). Peptide spectral data were searched against an *S. cerevisiae* protein database using Sequest (22).

Reporter Gene Assays for Subtelomeric Gene Silencing. For *URA3* reporter strains, logarithmic cultures of yeast were diluted, divided in two, and spread onto complete synthetic defined media (CSM) plates, or CSM plates containing 5-FOA. Resultant colonies were counted, and relative FOA resistance was calculated by dividing the number of colonies on CSM–FOA by the number on the CSM control media. For *ADE2* reporter assays, cells were diluted and spread onto CSM (or CSM-Leu plates). The percentage colony color was determined by calculating the percentage of red and white colonies for each strain.

DNA Adenine Methyltransferase Identification. DamID was performed as described (23), with minor modifications. Genomic DNA was isolated from three yeast strains: YTM38, which expresses a WT Asr1–Dam fusion; YTM39, which expresses a RING mutant Asr1–Dam fusion; and YTM40, which expresses Dam alone under the control of the *ASR1* promoter. After purification, DNA was either undigested, or digested with DpnI. The extent of cleavage was determined by using QPCR to amplify gene-specific segments that include a DpnII site. The ratio of methylated/nonmethylated DNA for each site was calculated by determining the extent of DNA cleavage in Dam fusion strains, compared with the unfused Dam strain.

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