Recruitment and allosteric stimulation of a histone deubiquitinating enzyme during heterochromatin assembly

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**ABSTRACT**

Heterochromatin formation in budding yeast is regulated by the silent information regulator (SIR) complex. The SIR complex comprises the NAD-dependent deacetylase Sir2, the scaffolding protein Sir4, and the nucleosome-binding protein Sir3. Transcriptionally active regions present a challenge to SIR complex-mediated \textit{de novo} heterochromatic silencing due to the presence of antagonistic histone PTMs, including acetylation and methylation. Methylation of histone H3K4 and H3K79 are dependent on mono-ubiquitination of histone H2B (H2B-Ub). The SIR complex cannot erase H2B-Ub or histone methylation on its own. The deubiquitinase (DUB) Ubp10 is thought to promote heterochromatic silencing by maintaining low H2B-Ub at sub-telomeres. Here, we biochemically characterize the interactions between Ubp10 and the SIR complex machinery. We demonstrate that a direct interaction between Ubp10 and the Sir2/4 sub-complex facilitates Ubp10 recruitment to chromatin via a co-assembly mechanism. Using hydrolyzable H2B-Ub analogs, we show that Ubp10 activity is lower on nucleosomes compared to H2B-Ub in solution. We find that Sir2/4 stimulates Ubp10 DUB activity on nucleosomes, likely through a combination of targeting and allosteric regulation. This coupling mechanism between the silencing machinery and its DUB partner allows erasure of active PTMs and the \textit{de novo} transition of a transcriptionally active DNA region to a silent chromatin state.

**INTRODUCTION**

Eukaryotes package their genome into specific chromatin structures to regulate gene expression in response to external stimuli. In contrast to open and transcriptionally active euchromatin, heterochromatin is a condensed form of chromatin that is refractory to gene transcription and contributes to genome stability. Despite being generally condensed, heterochromatin is subjected to multiple disruptive processes, including disassembly for DNA replication. The \textit{de novo} establishment of heterochromatin requires factors that act with silencing complexes to ensure heterochromatin fidelity.

The assembly of heterochromatin domains is facilitated by silencing proteins that recognize specific histone post-translational modifications (PTMs) or the absence thereof (1,2). The presence of particular combinations of histone tail PTMs is generally believed to “code” for regions of the genome that are active or inactive (3,4). A general unmodified state or absence of histone PTMs is the “code” that promotes heterochromatic silencing in budding yeast. This is exemplified by the specific recognition of the unmodified histone H4 tail by the silencing machinery (5-7). Additionally, methylation of histone H3 lysine 4 (H3K4me) and H3 lysine 79 (H3K79me)
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antagonize silencing (8-12). Heterochromatin assembly is coupled to enzymatic conversion of the histone code from the active to inactive state. For example, lysine deacetylase enzymes are frequently core components of gene repression complexes, removing histone acetylation, which otherwise promotes transcription.

Three general steps in establishing heterochromatin involve 1) recruitment of the silencing complex to the target locus, 2) nucleation via histone-modifying activities, and 3) spreading of the silencing complexes due to iterative cycles of recruitment and nucleation. In budding yeast, silent information regulator (SIR) complex-dependent heterochromatin is a well-studied and simplified silencing model (13-15). The SIR complex is composed of three proteins, Sir2, Sir3, and Sir4. Sir2 is the founding member of the Sir2p family and is a histone lysine deacetylase whose activity is necessary for nucleation of the SIR complex (16-18). Sir4 mediates protein-protein interactions within the complex and between other interacting partners. Sir3 is required for heterochromatic spreading and silencing due in part to strong association with unmodified H3 and H4 tails (6,7,19,20).

Active regions of the genome that will undergo heterochromatin remodeling contain euchromatin-promoting PTMs. Several active marks are known to be anti-silencing, such as H3K4me3 and H3K79me3, as they interfere with SIR complex-mediated silencing and prevent association of the SIR complex with euchromatic regions (8-12,21-24) (Fig 1A). Tri-methylation of H3K4 and H3K79 are dependent on Rad6/Bre1-mediated mono-ubiquitination of H2BK123 (H2B-Ub) (25-27). Collectively, H2B-Ub, H3K4me, and H3K79me are strongly associated with genomic regions that undergo transcription (11,22,28). Reduction of these euchromatin-promoting marks is important to the assembly and spreading of repressed heterochromatin domains.

Though the SIR complex is equipped to erase acetylation marks, which antagonize silencing, other proteins have been identified that work with the SIR complex to generate the fully “erased” heterochromatin histone code. One such protein is Ubp10, a deubiquitinase (DUB) with an identified role in silencing (29-32). Ubp10 regulates the levels of mono-ubiquitinated histone H2BK123 in yeast and localizes to sub-telomeric heterochromatin. A recent study identified that Ubp10 also acts within the nucleolus and interacts with factors that regulate ribosome biogenesis (33). Ubp10 genetically interacts with Sir4 and is mutually recruited with the SIR complex to the sub-telomeres (30,31,34). Changes to H2B-Ub levels when Ubp10 is deleted or overexpressed causes a disruption in telomeric silencing(32,35). Despite a genetic role for Ubp10 in silencing, the mechanism of Ubp10 targeting and regulation during SIR complex-mediated silencing is not well-understood.

In this study, we detail the molecular mechanism of how SIR complex recruits Ubp10 to chromatin and stimulates Ubp10 DUB activity to help generate the silent histone code. Using multiple biochemical approaches, we show that Ubp10 directly interacts with the Sir protein subcomplex, Sir2/4, independent of chromatin, and that this interaction likely supports the co-assembly of Ubp10 onto chromatin with SIR complex. We characterize Ubp10 DUB activity using substrates with homogenous H2B-Ub analogs. We discover that Sir4 recruits and allosterically enhances Ubp10 DUB activity on H2B-Ub mono-nucleosomes. Finally, we present a model for how this coupling mechanism allows for SIR complex-mediated heterochromatin to efficiently assemble and silence the target locus.

RESULTS

Ubp10 is recruited to SIR complex-mediated heterochromatin

We previously used stable isotope labeling of amino acids in cell culture and mass spectrometry (SILAC-MS) to identify proteins excluded from heterochromatin (36). We further analyzed these data and identified proteins that were preferentially recruited to heterochromatin and found the budding yeast deubiquitinase, Ubp10, as one of the top hits. Ubp10 was identified as a regulator of yeast heterochromatin by genetic analyses (29-31). Yeast two-hybrid analysis identified an interaction between Ubp10 and Sir4 suggesting a direct recruitment mechanism between the two proteins to chromatin (30,34). To further understand how Ubp10 is recruited to heterochromatin and contributes to silencing, we began by performing chromatin pulldown assays similar to the SILAC-MS experiments using whole cell extracts (WCE) in which Ubp10 is TAP-
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tagged endogenously. Briefly, chromatin was enzymatically assembled using purified budding yeast components including: unmodified histone octamers, Nap1 histone chaperone, Isw1a chromatin remodeler, and a biotinylated DNA template. Nucleosome spacing and periodicity were examined by performing a limited micrococcal nuclease (MNase) digestion (Fig. 1C). The chromatin substrates were then conjugated to magnetic streptavidin-coated beads for immobilization. Heterochromatin domains were further assembled by incubating recombinant SIR complex with chromatin (10,36). Assembled chromatin, in the presence or absence of SIR complex, was incubated in WCE with an ATP regeneration system. Incubations were followed by magnetic isolation of chromatin-beads and washes. Bound proteins were analyzed by Western (Fig. 1D). Ubp10 was differentially enriched on heterochromatin by roughly 5-fold in a SIR complex-dependent manner, re-confirming our previous SILAC-MS results (Fig. 1D and E).

Ubp10 recruitment to chromatin is stimulated by co-assembly with SIR complex
To determine whether Ubp10 is recruited to heterochromatin directly by the SIR complex, we performed chromatin and heterochromatin pulldowns in the presence of recombinant Ubp10 purified with a GST tag from E. coli. We utilized order of addition experiments to help determine the context in which Ubp10 gets to SIR-heterochromatin. As shown in Fig. 2A, heterochromatin was first assembled to mimic a maintained and stable state of heterochromatin before Ubp10 was added. Under these conditions, Ubp10 did not bind heterochromatin better than chromatin alone (Fig. 2B). One possible explanation for a lack of SIR complex-dependent enrichment of Ubp10 is that another protein is required to bridge Ubp10 to SIR complex. Another explanation is that Ubp10 can only be recruited to chromatin at the same time as the SIR complex, not after the SIR complex forms a stable assembly. We tested both possibilities simultaneously by changing the order of addition of the experiment (Fig. 2C). Ubp10 and SIR complex were pre-incubated and then added to chromatin. The pulldown under these conditions resulted in increased recruitment of Ubp10 by the SIR complex (6-fold increase) when compared to chromatin without SIR complex bound (Fig. 2D). These results demonstrate that Ubp10 co-assembles with SIR complex onto chromatin. We note that heterochromatin pulldowns using WCEs were performed using pre-assembled heterochromatin and resulted in an enrichment of Ubp10 binding (Fig. 1D). It is possible that pre-assembled heterochromatin is subject to activities such as chromatin remodeling which may increase the dynamics of the SIR complex, allowing Ubp10 to co-assemble. Overall, this co-assembly mechanism is strongly suggestive of Ubp10 recruitment by SIR complex during de novo formation of heterochromatin when H2B-Ub nucleosomes are most-likely to be encountered by the SIR complex in vivo.

Direct interaction with the Sir2/4 sub-complex underlies co-assembly of Ubp10 with SIR complex to chromatin
Previous yeast two-hybrid data suggested that Sir4 is a binding partner of Ubp10 in vivo (30,34) (Fig. 3A). To begin biochemical characterization of this interaction, we first tested if the purified Sir2/4 sub-complex mediates differential recruitment of Ubp10 to chromatin in WCE. The recombinant Sir2/4 sub-complex, rather than Sir4 alone, is used to maintain Sir4 integrity during the purification process (37). Sir2/4 stimulated Ubp10 association with chromatin in WCE (Fig. 3B, lanes 2 & 3). In this experiment, recombinant Sir2/4 is able to recruit Sir3 from the extract. To control for any background recruitment activity by endogenous Sir3 supplied in the extract, Sir3 was deleted in the Ubp10-TAP strain. In the absence of Sir3, Ubp10 was recruited to chromatin in a Sir2/4-dependent manner indicating that Sir3 is not required to recruit Ubp10 (Fig. 3B,C). We then tested whether Sir2/4-mediated recruitment of Ubp10 to chromatin also occurs in a purified system. Similar to the results with the full SIR complex components, Ubp10 is only differentially recruited to chromatin under conditions of co-assembly with Sir2/4 (Fig. 3, D and E). On its own, Sir3 did not preferentially recruit Ubp10 to chromatin under any condition (Fig. S-1B, lanes 5 & 6, and S-1C).

The chromatin pulldown results show that Ubp10 likely interacts with the SIR complex on chromatin through Sir4. It is possible that chromatin acts as a bridging substrate for the
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interactions in our pulldown and in previously reported yeast-two hybrid experiments that initially suggested an interaction between Sir4 and Ubp10 (30). To rule this out, we next conducted a co-immunoprecipitation (co-IP) experiment using only purified Ubp10 and Sir2/4. For the capture step, we took advantage of the calmodulin binding peptide (CBP) retained on Sir4 after Tandem Affinity Purification. Ubp10 was detected only in the presence of Sir2/4, indicating a direct interaction between Ubp10 and Sir2/4 (Fig. 3F,G). We next performed a reciprocal pulldown experiment and also tested the significance of the suggested Sir4-interacting region on Ubp10, which was previously mapped by yeast two-hybrid and shown to be necessary for telomeric silencing (34). Recombinant Ubp10 WT or Ubp10 Δ109-133 were immobilized on glutathione resin and Sir2/4 was added to the reaction. The loss of the Sir4-interacting region on Ubp10 resulted in the loss of a direct interaction with Sir2/4 (Fig. 3H, lanes 7 & 8), whereas Ubp10 WT pulled down Sir2/4 (lanes 4 & 5). In contrast, Sir3 weakly interacts with Ubp10 by co-IP indicating Sir3 does not play a significant role in Ubp10 recruitment (Fig. S-1D). Overall, these results strongly support that Ubp10 is brought to chromatin directly with SIR complex primarily through the interaction with Sir4.

**Ubp10 has independent histone-binding activity**

Ubp10 appears to exhibit limited chromatin binding in the absence of Sir proteins (Fig. 2). Ubp10 bound to chromatin better at high concentrations, however no binding to naked DNA was observed, suggesting that Ubp10 interacts with nucleosomes even in the absence of its ubiquitin substrate (Fig. 4A). Using recombinant proteins, including Ubp10, unmodified octamers, and octamers containing non-hydrolyzable H2B-Ub (H2B*Ub), we sought to determine which histones Ubp10 directly binds by performing a Glutathione pulldown. Histones are highly conserved from yeast to humans, including the site and functions of H2B ubiquitination in its C-terminal tail (K123 in *S. cerevisiae* and K120 in humans). We produced recombinant human histone octamers that contain stoichiometric H2B*Ub, where a non-hydrolyzable dithioether linkage attaches ubiquitin to lysine 120 (Fig. S-1E, Al-Afaleq and Yao, manuscript in preparation) (38,39). Importantly, these homogenous populations of octamers are free of post-translational modifications except for H2B ubiquitination.

Ubp10 pulled down not only H2A/H2B*Ub heterodimers as expected, but also unmodified H2A/H2B heterodimers and H3/H4 tetramers (Fig. 4B, lanes 4 and 7). In the pulldown conditions, H2A/H2B and H3/H4 form a stable dimer and tetramer, respectively. It is possible that Ubp10 can make simultaneous contacts with both the H2A/H2B dimer and H3/H4 tetramer, or Ubp10 may interact with either the H2A/H2B dimer or the H3/H4 tetramer. This result demonstrates that Ubp10 makes several interactions with the face of the nucleosome that permit limited binding on its own and may contribute to interactions during assembly with the SIR complex.

**Assembly of H2B-Ub into nucleosomes reduces Ubp10 deubiquitinating activity**

To determine the mechanism of Ubp10 activity regulation, we characterized Ubp10 DUB activity using ubiquitinated substrates. Ubp10 cleaves a variety of polyubiquitin chains of different linkages (K63, K11, K48) with the exception of a linear chain (linked through M1) (Fig. S-2A). To assess Ubp10 DUB activity on relevant ubiquitinated substrates, we performed in vitro DUB assays using recombinant histones that are chemically ubiquitinated at defined sites (H2AK119Ub or H2BK120Ub). These substrates contain hydrolyzable Ub-histone linkages that differ from the native isopeptide bonds by only one atom (Fig. S-1E, Al-Afaleq and Yao, manuscript in preparation).

We reconstituted H2B-Ub mono-nucleosomes by salt-dialysis and observed, as expected, reduced migration of mono-ubiquitinated nucleosomes on a native polyacrylamide gel compared to unmodified mono-nucleosomes (Fig. 5A). We tested the specificity of Ubp10 DUB activity for different mono-ubiquitinated nucleosomes and found that Ubp10 cleaves both H2BK120-Ub and H2AK119-Ub (Fig. 5B, lanes 5-8, and Fig. S-2C). Hydrolysis of H2B-Ub and H2A-Ub is a result of Ubp10 DUB catalytic activity as mono-ubiquitinated H2B remains un-cleaved in the presence of ubiquitin-aldehyde (Ub-Ald), a highly specific DUB inhibitor (Fig. S-2C, lane 5). Given
that Ubp10 binds free H2A/H2B-Ub heterodimers, we tested if Ubp10 also cleaves H2B-Ub outside of the nucleosomal context. There is a 6-fold increase in de-ubiquitinated H2B at 10 minutes when free H2A/H2B-Ub heterodimers are the substrate compared to H2B-Ub nucleosomes (Fig. 5B, lanes 4 and 8). The catalytic activity of Ubp10 is much lower when presented with a H2B-Ub nucleosome versus H2B-Ub dimers. As a control, the GST tag was cleaved from the N-terminus of Ubp10 and we tested DUB activity with H2A/H2B-Ub heterodimers and H2B-Ub mono-nucleosomes. In the absence of the GST tag, we found that Ubp10 rapidly loses catalytic activity (Fig. S-3). GST appears to either stabilize Ubp10 in a conformation that is required for catalytic activity or promotes the solubility/integrity of Ubp10. As such, all DUB activity assays were performed in the context of the GST tag.

Sir2/4 enhances Ubp10 DUB activity on H2B-Ub mono-nucleosomes

A number of DUBs require binding partners for targeting and enhanced stimulation of deubiquitinase activity (40-42). Since Sir4 and Ubp10 directly interact, we wanted to test if Sir4 binding stimulates Ubp10 DUB activity on H2B-Ub mono-nucleosomes. Indeed, when Sir2/4 was added equimolar to Ubp10, there was a substantial increase in Ubp10 DUB activity on H2B-Ub nucleosomes (Fig. 6A). At 10 minutes, there is a ~33-fold increase in de-ubiquitinated H2B mono-nucleosomes when Ubp10 and Sir2/4 are present compared to Ubp10 alone (Fig. 6B). As expected, Sir2/4 does not cleave H2B-Ub on its own, thus the increase in H2B-Ub cleavage observed is a Sir2/4-dependent effect on Ubp10 DUB activity (Fig. S-4A). Similarly, the SIR complex enhanced Ubp10 DUB activity (Fig. 6C), whereas Sir3 alone prevented Ubp10-dependent cleavage of nucleosomal H2B-Ub (Fig. S-1F). We also observed enhanced cleavage of free H2A/H2B-Ub heterodimers by Ubp10 in the presence of Sir2/4, indicating that stimulation can also occur outside the context of a nucleosome. However, Sir2/4 stimulation of Ubp10 DUB activity did not extend to a K11-linked di-ubiquitin chain substrate (Fig. S-2B), nor did Sir2/4 stimulate DUB activity of the SAGA DUB module, which contains Ubp8, another budding yeast DUB that targets H2B-Ub (Fig. S-4B).

Sir4 binds Ubp10 within the N-terminal region, distal from the C-terminal catalytic DUB domain (Fig. 3A) (30,34). We hypothesize that Sir4 allosterically stimulates Ubp10 DUB activity on H2B-Ub substrates. In support of an allosteric stimulation, we demonstrate that the loss of the Sir4-interaction site on Ubp10 results in the loss of stimulated DUB activity in the presence of Sir2/4 (Fig. 6E). Additionally, Sir2/4 does not directly interact with H2B-Ub (Fig. 6F, compare lanes 4 and 7), as this interaction could increase the local substrate concentration of both Ubp10 and H2B-Ub and thus, enhance DUB activity. From these results, we conclude that the enhancement in Ubp10 DUB activity on H2B-Ub substrates is a combination of direct recruitment and specific allosteric stimulation by Sir2/4 (Fig. 6G).

DISCUSSION

In this study, we employed multiple biochemical techniques to mechanistically determine how Ubp10 activity is coupled to the assembly of SIR complex heterochromatin. We demonstrate that Ubp10 co-assembles with SIR complex onto chromatin rather than to a pre-assembled heterochromatinic structure. We identify that Sir4 directly binds Ubp10 off chromatin and that this interaction ultimately facilitates enhanced de-ubiquitination of H2B-Ub substrates. The enhancement of Ubp10 de-ubiquitination of H2B-Ub mono-nucleosomes likely occurs through a combination of recruitment and allosteric stimulation. As discussed below, we propose that SIR complex-regulated targeting of Ubp10 to chromatin and stimulation of Ubp10 DUB activity on H2B-Ub nucleosomes is important for the initiation of de novo heterochromatic silencing (Fig. 7).

Ubp10 is not recruited to an established heterochromatin structure

We confirmed previous in vivo observations that Ubp10 is recruited to heterochromatin in a SIR complex-dependent manner using pre-assembled heterochromatin and whole cell extracts (Fig. 1) (30-32). SIR complex-mediated recruitment of Ubp10 to chromatin appears to be dependent on the state of SIR complex assembly. Ubp10 binding was minimal on pre-assembled heterochromatin, most likely due to the masking of the binding site for Ubp10 on Sir4. This suggests that once a stable
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...and compact heterochromatin structure, devoid of histone PTMs, is formed, Ubp10 recruitment is specifically limited. Our results may explain seemingly paradoxical observations in which *ubp10Δ* strains do not exhibit large increases in H2B-Ub or gene expression at the HM loci and sites of natural telomeric silencing (32,35,43). Similarly, pre-established, endogenous sites of heterochromatin (HML and HMR) in vivo appear to be maintained upon deletions of DOT1 or SET1 (24). At established heterochromatin, presumably Ubp10 has already de-ubiquitinated H2B during de novo assembly and is not required for further maintenance. In addition, Ubp10 DUB activity is also important for regulating non-histone ubiquitinated proteins, notably Rpa190 (the largest subunit of RNA polymerase I) (33). Thus, it is potentially important for Ubp10 to not be trapped at established heterochromatic regions in order to promote a soluble pool of Ubp10 to act elsewhere in the genome.

**Ubp10 co-assembly with SIR complex may facilitate de novo HC establishment**

Collectively, our order of addition experiments and complementary co-IP and pulldown results (Fig. 2 and Fig. 3) demonstrate that the direct interaction between Ubp10 and Sir4 likely underlies the co-assembly mechanism. Our studies confirm previous yeast 2-hybrid interaction data suggesting Sir4 and Ubp10 directly interact (30,34) and chromatin immunoprecipitation results that argue Ubp10 and SIR complex mutually recruit each other to regulate silencing at sub-telomeres (31). Our data extends this idea in that an upstream event: Sir2/4 binding Ubp10 in the nucleoplasm prior to chromatin association, may reflect the recruitment of Ubp10 during the initiation of heterochromatin assembly in vivo.

A de novo silencing event of an active locus, where the SIR complex would assemble in a genomic region for the first time, may be the context where Ubp10 is most important. H2B-Ub appears to be important for nucleosome re-assembly after chromatin-disruptive processes, such as DNA replication and RNA transcription (44,45). A recent study suggests that Asf1-mediated incorporation of H2B-Ub during nucleosome assembly is needed to regulate heterochromatin establishment (46). Based on our model, Ubp10 recruitment would act downstream to clear H2B-Ub from chromatin to facilitate SIR complex-mediated compaction of nucleosomes. This mechanism likely occurs during each round of re-assembly of SIR-mediated heterochromatin, even if ubiquitinated H2B is only encountered infrequently.

**Sir2/4 specifically targets Ubp10 to H2B-Ub nucleosomes**

On its own, Ubp10 binds to chromatin only at relatively high concentrations, and associates with unmodified H2A/H2B heterodimers and surprisingly, H3/H4 tetramers. These results indicate Ubp10 can interact with a nucleosome through low affinity histone interactions in the absence of its ubiquitin substrate (Fig. 4). However, we are unable to distinguish whether one molecule of Ubp10 makes simultaneous or independent contacts with H2A/H2B and H3/H4. In contrast, we show that Ubp10 is recruited to chromatin and targeted to H2B-Ub nucleosomes when in complex with the main silencing machinery in budding yeast. A parallel targeting mechanism to nucleosomes is seen with the targeting of Ubp8 to regions of active transcription by the SAGA DUB module (41,47). Ubp8 displays little DUB activity independent of the SAGA DUB module. However, Ubp8 is incorporated into the DUB module off chromatin, targeted to H2B-Ub nucleosomes in a Sgf11-dependent manner, and exhibits DUB activity on H2B-Ub chromatin. Overall, our results support the theme of incorporating DUBs into larger protein complexes for recruitment and precise targeting (48). This mechanism appears to be an important layer of regulation in DUB biology in light of the diverse number of substrates that are ubiquitinated (49).

**Sir2/4 allosterically stimulates Ubp10 activity to generate the silent histone code**

The "histone code" that drives heterochromatin establishment in budding yeast requires simply the absence of histone PTMs. Sir2-dependent deacetylation is the sole erasure activity of the SIR complex, yet SIR complex binding is not severely affected by acetylated chromatin in the absence of deacetylase activity (10). Instead Sir3, the Sir protein important for spreading of the SIR complex, is particularly sensitive to histone acetylation and H3K79me (6,8,9,11,12,23,50). It
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remains unknown if H2B-Ub alone can directly inhibit or reduce SIR complex binding to the nucleosome. However, H2B-Ub indirectly inhibits SIR complex silencing through the promotion of H3K4 and H3K79 methylation. The kinetics of establishing heterochromatin is delayed in the absence of Jhd2, the H3K4 demethylase, indicating that erasing methylation marks is a key feature in heterochromatin assembly (24). Whereas Jhd2 is known as a H3K4 demethylase (51), there remains no known demethylase for H3K79me. Instead, the removal of this stable methylation mark is likely to be dependent on multiple cycles of DNA replication to be diluted.

We propose a model in which the SIR complex, in conjunction with Ubp10, can overcome the challenge of anti-silencing H3 methylation marks on chromatin. Alone, Ubp10 is an inefficient DUB in the presence of its target substrate as it is less active on a H2B-Ub nucleosome compared to a H2A/H2B-Ub dimers (Fig. 5). However, when in complex with Sir2/4, Ubp10 DUB activity is greatly stimulated on H2B-Ub substrates, both mono-nucleosomes and H2A/H2B-Ub dimers (Fig. 6). Furthermore, the data strongly argues that this increase in DUB activity is regulated by allosteric stimulation and is specific to Ubp10 (Fig. 6). The observed resistance of H2B-Ub nucleosomes to Ubp10 DUB activity may be multi-faceted. First, the mono-nucleosomal context of H2B-Ub may structurally present a challenge to Ubp10 regarding the accessibility of the isopeptide bond. Sir2/4 recruitment of Ubp10 to the nucleosome may better position the DUB domain of Ubp10 to cleave mono-ubiquitin. Second, a Sir2/4-bound Ubp10 may have a decreased off rate on the nucleosome versus Ubp10 alone. This Sir2/4-dependent tethering of Ubp10 increases the probability of cleaving mono-ubiquitinated H2B. Last, Ubp10 DUB activity is allosterically stimulated by Sir2/4. Allosteric stimulation appears to be a common means to regulate and restrict DUBs to specifically act on their substrate (48,52,53). None of the above-mentioned mechanisms for Ubp10 and the SIR complex are mutually exclusive and likely work in combination to achieve specific targeting of Ubp10 to H2B-Ub chromatin in regions that will undergo silencing.

Our study demonstrates how the SIR complex can coordinate with other epigenetic erasure proteins to transition an active domain into a silent domain. This specific coupling of deacetylation and H2B de-ubiquitination by a chromatin-modifying complex to achieve gene repression has also been recently shown in a mammalian system (54). We propose that Ubp10 is important for de novo heterochromatin establishment and that Sir2/4 enhancement of Ubp10 DUB activity on H2B-Ub nucleosomes represents a mechanism that minimizes the opportunity for subsequent H3 methylation (Fig. 7). The eventual dilution of the anti-silencing mark H3K79me and the prevention of new H3K79 methylation by removing H2B-Ub would facilitate heterochromatin establishment, as has been observed previously for de novo silencing kinetics (9,24,55). In conjunction with previous genetic analyses of Ubp10 in silencing, we present a more refined and mechanistically detailed model for how Ubp10 promotes assembly of SIR complex-mediated heterochromatin.
Experimental Procedures

DNA Templates
The 3-kb DNA template used for chromatin pulldowns was PCR-generated from plasmid pUC18-G5cyc1, bearing five Gal4 binding sites upstream of a CYC1 promoter-driven G-less cassette, using a biotinylated primer as described in (10). DNA used for reconstituting mononucleosomes was PCR-generated from plasmid 601 using primers that primed 147-bp with the Widom 601 sequence in the center of the template. PCR products were purified using Macherey-Nagel Nucleospin DNA Purification kit.

Cloning and purification of GST-Ubp10 (full length and mutant)
PCR was used to amplify the Ubp10 ORF from S. cerevisiae genomic DNA. pGEX-6-P1 and the Ubp10 PCR Insert were digested with XhoI and EcoRI and followed by T4 DNA ligation to make pAJ285. For the Sir-interaction Ubp10 mutant, pAJ285 was digested with HindIII and SacI. A Gibson assembly reaction was then performed using digested pAJ285 and a Ubp10 Δ109-133 gBlock (IDT) fragment to make GST-Ubp10 Δ109-133, pAJ342. GST-Ubp10 WT or mutant was expressed in E. coli BL21 Codon-Plus cells as described in (34). Cells were harvested and lysed in Lysis Buffer containing PBS (pH 7.4) additionally containing 350 mM NaCl, 1 mM EDTA, 1 mM EGTA, 15 mM DTT, 0.5% Triton X, 1 mM PMSF, 2 mg/mL lysozyme. The soluble extract was added to Glutathione resin (Thermo Scientific) and eluted off with 10 mM Glutathione. A monoQ column was used to further purify GST-Ubp10. Fractions were dialyzed in PB150 and aliquots were stored at −80°C.

Sir Protein Purifications
Recombinant Sir2, Sir3, and Sir4 were purified as described in (10), except that Sir2/4 was tandem affinity-purified from strain AJY136. The integrated Sir2/4 yeast strain (AJY136) was generated by first removing the CEN6 from 3XHA-Sir2/LEU2 and TAP-Sir4/URA3 using Gibson assembly. pAJ327 (TAP-Sir4/URA3 – CEN6) and pAJ328 (3XHA-Sir2/LEU2 –CEN6) were linearized and integrated into AJY65 using the lithium acetate transformation to generate AJY136.

Chromatin Reconstitution and Nucleosome Assembly
Enzymatic assembly of chromatin templates, MNase analysis, and quantification of DNA were performed as described in (10). Mononucleosomes were reconstituted using salt-dialysis as described previously (56) and stored at 4°C for no longer than a month.

Whole Cell Extract Preparation
The Ubp10-TAP yeast strain was obtained from the yeast TAP collection (Open Biosystems). Cells were grown to ~1 x 10⁷ cells/ml in YPD at 30°C with shaking. Cells were pelleted at 4000 rpm and washed in Wash Buffer: 20 mM Tris-HCl pH 7.5, 150 mM NaCl. Cells were spun, re-suspended in 1 mL Wash Buffer, pelleted, and flash frozen. Pellets were thawed and re-suspended in Lysis Buffer (50 mM HEPES-KOH pH 7.5, 325 mM NaCl, 10% Glycerol, 0.5% NP-40, 1 mM EDTA, 1 mM PMSF, 1 µg/mL bestatin/leupeptin/pepstatin, and 1 mM benzamidine). Glass beads were added to the biomass in a final volume of 1 mL and lysed using bead mill (Mini-Beadbeater, BioSpec): 2 x 45 second bead beating at 4°C with a 2-minute rest in between on ice. The lysate was collected and spun 14K rpm for 5 minutes at 4°C. Soluble lysate was transferred to a new tube, spun for 15 minutes, and collected in a new tube. Bradford readings were taken and lysates flash frozen and stored at −80°C.

Chromatin and Heterochromatin Pulldowns
Bead-conjugated 3.0-kb pUC18-G5cyc1 chromatin template (113 ng DNA) was incubated with Sir complex: Sir3 (23 pmol for WCE experiments or 2.3 pmol for experiments using all recombinant proteins) and Sir2/4 (0.6 pmol), for 1 hr at room temperature with rotation in 30 µL Pulldown Buffer 150 (PB150): 50 mM HEPES pH 7.5, 10 mM Magnesium acetate, 5 mM EGTA, 0.1 mM EDTA, 0.02% NP-40, 5% Glycerol, 150 mM Potassium acetate, 1 mM DTT, 1 mM PMSF, 1 µg/mL bestatin/leupeptin/pepstatin, and 1 mM benzamidine. For experiments in whole-cell extracts (WCEs), SIR complex-bound chromatin was incubated in 100 ng of WCE, an ATP regeneration system (30 mM creatine phosphate, 3 mM ATP, 4.1 mM magnesium acetate, and 6.4 µg/ml creatine kinase, final concentration), and...
PB150 in 20 µL for 1 hr at room temperature with rotation, followed by 1 hr rotation at 4°C. Samples were washed in cold PB100 twice in 500 µL and once in 150 µL. In the purified in vitro system, pulldown experiments were performed as in the above procedure with several modifications: 2.25 pmol GST-Ubp10 was used in place of WCE and the ATP regeneration system was not used. Samples were boiled, separated from magnetic beads, run on a 12% SDS-PAGE gel, and transferred to a nitrocellulose membrane. Proteins were detected by Western analysis.

**Generation of hydrolyzable and non-hydrolyzable mono-ubiquitinated histone octamers**

The non-hydrolyzable ubiquitinated histone mimics (H2B*Ub) were prepared by crosslinking of recombinant human H2BK120C and His-tagged UbG76C, as described in (38). The hydrolyzable mimic (H2BK120Ub) were prepared from recombinant human H2BK120C and His-tagged Ub. An isopeptide bond was formed between the C-terminus of Ub and a derivatized aminothylcysteine side chain at position 120. A series of blocking and de-blocking steps were taken to ensure site specificity of the reaction, which were confirmed by mass spectrometry analysis. Detailed method will be reported elsewhere (Al-Afaleq and Yao, manuscript in preparation).

**Co-immunoprecipitations**

After tandem affinity purification of Sir2/4, Sir4 retains the calmodulin binding peptide (CBP) sequence. α CBP (ICL RCBP-45A-Z) was conjugated to ProteinA Dynabeads (LifeTech, 10002D) for 30 min at room temperature. Beads were washed with PBS and re-suspended in Pulldown Buffer100 (PB100): 50 mM HEPES pH 7.5, 10 mM Magnesium acetate, 5 mM EGTA, 0.1 mM EDTA, 0.02% NP40, 5 % Glycerol, 100 mM Potassium acetate, 1 mM DTT, 1 mM PMSF, 1 µg/mL bestatin/leupeptin/pepstatin, and 1 mM benzamidine. 0.6 pmol Sir4 and 2 pmol Ubp10 were added to beads in a 20 µL reaction. When histone octamers (4-times molar amounts of Sir2/4 or Ubp10) were used, tubes were blocked with buffer containing 0.1 mg/mL BSA and 0.1% NP-40, and reactions were performed in 250 µL. Proteins were incubated for 60 mins on ice. Beads were added to the proteins and rotated end-over-end for 60 minutes at 4°C. Beads were magnet separated, re-suspended in SDS-Laemmli Buffer, and boiled at 95°C. Ubp10 and Sir2/4 samples were run on an 8% polyacrylamide-SDS gel and Sir2/4 or Ubp10 with histone octamers were run on a 4-20% gradient gel (BioRad). Sir2/4 and Ubp10 were transferred to a nitrocellulose membrane while histones were transferred onto an Immobilon P SQ PVDF membrane. Proteins were detected by Western analysis.

**GST Pulldown**

For Ubp10 pulldowns with Sir2/4, 2 pmol of GST-Ubp10 or GST-Ubp10 Δ109-133 were incubated with Glutathione SuperFlow Agarose resin (Pierce cat. 25237) for 120 minutes at 4°C with rotation in Pulldown Buffer 150 (PB150): 50 mM HEPES pH 7.5, 10 mM Magnesium acetate, 5 mM EGTA, 0.1 mM EDTA, 0.02% NP40, 5 % glycerol, 150 mM Potassium acetate, 1 mM DTT, 1 mM PMSF, 1 µg/mL bestatin/leupeptin/pepstatin, and 1 mM benzamidine. Ubp10-bound resin was washed once in 200 µL PB150. Sir2/4 was added at 2 pmol (1x) or 4 pmol (2x) to Ubp10-resin and incubated for 30’ at 4°C with rotation in PB150. Beads were spun at 500xg for 1 minute at 4°C and washed in 50 µL PB150. Samples were boiled in SDS-Laemmli Buffer and supernatants were run on an 8% polyacrylamide-SDS gel. Proteins were transferred onto a nitrocellulose membrane and detected by Western.

For Ubp10 pulldowns with histones, 2.25 pmol recombinant GST-Ubp10 was incubated with 4.5 (2X) pmol recombinant *H. sapiens* histone octamer in PB150 for 60 minutes rotating at 4°C. Glutathione resin was added to IP sample and incubated for 60 minutes rotating at 4°C. Beads were spun 700xg for 1 minute 4°C and washed once in PB500 (0.5 M Potassium acetate). Beads were spun, re-suspended in SDS-Laemmli Buffer, and boiled at 95°C. Samples were run on a 4-20% polyacrylamide-SDS gel and Ubp10 was transferred to a nitrocellulose membrane and histones were transferred to a PVDF P SQ (0.22 µM). Proteins were detected by Western analysis.

**In vitro Deubiquitination Assays**

In vitro DUB Assays were performed in 15 µL reactions with DUB Buffer (40 mM Tris pH 7.6, 100 mM NaCl, 0.1 mg/mL BSA, 5% Glycerol, and...
2 mM DTT). Low-retention tubes were blocked with 2 mg/mL BSA and 0.1% NP-40. Recombinant proteins were dialyzed in DUB buffer with 100 μM ZnSO₄ prior to experiments. 100 nM free H2A/H2B-Ub heterodimers or 100 nM nucleosomal H2A/H2B-Ub heterodimer (50 nM nucleosome sample with two copies of H2A/H2B-Ub) were incubated with 5 nM recombinant GST-Ubp10 or GST-Ubp10 Δ109-133. When indicated, SIR complex or Sir proteins were incubated with GST-Ubp10 at equimolar concentrations for 1 hr on ice prior to experiment. Ub-aldehyde was used for inhibited control samples at 2.5 μM final. Reactions were incubated at 23ºC in a thermoshaker and time point samples were quenched in 5X SDS-Laemmlli Buffer. Boiled samples were run on a 15% SDS-PAGE gel and either stained with Coomassie Blue or transferred to Immobilon P™ PVDF membranes for western analysis. Proteins were probed by Western analysis.

We observed differences in detection and transfer efficiencies between mono-ubiquitinated H2B and H2B that influenced the detection signal by the H2B antibody. Mono-ubiquitin potentially influences access of the H2B antibody to its epitope, which resides within the last 25 amino acids of the C-terminal tail of H2B. We thus used arbitrary units as a mode of measure to compare volumetric intensities of de-ubiquitinated H2B between different conditions within the same experiment. Quantifications of westerns were performed using ImageLab (BioRad). For data analysis, volumetric signal intensity for H2B (cleaved H2B-Ub product) was determined by subtracting background signal from the H2B signal. This provided de-ubiquitinated H2B signal that was then normalized to H3 signal per lane (loading control) and divided by 10^7. Arbitrary units (a.u. 10^7) were then plotted as a function of time. Error bars were determined from three individual experiments and p-values were generated using Welch’s T-test.

### Table 1. Antibodies

| Antibody | Company | Catalog # | Lot #     | Dilution | Species |
|----------|---------|-----------|-----------|----------|---------|
| αH2B     | Abcam   | ab1790    | GR181765-1| 1:1000   | Rabbit  |
| αH3-HRP  | Abcam   | ab21054-200| 1:10K    | Rabbit   |
| αGST     | Santa Cruz | sc-459 (Z-5) | J1515 | 1:1000 | Rabbit |
| αCBP     | ICL     | RCBP-45A-Z | 1         | 1:5000   | Rabbit  |
| αPAP     | Sigma   | P1291     |           | 1:5000   | Rabbit  |
| αFLAG    | Sigma   | F1804 (M2) | 1         | 1:1500   | Mouse   |
| αHA      | Santa Cruz | sc-7392 (F-7) | K0215 | 1:1500 | Mouse   |

### Table 2. Plasmids and Yeast strains

| Plasmid | Genotype or description | Source |
|---------|-------------------------|--------|
| pAJ285  | pGEX-6P-1_GST-UBP10     | This study |
| pAJ342  | pGEX-6P-1_GST-UBP10Δ109-133 | This study |
| pAJ327  | pGAL-TAP-Sir4/URA3 (–CEN6) | This study |
| pAJ328  | pGAL-HA-Sir2/Leu2 (–CEN6) | This study |

**Yeast strain**

| Genotype or description | Source |
|-------------------------|--------|
| AJY106 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ubp10-TAP | Open Biosystems |
| AJY143 AJY65 Ubp10-TAP, sir3Δ::KanMX | This study |
| AJY136 AJY65 3XHA-Sir2 leu2Δ1::LEU2, TAP-Sir4 ura3-52::URA3 | This study |
| AJY65 BJ5459, MATa ura3-52 trp1 lys2-801 leu2Δ1 pep4::HIS3 prb11.6R can1 | (57) |
SIR complex stimulates Ubp10 assembly and activity

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Conflict of Interest
The authors declare that they have no conflicts of interests with the contents of this article.

Author Contributions
AZ and AMJ designed and wrote the paper. AZ designed, performed, and analyzed all experiments. ED expressed and purified Ubp10 proteins. NA and TY designed, expressed, and assembled H2B-Ub and H2B*Ub octamer proteins. All authors reviewed the results and approved the final version of the manuscript.
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Footnotes
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FIGURE LEGENDS

Figure 1. Ubp10 is recruited to SIR complex-mediated heterochromatin. A) Diagram of post-translational modifications that affect SIR complex binding to nucleosomes. SIR complex has inherent histone deacetylase activity (HDAC) by virtue of Sir2. SIR complex cannot erase histone ubiquitination or histone methylation on its own. Histone ubiquitination indirectly inhibits SIR complex association by promoting H3 lysine methylation. B) Experimental design for chromatin/heterochromatin pulldowns using whole cell extracts. WCEs were made from a strain expressing endogenous Ubp10 that is C-terminally TAP-tagged. Bead-conjugated chromatin or SIR complex-assembled heterochromatin were incubated in Ubp10-TAP extracts. Beads were magnetically isolated and washed. Bound proteins were detected by western analysis. C) Analysis of reconstituted chromatin by limited MNase digestion. From left, titration of MNase from high to low (tick marks represent 2-log ladder markers). D) Western analysis of heterochromatin pulldown compared to chromatin from whole cell extracts. SIR complex and chromatin are recombinant proteins and were detected by Western. SIR: SIR complex, recombin.: recombinant, PAP: Peroxidase-anti-peroxidase. E) Western quantification of Ubp10-TAP enrichment on chromatin versus heterochromatin. Ubp10 signal in mock reaction quantified as 1 and Ubp10 signal in +SIR complex sample is relative to mock. Mock and relative signals were normalized to H3. An integration of both a Box plot and scatter plot. Error bars determined from 3 independent experiments. Asterisk indicates p-value < 0.05.

Figure 2. Ubp10 co-assembles with SIR complex onto chromatin. A) Schematic for testing Ubp10 binding to pre-assembled heterochromatin using recombinant proteins: SIR complex and Ubp10. SIR complex was pre-incubated with chromatin-beads before the addition of Ubp10 to the reaction. B) Western analysis of pre-assembled heterochromatin pulldown. Ubp10 signal in mock reaction quantified as 1 and Ubp10 signal in +SIR complex sample is relative to mock. Mock and relative signals were normalized to H3. Proteins were detected using antibodies specific for epitope tags: GST-Ubp10, 3XHA-Sir2, Sir3-3XFLAG, whereas H3 was detected by its native epitope. C) Schematic for testing co-assembly of Ubp10 and SIR complex to chromatin using recombinant proteins: SIR complex and Ubp10. SIR complex was pre-incubated with Ubp10 before the addition of chromatin-beads to the reaction. D) Western analysis of co-assembly of Ubp10 and SIR complex on chromatin, analyzed as in B.

Figure 3. Sir2/4 sub-complex directly interacts with Ubp10 during co-assembly onto chromatin. A) Depiction of the domains and protein binding sites on Ubp10 and Sir4. Ubp10 C371 is the active cysteine involved in deubiquitinase activity. IDR: intrinsically disordered region, USP: ubiquitin-specific protease. The dotted line indicates the broad interaction region on Sir4 that Ubp10 is expected to bind as mapped by previous yeast 2-hybrid data (28,32). B) Western analysis of endogenous Ubp10-TAP recruitment to chromatin or Sir2/4-coated chromatin in whole cell extracts made with sir3Δ. Lane 4: input for both
SIR complex stimulates Ubp10 assembly and activity

recombinant Sir2/4 and Ubp10-TAP (sir3Δ cells) were combined. C) Quantification of B. Signal for Ubp10-TAP is relative to input signal and then normalized to H3 signal. An integration of both a Box plot and scatter plot. Error bar indicates at least 2 independent experiments. D,E) Similar experiments performed as in Figure 2 except recombinant SIR complex is replaced with recombinant Sir2/4 subcomplex. Western analysis of recombinant GST-Ubp10 recruitment to pre-bound Sir2/4 chromatin (D) or co-assembly with Sir2/4 onto chromatin (E). Ubp10 signal in mock reaction quantified as 1 and Ubp10 signal in +SIR complex sample is relative to mock. Mock and relative signals were normalized to H3. Note: Image (E) cropped from the same western blot exposure as depicted in Figure 2D. F) Western analysis of Sir2/4 co-IP of Ubp10. Ubp10 and Sir2/4 were incubated together prior to immunoprecipitation with α CBP-conjugated to Protein A beads. G) Quantification of F. An integration of both a Box plot and scatter plot. Error bar indicates 3 independent experiments. H) Western analysis of GST pulldown of Sir2/4 sub-complex using GST-Ubp10 or GST-Ubp10 Δ109-133 bound to glutathione resin. Sir2/4 was added 1x or 2x times the picomoles of Ubp10 WT or mutant in the reaction.

Figure 4. Up10 has independent histone-binding activity. A) Western blot of the titration of recombinant GST-Ubp10 with naked DNA or chromatin. Naked DNA and chromatinized DNA conjugated to beads were quantified and matched for pulldowns. B) Western of glutathione pulldown using recombinant Ubp10, unmodified octamers, and H2B*Ub-containing octamers.

Figure 5. H2B-Ub nucleosomes are resistant to Ubp10 DUB activity. A) Native gel of reconstituted mono-nucleosomes containing either unmodified human histone octamer (H2B) or H2B-Ub assembled octamer. Mono-nucleosomes were reconstituted via salt dialysis using 147-bp DNA template containing a 601 Widom sequence. DNA-protein complexes were run on a 5% native polyacrylamide gel and stained with SYBR GOLD. B) An in vitro deubiquitinase assay (DUB assay) was performed using 5 nM recombinant Ubp10 and 100 nM nucleosomal H2A/H2B-Ub or 100 nM free H2A/H2B-Ub heterodimers. Deubiquitination activity was determined by SDS-PAGE followed by western analysis using α H2B and looking at formation of cleaved H2B. Samples were quenched at indicated time points.

Figure 6. Sir2/4 allosterically stimulates Ubp10 DUB activity on H2B-Ub nucleosomes. A) In vitro DUB assays performed as in Fig 5. 5 nM Sir2/4 was added to 5 nM Ubp10 and 100 nM nucleosomal H2A/H2B-Ub. Samples were quenched at indicated time points. Ub-ald above lane indicates ubiquitin-aldehyde (DUB inhibitor) was added to reaction. B) Quantification of A. Generation of H2B signal was plotted as a function of time. H2B signal (volume intensity) was normalized to H3 signal (loading control) to generate arbitrary values (10^7). Error bars indicate three independent experiments. Asterisks indicates a p-value < 0.05. C) DUB assays as in A with 5 nM SIR complex, 5 nM Ubp10, and 100 nM nucleosomal H2A/H2B-Ub D) In vitro DUB assays performed as in Fig 5. 5 nM Sir2/4 was added to 5 nM Ubp10 and 100 nM H2A/H2B-Ub heterodimers. E) In vitro DUB assays performed as in Fig 5. 5 nM Sir2/4 was added to 5 nM Ubp10 Δ109-133 and 100 nM H2A/H2B-Ub heterodimers. F) Sir2/4 co-IP in the presence of either unmodified core histone octamer (H2B) or H2B-Ub assembled octamer. Sir4 was immunoprecipitated by bead- conjugated α CBP (calmodulin-binding peptide) and associated proteins were detected by western. G) Diagram depicting Sir2/4-dependent recruitment and allosteric stimulation of Ubp10 for enhanced DUB activity on a H2B-Ub nucleosome.

Figure 7. A model for Ubp10 function during de novo heterochromatin assembly. A) SIR complex recruits a H2BK123ub1 deubiquitinase, Ubp10, off chromatin and targets Ubp10 to mono-ubiquitinated chromatin (an active locus). Sir2/4 allosterically enhances (thick orange arrow) Ubp10 de-ubiquitination.
SIR complex stimulates Ubp10 assembly and activity of H2BK123-Ub, which directly prevents subsequent H3 methylation.

B) De-modification of the adjacent nucleosome allows SIR complex to engage the nucleosome, recruit another SIR-Ubp10 complex via self-self interactions (double-headed arrow), and promote de-modification of the next nucleosome. C) An established heterochromatin domain is devoid of post-translational modifications and does not recruit Ubp10.
**A**

SIR complex (HDAC)

Target locus for de novo silencing

**B**

Ubp10-TAP whole cell extract

Magnetic Isolation

Washes

Western Analysis

**C**

MNase

**D**

| Lane | Input | Ubp10-TAP |
|------|--------|-----------|
| 1    | SIR    | -         |
| 2    | Ubp10  | -         |
| 3    | -      | +         |
| 4    | -      | +         |

| Lanes | SIR (recomb.) | PAP (Ubp10) | αFLAG (Sir3) | αHA (Sir2) | αH3 |
|-------|---------------|--------------|--------------|------------|-----|
| 1     | 135 kD        | 135 kD       | 100 kD       | 75 kD      | 63 kD |
| 2     | 135 kD        | 135 kD       | 100 kD       | 75 kD      | 63 kD |
| 3     | 17 kD         | 17 kD        | 11 kD        | 11 kD      | 11 kD |
| 4     | 17 kD         | 17 kD        | 11 kD        | 11 kD      | 11 kD |

**E**

Relative Ubp10 normalized to H3
**A**

1. SIR complex + chromatin-beads, 60' RT
2. Add Ubp10 to heterochromatin, 60' 4ºC
3. Washes
4. Western analysis

**B**

| Input   | 50% Ubp10 | 20% SIR | Ubp10 |
|---------|-----------|---------|-------|
| SIR (recomb.) |          |         |       |
| αGST (Ubp10) |          |         |       |
| αHA (Sir2) |          |         |       |
| αFLAG (Sir3) |          |         |       |
| αH3 |          |         |       |

**C**

1. SIR complex + Ubp10, 60' 4ºC
2. Add chromatin-beads, 60' RT
3. Washes
4. Western analysis

**D**

| Input | 20% SIR | 50% Ubp10 | chromatin |
|-------|---------|-----------|-----------|
| SIR (recomb.) |         |          |           |
| αGST (Ubp10) |         |          |           |
| αHA (Sir2) |         |          |           |
| αFLAG (Sir3) |         |          |           |
| αH3 |         |          |           |

**Legend**

- αGST (Ubp10)
- αHA (Sir2)
- αFLAG (Sir3)
- αH3

**Notes**

- GST signal/H3
- 135 kD –
- 75 kD –
- 63 kD –
- 17 kD –
- 11 kD –
- 75 kD –
- 63 kD –
- 135 kD –
- 100 kD –

**Graphical Data**

- Input
  - 1.00
  - 0.42
  - GST signal/H3
- Input
  - 1.00
  - 6.06
  - GST signal/H3
A. Sir2/4 + Chromatin

B. Ubp10-TAP WCE

C. Relative Ubp10 normalized to H3

D. Input 1. Sir2/4 + Chromatin 2. Ubp10

E. 1. Sir2/4 + Ubp10 2. Chromatin

F. Input IP: αCBP (Sir4)

G. Ubp10 relative to Input

H. GST Pulldown

Lanes: 1. Sir2/4 + Ubp10 2. Chromatin

Graphs and images show various experiments involving Ubp10, Sir2/4, and H3, with analyses of GST signal/H3, αPAP (Ubp10), αHA (Sir2), αH3, αGST (Ubp10), and αCBP (Sir4).
A

Naked DNA

Chromatin

Input
100%

Ubp10

Ubp10

αGST (Ubp10)

αH3

B

H2A/H2B dimer

H2A/H2B*Ub dimer

Ubp10 Input

2.5% Input

Beads

2.5% Input

Beads

αH3

H2B

Lanes 1 2 3 4 5 6 7 8 9
A

Octamer

DNA  H2B  H2B-Ub

H2B-Ub Nucleosome
H2B Nucleosome
Free DNA

B

| t(min):           | H2A/H2B-Ub dimer | H2B-Ub nucleosome |
|-------------------|------------------|-------------------|
|                   | 0 2.5 5 10       | 0 10 20 40        |
| Lanes             | 1 2 3 4 5 6 7 8  |                   |

- 25 kD –
- 17 kD –
- 17 kD –

DUB activity

H2A
H2B
Ub
Ubp10
H4
H2A/H2B
H3
Ub
Ubp10

B

Ubp10

DUB activity

H3  H4  H2A  H2B

αH2B  αH3
A

SIR + Ubp10

B

Established silent locus
(unmodified histones)

C
Recruitment and allosteric stimulation of a histone deubiquitinating enzyme during heterochromatin assembly
Alexis Zukowski, Nouf Omar Al-Afaleq, Emily D Duncan, Tingting Yao and Aaron M. Johnson

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