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Proteomic Profiling of Yeast Heterochromatin Connects Direct Physical and Genetic Interactions

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Abstract

Heterochromatin domains are stably-repressed chromatin structures composed of a core assembly of silencing proteins that condense adjacent nucleosomes. The minimal heterochromatin structure can serve as a platform for recruitment of complementary regulatory factors. We find that a reconstituted budding yeast heterochromatin domain can act as a platform to recruit multiple factors that play a role in regulating heterochromatin function. We uncover the direct interaction between the SIR heterochromatin complex and a chromosomal boundary protein that restricts the spread of heterochromatin. We find that the SIR complex relieves a mechanism of auto-inhibition within the boundary protein Yta7, allowing the Yta7 bromodomain to engage chromatin. Our results suggest that budding yeast shares with other eukaryotes the ability to establish complex heterochromatin domains that coordinate multiple mechanisms of silencing regulation through physical interactions.

Keywords

heterochromatin; SIR complex; proteasome; Yta7

INTRODUCTION

Heterochromatin was initially observed by light microscopy as nuclear material that maintained a constitutively dense state through the cell cycle. This observation, along with

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the demonstration that purified chromatin fibers alone could undergo compaction, led to the early proposal that heterochromatin was an inert structure composed simply of condensed nucleosomes. There has been a significant shift in our understanding of heterochromatin domains of different organisms in the last few decades. The perception of a simple, static domain has been replaced with the realization that heterochromatin is a complex and at times dynamic structure bound by transcriptional repressor proteins and histone-modifying enzymes. Collectively, these factors act to maintain specific heterochromatic post-translational modifications as well as confer/enable heterochromatic silencing. In addition to these core heterochromatin factors that are necessary for stably repressing transcription at the stage of initiation and elongation, other pathways have been found to be harnessed by heterochromatin domains through recruitment of accessory proteins to contribute to silencing. These additional layers of silencing have been well-established in many organisms. For example, the RNAi machinery are known to contribute directly to endogenous heterochromatic gene silencing in fission yeast, worms, plants, and fruit fly (Martienssen and Moazed 2015). The heterochromatin protein 1 (HP1) family of proteins, present from fission yeast to humans, have been found to coordinate many of the secondary interactions that contribute to heterochromatin regulation (Kwon and Workman 2011).

The budding yeast gene silencing system has served as a model to understand fundamental principles of heterochromatin, including how histone modifications regulate formation of a specific repressive heterochromatin structure (Kueng, Oppikofer, and Gasser 2013). Budding yeast heterochromatin is composed of a core silencing complex, the Silent Information Regulator (SIR) complex, made up of the founding member of the sirtuin family, Sir2, and two chromatin-interacting proteins, Sir3 and Sir4. The SIR complex is recruited to specific sites in the genome by chromatin-associated proteins that allow Sir2 enzymatic activity to deacetylate histones to allow Sir3 and Sir4 to bind tightly. Self-association of the SIR complex then allows chromatin deacetylation coupled with continuous spreading away from the initiating site in a sequence-independent manner. The SIR complex is stably-associated with nucleosomes within the heterochromatin domain, generating a distinct compact structure, which is required for gene repression.

In general, the SIR-heterochromatin structure prevents the association of most chromatin-interacting proteins, including those that are necessary for activation of transcription (Johnson et al. 2013). However, genetic studies have suggested positive interactions between Sir proteins and specific nuclear factors (Figure 1A-D). Three basic designs have been used to screen for modifiers of silencing, all of which take advantage of the fact that reporter genes can be silenced by the normal pathway when inserted within normally heterochromatic regions, including the subtelomeric regions (Figure 1B) and the HM loci (Figure 1C). Variations on the silencing screen include testing the genetic requirements for the barrier to spreading of silencing by inserting a reporter gene downstream of a barrier (Figure 1D). In some cases, these genetic interactions have been demonstrated to involve physical association between the heterochromatin proteins and a regulatory factor. However, it remains unclear in many cases what the underlying molecular mechanism is that leads to these genetic interactions.

We set out to test whether the SIR-heterochromatin assembly can serve as a platform for direct recruitment of protein factors that may regulate silencing. We previously developed a system that reconstitutes functional budding yeast heterochromatin that identified a particular assembly pathway and chromatin structure necessary for transcriptional repression (Johnson et al. 2013; Johnson et al. 2009). In the current study, we used this reconstituted heterochromatin assembly as a platform to recapitulate physical interactions from the yeast proteome, using a quantitative mass spectrometry screen. We identified a number of heterochromatin-enriched factors, many of which have been identified to have genetic association with yeast silencing. We identify the chromatin boundary factor, Yta7, as being directly recruited by the heterochromatin structure. This physical interaction suggests a self-regulated competition mechanism underlies the ability of Yta7 to stop the spread of heterochromatin. This work emphasizes the fact that budding yeast heterochromatin can recruit a network of additional regulatory factors to assemble and/or regulate a complex chromatin domain, functionally similar to heterochromatin domains in other organisms.

RESULTS

SILAC-Mass Spectrometry identifies yeast proteins specifically recruited to yeast heterochromatin

We assembled a repressive heterochromatin domain from purified components, conjugated to magnetic beads, as previously described. This conjugated heterochromatin domain, or a control chromatin domain without SIR complex bound, was incubated with differentially labeled yeast cell extracts grown in either light or heavy lysine. After isolation of proteins that stably bound to the chromatin domains, the bound protein was stripped with high salt, heavy and light samples combined, protein precipitated with trichloroacetic acid (TCA), protease-digested, and processed for tandem mass spectrometry as previously described (Figure 2A) (Johnson et al. 2013).

We observed a number of nuclear proteins enriched more than four-fold in the heterochromatin fraction (Figure 2B). Impressively, 13 of the 18 enriched nuclear proteins have been previously-identified as genetically associated with yeast silencing (Figure 2B,C). Out of these proteins with genetic interactions, less than half have been shown to occur through physical association with the Sir proteins themselves, though some interact with each other, based on STRING analysis (Figure 2D) (Szklarczyk et al. 2017). This result suggests that the orthogonal approach of biochemical reconstitution is capable of reproducing relevant *in vivo* interactions, similar to genetics, as well as capturing physical interactions that may only be fostered in the context of an assembled heterochromatin structure.

We identified Ubp10, a deubiquitinase previously identified as a heterochromatin regulator that is physically recruited by the SIR complex (Emre et al. 2005; Gardner, Nelson, and Gottschling 2005; Zukowski and Johnson 2018). We recently dissected the mechanism by which Ubp10 works during the heterochromatin assembly and how direct physical protein-protein interactions play an important role in Ubp10 activity (Zukowski et al. 2018).

All components of the trimeric Hst1/Sum1/Rfm1, involved in meiotic gene repression(Xie et al. 1999), were also found enriched in the heterochromatin sample. Hst1 is an HDAC that is closely-related to Sir2, so much so that Sir2 can physically interact with Sum1/Rfm1 to partially compensate for loss of Hst1(Hickman and Rusche 2007). This interaction may underlie the physical association of this complex with the SIR-mediated heterochromatin platform.

Esc1, a protein associated with the nuclear periphery(Andrulis et al. 2002) was enriched on the reconstituted heterochromatin platform. Esc1 was identified first as a protein that, when tethered directly to the genome, can initiate heterochromatin formation through interaction with Sir4(Andrulis et al. 2002). This interaction was found to underlie a mechanism by which heterochromatin associates with the nuclear periphery(Andrulis et al. 2002), a common phenomenon from yeast to human.

Validation of proteins enriched in heterochromatin proteomic results

We next validated proteins that were associated preferentially with the heterochromatin platform in our SILAC results, some of which were not previously shown to make physical interactions with the Sir proteins. We generated yeast extracts from cells bearing C-terminal TAP tags of these proteins to test recruitment in heterochromatin pulldown experiments analogous to the SILAC protocol (Figure 3A). Interestingly, we found that the chromosomal boundary protein Yta7 is recruited preferentially to the heterochromatin substrate (Figure 3B). Yta7 has been shown to act as a barrier to the spread of heterochromatin, but no specific SIR-based recruitment mechanism was reported. We pursue the relationship of this interaction later in our study.

We have previously used this SILAC approach to study the association of transcriptional machinery to chromatin and how the SIR-dependent structure interferes with recruitment of transcription and chromatin factors via an acidic activator. Along with the multiple co-activator complexes that were prevented from interaction with the heterochromatin platform, the most-depleted protein in heterochromatin was Leo1, a subunit of the PAF1 complex(Tomson and Arndt 2013). Interestingly, the rest of the PAF1 complex was not dramatically affected in the SILAC assay. Upon validation with Leo1 and another PAF1 complex subunit Rtf1, we observed little Leo1-TAP association with either chromatin or heterochromatin, whereas the Rtf1-TAP signal was observed with chromatin and increase with heterochromatin (Figure 3C). The ability for Rtf1-TAP and, presumably, the remaining subunits of PAF1 complex except Leo1, to interact with chromatin is likely mediated through the HMD domain of Rtf1 which has been shown to interact with chromatin in locations where Pol II does not(Piro et al. 2012). It is unclear what the significance of the SIR-PAF1 complex physical interaction is, but it may relate to the genetic relationships previously established between these two complexes that operate at telomeric regions(Cusanelli, Romero, and Chartrand 2013; Rodrigues and Lydall 2018; Krogan et al. 2003; PerezRomero et al. 2018).

Interaction of proteasome lid components with heterochromatin

Of the proteins enriched in the heterochromatin sample, Rpn5, Rpn6, Rpn7, Pre9, and Ubp6 are all components of the proteasome. Additional proteasome components, Rpn8, Rpn9, Rpn11, and Rpn12 were enriched as well, just under the four-fold cutoff (Figure 4A). Nearly all of these proteasomal components are associated with the dissociable “lid” component of the proteasome (Figure 4B)(Park et al. 2010), which has been shown to act independently of the core particle of the proteasome. Protein A epitope-tagged Rpn11 in a whole cell extract (strain generated in(Leggett, Glickman, and Finley 2005)) is also stimulated to interact with chromatin when the SIR complex is present(Figure 4C), suggesting that heterochromatin recruits the proteasome, potentially the lid by itself. Recent evidence suggests that Esc1, which interacts with Sir4, interacts with the proteasome(Niepel et al. 2013) and this may be the bridge to heterochromatin. Using purified yeast proteasome, rather than from cell extract, we found no significant interaction with either chromatin or heterochromatin (Figure 4D), suggesting that Esc1 may be an important bridge for these interactions.

Yta7 is directly recruited to the heterochromatin platform

We further investigated the physical interaction identified in our proteomic screen between SIRheterochromatin and Yta7, an AAA+ and bromodomain-containing protein (Figure 5A). Yta7 was identified as a chromosome boundary factor that could prevent the spread of SIR-heterochromatin(Jambunathan et al. 2005; Tackett et al. 2005). Although there is a clear functional relationship between the SIR complex and Yta7, no physical interaction had been proposed. Interestingly, we find that the SIR complex promotes association of Yta7 with chromatin in a cellular extract, suggesting that the silencing machinery may recruit its own negative regulator (Figure 3B and5B). This stimulation was observed when using a sub-complex of Sir2 and Sir4 alone using Yta7 from extract (data not shown), suggesting that Yta7 may directly interact with Sir2/4. We tested whether there may be a direct physical interaction between Sir2/4 and Yta7 using a GST-tagged Yta7 construct purified in *E. coli* and composed of AAA+ domains and the bromodomain near the C-terminus. We refer to this construct as Yta7-AAC (Figure 5A). With all purified components, the SIR complex and Sir2/4 subcomplex recruited Yta7-AAC to unmodified chromatin (Figure 5D,E). Yta7-AAC was unable to bind to chromatin on its own, under these conditions, compared to the stimulation observed when Yta7 is titrated with chromatin in the presence of Sir2/4 (Figure 5F,G). This direct interaction with Sir2/4 is only observed in the context of chromatin, as Yta7-AAC is unable to pull down Sir2/4 in solution (Figure 5H). The bromodomain contained in this construct can bind to free unmodified or acetylated histones H3 and H4(Gradolatto et al. 2009; Jambunathan et al. 2005), but the affinity for chromatin has not been tested. In order to further understand the role of the Yta7 bromodomain in this Sir2/4-dependent recruitment, we performed similar chromatin pulldown experiments using a recombinant version of the Yta7 bromodomain only (Yta7 BD). On its own, the Yta7 BD binds unmodified chromatin (Figure 5I). This result is in contrast to the Yta7-AAC construct, which has the two AAA+ domains in addition to the bromodomain. This suggests that the presence of the two AAA+ domains negatively regulates the bromodomain interaction with histones. Yet the presence of the two AAA+ domains promote Sir2/4-dependent recruitment of Yta7 onto chromatin (Figure 5I).

DISCUSSION

We find that the minimal biochemical system that we have developed to study yeast heterochromatin recapitulates many relevant interactions previously identified through genetic screens. Many previously-identified physical interactions are captured when the reconstituted heterochromatin domain is introduced into the entire yeast proteome extract. In addition, we capture physical interactions that had previously only been characterized through functional genetics. This is exemplified by the direct recruitment of Yta7 to chromatin, likely by Sir4. While many of these interactions are not essential for SIR complex function, some are known to contribute in various ways to the regulation of silencing and others have potential undiscovered roles.

In this study, we focused on the interactions of heterochromatin with factors where physical interactions had not been previously shown, though a genetic interaction had. The proteasome has been implicated in a number of aspects of genome regulation, including transcription and heterochromatin in both budding and fission yeast (McCann and Tansey 2014; Ezhkova and Tansey 2004; Seo et al. 2017; Seo, Kwon, and Lee 2018; Qin et al. 2009). The likely mechanism of physical recruitment of the budding yeast proteasome to heterochromatin is through the Esc1 protein, which binds to both (Andrulis et al. 2002; Niepel et al. 2013). This agrees with the inability of the purified heterochromatin machinery to directly interact with purified proteasome (Figure 4D), suggesting a bridging factor is required. Whether and how the proteasome contributes to heterochromatin regulation is not clear and requires further investigations, though some evidence suggests a role in maintenance of heterochromatin structure (Qin et al. 2009).

The direct physical interaction between Yta7 and heterochromatin that we demonstrate here highlights a mechanism by which heterochromatin may limit its own spreading. We hypothesize that both the bromodomain and the AAA+ domains of Yta7 play a role in this, as both have been shown to be required to restrict heterochromatin spreading (Gradolatto et al. 2009; Lombardi, Ellahi, and Rine 2011). The bromodomain may directly interfere with SIR complex function through competing for the histone interactions that the SIR complex makes. For example, a bromodomain-containing protein has been shown to interfere with *S. pombe* Sir2 (Wang et al. 2013) or this could occur via competition with Sir3 or Sir4 histone binding. One or both AAA+ domains may mediate interactions with Sir4, perhaps through similar interactions that allow Sir4 to interact with the AAA+ domain of Sir3 (Ehrentauf et al. 2011). If Yta7 competes with Sir3 to bind Sir4, this may also be a mechanism of potential interference with the spread of silencing. ATP was omitted from our experiments in Figure 5, suggesting that the interactions of the SIR complex with Yta7 do not require the ATP-related functions of the Yta7 AAA+ region that have been shown to be important for boundary function (Lombardi, Ellahi, and Rine 2011). Future studies will be needed to piece apart these potential complex regulatory interplays. We also uncovered a potential mode of auto-inhibition within Yta7 where the AAA+ domains interfere with the ability of the bromodomain to engage chromatin (Figure 5F-J). Binding of Sir2/4 relieves this inhibition, allowing for multi-valent engagement of chromatin through interactions with chromatin-bound Sir2/4, Yta7 AAA+ domains, and Yta7 bromodomain-histones (Figure 5J).

We identified a small number of proteins that were highly-enriched in binding selectively to heterochromatin that have not been previously associated with the regulation of silencing. These included the 14-3-3 proteins Bmh1 and Bmh2, as well as Lsm12, a component of a stress granule complex (Swisher and Parker 2010). Deletion of the genes encoding these proteins does not disrupt telomeric silencing and Bmh protein validation with TAP-tagged strains was inconclusive, perhaps due to tag interference (data not shown), leaving for future investigations the question of how they may contribute to heterochromatin regulation.

Using proteomic approaches to study complex physical associations with chromatin has been used to identify new players in chromatin pathways and it promises to be a powerful tool for the future. Our work demonstrates that this technique can serve as a useful complementary approach to yeast genetics, even allowing for novel interaction to be identified in a system that has been studied through extensive genetics and high-throughput proteomics.

EXPERIMENTAL PROCEDURES

Proteins and DNA templates

DNA templates used in activator binding and proteomic profiling experiments were made by PCR from the plasmid pUC18-G5cyc1^G bearing five Gal4 binding sites upstream of a CYC1 promoter-driven G-less cassette with two predicted start sites producing transcripts of ~250 and 277 nt (see (Sawadogo and Roeder 1985; Woontner et al. 1991) for details on use of G-less templates). PCR-generated templates were produced with a biotinylated primer on the end furthest from the transcription cassette. The transcription cassette begins 582 bp from the non-biotinylated end. The size of PCR product used is indicated in the description of the experiment. *S. cerevisiae* YTA7 gene sequence was cloned from genomic DNA and a PCR construct containing coding sequence of amino acids 400–1379 (AAC) or 999–1101 (bromodomain) was made, with homology ends for pGEX-6P-1 insertion with isothermal assembly. The resulting plasmid constructs, “Yta7-AAC” and “Yta7 bromodomain” were sequenced within the manipulated region to confirm.

Yeast histones, chromatin assembly factors, and Sir proteins were purified as described (Johnson et al. 2009; Zukowski et al. 2018). GST-Yta7-AAC construct was expressed in pLysS cells with overnight induction at 16° C. Protein was lysed by sonication and purified with Glutathione SuperFlow Agarose resin (Pierce cat. 25237) in PBS containing 500 mM NaCl (total), 0.5 M Urea, 1% Triton X-100, 5% glycerol, 10 mM DTT, 1 mM EDTA, and 1 mM PMSF. Protein was eluted with 50 mM glutathione, dialyzed in 50 mM Tris pH 8.0, 150 mM NaCl, 5% glycerol, 1 mM DTT. The construct was subsequently purified by FPLC on a 1 mL MonoS column (GE Healthcare) with a 0.1–1.0 M NaCl gradient. Fractions were pooled and flash-frozen in aliquots. GST-Yta7 bromodomain construct (amino acids 999–1101) was induced in Codon Plus cells for 3 hr at 37° C and purified under standard conditions in PBS 500 mM NaCl (total), 0.5% Triton X-100, 10 mM DTT, 1 mM EDTA, and 1 mM PMSF. Protein was eluted in 50 mM Tris pH 8.0, 350 mM NaCl, 10 mM glutathione, 1 mM DTT. Elution fractions were pooled, dialyzed against buffer without glutathione and flash-frozen in aliquots.

Proteasome purification

Proteasome purification was performed as described (Leggett, Glickman, and Finley 2005).

Chromatin reconstitution and Nucleosome Assembly

Long (>four nucleosomes) nucleosome arrays were assembled enzymatically as previously described (Johnson et al. 2009). Conjugation of chromatin fragments to magnetic beads and acetylation with Piccolo acetyltransferase was also done as previously described (Johnson et al. 2009).

Quantitative Mass Spectrometry Profiling of Chromatin Domains

Yeast cultures, strain SF10 (Hoppe et al. 2002), were grown in synthetic medium with either 32 mg/L $^{12}\text{C}_6^{14}\text{N}_2$ lysine-HCl (Sigma) or $^{13}\text{C}_6^{15}\text{N}_2$ lysine-HCl (Cambridge Isotope Laboratories) in 3% glucose to $\text{OD}^{600} = 2.0$. Extracts were prepared from cell pellets as described previously (Woontner et al. 1991).

Bead-conjugated 3.1 kb pUC18-G5cyc1^G- chromatin template (1.5 μg DNA was incubated with the SIR complex (13.6 pmol Sir2/4, 52 pmol Sir3) for one hour with rotation in 400 μL 50 mM Hepes pH 7.5, 10 mM magnesium acetate, 5 mM EGTA, 0.1 mM EDTA, 0.02% NP-40, 5% glycerol, 1 mM DTT, 1 mM PMSF, 1 $\mu\text{g}/\text{mL}$ bestatin/leupeptin/pepstatin, and 1 mM benzamidine. Subsequently, 150 ng Gal4-VP16, an ATP regeneration system (30 mM Creatine phosphate, 3 mM ATP, 4.1 mM magnesium acetate, and 6.4 $\mu\text{g}/\text{mL}$ creatine kinase final concentration), and 1.5 mg light or heavy yeast extract were added to 500 μL and incubated for one hour at room temperature and one hour at 4°C with rotation. Beads were then washed one time with 1.5 mL cold IP Buffer. Beads were then stripped of protein with a solution of 50 mM Hepes pH 7.5 and 2 M NaCl. Samples were diluted to 400 mM NaCl in 50 mM Hepes pH 7.5, light and heavy were combined, and precipitated with trichloroacetic acid.

TCA pellets were resuspended and digested with Lys-C protease. The resulting peptides were purified using tC18 SepPak cartridge (Waters, Milford, MA). The dried peptides were resuspended in the solvent of 5% acetonitrile and 4% formic acid and were loaded onto a microcapillary column packed with C18 beads (Magic C18AQ, 5 μm , 200 Å) using a Famos autosampler (LC Packings, San Francisco, CA). The samples were separated by on-line reversed-phase chromatography using an Agilent 1100 binary pump with a 70-min gradient of 5–30% acetonitrile (in 0.125% formic acid) and detected in a hybrid quadrupole linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap XL, ThermoFisher).

All recorded MS/MS spectra were searched using the Sequest algorithm (version 28) (Yates et al. 1995). Spectra were matched against a database encompassing sequences of all proteins in the yeast open reading frame (ORFs) database downloaded from the Saccharomyces Genome website (<http://www.yeastgenome.org/>). Each protein sequence was listed in both forward and reversed orientations to facilitate estimation of peptide and protein identification false discovery rates (FDRs). The following parameters were adopted: 10 ppm precursor mass tolerance; 1.0 Da product ion mass tolerance; up to two missed cleavages; variable modifications: oxidation of methionine (15.9949) and carbamidomethylation of

cysteine (57.0214). The target-decoy method was employed to distinguish correct and incorrect identifications and thus control peptide and protein level FDRs (Elias and Gygi 2007). The final list of peptides and proteins were selected by Linear discriminant analysis (LDA) in which numerous parameters, such as Xcorr, Cn, precursor mass error, and charge state, were considered (Huttlin et al. 2010). 1% FDR was strictly controlled at the protein level. Significant protein hits were listed in Systematic ORF nomenclature as ratio of heterochromatin to euchromatin sample, with recombinant proteins added to the experiment filtered (Table in Online Resource 1).

Whole Cell Extract Preparation

The TAP-tagged yeast strains were obtained from the yeast TAP collection (Open Biosystems). Cells were grown to $\sim 1 \times 10^7$ 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): 2×45 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^{G-} 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, 0.1 mg/mL BSA, 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 µg 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. Pulldown experiments with purified proteasome were performed as in the above procedure with several modifications: 750 ng affinity-purified proteasome 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 (see table in Online Resource 2 for antibody information).

Chromatin Pulldown of Yta7 Constructs

Bead-conjugated 3.0-kb pUC18-G5cyc1^{G-} chromatin template (113 ng DNA) was incubated with recombinant GST-Yta7-AAC in PB₁₅₀ for 60 minutes at 23°C with rotation. GST-Yta7-

AAC was added at increasing amounts (0.725, 2.175, and 4.35 pmol). Beads were magnetically isolated and washed twice in 100 μ L PB₁₅₀. Beads were resuspended in 15 μ L SDS-Laemmli Buffer and analyzed by Western. For GST-Yta7-AAC recruitment to Sir2/4-coated chromatin, 0.45 pmol Sir2/4 was incubated with chromatin (113 ng DNA) for 60 minutes at room temperature (23°C) with rotation in PB₁₅₀. GST-Yta7-AAC was added at increasing amounts (0.725, 2.175, and 4.35 pmol) to the reaction for a total 30 μ L for 60 minutes at 23°C with rotation. Beads were magnetically isolated and washed twice in 100 μ L PB₁₅₀. Beads were resuspended in 15 μ L SDS-Laemmli Buffer and analyzed by Western. Recombinant GSTYta7 BD was added (0.725 pmol) at increasing concentrations (1X, 3X and 6X) to a reaction mixture containing chromatin or Sir2/4-bound chromatin. Pulldowns were performed as described above.

GST Pulldowns

For Yta7 pulldowns with Sir2/4, 2 pmol of GST-Yta7-AAC and 2 pmol of Sir2/4 were incubated on ice for 30 minutes. Glutathione SuperFlow Agarose resin (Pierce cat. 25237) was added to the protein mixture for 60 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, 0.1 mg/mL BSA, 1 μ g/mL bestatin/leupeptin/pepstatin, and 1 mM benzamidine. Beads were spun at 500 x g for 1 minute at 4°C and washed once in 200 μ L 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Functional Interactions with SIR-HC

B. Telomeric Genetic Reporter Screens

C. Mating Type Maintenance Screen

D. Spreading/Barrier Screen

Figure 1. Yeast heterochromatin interactions.
 A) Summary of proteins that interact functionally with yeast SIR-heterochromatin (SIR-HC). B) Telomeric silencing reporter systems and genetic assays for disruptors of silencing. C) Mating type silencing and genetic screen approaches. D) Setup of genetic systems to identify genes that regulate heterochromatin spreading by barrier function. *HMT* histone methyltransferase, *Ub* ubiquitin, *Ac* acetylation, *Me* methylation, *RF* recruitment factor.

Figure 2. Proteomic profiling of yeast heterochromatin.

A) Scheme for SILAC proteomic approach to identify differentially-enriched factors associated with a reconstituted yeast heterochromatin platform. B) Results of SILAC analysis displaying top enriched nuclear proteins associated preferentially with the heterochromatin platform (HC) compared to the chromatin alone, “euchromatin” like (EC). Both platforms contain a transcriptional activator, which is active in EC and inactive in HC. C) Key for top proteomic hits, including their annotations in the *Saccharomyces Genome Database* (SGD) or manually curated from published studies(non-SGD). D) STRING

analysis of known physical interactions according to “Experiment” (pink connectors) and “Database” (blue connectors) curated interactions.

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Figure 3. Validation of proteomic screen hits.

A) Scheme for using endogenously-tagged versions of target proteins to validate interaction in a comparable yeast extract to the proteomic screen in Figure 2. B,C) Chromatin pulldowns with and without SIR complex pre-assembly with TAP-tagged versions of indicated proteins in yeast whole cell extract.

Figure 4. Physical interaction of the proteasome with SIR heterochromatin.

A) Highlighted proteasome subunits (blue) from the proteomic screen results. Esc1 (maroon) is also highlighted. B) Scheme of the yeast proteasome with subunits enriched ≥ 3 -fold in heterochromatin highlighted. C) Validation of proteasome “lid” component interaction with heterochromatin using a yeast extract from endogenously Protein A-tagged Rpn11 strain. D) Purified yeast proteasome does not significantly interact directly with SIR heterochromatin.

Figure 5. Direct interaction between SIR-heterochromatin and the spreading barrier protein Yta7.

A) Scheme of the domain structure of the Yta7 protein and the Yta7-AAC truncated construct. Two AAA+ domains and one bromodomain (BD) are predicted from sequence similarity. A putative histone binding domain is predicted within the N-terminal region. B) Heterochromatin pulldown of Yta7 from Yta7-TAP whole cell extracts (WCE). C) Quantification of **B** represented as a box-dot plot. Error bars indicate three independent experiments performed; p-value = 0.05. D) The Sir2/4 subcomplex directly recruits

recombinant Yta7-AAC to chromatin independent of WCE. E) Quantification of **D** represented as a box-dot plot. Error bars indicate at least two independent experiments were performed. F,G) The presence of Sir2/4 stimulates the recruitment of Yta7-AAC to chromatin. GST-Yta7-AAC was titrated (1X, 3X, 6X) with chromatin with or without Sir2/4 pre-assembled. H) A GST pulldown demonstrating that Sir2/4 does not interact with Yta7 stably in the absence of a chromatin context. Asterisk denotes un-cleaved TAP-Sir4 present in the purified Sir4 sample. I) Western analysis of chromatin pulldowns using GST-Yta7 BD (bromodomain). GST-Yta7 BD was titrated (1X, 3X, 6X) with chromatin with or without Sir2/4 pre-assembled. Yta7 BD was added in equimolar amounts compared to Yta7-AAC, as was performed in Panels F and H. J) Model for autoinhibition of Yta7 bromodomain binding to chromatin. (Top) the presence of the AAA+ domains inhibits bromodomain binding to histones and prevents binding of Yta7 to chromatin. (Bottom) when Sir2/4 is present on chromatin, the AAA+ domains may undergo a conformational change through weak interactions (dashed lines) with Sir2/4. This frees the bromodomain from an auto-inhibitory state and enables interaction with histone tails.