A Novel Proteomics Approach for the Discovery of Chromatin-associated Protein Networks*[S]

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Protein-protein interaction mapping has progressed rapidly in recent years, enabling the completion of several high throughput studies. However, knowledge of physical interactions is limited for numerous classes of proteins, such as chromatin-bound proteins, because of their poor solubility when bound to DNA. To address this problem, we have developed a novel method, termed modified chromatin immunopurification (mChIP), that allows for the efficient purification of protein-DNA macromolecules, enabling subsequent protein identification by mass spectrometry. mChIP consists of a single affinity purification step whereby chromatin-bound protein networks are isolated from mildly sonicated and gently clarified cellular extracts using magnetic beads coated with antibodies. We applied the mChIP method in *Saccharomyces cerevisiae* cells expressing endogenously tandem affinity purification (TAP)-tagged histone H2A or the histone variant Htz1p and successfully co-purified numerous chromatin-bound protein networks as well as DNA. We further challenged the mChIP procedure by purifying three chromatin-bound bait proteins that have proven difficult to purify by traditional methods: Lge1p, Mcm5p, and Yta7p. The protein interaction networks of these three baits dramatically expanded our knowledge of their chromatin environments and illustrate that the innovative mChIP procedure enables an improved characterization of chromatin-associated proteins. ***Molecular & Cellular Proteomics 8:870–882, 2009.***

Chromatin, the complex packaging of DNA with proteins, is in many ways the master control center for the cell. At the most fundamental level, DNA is wrapped around histone proteins in a structure referred to as the nucleosome (1). Histones H2A, H2B, H3, and H4 form the core of the nucleosome around which the DNA is wrapped (2). In addition to core histones variant forms, such as histone Htz1p in yeast or H2A.Z in mammalian cells, also contribute to the diverse range of biological processes regulated by chromatin (3). Numerous post-translational modifications of histone proteins modulate DNA-protein interactions as well as regulate the intricate and temporal associations of other proteins and protein complexes, such as chromatin-remodeling or -modifying proteins and transcription factors, with chromatin (for a review, see Ref. 4). To truly understand the regulatory role that chromatin plays in the cell and disease states will require a detailed understanding of the intricate protein-protein interactions that occur on it.

The study of protein-protein interactions has grown by leaps and bounds in recent years. We and others have successfully performed large scale studies of protein-protein interactions in both yeast and human cells by immunopurification and high throughput mass spectrometry (5–10). These studies, along with numerous small scale studies, have provided the scientific community with a wealth of information regarding the elaborate protein networks that function as the building blocks of life. Although technical developments resulting in improved throughput and sensitivity have been achieved (for reviews, see Refs. 11 and 12), hurdles remain in the discovery of protein-protein interactions of chromatin-bound proteins/complexes. Proteins associated with DNA have the dual natures of participating in conventional soluble protein-protein interactions as well as participating in larger DNA-protein macrocomplexes. In conventional immunopurification protocols, these macrocomplexes are often lost during the clarification steps as they pellet along with the DNA/chromatin and hence are not available for the downstream immunopurification step. Consequently protein interaction networks have been discerned for the soluble fraction of chromatin-binding proteins; however, these networks may not be representative of the chromatin-bound protein environment. A holistic view of cellular protein-protein interactions occurring on chromatin requires the development of new purification techniques.

Over the years, a number of proteomics methodologies have been developed to study chromatin-bound proteins. Acid extraction, a well established protocol for the study of histones, utilizes the low solubility of the histone proteins that are complexed with DNA to specifically precipitate them from the cellular matrix; histones are subsequently resolubilized for

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TABLE I
Strains used in this study

Strain	Genotype	Source or Ref.
YPH499	*MATa ura3–52 lys2–801 ade2–101 trp1-Δ63 his3-Δ200 leu2-Δ1*	70
YSC1178-7499158	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 H2A.2-TAP::HIS*	TAP collection
YSC1178-7502629	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 HTZ1-TAP::HIS*	TAP collection
YSC1178–7503047	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 LGE1-TAP::HIS*	TAP collection
YSC1178-7501832	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MCM5-TAP::HIS*	TAP collection
YSC1178-7500768	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YTA7-TAP::HIS*	TAP collection
YKB509	*MATa ura3–52 lys2–801 ade2–101 trp1-Δ63 his3-Δ200 leu2-Δ1 swr1Δ::kanMX*	This study
YKB963	*MATa HTZ1-TAP::HIS swr1Δ::KanMX*	This study

analysis (13). This protocol has enabled the detailed study of histone composition and their associated post-translational modifications in a variety of organisms (14). However, the denaturing conditions inherent to this method are not amenable to the identification of protein-protein interactions. Recently a number of methods have been reported that facilitate the identification of interaction partners of proteins bound to chromatin (for a review, see Ref. 15). A common approach in these methods is to drastically reduce, or entirely remove, genomic DNA in the cellular extract by various mechanical or enzymatic means, thereby enhancing the solubility of the protein of interest. Such procedures have provided insight into the interaction partners of a number of histone variants and their interaction partners in both mammalian (16, 17) and yeast cells (18). However, these methods drastically reduce the number of associated proteins purified with the bait of interests and hence provide an incomplete picture of the physical interactions for any given bait.

Here we report a novel method, termed modified chromatin immunopurification (mChIP),[1] for the purification and analysis of chromatin-bound proteins and their associated protein interaction networks. This method is similar to chromatin immunoprecipitation procedures used to define DNA binding sites for transcription factors and other DNA-binding proteins (for a review, see Ref. 19). The mChIP protocol involves DNA shearing by sonication, gentle clarification, and affinity purification of protein-DNA complexes. This method allows the purification of chromatin-bound proteins and their associated DNA in quantities sufficient for analysis by mass spectrometry. To optimize the mChIP protocol we initially focused on the identification of associated proteins of histone H2A (Hta2p) and its variant (Htz1p) in the budding yeast *Saccharomyces cerevisiae*. The resulting Hta2p and Htz1p mChIP protein networks include an array of chromatin-bound proteins and serve to define the "chromatin background" of the new mChIP procedure. We subsequently used the new mChIP procedure to explore the cellular function of Lge1p, Mcm5p, and Yta7p, three known chromatin-binding proteins with differential cellular roles. Although traditional tandem affinity purification (TAP) with these baits revealed limited insight into these proteins, mChIP identified novel protein interactions providing unique biological insights into the function of these proteins and illustrating the potential worth of our procedure.

[1] The abbreviations used are: mChIP, modified chromatin immunopurification; IP, immunopurification; ChIP, chromatin immunopurification; TAP, tandem affinity purification; MCM, minichromosome maintenance; SPB, spindle pole body; E3, ubiquitin-protein isopeptide ligase.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Genetics Methods—All yeast strains used in this study are listed in Table I. Growth media and strains were prepared following standard practices. Strains from the tandem affinity purification (TAP) collection were obtained from Open Biosystems (Huntsville, AL). Genomic deletions and epitope tag integrations made for this study were designed with PCR-amplified cassettes as described previously (20, 21) and confirmed by either PCR analysis or immunoblotting for tag expression.

Modified Chromatin Immunopurification—One-step affinity immunopurification was performed using TAP-tagged proteins and M-270 epoxy Dynabeads (Invitrogen) coated with rabbit IgG according to the manufacturer's instructions. Briefly 700 ml of cultured yeast cells grown in yeast, peptone, dextrose (YPD) medium to an A_{600} of 1 were pelleted and washed with water. Cells were resuspended in lysis buffer (100 mM HEPES, pH 8.0, 20 mM magnesium acetate, 10% glycerol (v/v), 10 mM EGTA, 0.1 mM EDTA + fresh yeast protease inhibitors mixture (Sigma-Aldrich)), frozen in liquid nitrogen in small droplets, and lysed using a coffee grinder half-filled with dry ice for 1 min. The dry ice from the ground cells was allowed to evaporate, and the resulting whole cell extract was sonicated three times for 30 s with at least 1 min on ice between each pulse. Nonidet P-40 was added to a final concentration of 0.4%, and the sample was mixed by hand for 30 s. The extract was gently clarified by centrifugation at 1800 relative centrifugal force for 10 min (4 °C), and the supernatant was transferred to a fresh tube. Freshly prepared rabbit IgG (Sigma-Aldrich)-coated Dynabeads were added (200 μl/sample), and the samples were incubated with end-over-end rotation for 3 h at 4 °C. Using a Dynal MPC-S magnet (Invitrogen) the beads were collected on the side of the sample tubes, and the supernatant was discarded. The beads were washed three times by resuspension in 1 ml of wash buffer (100 mM HEPES, pH 7.4, 20 mM magnesium acetate, 10% glycerol (v/v), 10 mM EGTA, 0.1 mM EDTA, 0.5% Nonidet P-40) and subsequently transferred to a fresh tube following each wash. Finally the beads were resuspended in 1 ml of elution buffer (0.5 M NH_4OH, 0.5 mM EDTA) and incubated with end-over-end rotation for 20 min at room temperature. The protein eluates were transferred to a fresh tube and were evaporated to dryness using a SpeedVac.

The protein sample was resuspended in 1× loading buffer (50 mM Tris-HCl, pH 8, 2% SDS, 100 mM DTT, 10% glycerol) and resolved on a NuPAGE 4–12% SDS-PAGE gel. For protein visualization, the gels were silver-stained. For Western blot analysis, the proteins were

transferred onto nitrocellulose membrane, blocked in 5% nonfat milk in TBST (20 mM Tris base, 150 mM NaCl, 0.1% Tween 20), and probed with polyclonal rabbit anti-TAP antibodies (Open Biosystems) or with polyclonal rabbit anti-histone H3 antibodies (Abcam Inc.). When stated, standard immunopurification was performed without the use of sonication and with clarification of the yeast extracts at 14,000 rpm for 15 min (4 °C) while maintaining the same buffer and bead conditions as for the modified ChIP method outlined above.

Micrococcal Nuclease S7 and DNase I Digestion—Following cell lysis and sonication, $MgCl_2$ and $CaCl_2$ were adjusted to a final concentration of 10 and 3 mM, respectively. 500 units/ml micrococcal nuclease S7 (BioShop Canada Inc.) or 15 μg/ml DNase I (Sigma) were added, and samples were incubated for 20 min at room temperature. The reminder of the sample preparation was performed as described above.

PCR Analysis of Immunopurified DNA by Modified ChIP—Aliquots of the final eluates (DNA sample) or genomic DNA prepared by phenol/chloroform extraction of cell extracts (input) were resuspended in 100 μl of 10 mM Tris, EDTA, pH 8.0 and treated with 40 μg of proteinase K for 2 h at 37 °C. DNA fragments were then purified using the GFX PCR DNA and gel band purification kit (GE Healthcare) according to the supplier's protocols. DNA was eluted with 50 μl of water, and 1 μl was used for each reaction. PCR was performed in 25-μl batches using the iQ SYBR Green Supermix (Bio-Rad) following the supplier's instructions. Immunoprecipitated DNA was amplified by PCR with the following primers pair: *GAL1* F, 5′-GAAGAGTCTCTCGCCAATAAGAAACAGG; *GAL1* R, 5′-GAACATTCGTAAAGTTTATCGCAAG (22). PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

Mass Spectrometry Analysis—Gel bands were excised, reduced, alkylated, and digested as described previously (23) based on the original protocol of Wilm *et al.* (24). Peptide solutions were dried in a SpeedVac and stored at −20 °C until mass spectrometric analysis. LC-MS/MS was performed by dissolving the peptide samples in 5% formic acid and loading them into a 200-μm × 5-cm precolumn packed in house with 5-μm YMC ODS-A C_{18} beads (Waters) using a micro Agilent 1100 HPLC system (Agilent Technologies). The peptides were desalted on line with 95% water, 5% acetonitrile, 0.1% formic acid (v/v) for 10 min at 10 μl/min. Then the flow rate was split before the precolumn to produce a flow of rate of ~200 nl/min at the column. Following their elution from the precolumn, the peptides were directed to a 75-μm × 5-cm analytical column packed with 5-μm YMC ODS-A C_{18} beads. The peptides were eluted using a 1-h gradient (5–80% acetonitrile with 0.1% formic acid) into an LTQ linear ion trap mass spectrometer (Thermo-Electron). MS/MS spectra were acquired in a data-dependant acquisition mode that automatically selected and fragmented the five most intense peaks from each MS spectrum generated.

Peak lists were generated from the MS/MS .raw file using Mascot Distiller 2.0.0.0 (Matrix Science) to produce a .mgf file with default parameters with the exception that for each MS/MS individual peak lists were generated assuming a 2+ and a 3+ charge. All .mgf files from one sample were merged into a single file and then analyzed and matched to the 6298 *S. cerevisiae* protein sequences in the National Center for Biotechnology Information (NCBI) database (released April 2007) using the Mascot 2.1.04 database search engine (Matrix Science) with trypsin as digestion enzyme, carbamidomethylation of cysteine as a fixed modification, and methionine oxidation as a variable modification. Peptide and MS/MS mass tolerances were set at ±2 and ±0.8 Da, respectively, with one miscleavage allowed and the significance threshold set to 0.01 ($p < 0.01$). Finally an ion score cutoff of 30 was chosen to produce a false-positive rate of less than 1% in the MS data (25). A protein hit required at least two "bold red peptides," *i.e.* the most logical assignment of the peptide in the database selected, to be reported. Furthermore when peptides matched to more than one database entry, only the highest scoring protein was considered.

RESULTS

mChIP Facilitates the Purification of Protein Networks Bound to DNA—The study of proteins associated with chromatin is difficult. Well established protocols, such as TAP (21, 26), failed to identify proteins shown previously to associate with the *S. cerevisiae* histone Hta2p and its variant, Htz1p (data not shown and Refs. 7 and 10). In our hands, we found that most of the proteins expected to be associated with chromatin were lost during the sample preparation, particularly in the centrifugation steps. We therefore postulated that the poor solubility of large protein-DNA complexes, further aggravated by the necessary centrifugation steps, was the cause of this problem. To address this issue, we have developed a novel method based on the principle of ChIP, an established method for the purification of DNA associated with specific proteins (for a review, see Ref. 19). The modified ChIP (or mChIP) method ensures that chromatin-bound protein complexes remain in solution by shearing DNA using sonication prior to the immunopurification (Fig. 1). This step breaks DNA into fragments that are sufficiently small (<2000 bp) to remain soluble following mild centrifugation. Not surprisingly, we found that by limiting the clarification of the samples before performing the IP more contaminant proteins were found bound to the IgG-agarose beads (data not shown). We resolved this problem by using magnetic beads that dramatically reduce the binding of nonspecific proteins to the affinity matrix (see "Materials and Methods" for details).

The mChIP method was used to identify chromatin-bound protein networks from yeast cells expressing the endogenously TAP-tagged histone Hta2p (Hta2-TAP) or Htz1p (Htz1-TAP) (Fig. 2). To ensure that our novel mChIP protocol was successfully purifying genuine protein-DNA complexes and not experimental artifacts, we also performed mChIP from cells expressing Htz1-TAP mChIP in an *swr1*Δ mutant background. Swr1 is a component of the SWR1 complex that is required for the incorporation of Htz1 into nucleosomes (18, 27, 28), and *swr1*Δ cells display a dramatic reduction of Htz1p incorporation in chromatin (27, 28). In addition, a traditional immunopurification method was used to purify Htz1-TAP (Fig. 2, *IP*; see "Materials and Methods" for details). Although both protocols resulted in the purification of roughly equal amounts of Hta2-TAP or Htz1-TAP as assessed by Western blot (Fig. 2*B*), the mChIP of both Hta2-TAP and Htz1-TAP resulted in the purification of an intricate mixture of proteins that were not present in purifications from untagged control cells or Htz1-TAP *swr1*Δ cells as wells as a conventional Htz1-TAP IP (Fig. 2*A*; see supplemental Table 1 for a complete list of identified proteins). Because of the use of gradient gels to resolve the mChIP proteins, small proteins, including core histones, are poorly silver-stained but observed in large amounts by Coo-

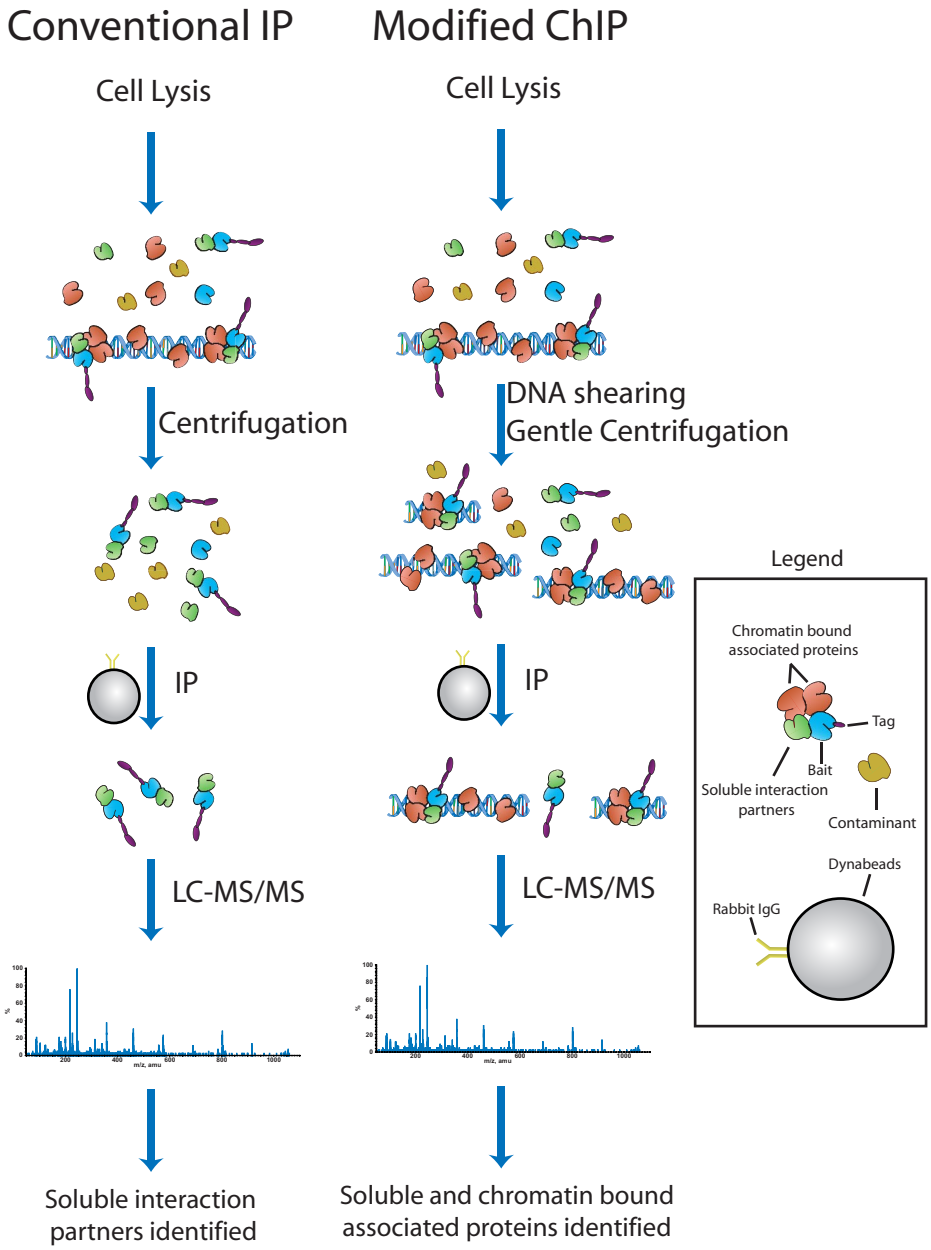

FIG. 1. **Experimental protocol for immunopurification of chromatin-bound protein complexes using the mChIP approach as compared with conventional IP methods.**

massie (data not shown), Western blot (Fig. 2*C*), and mass spectrometry analysis (supplemental Table 1). Histone H3 was only co-purified in the Hta2-TAP and Htz1-TAP using mChIP suggesting that this method is purifying intact nucleosomes and possibly chromatin (Fig. 2*C*). Htz1p has been shown to localize to the *GAL1* promoter (22), and we demonstrated that the *GAL1* promoter region can be amplified from our Hta2-TAP and Htz1-TAP mChIPs eluates but not from the Htz1-TAP *swr1*Δ mChIP or Htz1-TAP traditional IP (Fig. 2*D*). These results demonstrate that the mChIP protocol can purify chromatin from sonicated whole cell extract (Fig. 2*E*).

Mass spectrometry analysis also indicates that the mChIP method can successfully identify chromatin-bound protein networks (see supplemental Table 1 for a complete list of identified proteins). Hta2-TAP and Htz1-TAP mChIPs along with the untagged control mChIP purification were performed in triplicate. Ribosomal proteins and other common contaminant proteins as well as proteins observed only in a single mChIP experiment were removed from the final high confidence data sets (supplemental Table 2). The high confidence data set of Hta2-TAP and Htz1-TAP includes several well characterized chromatin proteins such as the core histones, the topoisomerase Top2 (29), RNA polymerase subunits, and replication factors A and C (30, 31) among others. Numerous chromatin-remodeling complexes also co-purified with both Hta2-TAP and Htz1-TAP including the imitation-switch (ISWI) chromatin-remodeling complex (32). In contrast, Swr1 and Rvb1, components exclusively found in the SWR1 complex

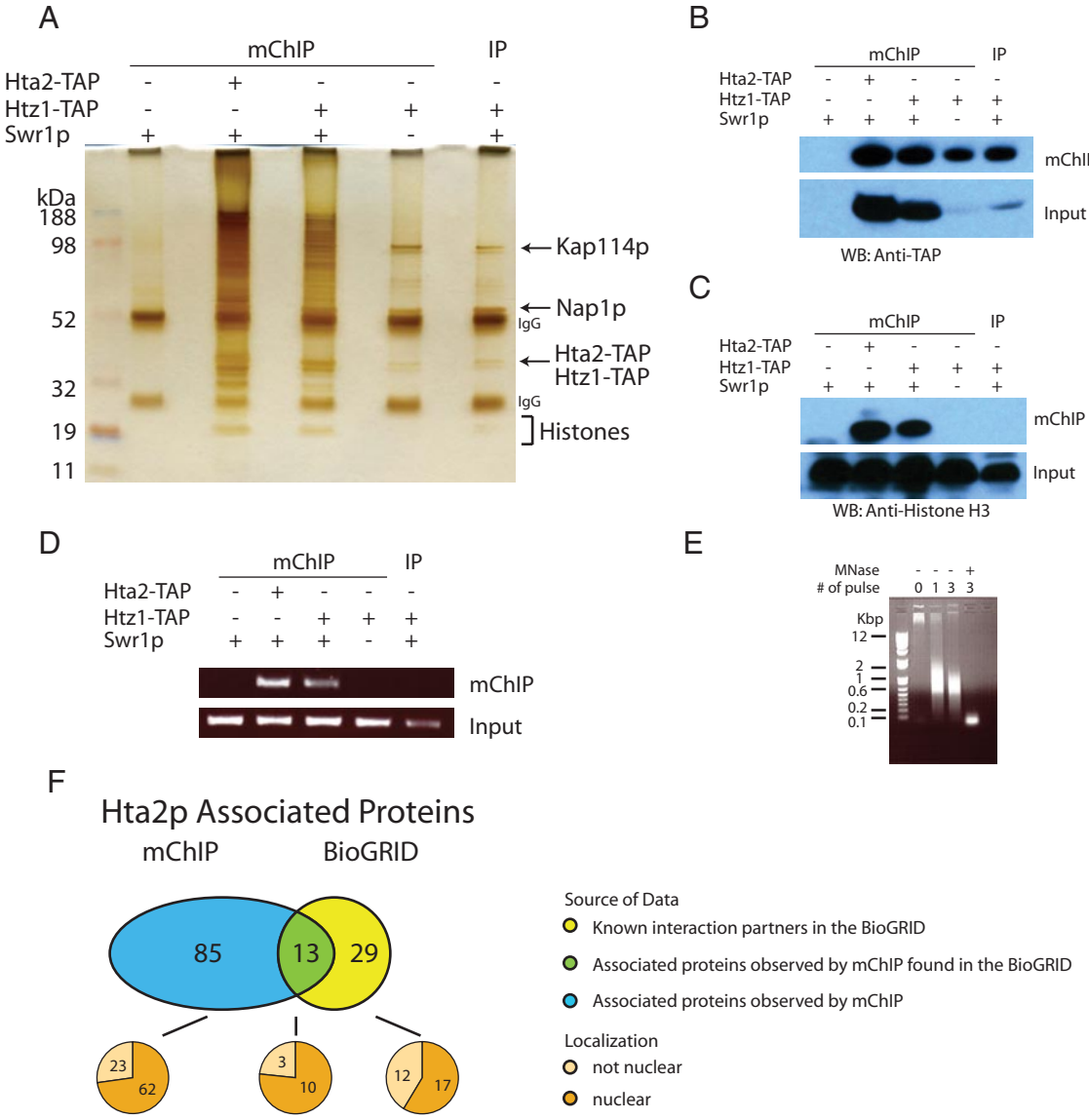

FIG. 2. **Purification of chromatin-bound protein associated with Hta2p and Htz1p by mChIP.** *A*, silver-stained SDS-PAGE gel of protein network associated with H2A-TAP (YSC1178-7499158), Htz1-TAP (YSC1178-7502629), and Htz1-TAP *swr1*Δ (YKB963) purified by mChIP compared with an untagged control (YPH499) and with a traditional Htz1-TAP (YSC1178-7502629) purification (*IP*). An aliquot of the protein samples purified was also transferred on nitrocellulose membrane for Western blot (*WB*) analysis against TAP-tagged (*B*) or histone H3 proteins (*C*). *D*, PCR analysis for *GAL1* locus (+1039 to +1331) following mChIP showing the presence of chromatin co-purifying with the histones H2A and Htz1p. *E*, DNA isolated before and after DNA shearing by sonication and micrococcal nuclease S7 (*MNase*) treatment resolved on a 1.5% agarose gel stained with ethidium bromide. The sample prepared for mChIP usually contains chromatin fragments of 500–1500 base pairs. Results shown are representative of three experiments. *F*, comparison of histone H2A-associated proteins observed by mChIP and those previously reported in IP-MS experiments with H2A as a bait (literature data from BioGRID) and of their nuclear localization (69).

(18, 27, 28), only co-purified with Htz1-TAP, whereas Rvb2p, as SWR1 complex component that also functions in additional chromatin-associated complexes, co-purified also with Hta2-TAP. Htz1-TAP also co-purified the SWR1 complex proteins Arp4p, Arp6p, Swc4p, Swc6p, and Vps71p in one of three replicates (supplemental Table 1). As expected the absence of Swr1p caused a marked decrease in the number of proteins co-purified by Htz1p-TAP; in fact, the protein interaction profile greatly resembled that of Htz1-TAP generated using traditional IP methods (Fig. 2*A*). In both cases, the Htz1p interaction map included Nap1p and Kap114p, two proteins shown previously to interact with Htz1p in the soluble (cytosolic) fraction of the cell (33). The remarkable differences in associated protein profiles between the mChIP and traditional IP support the use of the mChIP procedure in generating protein interaction networks of chromatin-bound proteins. This can be further demonstrated by comparing the 98 protein interactions observed for Hta2-TAP by mChIP against the 42

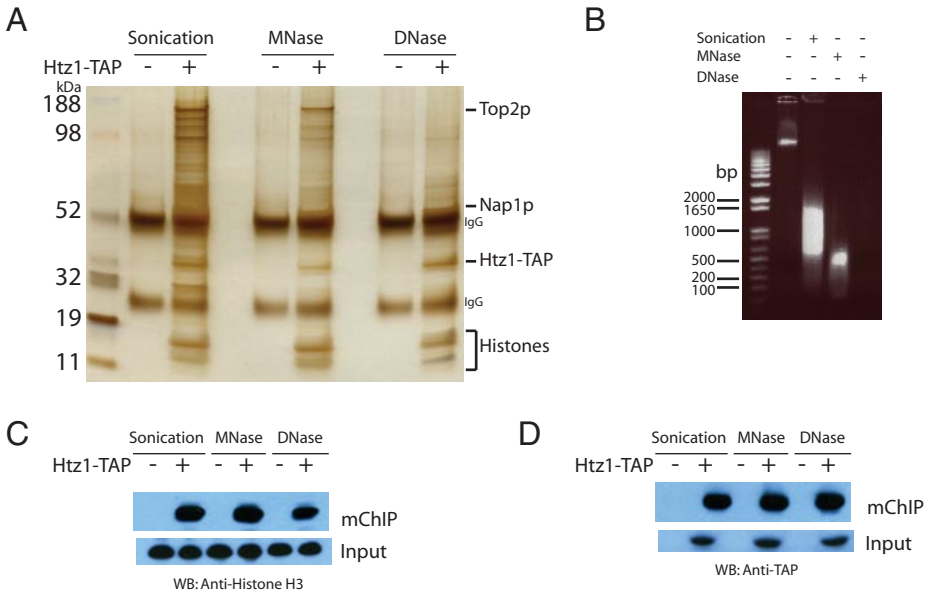

FIG. 3. **The size of chromatin fragments in whole cell extract determines the magnitude of the protein networks co-purifying with Htz1-TAP by mChIP.** The mChIP procedure was performed from the Htz1-TAP strain (YSC1178-7502629) or untagged control strain (YPH499) previously treated with sonication, micrococcal nuclease S7 (*MNase*) or DNase I (see "Materials and Methods" for details). *A*, 90% of the protein eluted for each sample was resolved on a NuPAGE 4–12% SDS-PAGE gel and silver-stained. *B*, phenol/chloroform extractions from the whole cell extracts used in *A* were resolved on a 1% agarose gel, and the chromatin fragments were visualized by ethidium bromide to define their size. 2.5% of each input and mChIP was analyzed by Western blot (*WB*) using antibodies against histone H3 (*C*) or the TAP tag (*D*).

protein interactions for Hta2-TAP IP-MS purifications curated in the BioGRID (34) (Fig. 2*F*). We observed an overlap of 13 protein interactions between traditional IP and mChIP. Furthermore it was found that the mChIP-derived protein interactions for Hta2-TAP have a greater proportion of nuclear localization than those found in the BioGRID only, indicating an improved accessibility to chromatin protein networks in mChIP purifications compared with classical methodologies (Fig. 2*F*).

mChIP Protein Networks Are Affected by DNA Size—We were interested in determining how the size of DNA fragments in the cellular extracts affects the proteins co-purified by mChIP. As our method purifies large chromatin pieces (500–2000 base pairs; Fig. 2*E*), it was expected that some co-purified proteins would not interact directly with the bait protein, but rather the interactions would be mediated through DNA. This was tested by adjusting the size of DNA in the cellular extracts with one of two enzymes: micrococcal nuclease S7, which cleaves DNA between nucleosomes, or DNase I, which digests most DNA in solution given enough time (16). This makes for a system where the DNA in an extract can be large (sonication), medium (micrococcal nuclease S7), or small/absent (DNase I) (Fig. 3*B*). Htz1-TAP and untagged control mChIPs were performed from extracts treated by sonication, micrococcal nuclease S7, or DNase I, and the purified proteins were resolved on a 4–12% NuPAGE gel and silver-stained (Fig. 3*A*). We found that as the DNA size was reduced in the cellular extract (Fig. 3*B*) the amount of co-purifying proteins was concomitantly reduced as assessed by silver stain (Fig. 3*A*). Notably DNA fragment size did not significantly affect the purification efficiency of Htz1-TAP and the associated core histones, in particular histone H3 (Fig. 3, *C* and *D*), reflecting the fact that the mChIP method can efficiently purify large protein-DNA macromolecules. These data support the view that the mChIP method isolates both proteins that directly or indirectly interact with the bait and proteins that are not necessarily physically interacting with the bait but are bound to the DNA/chromatin that is co-purified by the bait. A specific example of this phenomenon is the association of Top2p and Nap1p with Htz1-TAP by mChIP. As seen in Fig. 3*A*, the histone chaperone Nap1p is strongly associated with Htz1-TAP independently of the DNA shearing method used reflecting their strong direct interaction. On the other hand, Top2p, a DNA topoisomerase, is dependent on the presence of DNA for its associations with Htz1-TAP and is lost upon DNase I treatment. Thus manipulating the size of the DNA fragments can be used as a tool to further dissect the protein networks associated with a bait of interest. But for the purpose of this work, all the subsequent experiments were done using sonication as the DNA shearing method because it provided us with the largest number of associated proteins upon mChIP.

The mChIP procedure functions efficiently for histone baits (Fig. 2) because these proteins are tightly associated with DNA. The question of whether other classes of chromatin-associated proteins would be as easily amenable to mChIP purifications remained open. To answer this, three non-histone baits that are difficult to purify by the traditional IP method were selected for mChIP purification. The baits chosen were Lge1p, Mcm5p, and Yta7p, and each was purified in triplicate following the mChIP protocol developed previously.

Efficient Purification of the Poorly Characterized Lge1p and of Its Associated Proteins by mChIP—Lge1p, which stands for large cells 1, is a protein of unknown function whose deletion results in the formation of abnormally large cells, a rare phenotype in *S. cerevisiae* (35). Using traditional TAP and MS techniques, Lge1p was observed previously to interact with only two proteins: Bre1p (9, 10, 36), an E3 ubiquitin ligase

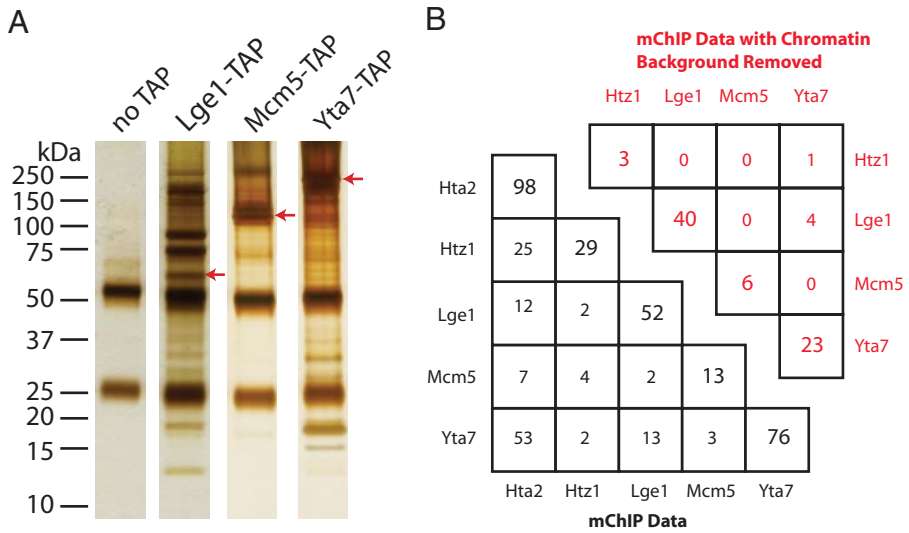

FIG. 4. **mChIP efficiently purifies chromatin-associated protein networks when non-histone protein are used as baits.** *A*, silver-stained 4–12% NuPAGE gels of the protein networks associated with Lge1-TAP (YSC1178-7503047), Mcm5-TAP (YSC1178-7501832), and Yta7-TAP (YSC1178-7500768). mChIP purifications performed according to the "Materials and Methods" are shown as compared with an untagged control (YPH499). The *red arrows* indicate the TAP-tagged bait protein. Results shown are representative of three experiments. *B*, summary of the overlap between the associated proteins observed in the high confidence data set (supplemental Table 2) and each mChIP bait (*bottom left triangle*) and with the chromatin background or Hta2-TAP high confidence data set removed (*top right triangle*).

required for monoubiquitination of histone H2B (37), and Isw1 (10), a component of the chromatin-remodeling complex Isw. Interestingly histone H2B monoubiquitination at lysine 123 is required for the methylation of histone H3 at lysine 4 (38–40). The exact role of Lge1p in this "cross-talk" is still undefined (41, 42). Lge1p was also shown to participate in the induction of *PDR3* transcript independently of its association with Bre1p and of its role in histone H2B ubiquitination (43). *lge1*Δ cells display genetic interactions with more then 369 genes indicating that its function impacts a myriad of cellular functions. These puzzling aspects of Lge1p biology and its apparent association with chromatin make it a prime candidate for mChIP analysis in hopes of further refining its cellular functions.

Although traditional methods only identified Isw1 (10), mChIP of Lge1-TAP co-purified an unexpectedly large number of associated proteins (Fig. 4*A*). We reproducibly identified 52 proteins co-purifying with Lge1-TAP in two of three purifications, 40 of which were unique to Lge1-TAP and not identified in the Hta2-TAP mChIPs that serve as a chromatin background control (Fig. 4*B* and see supplemental Tables 1 and 2 for complete lists). Bre1p was observed as the top hit in each of the Lge1p mChIP experiments, confirming that Lge1p and Bre1p strongly interact. In addition, a plethora of new associated proteins were found. Surprisingly Kog1p, for kontroller of growth, was the second MS hit in two of the three Lge1p mChIP experiments and thus strongly associated with Lge1p. Kog1p is a member of the TOR1 complex, a protein complex containing the Tor1p kinase, and is involved in control of cell growth in response to nutrient level (44). Two more subunits of the TOR1 complex (Tco89p and Tor1p) reproducibly were also co-purified with Lge1p by mChIP, and a third member, Lst8p, was identified in one experiment. The association between Lge1p and the TOR1 complex raises the possibility that Lge1p could affect cell size via interplay with the action of the TOR1 complex. Furthermore a significant amount of Ssd1p was co-purified in the three Lge1p mChIPs, solidifying the connection between Lge1p and the TOR1 complex made in a previous report linking the TOR1 complex component to Ssd1p (45). Another subset of Lge1p-associated proteins (Msh2p, Msh3p, Msh6p, and Rad52p) was found to be involved in DNA repair. These associations are consistent with previous reports showing the sensitivity of *lge1*Δ cells to UV radiation (46). Finally spindle pole body (SPB) components make up a large fraction of proteins co-purifying with Lge1p by mChIP. This includes core SPB components such as Spc29p, Spc110p, Spc97p, Spc98p, Spc42p, Cdc31p, Nud1p, and Cnm67p but also associated kinases Kin3p and Kin4p and the mitotic exit network protein Bub2 (for a review, see Ref. 47). Both the number of SPB components and their high scores in our MS analysis suggest that a significant fraction of the cellular Lge1p may be present at the SPB and may contribute to spindle dynamics.

Purification of the Minichromosome Maintenance (MCM) Helicase and of Its Associated Interaction Partners by mChIP—The MCM complex is composed of six highly conserved components termed Mcm2p through Mcm7p (for a review, see Ref. 48). The MCM complex participates in DNA replication and elongation, and each subunit is essential for cell survival in *S. cerevisiae*. To effectively function, the MCM complex associates with a number of distinct proteins such as the DNA replication initiation factor Cdc45p as well as other

chromatin-associated proteins such as Ctf4p at specific cell cycle stages and chromatin locations (49–51). To date, successful affinity purification of the MCM complex has required a large amount of cells (up to 10 liters) (50), and high throughput studies failed to purify the highly abundant MCM complex (7, 9, 10). We hypothesized that the tight interactions of the MCM complex with chromatin might be the cause of the low efficiency in previous immunopurifications and therefore tested the mChIP method with a component of the complex, Mcm5-TAP. The three Mcm5p mChIP experiments were successful at reproducibly co-purifying 13 proteins, six of which were only identified in the Mcm5-TAP (Fig. 4*B*). High levels of the six core MCM components (Mcm2p, Mcm3p, Mcm4p, Mcm5p, Mcm6p, and Mcm7p) were found in the three Mcm5-TAP mChIP experiments despite the relatively small quantity of cells used for the purifications (700-ml cultures). Mcm3p and Mcm6p were identified in only the Mcm5-TAP, whereas Mcm2p, Mcm4p, Mcm5p, and Mcm7p were identified in both Mcm5-TAP and Hta2-TAP mChIPs. Furthermore additional non-core MCM complex components were also co-purified by mChIP, Tof1p and Ctf4p. Sap185p was identified as a novel interaction partner for Mcm5p. Sap185p is a phosphatase shown previously to interact with Sit4p, another phosphatase, and is involved in the G_1 to S cell cycle progression (52). The co-purification of Sap185p with Mcm5-TAP by mChIP raises the possibility that Sap185p may interact with one or more MCM core subunits and dephosphorylate them in a cell cycle-dependent manner.

Determination of Yta7p Interaction Partners on Chromatin by mChIP—One protein that was identified in both Hta2-TAP and Htz1-TAP mChIP experiments was Yta7p. Yta7p contains three domains: two AAA ATPase domains of unknown function and one bromodomain, a domain known to bind acetylated histones (53). Yta7p had been observed previously only at chromatin boundary regions between transcriptionally active and silent chromatin (54). Recently Yta7p was also reported to bind to the promoter regions of histone genes and to regulate their transcriptional expression (55). Therefore, we were surprised to observe Yta7p as one of the top preys associated with both Hta2-TAP and Htz1-TAP, hinting to a broad distribution for this protein on chromatin and a potentially global regulation of chromatin structure for Yta7p in *S. cerevisiae*. To further investigate, the mChIP method was applied to identify the chromatin-associated protein networks of Yta7p (Fig. 4*A*).

Yta7-TAP was successfully purified using the mChIP method, and 76 proteins were reproducibly identified (Fig. 4). The overlap between Yta7-TAP and Hta2-TAP protein interaction profiles was large at 53 proteins, suggesting a wide distribution of Yta7 along chromosomes (Fig. 4*B*). However, 23 proteins were specific to Yta7-TAP (Fig. 4*B*). Previously reported interaction partners of Yta7p such as the core histones, Htz1p, Top2p, Spt16p, Sas3p, and Ylr455wp (54) were observed upon mass spectrometric analysis (see supplemental Table 1 for a complete list of interaction partners). In addition our mChIP method identified numerous other proteins including Pob3p, a subunit of the Facilitates Chromatin Transcription (FACT) complex known to interact with Spt16p (56); Chd1p, a nucleosome-remodeling factor containing a chromodomain (57); Nto1p, a subunit of the NuA3 acetyltransferase complex (for a review, see Ref. 58), and others. This suggests that despite the broad distribution of Yta7 it is likely found in specific chromatin microenvironments.

DISCUSSION

In this study, we present a novel proteomics method, termed mChIP, for the purification of proteins that interact with chromatin. Our method increases the solubility of protein-DNA complexes by introducing a DNA shearing step and subsequently minimizing centrifugation in the sample preparation procedure. Together these steps ensure the maintenance of protein-DNA macromolecules in the soluble fraction of the whole cell extract and hence make them available for purification in sufficient quantities for mass spectrometric analysis. Using Hta2-TAP and Htz1-TAP as tests baits we applied our method to build a chromatin-bound protein network in yeast. To further demonstrate the power of this method, we applied it to three non-histone proteins and demonstrated that the mChIP method is specific and sensitive for these functionally distinct baits.

mChIP Method Expands Chromatin-bound Protein Networks—We initially tested the mChIP method for its ability to purify chromatin-bound protein networks associated with the canonical histone H2A (Hta2p) and its variant (Htz1p) in *S. cerevisiae*. We observed that our newly developed method minimized the co-purification of nonspecific interacting proteins (supplemental Table 1) while providing a wealth of new information concerning protein interactions of the histone baits (Fig. 2*F* and supplemental Table 1). Additionally we determined that contrary to classical TAP (26) the mChIP method efficiently purifies both chromatin-bound and cytosolic interaction partners of Htz1-TAP. This was accomplished by purifying Htz1-TAP in cells lacking *SWR1*. Swr1p is the enzyme responsible for the insertion of Htz1p into the nucleosome, and its deletion greatly reduces Htz1p interaction with chromatin (18, 27, 28). Notably *SWR1* deletion causes a significant reduction in the cellular level of Htz1p as compared with wild type cells (59). However, because of the efficiency of the mChIP method, Htz1-TAP and its interaction partners were still immunopurified in *swr1*Δ cells in sufficient quantity to allow for MS analysis. We observed that in the absence of Swr1p the chromatin-bound interaction partners of Htz1p were lost, whereas the interaction with the two histone chaperones Nap1p and Kap114p, present in the cytoplasm and nucleus, were maintained (Fig. 2*A*). This supports our view that the DNA and chromatin-bound proteins co-purified with our baits are not artifacts produced in the sample preparation but represent genuine interactions. Moreover we demon-

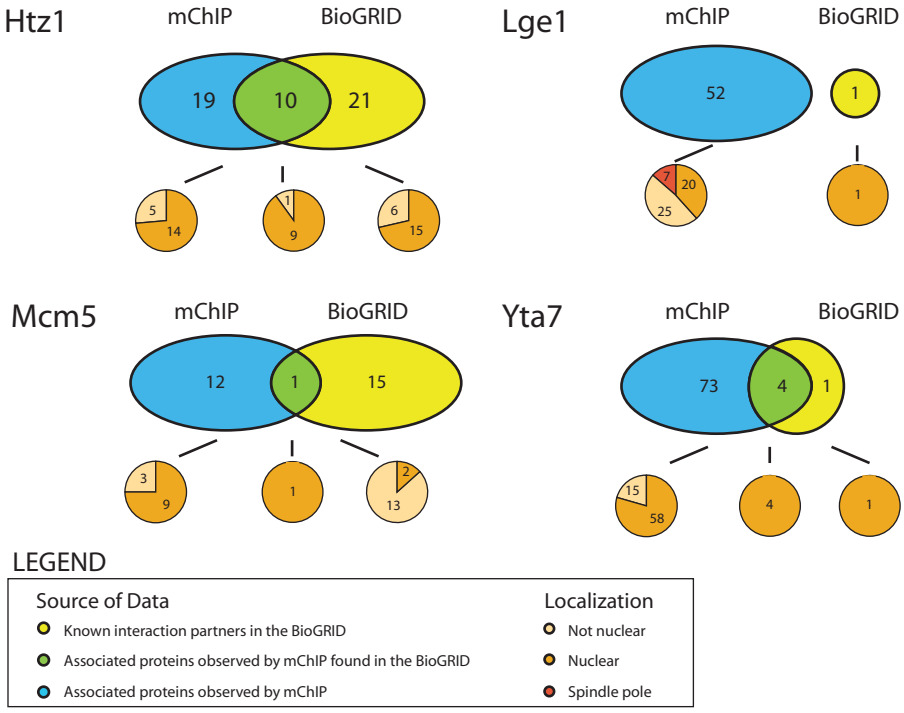

FIG. 5. **mChIP improve the depth and nuclear coverage of protein networks associated with chromatin-bound proteins.** Shown are Venn diagrams of unique and shared associated proteins found in two of three mChIP purifications and traditional TAPs as curated in the BioGRID (supplemental Table 2). The localization of each associated proteins was assessed by using the yeast green fluorescent protein fusion localization database (69).

strated that by adjusting the DNA fragment size in the cellular extracts through the use of enzymes we can modulate the extent of interaction partners that co-purify with Htz1-TAP bait (Fig. 3). This technique may be used to precisely define chromatin-bound protein environments on chromatin that to date have not been extensively characterized.

An interesting side product observed in the development of the mChIP is the ability to efficiently co-purify DNA associated with the bait of interest with this new method (Fig. 2*D*). We recently exploited this to localize Msn4p to the *HSP12* gene under stress conditions by mChIP (60). Chromatin immunoprecipitation that circumvents the use of formaldehyde, often named native ChIP (or NChIP), have been described previously (61), but these protocols are lengthy and restricted to histone proteins. In contrast the mChIP method produced good results for the isolation of DNA associated with both histone (Fig. 2*D*) and non-histone proteins (60) while remaining a relatively fast and convenient protocol.

mChIP experiments performed using histone baits provide an efficient means to define the chromatin protein background, or base line, in *S. cerevisiae* with which other mChIP purifications may be compared (Fig. 4*B*). In addition, it enabled us to define the depth of the current literature regarding chromatin-based protein interactions. When comparing the Hta2-TAP mChIP data with previously reported Hta2-TAP IP-MS experiments, it became obvious that the mChIP procedure significantly expanded the number of associated proteins detected (Fig. 2*F*). Furthermore Hta2-TAP interaction partners obtained through mChIP had a higher proportion of nuclear localization (Fig. 2*F*) reflecting an increase in sensitivity and specificity of the mChIP method because of the special care taken to maintain the integrity of macromolecules during the purification process (Fig. 1).

Efficient mChIP Purifications of Non-histone Chromatin Baits—The new protocol we developed was optimized using histones as the archetype of chromatin-associated baits. These baits by their very nature are strongly bound to DNA and constitute the foundation of chromatin. A crucial test of the mChIP protocol was to efficiently purify non-histone baits associated with chromatin. Yta7p, Mcm5p, and Lge1p were successfully purified by mChIP without the need of any bait-specific optimization (Fig. 4*A*). Furthermore the protein networks identified for each bait are significantly different, demonstrating the specificity of the mChIP procedure (Figs. 4*B* and 6). For example, Mcm5p is known to be present at replication forks, which are very specialized chromatin environments that are devoid of most common chromatin protein (62). This is reflected by the mChIP data for Mcm5p that contain a small fraction of the previously defined chromatin background by histone mChIP (supplemental Table 2). On the other hand, Yta7p has been shown recently to be bound at the promoter sequence of a large number of genes (55). This is in agreement with the mChIP data obtained for Yta7p that contain a large proportion of the proteins associated with histone baits by mChIP, reflecting its broad chromatin localization. Thus, mChIP data can be use to provide an indirect readout of the distribution of a bait protein on chromatin.

When the mChIP data were compared with protein-protein interaction data contained in the BioGRID when the five proteins were used as baits, we observed that the overlap was particularly low for Mcm5p and Lge1p (Fig. 5). This is consistent with our view that traditional TAPs are not effective for

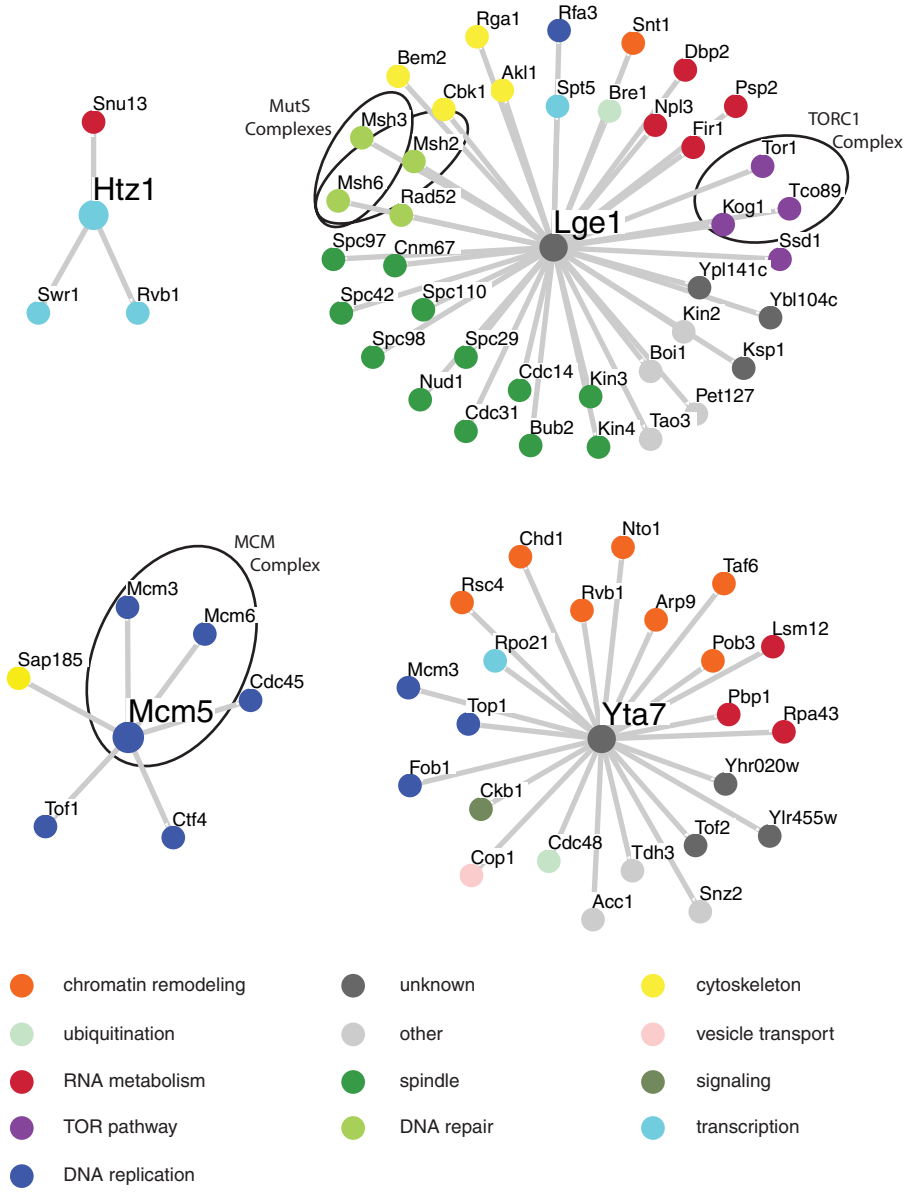

FIG. 6. **Distinct protein networks co-purify by mChIP with histone Htz1-TAP, Lge1-TAP, Mcm5-TAP, and Yta7-TAP (baits are shown in *bold* text).** Associated preys displayed are those reproducibly identified (minimum of two of three mChIPs) that were not identified in Hta2p-TAP mChIP (supplemental Table 2). Osprey was used to display proteins identified in one or more mChIP purifications for each bait protein. *Gray edges* represent physical interaction observed by mChIP, and *node color* corresponds to gene ontology annotations as listed in the *Saccharomyces* Genome Database. Some known protein complexes are denoted by a *black circle*.

numerous chromatin-associated proteins and thus poorly represented in the literature. Mcm5p provide a clear example of this situation. When Mcm5p was used as a bait, a total of 16 distinct interaction partners have been curated in the BioGRID (supplemental Table 2). Interestingly only three of these interaction partners are known to be present in the nucleus (supplemental Table 2). On the other hand, 13 interaction partners were found for Mcm5p in at least two of three mChIP purifications (supplemental Table 2) with 10 of these (~75%) present in the nucleus. This effectively demonstrates the improvement in the coverage of the Mcm5p nuclear interactome by mChIP as compared with classical methodologies.

Gain of Novel Protein-Protein Interaction Information through mChIP Purifications—The mChIP method was also shown to be successful at purifying large protein networks associated with poorly characterized non-histone proteins (Fig. 4). The most striking example of this is the success of the mChIP procedure at purifying Lge1p. To our knowledge, the only reported protein-protein interactions for Lge1p is with Bre1p (7, 9, 10), Isw1p (10), and Ybr296c-Ap (63). Despite this apparently limited interactome of Lge1p, we observed over 50 proteins co-purifying with Lge1p in at least two distinct mChIP purifications (Fig. 4*B*), most of which were not part of the chromatin background defined by Hta2-TAP (Fig. 6). In particular, various protein complexes, such as TORC1 and the SPB, were observed to associate with Lge1p in a reproducible manner by mChIP. This new layer of information now accessible through the use of the mChIP procedure can help explain some of the known phenotypes associated with the

LGE1 gene. For instance, the most drastic phenotype of *lge1*Δ cells is their size (35). Lge1p mChIP revealed a significant enrichment for the TOR1 complex (Tor1p, Kog1p, Tco89p, and Lst8p). The TOR1 complex has been shown to regulate the cell cycle by integrating a number of cell signals (nutrients, hormones, and growth factors levels) (64). The exact mechanism of the TOR1 complex regulation of the cell cycle remains nebulous, but it was observed in fission yeast (*Schizosaccharomyces pombe*) that TOR1 inhibition through rapamycin treatment results in a significant reduction of cell size (65). In *S. cerevisiae*, *lge1*Δ cells were observed to tolerate rapamycin treatment better than wild-type cells in a genome-wide study (66). This partial rescue to TOR1 inhibition by rapamycin through the deletion of *lge1*, in addition to their association in mChIP purification, points to their participation in common cellular events. One such event could be in the control of cell cycle through the monoubiquitination of histone H2B at lysine 123. Hwang *et al.* (37) showed that mutation of histone H2B at lysine 123 to an arginine (K123R), preventing its monoubiquitination, resulted in an increase in cell size. The authors speculated that histone H2B monoubiquitination could function as part of a "biochemical calculation" that could participate in determining cell size and impact cell cycle check, such as START initiation (37). The resistance of *lge1*Δ cells to rapamycin is also shared by *bre1*Δ and *rad6*Δ cells (66); these proteins are directly involved in histone H2B Lys-123 monoubiquitination. In light of our Lge1p mChIP and of the previous literature regarding *LGE1* and the TOR1 complex, the TOR1 complex could participate in this biochemical calculation to efficiently regulate the cell cycle.

A few years ago, Zhang *et al.* (43) demonstrated that Complex Proteins Associated with Set1 (COMPASS) was capable of dimethylating lysine 233 of Dam1p, a protein localizing to the kinetochore that is required for proper chromosome segregation and spindle integrity (67). This process was shown to be independent of Bre1p-Rad6p and of H2B Lys-123 monoubiquitination (43), but the possibility of a cross-talk between Complex Proteins Associated with Set1 (COMPASS) and Bre1p-Rad6p on non-histone proteins was raised (68). Although this possibility has not been demonstrated to be correct yet, it remains an attractive hypothesis in light of the large Lge1p interactome observed by mChIP that would locate Lge1p (and potentially Bre1p) to the spindle pole body. Whether Bre1p possesses an enzymatic activity toward any spindle pole body constituents is still unknown, but this process could be linked to proper cell cycle progression and thus be related to the known *lge1*Δ cell phenotype.

In summary, we have developed a novel method for the purification of protein networks associated with chromatin. The mChIP method was shown to be capable of purifying a large amount of chromatin-bound protein complexes associated with the histones Hta2p and Htz1p in an efficient and specific manner. The mChIP protocol was also capable of establishing protein networks of chromatin-associated baits that are hard to purify by traditional IPs. Importantly we demonstrated that this method is not limited to purifying proteins that are physically linked to the bait but can purify proteins that interact with the DNA or chromatin with which the bait interacts. This represents an advance in the identification of protein-protein interactions from the detection of direct interactions to a more global association between complexes or macromolecular environments. As we have shown in this report, the ability to efficiently and specifically purify protein networks on chromatin can provide a novel means to improve our understanding of protein functions. We are currently using the new mChIP procedure to explore the chromatin networks associated with poorly characterized DNA-binding proteins in *S. cerevisiae* and to improve our comprehension of their individual functions.

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