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The Snf2 Homolog Fun30 Acts as a Homodimeric ATP-dependent Chromatin-remodeling Enzyme*[S]

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The *Saccharomyces cerevisiae* Fun30 (Function unknown now 30) protein shares homology with an extended family of Snf2-related ATPases. Here we report the purification of Fun30 principally as a homodimer with a molecular mass of about 250 kDa. Biochemical characterization of this complex reveals that it has ATPase activity stimulated by both DNA and chromatin. Consistent with this, it also binds to both DNA and chromatin. The Fun30 complex also exhibits activity in ATP-dependent chromatin remodeling assays. Interestingly, its activity in histone dimer exchange is high relative to the ability to reposition nucleosomes. Fun30 also possesses a weakly conserved CUE motif suggesting that it may interact specifically with ubiquitinylated proteins. However, *in vitro* Fun30 was found to have no specificity in its interaction with ubiquitinylated histones.

The process of eukaryotic gene regulation is intimately associated with the manipulation of chromatin structure. This is accomplished via a range of strategies that include protein complexes that remodel the structure of chromatin using the energy of ATP hydrolysis (for review, see Ref. 1) or covalently modify the core histones by acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation (for review, see Ref. 2). ATP-dependent chromatin remodeling enzymes share a catalytic subunit with homology to the yeast Snf2 protein. The helicase-related motifs within this region are thought to function as a DNA translocating motor (3). Based on the homology within this region, Snf2-related proteins can be assigned to 24 subfamilies, many of which have been broadly conserved during the evolution of eukaryotes (4). Snf2 family proteins have a diverse range of functions with many, but not all, acting to alter chromatin structure. These ATP-dependent chromatin remodeling enzymes can generate a spectrum of different types of transition in chromatin structure ranging from nucleosome eviction or sliding to the exchange of histone dimers (3).

In this report, we investigate the *Saccharomyces cerevisiae* Snf2 family protein, Fun30 (Function unknown now 30). Fun30 was originally identified as a result of genome sequencing (5) and shares most sequence homology with the Swr1 and Ino80 chromatin remodeling enzymes (4), both of which are implicated in histone dimer exchange (6, 7). Previous studies have shown that yeast *fun30* deletions are viable, but temperature sensitive (5), and are resistant to ultraviolet (UV) radiation (8). The overexpression of Fun30 has been shown to affect chromosome stability, integrity, and segregation (9). Fun30 has also been shown to be a potential cyclin-dependent kinase (Cdk1)/Cdc28 substrate (10). More recently, Fun30 has been found to play a role in gene silencing (11).

Fun30 is conserved through evolution and its mouse homologue, Etl1 (Enhancer Trap Locus 1), has been identified as being expressed during early development (12). Etl1 is widely expressed but non-essential, although deletion is associated with developmental defects such as skeletal dysplasia, growth retardation, and impaired fertility (13, 14). The human homolog, *SMARCAD1* (previously known as human helicase 1 (hHel1)), has been mapped to the chromosome 4q22–q23 region, which is rich in breakpoints and deletion mutants of genes involved in several human diseases, notably soft tissue leiomyosarcoma, hepatocellular carcinoma, and hematologic malignancies (15). It has been recently reported that the binding sites of endogenous SMARCAD1/KIAA1122 are frequently found in the vicinity of transcriptional start sites (16).

To gain insight into the function of Fun30, we have purified it from tagged yeast strains. We obtain Fun30 as a homodimeric complex. This complex displays activity in a range of chromatin remodeling assays. Interestingly, the Fun30 complex displays increased activity in histone dimer exchange assays in comparison to nucleosome sliding. These results suggest that Fun30 function may involve the manipulation of the histone content of nucleosomes.

EXPERIMENTAL PROCEDURES

Purification and Characterization of SWI/SNF, RSC, and Fun30—The SWI/SNF and RSC complexes as well as Fun30 were purified from yeast whole cell extract by tandem affinity

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purification (TAP)[4] over two affinity columns (17). The Snf6TAP tag strain used for the purification of the SWI/SNF was generated by M. Carroza, for RSC, strain BCY211 was kindly supplied by Brad Cairns, and the Fun30 TAP tag strain was purchased from Euroscarf. Complexes were purified as described previously (18). Purification was monitored by Western blotting, using anti-TAP antibody (Open Biosystems, Huntsville, AL) as well as silver staining. Equivalent quantities of the enzymes were used in assays after normalization of the amounts of purified protein. For the co-immunoprecipitation experiments, the TAP-tagged strain was transformed with a GAL hemagglutinin (HA)-tagged Fun30 expression plasmid (Open Biosystems). Following induction, total cell protein was purified from 500 ml of minimal media culture inoculated with either the single or double-tagged yeast strains. The protein concentration was measured by Bradford assay, and 60 μg of total protein was incubated with either IgG-Sepharose beads (GE Healthcare) or Protein G Plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) coupled to anti-HA high-affinity rat monoclonal antibody 3F10 (Roche Applied Science) in a pulldown buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml of leupeptin, and 2 μg/ml of pepstatin A) for at least 2 h at 4 °C while mixing on a rotating wheel. The supernatants were collected, and the beads were then washed with pulldown buffer three times and left as a 50% slurry after the final wash. Equal fractions of both supernatants and beads were loaded on a 10% SDS-PAGE gel, and Fun30 was detected by immunoblotting.

Expression and Purification of Recombinant His-tagged Fun30—The *fun30* gene was amplified from yeast genomic DNA and subsequently subcloned into the pGEX-6P1 expression plasmid (pGF30). pGF30 was then engineered to contain an in-frame hexa-histidine tag (SSHHHHHH; His_6) at the C terminus of the *fun30* gene (pGF306H). Double-tagged Fun30 (N-terminal GST-tag, C-terminal His_6; GST-Fun30-His_6) was expressed from pGF306H in the Rosetta2 *Escherichia coli* strain (Novagen) at 20 °C overnight by induction with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris, pH 7.5, 350 mM NaCl, 0.05% β-mercaptoethanol) containing protease inhibitors (0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 2 μM E64, 2.6 mM aprotinin, 1 μM pepstatin) and with or without 0.1% Tween 20. Cells were lysed by freeze/thawing in liquid nitrogen and sonication and the soluble fraction extracted by centrifugation at 35,000 $\times$ *g* at 4 °C for 30 min. GST-Fun30-His_6 was then purified in batches over HisPur cobalt resin, washed in lysis buffer, and eluted in lysis buffer containing 250 mM imidazole. The HisPur eluate was then applied to SuperGlu-glutathione resin (Generon) and washed in lysis buffer, then Prescission protease buffer (20 mM Tris, pH 7.5, 350 mM NaCl, 1 mM DTT, 1 mM EDTA) with or without the inclusion of 0.1% Tween 20. Fun30-His_6 was then released from the GST tag by cleavage overnight with Prescission protease and subsequently eluted in Prescission protease buffer. Fun30-His_6 was then concentrated in 50-kDa molecular mass cut-off centrifugal concentrators and dialyzed against 20 mM Tris, pH 7.5, 350 mM NaCl, 10% glycerol, 1 mM DTT, and stored at −80 C.

Chromatin Assembly—Chromatin was assembled onto the GUB template from purified HeLa oligonucleosomes by dilution from high salt as described previously (18). In Fig. 6 histone octamers were assembled from individual *Xenopus laevis* histones expressed in bacteria (19). Octamers were reconstituted into nucleosomes using PCR prepared from DNA derived from the murine mammary tumor virus nucleosome A (NucA) (20). Reconstitutions were performed at 1 μM concentration and pH 7.5 by stepwise dialysis from 2 M NaCl or KCl to 0.85, 0.65, and 0.5 M and finally 0 M. H2B was fluorescently labeled by attachment of monomaleimide dye (GE Healthcare) as described previously (21).

Immobilized Template Binding Assay—A 2.5-kb fragment excised from plasmid pG5E4-5S that contains a dinucleosome length G5E4 fragment flanked on both sides by five 5 S sequences was prepared as described (22). Following reconstitution by step dilution as indicated the arrays were coupled to streptavidin Dynabeads (Dynal). 10 nM SWI/SNF or Fun30 was added to 200 ng of the above template in 20 μl of binding buffer and incubated for 1 h at 30 °C. The templates were then concentrated using a magnet, the supernatant was removed, and the beads were washed twice. The presence of SWI/SNF and Fun30 was determined by Western blotting using the anti-TAP antibody.

Quantitative Electrophoretic Mobility Shift Assay—Fun30-His_6 was serially diluted in 1× HMA buffer (20 mM HEPES, pH 7.6, 25 mM KOAc, 5 mM MgAc) containing 0.1% Tween 20. Binding reactions were established in a final volume of 10 μl containing 0.5× HMA buffer, 0.1 mg/ml of bovine serum albumin, 1 mM DTT, 5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 30 nM Cy3-labeled nucleosome or DNA, and 2 μl of Fun30-His_6 diluted to the concentrations described in the figures. Reactions were allowed to equilibrate on ice for 30 min before electrophoresis on 0.5× Tris borate-EDTA native polyacrylamide (49:1) gels (5% for nucleosomes, 7.5% for DNA) at 150 V for 60 min at 4 °C. Gels were scanned on a FLA-5100 laser scanner (Fujifilm) for the Cy3 signal and the nucleosome/DNA bands were quantified using AIDA image analysis software (version 3.27.001) and curve fitting was performed in Microcal Origin 7 software.

ATPase Assays—TAP purified SWI/SNF and Fun30 were incubated at 30 °C in 5 μl of reaction buffer (13 mM Na-HEPES, pH 7.9, 3 mM Tris-HCl, pH 8.0, 60 mM KCl, 9 mM NaCl, 7 mM $MgCl_2$, 6% glycerol, 0.6 mM DTT, 0.3 mM EDTA, 2 μM unlabeled ATP, 30 nM [γ-^{32}P]ATP) with 6 nM SWI/SNF and 20 nM Fun30 in the presence of 2 ng of chromatin, double-stranded DNA, and single-stranded DNA. Reactions were quenched by 2 μl of stop solution (100 mM EDTA, 50 mM Tris-HCl, pH 7.5). 0.5 μl of the reaction was spotted onto a polyethyleneimine-cellulose thin-layer chromatography (TLC) plates (JT Baker, Inc.). Inorganic phosphate was separated from unreacted ATP by running the TLC plates in 0.5 M LiCl and 1 M formic acid. The plate was dried when the solvent front reached three quarters the height of the plate and was subjected to autoradiography. For the real time ATPase assay, we used the Invitrogen P_i sensor assay essentially following the manufacturer's protocols as described previously (23).

[4] The abbreviations used are: TAP, tandem affinity purification; HA, hemagglutinin; DTT, dithiothreitol; GST, glutathione *S*-transferase; IP, immunoprecipitation.

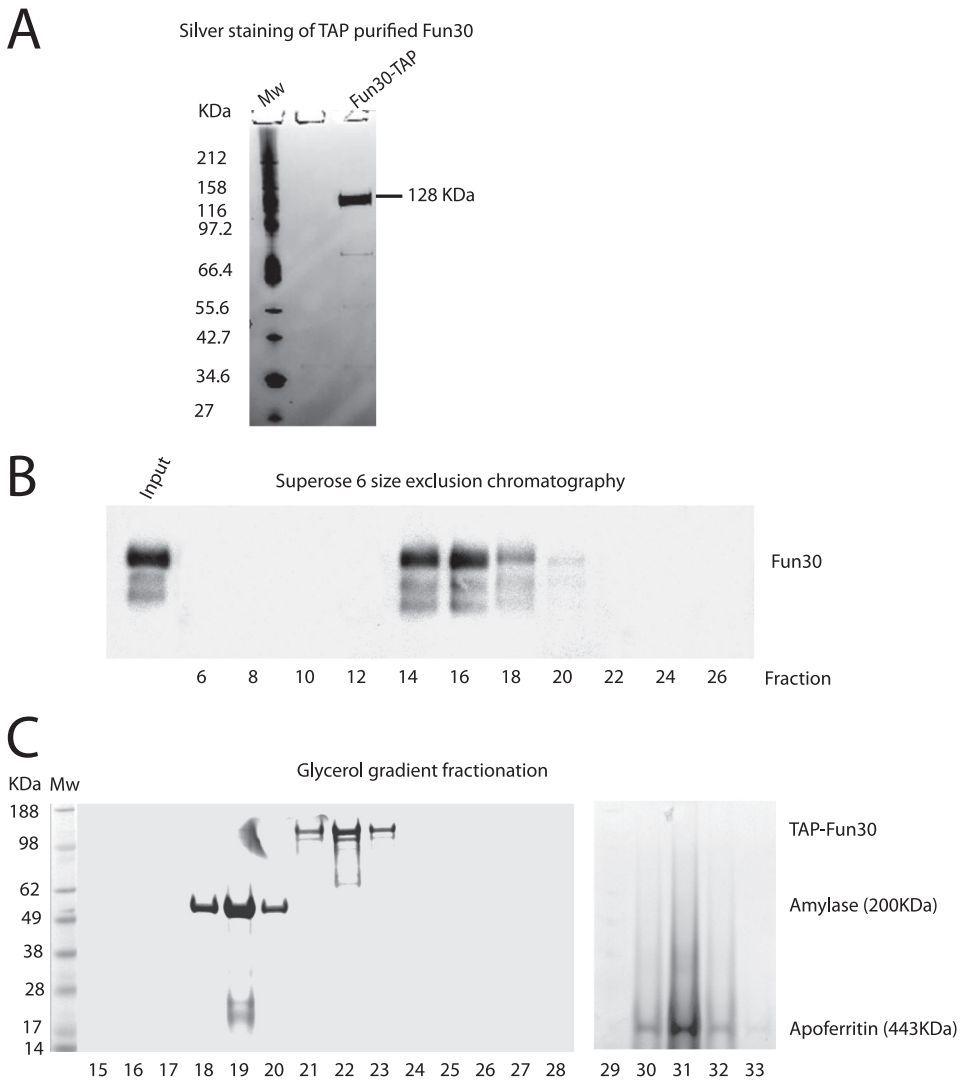

FIGURE 1. **Purification of the Fun30 complex.** *A*, SDS-PAGE and silver staining of TAP-purified Fun30. *B*, Superose-6 chromatography of TAP-purified Fun30. Fractions containing Fun30 were detected by Western blotting with anti-TAP antibody. The Fun30 peak (fraction 16) corresponds to a molecular mass of 250 kDa calculated from a standard curve of molecular mass standards run on the same column (not shown). *C*, Fun30 was resolved by 10–40% glycerol gradient sedimentation together with amylase and apoferritin as molecular weight markers. Coomassie Blue staining followed SDS-PAGE of the fractions indicated where each protein elutes. The elution profile for Fun30 peaked in fraction 22, which was consistent with a complex of about 250 kDa.

SalI Accessibility Assay—10 ng of GUB nucleosomes were incubated with 4 nM SWI/SNF or 10 nM Fun30 for 1 h at 30 °C, the binding reactions were then treated with 10 units of SalI for 30 min at 30 °C and processed as described previously (18).

Nucleosome Repositioning/Dimer Exchange Assay—Murine mammary tumor virus nucleosome A DNA fragments were generated by PCR with the 5′ primer labeled with the Cy3 dye to allow substrates to be visualized as described before (23). Nucleosomes in which H2B was labeled with Cy5 were assembled onto the fragments. Remodeling reactions contained 0.25 pmol of donor nucleosome, 0.75 pmol (3-fold excess) of 146-bp acceptor template (derived from the 601 positioning sequence (24)) assembled with an equimolar ration of $(H3/H4)_2$, 1 mM ATP, 3 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris-Cl, pH 8.0, and RSC or Fun30 as indicated. Reactions were incubated at 30 °C for 30 min and terminated by addition of 0.5 μg of λHindIII-digested DNA and quenching on ice. Sucrose was added to a final concentration of 2% as a loading buffer and reactions were run for 3.5 h on a 0.2× Tris borate-EDTA, 5% polyacrylamide gel at 4 °C and 300 V and visualized using a Fluorimager (FLA5000, Fuji).

Western Transfer of Native Gels—Western blotting of native gels was performed by transfer to a 0.2 μM polyvinylidene difluoride membrane (Bio-Rad) in 20% methanol, 1.44 M glycine, 0.033 M Tris base, 0.1% SDS, overnight at 30 V at 4 °C. Following transfer Western blotting was carried out using standard procedures.

RESULTS

Purification of the Fun30 Protein Complex—To gain insight into the function of the Fun30 protein and study its biochemical activity, Fun30 protein was purified using the TAP technique (17). Silver staining of the purified Fun30 shows a highly pure single band at 128 kDa corresponding to the size of Fun30 (Fig. 1*A*). When the TAP-purified protein was subject to gel filtration chromatography, it was found to elute in fractions consistent with a molecular mass of ~250 kDa (Fig. 1*B*). Purified Fun30 was also fractionated on a 10–40% glycerol gradient by velocity centrifugation (Fig. 1*C*). Again the size of the TAP-purified Fun30 protein peak was estimated to be 250 kDa. Recombinant Fun30 expressed in *E. coli* was found to have similar properties in glycerol gradients and during gel filtration indicating that no other yeast proteins are required for this anomalous behavior (data not shown). As the silver stain revealed a single major band of 128 kDa, yet the size of the native complex is almost twice this, it is possible that Fun30 forms a homodimer.

To test this, yeast strains were made in which Fun30 was expressed as either a TAP-tagged form only or both TAP-tagged and HA-tagged forms of Fun30 were co-expressed. Immunoprecipitation with the TAP antibody enriched for TAP-Fun30 on the beads in both strains (Fig. 2). Probing the immunoprecipitates with an antibody against the HA epitope after an IP with the TAP antibody showed that HA-Fun30 was precipitated with the TAP antibody only in the coexpressing strain (Fig. 2*A*, *second panel*, *lane 3*). Moreover, the reciprocal immunoprecipitation confirms these findings, following immunoprecipitation with the HA antibody, TAP-fun30 was only

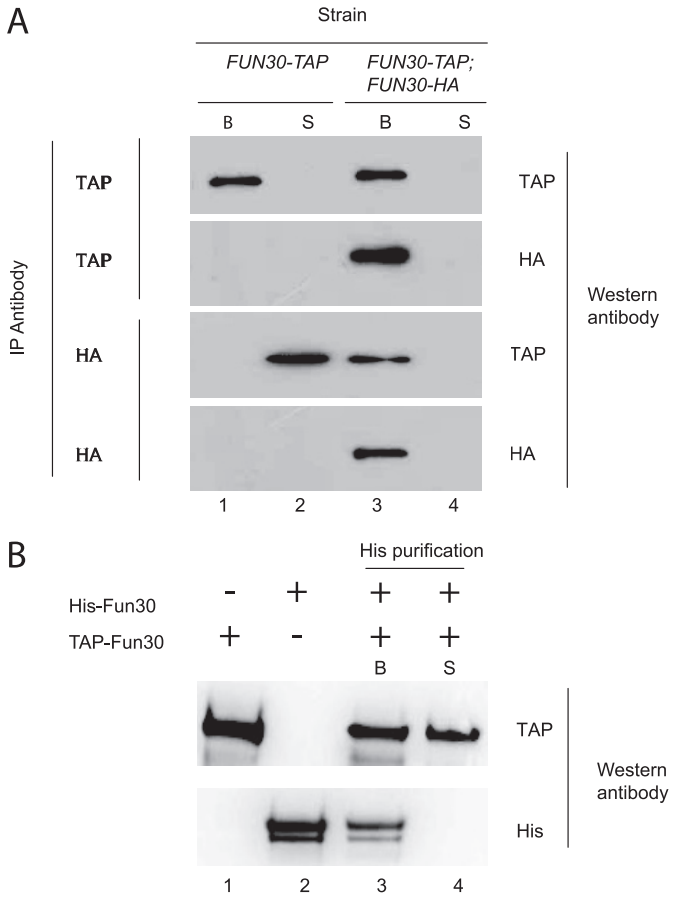

FIGURE 2. **TAP-purified Fun30 is a homodimer.** *A*, Western blots of Fun30 recovered following reciprocal coimmunoprecipitation from a yeast strain expressing either TAP-tagged Fun30 (*lanes 1* and *2*) or both HA- and TAP-tagged Fun30 (*lanes 3* and *4*). Detection of HA-tagged protein in the bound fraction (*B*) following TAP IP and TAP following HA IP (*lane 3*) indicates that the two forms of Fun30 interact. Immunopurification effectively removed Fun30 from the supernatant (*S*). *B*, preparations of Fun30 purified by TAP tag and His tag were mixed together and subject to IP with nickel-agarose beads. The presence of TAP-tagged Fun30 on the nickel beads indicates that these two proteins can interact.

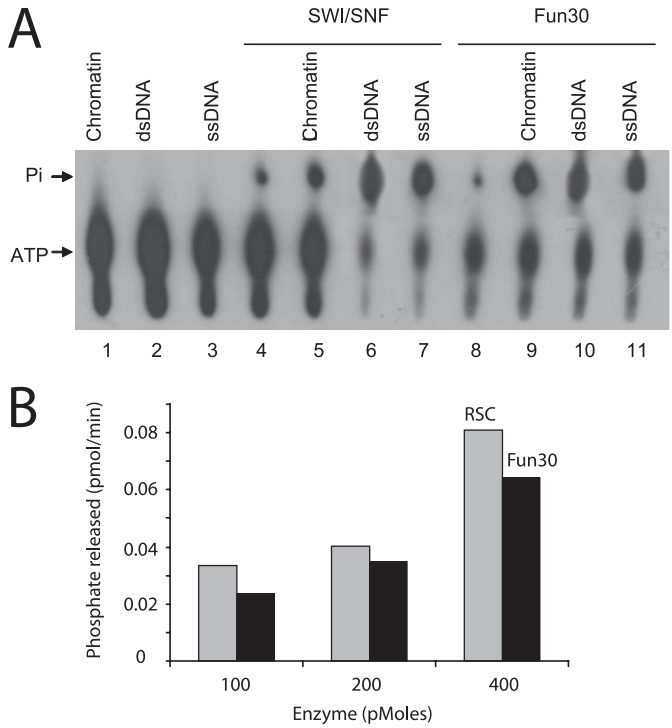

FIGURE 3. **Fun30 has ATPase activity.** *A*, TLC analysis showing ATPase activity of the Fun30 complex. The ATPase activity of ~12 nM Fun30 (*lanes 8–11*) is compared with that of the ~5 nM SWI/SNF complex (*lanes 4–7*) using 2 ng of either single-stranded (*ss*) or double-stranded (*ds*) DNA, or 2 ng of HeLa chromatin as the substrate. In *lanes 1–3*, no chromatin remodeling proteins are added, and in *lanes 4* and *8*, no substrate is included. *B*, Fun30 has a specific activity comparable with RSC. Rates of ATP hydrolysis were assessed using a real time ATPase assay using the indicated quantities of enzyme in the presence of 2 ng of chromatin.

detected in the co-expressing strain. These observations indicate that the two differently tagged forms of Fun30 interact in yeast nuclear extracts. As it is possible that additional proteins might mediate this interaction within an extract, we next investigated whether highly purified Fun30 prepared with different tags and from different sources could interact. To this end, we expressed a recombinant form of Fun30 in *E. coli* that contained a C-terminal histidine tag (Fun30-His_6). Fig. 2*B* shows that when purified TAP-Fun30 and Fun30-His_6 were mixed and Fun30-His_6 pulled down on Ni^{2+} beads, both forms of the protein were precipitated. This indicates that Fun30 is capable of directly interacting with itself. In concert with the observation that the size of the native TAP-Fun30 complex is twice that of a Fun30 monomer, these data provide strong evidence that the Fun30 protein exists predominantly as a homodimer of ~250 kDa.

Fun30 Has DNA and Nucleosome-stimulated ATPase Activity—All Snf2 family proteins studied to date have ATPase activity that is stimulated by the presence of DNA and/or chromatin. To investigate whether Fun30 also has ATPase activity, TAP-Fun30 was incubated with radiolabeled ATP in the presence or absence of DNA or chromatin. Radiolabeled P_i was then separated from ATP by thin layer chromatography (TLC). This showed that the ATPase activity of Fun30, like SWI/SNF is stimulated by both DNA and chromatin (Fig. 3*A*). Using a real time assay for phosphate release (25), the specific activity of TAP-Fun30 was also found to be comparable with that of the RSC complex (Fig. 3*B*). This indicates that the Fun30 complex we have purified from yeast has an ATPase activity comparable with that of other chromatin remodeling enzymes.

Fun30 Binds to DNA and Chromatin—To remodel chromatin, chromatin-remodeling complexes have to be able to recognize and bind to their substrate as a first step. To investigate the ability of Fun30 to bind chromatin, a DNA fragment of ~2.5 kb in length was immobilized to paramagnetic beads either as free DNA or following chromatin assembly. Association of Fun30 and SWI/SNF complex with these immobilized templates was then monitored by Western blotting. Fun30 was found to be capable of binding to DNA and chromatin fragments with comparable efficiency (Fig. 4*A*, *lanes 7–10*).

To gain further insight into the nature of the complex between Fun30 and nucleosomes, quantitative electrophoretic mobility shift assays were performed using Fun30-His_6 and mononucleosomes assembled at defined locations on DNA fragments derived from the 601 sequence (26). Titration of Fun30-His_6 into reactions containing Cy3-labeled mononucleosomes possessing 47 bp of linker DNA on one side (0W47) resulted in a shifting of the nucleosome signal that accumulates

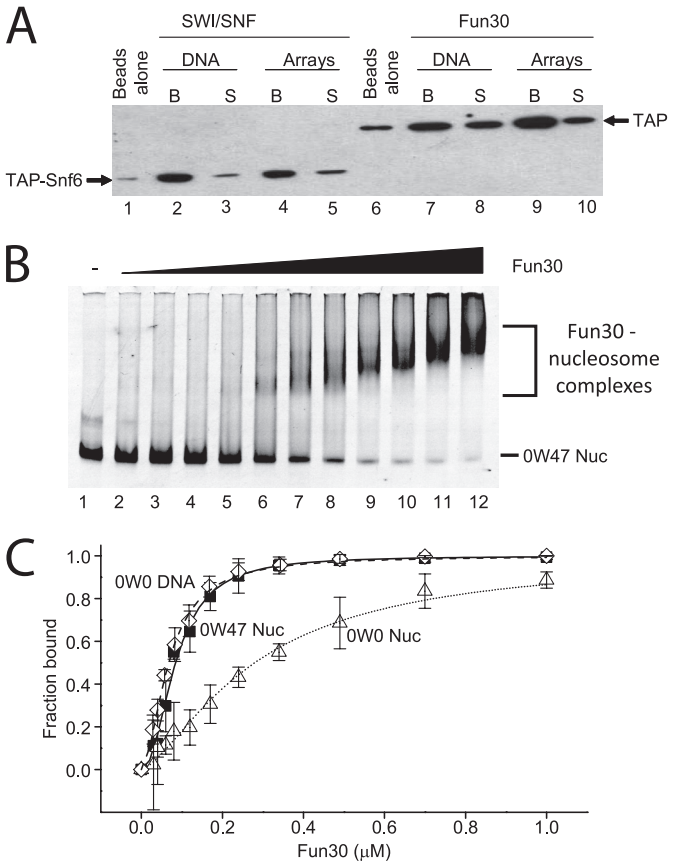

FIGURE 4. **The Fun30 binds DNA and chromatin.** *A*, Fun30 binds to nucleosome arrays efficiently. Immobilized G5E4 (either DNA or reconstituted into nucleosomal arrays), generated as described under "Experimental Procedures," was incubated with an equal amount of Fun30 (*lanes 7–10*) or the SWI/SNF complex (*lanes 2–5*, as control) based on anti-TAP Western blotting normalization. The amount of bound protein (SWI/SNF complex or Fun30) was determined by separating the supernatants (*S*) from the beads (*B*), washing the beads, and running them on a 12% SDS gel followed by Western blot analysis using the anti-TAP antibody for detection of the proteins. The background binding of SWI/SNF or Fun30 to the magnetic Dynabeads alone are shown in *lanes 1* and *6*, respectively. *B*, nucleosomes were assembled on the fragment 0W47 in which the 601 nucleosome positioning sequence directs assembly of a nucleosome such that it is flanked by 47 bp of linker DNA on one side. Incubation of 30 nM 0W47 nucleosomes with increasing concentrations of His_6 Fun30 (28 nM to 1 μM, *lanes 2–11*) resulted in a gel-shifted species (Nuc/Fun30). *C*, binding curves indicating the fraction of the Fun30-bound template following incubation with the indicated concentrations of Fun30. Quantification was based on the material remaining unbound in gel shifts such as that shown in *B*. ◇, corresponds to 0W0 DNA; ■, corresponds to 0W47 nucleosomes; and △, corresponds to 0W0 nucleosomes.

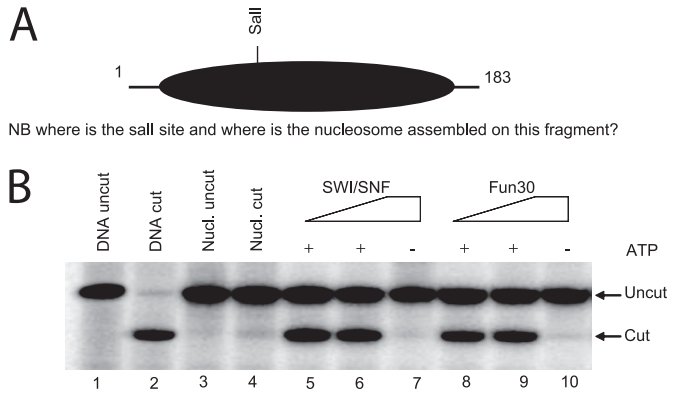

FIGURE 5. **Fun30 is an ATP-dependent chromatin remodeling enzyme.** *A*, schematic illustration of the template used for restriction enzyme accessibility assays. *B*, restriction enzyme accessibility assay. Increasing amounts of SWI/SNF (~2–8 nM, *lanes 5–7*) and Fun30 (~10–40 nM, *lanes 8–10*) based on normalization were added to ~10 ng of the GUB template in the presence or absence of 2 mM ATP as indicated. The binding reactions were then treated with 10 units of SalI for 30 min at 30 °C and the proportion of DNA cleaved was assessed by electrophoresis.

in a concentration-dependent fashion (Fig. 4*B*). This shift is diffuse and appears to go through a transition from faster migrating (Fig. 4*B*, *lane 6*) to slower migrating complexes (Fig. 4*B*, *lane 12*). A binding curve was constructed by quantifying the disappearance of the nucleosome band. Fits to this data using a simple monomer or dimer binding model were poor (data not shown), however, fitting a cooperative binding model to the data yielded a much better fit, revealing a K_d of 84 ± 2.7 nM and a cooperativity (Hill) coefficient of 2.17 ± 0.14 (Fig. 4*C*). This suggests that two molecules of Fun30 can bind to a single nucleosome with high affinity and in a cooperative fashion, and this is again consistent with Fun30 being able to associate with itself. Previous work has shown that some chromatin remodeling enzymes interact with linker DNA flanking the nucleosome core (27–30). We sought to test whether this was also the case for Fun30 by measuring the binding affinity of Fun30 for nucleosomes that had no flanking linker DNA (0W0) (Fig. 4*C*). Fitting the same model used for the 0W47 data reveals a more than 3-fold weaker K_d (279.8 ± 9.9 nM) and a significant decrease in the cooperativity of binding (1.47 ± 0.07). Using the same assay again we also measured the affinity for Fun30-His_6 for DNA, using the same DNA fragment used to assemble 0W0 nucleosomes. Interestingly, the binding curve for this DNA is almost identical to that of the 0W47 nucleosomes and gives a K_d of 67.3 ± 1.2 nM and cooperativity coefficient of 1.81 ± 0.06. This data clearly shows that the association of Fun30 with chromatin is strongly influenced by interactions with DNA flanking the nucleosome core. Consistent with this are the gel shifted species observed with nucleosome cores that are less discrete (supplemental Fig. S1).

Fun30 Has ATP-dependent Chromatin Remodeling Activity—The GUB fragment of DNA directs the assembly of nucleosomes such that the SalI site is occluded (Fig. 5*A*). However, when nucleosomes assembled onto this fragment were incubated with either SWI/SNF or Fun30, SalI was able to gain access to and cleave a significant proportion of the templates (Fig. 5*B*). This effect required the presence of ATP as no increase in cleavage was detected when ATP was omitted (Fig. 5*B*, *lanes 7* and *10*). These observations indicate that like other Snf2 family proteins (*i.e.* SWI/SNF), Fun30 can act to increase access to DNA within nucleosomes.

Fun30 Is More Efficient in Exchanging Histone Dimers Than Repositioning Nucleosome—Although the restriction enzyme accessibility assay described above provides evidence of an alteration to chromatin structure, it does not provide significant insight as to how the structure of chromatin is altered. To investigate this further, we initially tested the ability of Fun30 to catalyze the repositioning of nucleosomes from one location to another. Although we could detect some repositioning activity this was relatively modest (data not shown). We next sought to study the ability of Fun30 to direct the removal of histone H2A/H2B dimers from nucleosomes and their transfer onto tetra-

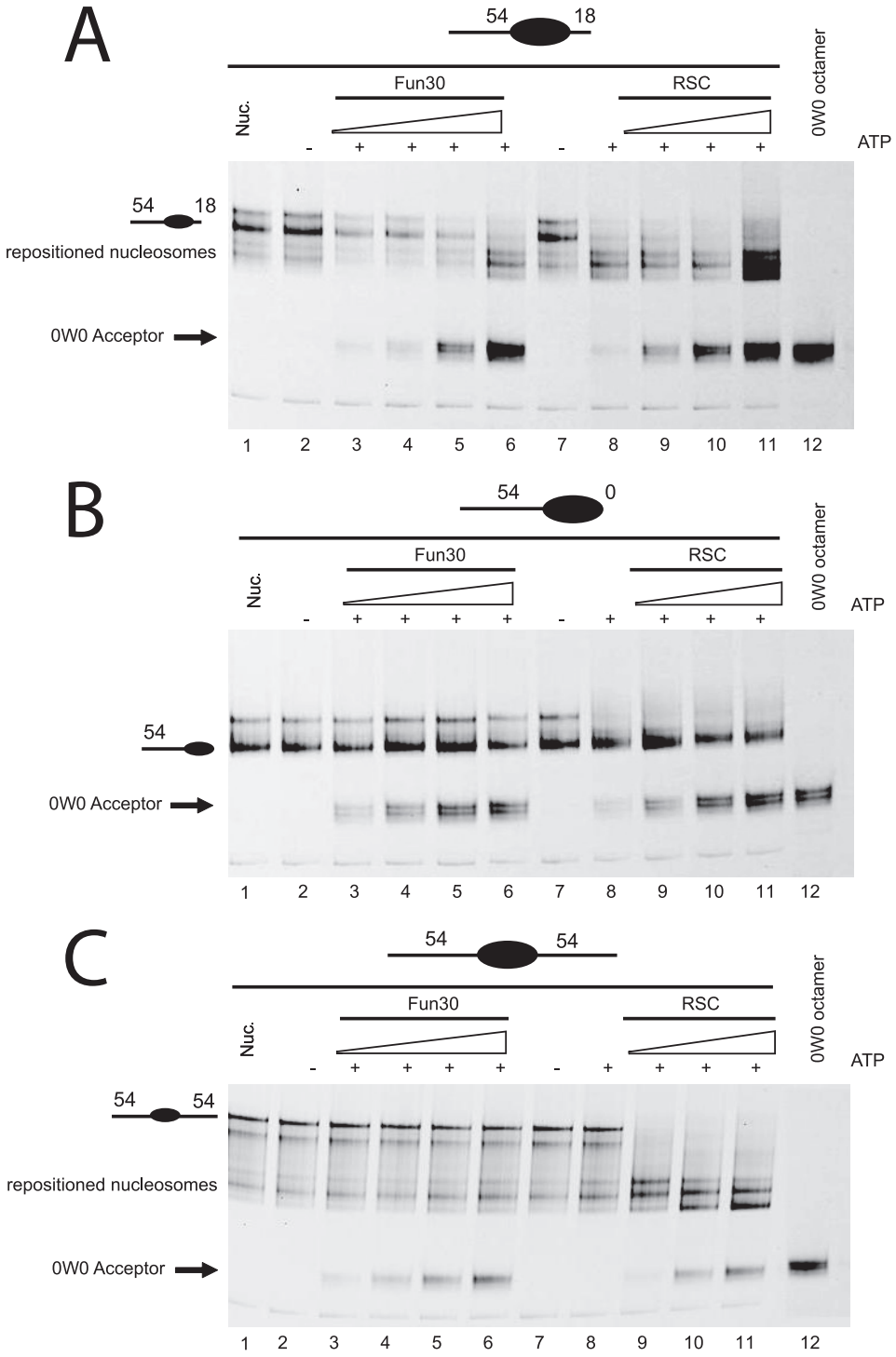

FIGURE 6. **Fun30 has higher activity in histone dimer exchange than nucleosome repositioning.** 10-μl reactions containing 0.25 pmol of nucleosomes (250 nM) in which H2B is fluorescently labeled with Cy5 assembled at the murine mammary tumor virus nucleosome A (*NucA*) positioning sequence flanked by the indicated lengths of linker DNA were incubated with Fun30 (100, 200, 300, and 400 pM, *lanes 3–6*) or RSC (100, 200, 300, and 400 pM, *lanes 8–11*) in the presence of 0.75 pmol of histone tetramers assembled onto 147-bp DNA (0W0). In each panel, *lane 1* contains nucleosomes assembled on the appropriate donor DNA fragment and *lane 12* contains the 0W0 fragment assembled with an octamer including fluorescently labeled H2B. Following native gel electrophoresis, the fate of H2B was monitored by fluorescent scanning of the gels. In some cases the signal moves to a location consistent with repositioning of nucleosomes on the donor DNA fragment. In others, transfer to the 0W0 acceptor DNA, which has a distinct mobility, could be detected. Fun30 was observed to cause dimer exchange even in circumstances where repositioning was inefficient. *A*, the donor nucleosome has an asymmetric linker DNA of 54 bp on one end and 18 bp on the other (54A18). *B*, the donor nucleosome has a 54-bp linker DNA on one end and 0 bp on the other (54A0). *C*, the donor nucleosome has 54-bp linkers on either side (54A54).

mers of histones H3 and H4 assembled on DNA. To do this, we made use of an assay in which histone dimers are labeled through attachment of the fluorescent dye Cy5 (21). When chromatin was subject to remodeling with increasing concentrations of Fun30, the Cy5 signal was removed from the nucleosomes and accumulated on the acceptor (Fig. 6, *lanes 3–6*). When the donor nucleosomes were assembled such that they were asymmetrically positioned with 54 bp on one side and 18 bp on the other some repositioning of nucleosomes on the donor DNA could also be observed (Fig. 6*A*, *lane 6*). However, the extent of repositioning was less than that observed with the RSC complex (Fig. 6*A*, *lanes 8–11*). This suggested that the Fun30 complex was more efficient in exchanging histone dimers than repositioning nucleosomes when compared with RSC. This effect was most striking when nucleosomes were assembled onto donor fragments such that they had 54-bp extensions on either side (Fig. 5*C*). In this case, RSC caused extensive repositioning of these nucleosomes, but Fun30 had relatively little effect, yet in both cases dimer exchange was comparable. When we used donor nucleosomes that had 54-bp extensions only on one side, again dimer exchange was observed, whereas nucleosome repositioning was limited (Fig. 5*B*). We have also observed that Fun30 is capable of catalyzing the transfer of histone octamers (supplemental Fig. S2). However, the activity of Fun30 is estimated to be an order of magnitude lower in this assay.

Fun30 Does Not Exhibit Specificity for Ubiquitinylated HeLa Histones—N-terminal to the region sharing homology with Snf2-related ATPases, Fun30 shares weak homology with conserved CUE and HHH domains (Fig. 7*A*) (11). CUE motifs in other proteins have been observed to interact with ubiquitin (31). Although the Fun30 CUE motif differs from related domains in well characterized ubiquitin-binding proteins, this region is con-

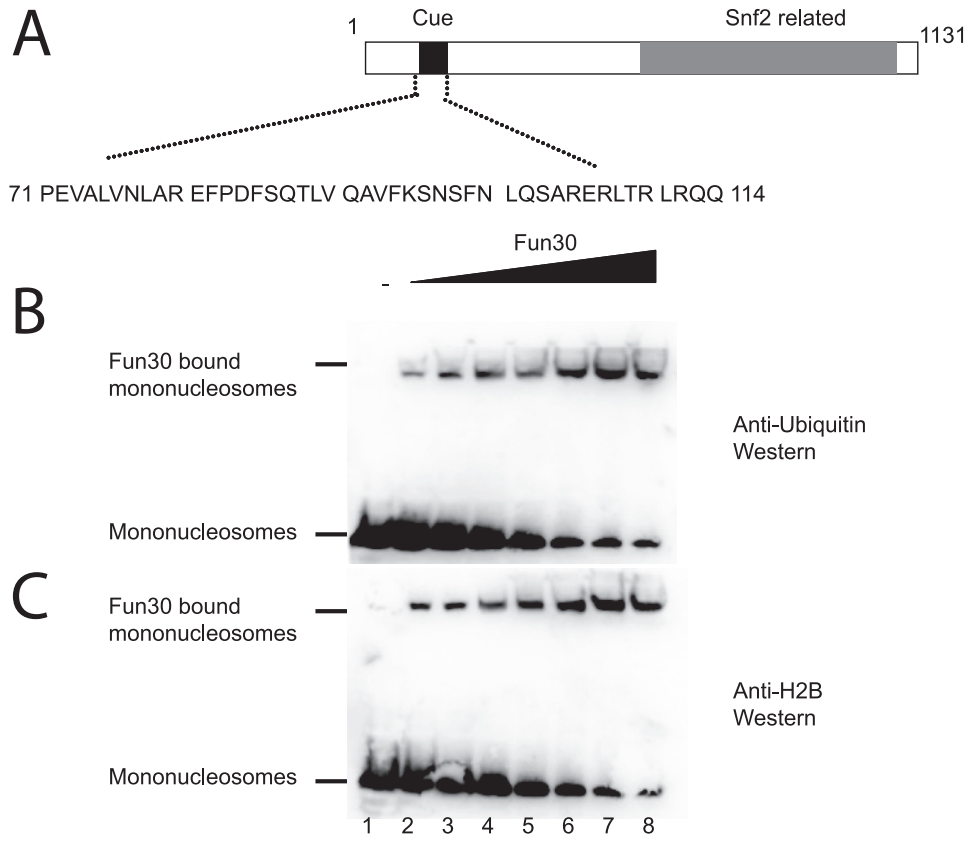

FIGURE 7. **Fun30 contains a CUE motif, but no specific interaction with ubiquitinylated histones can be detected.** *A*, schematic representation of CUE domain location within Fun30. A native gel in which ~200 nM HeLa mononucleosomes were incubated with increased quantities of Fun30 (~5–320 nM, *lanes 2–8*) was transferred to a polyvinylidene difluoride membrane. *B*, the transfer of ubiquitinylated histones was monitored by Western blotting using an anti-ubiquitin antibody. *C*, Western blotting to detect the transfer of total H2B. No difference in the efficiency with which ubiquitinylated or total H2B is transferred could be detected.

served in closely related yeast species, suggesting that it assumes a CUE/UBA-fold and raising the possibility that it might bind ubiquitin.

With the aim of identifying a specific interaction with ubiquitinylated histones, increasing concentrations of Fun30 were incubated with mononucleosomes purified from HeLa cells. Consistent with the binding of Fun30 to these nucleosomes, they were observed to be supershifted following native gel electrophoresis (not shown). To investigate whether Fun30 preferentially binds to ubiquitinylated chromatin, the proteins in the native gel were transferred to a nitrocellulose membrane and subjected to Western blotting with anti-ubiquitin antibody (Fig. 7*B*). Although Fun30 was capable of binding ubiquitinylated chromatin, no difference in the interaction of Fun30 with ubiquitinylated chromatin was observed in comparison to total H2B (Fig. 7*C*). This suggests that Fun30 does not have a preference in binding to ubiquitinylated histones.

DISCUSSION

The *S. cerevisiae* Fun30 protein has been purified and found to exist predominantly as a homodimer. Like other Snf2 family proteins, it is capable of binding nucleosomes, hydrolyzing ATP, and disrupting nucleosomes in an ATP-dependent reaction. Fun30 was found to be especially proficient in catalyzing the exchange of histone dimers between nucleosomes in comparison, for example, to nucleosome sliding. This is consistent with the fact that based on sequence homology Fun30 is most closely related to the Swr1 and Ino80 proteins (4), which have been reported to have activity in histone exchange (6, 7). The observation that Fun30 is relatively inefficient in repositioning nucleosomes supports previous work that suggests the mechanisms for dimer exchange and nucleosome sliding are distinct (32).

The Swr1 complex exhibits specificity in histone exchange directing the incorporation of the histone variant Htz1 (6). It is possible that Fun30 also has specificity in directing exchange of specific histone subtypes; however, in our preliminary studies we obtained no evidence for this. Given that Fun30 contains a weak CUE motif potentially capable of interacting with ubiquitin, one hypothesis we investigated was that Fun30 directs the incorporation or removal of ubiquitinylated histones. However, we could obtain no evidence for specific binding of Fun30 to ubiquitinylated histones (Fig. 7), or the ability to exchange ubiquitinylated histones (data not shown). A caveat to this experiment is that HeLa cells were used as a source of chromatin and the possibility remains that there is specificity for a feature of yeast chromatin we may have missed. It is also worth noting that Fun30 binds DNA better than nucleosome core particles (Fig. 4*C*) and that this could potentially target the action of Fun30 to accessible regions of the genome.

The observation that Fun30 elutes from gel filtration columns in a volume corresponding to a mass of 250 kDa could be interpreted as indicating the presence of a stable dimer. However, as elution volumes were observed to increase progressively when lower concentrations of Fun30 were loaded (data not shown) and TAP- and His-tagged Fun30 preparations interacted with each other *in vitro*, we favor the existence of a rapid equilibrium between monomeric and dimeric forms in solution. For some other remodeling enzymes, it is clear that the Snf2-related subunit is present as one copy (33). However, in other cases the involvement of a pair of catalytic subunits is an emerging theme (29, 34). In the case of Fun30, further investigation will be required to confirm that two molecules of Fun30 interact with a single nucleosome, although this is our favored interpretation of the cooperativity observed.

The previously reported phenotypes of increased resistance to DNA damage induced by UV (8) together with our own observation that the deletion of Fun30 results in resistance to UV, ionizing radiation, and resistance to 6-azauracil (data not

shown) might indicate an involvement in DNA repair. Interestingly, genome-wide surveys indicate physical and genetic interactions between Tel1 and Rad3, respectively (35, 36). Mutations to the histone variant Htz1 also have repair phenotypes, and Fun30 exhibits synthetic lethality with Htz1 and several components of the Swr1 complex, which directs its incorporation (Vps71, Vps72, and Arp6) (37). Thus, despite the lack of specificity for H2AZ *in vitro*, Fun30 functionally interacts with Htz1 in some way. The recent observation that Htz1 is targeted to nucleosomes adjacent to nucleosome-free regions resulted in speculation that the action of Swr1 might be targeted to regions of exposed DNA (38). Nonspecific histone dimer exchange especially with S-phase could potentially contribute to this process. Fun30 has the potential to accelerate this process and based on its *in vitro* DNA binding properties could be targeted to nucleosome-free regions. Interactions between Fun30 and proteins involved in transcription (Taf13, Rpo21, Rpc40, and Rpc34) (36), cell cycle progression (Orc2 and Orc5, (39), Cks1 (36), Clb2, and Cdc28 (10) have also been identified. Although it is intriguing that Fun30 is linked to the process of DNA replication, repair, and transcription, which all involve histone exchange, further studies are required to reveal the nature of this association.

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