Assembly of the Arp5 (Actin-related Protein) Subunit Involved in Distinct INO80 Chromatin Remodeling Activities*

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ATP-dependent chromatin remodeling, which repositions and restructures nucleosomes, is essential to all DNA-templated processes. The INO80 chromatin remodeling complex is an evolutionarily conserved complex involved in diverse cellular processes, including transcription, DNA repair, and replication. The functional diversity of the INO80 complex can, in part, be attributed to specialized activities of distinct subunits that compose the complex. Furthermore, structural analyses have identified biochemically discrete subunit modules that assemble along the Ino80 ATPase scaffold. Of particular interest is the identified biochemically discrete subunit modules that assemble processes, including transcription, DNA repair, and replication. The functional diversity of the INO80 complex can, in part, be attributed to specialized activities of distinct subunits that compose the complex. Furthermore, structural analyses have identified biochemically discrete subunit modules that assemble along the Ino80 ATPase scaffold. Of particular interest is the Saccharomyces cerevisiae Arp5-Ies6 module located proximal to the Ino80 ATPase and the Rvb1-Rvb2 helicase module needed for INO80-mediated in vitro activity. In this study we demonstrate that the previously uncharacterized Ies2 subunit is required for Arp5-Ies6 association with the catalytic components of the INO80 complex. In addition, Arp5-Ies6 module assembly with the INO80 complex is dependent on distinct conserved domains within Arp5, Ies6, and Ino80, including the spacer region within the Ino80 ATPase domain. Arp5-Ies6 interacts with chromatin via assembly with the INO80 complex, as IES2 and INO80 deletion results in loss of Arp5-Ies6 chromatin association. Interestingly, ectopic addition of the wild-type Arp5-Ies6 module stimulates INO80-mediated ATP hydrolysis and nucleosome sliding in vitro. However, the addition of mutant Arp5 lacking unique insertion domains facilitates ATP hydrolysis in the absence of nucleosome sliding. Collectively, these results define the requirements of Arp5-Ies6 assembly, which are needed to couple ATP hydrolysis to productive nucleosome movement.

Eukaryotic genomes are organized within chromatin, making chromatin manipulation essential to DNA-templated processes. Included among chromatin modifiers are enzymes that post-translationally modify histones and chromatin remodelers that alter the position and composition of nucleosomes. ATP-dependent chromatin remodeling is a conserved essential process, and deficiencies in these enzymes result in viability defects, developmental abnormalities, and disease initiation in different species (1, 2). The form and structure of nucleosomes influence many chromatin-associated functions including, but not limited to, the recruitment of factors involved in transcription, replication, and DNA repair.

The INO80 chromatin remodeling complex is highly conserved with roles in transcription, genome stability, embryonic stem cell identity, and disease pathogenesis (1, 3–7). In vitro, the INO80 complex moves nucleosomes into regularly spaced arrays (8), which is reflected in the deregulation of nucleosome abundance and positioning in vivo (9, 10). Previous research has demonstrated that the large multisubunit INO80 complex influences diverse DNA-templated processes through subunit specification of distinct chromatin remodeling activities (3, 11). Indeed, each chromatin remodeling event is a complex multistep process involving chromatin recruitment and substrate selection followed by ATP hydrolysis and nucleosome positioning (2). All chromatin remodelers contain SNF2-type ATPases that “couple” ATP hydrolysis to nucleosome sliding activity (12–14), with “coupling efficiency” indicative of the amount of ATP hydrolyzed per sliding event (15). Each chromatin remodeling step needs to be tightly controlled for optimal enzyme activity and subsequent chromatin in diverse DNA-templated processes.

Recently, the structure of the 1.3-MDa Saccharomyces cerevisiae INO80 complex was described (16, 17), providing clues to the functional organization of this remodeler. The complex was found to be modular with biochemically separable

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subcomplexes consisting of distinct subunits assembling onto the Ino80 ATPase scaffold. Thus, it is likely that the Ino80 complex elicits diverse functions that are attributed to unique modules within the larger complex. Of particular interest are the actin-related proteins (Arps) that are critical for chromatin remodeling function (18). Of the 10 Arps in S. cerevisiae, four are primarily cytoplasmic with cytoskeleton functions, whereas the remaining six are in chromatin remodeling complexes. The INO80 complex contains Arp4, Arp8, and Arp5, all of which are required for in vitro chromatin remodeling (19). Arp4 and Arp8 assemble as a separate module in the helicase-SANT-associated domain of Ino80 (16, 20) and are proposed to facilitate interactions between the nucleosome and remodeler (19, 21). The N-terminal domain of Ino80 ATPase assembles subunits that are less conserved among species (16), yet some of these subunits have directed functions in DNA damage recognition (22) and telomere stability (23) in S. cerevisiae.

The Ino80 subfamily of chromatin remodelers is uniquely distinguished by an insertion region that splits the ATPase domain (3, 24). The insertion region is evolutionarily conserved and necessary for the recruitment of mammalian RuvBL1-RuvBL2 (Rvb1-Rvb2 in yeast) (25), which are A++ helices with homology to the bacterial RuvB helicase involved in Holliday Junction migration (26), yet their roles in chromatin remodeling remain unclear. In addition, the insertion region of mammalian Ino80 is needed for ACTR5-INO80C module (Arp5-les6 in yeast) assembly within the INO80 complex (25), although its requirement for the yeast complex has not previously been reported. However, Rvb1-Rvb2 module is necessary for Arp5-les6 association with the INO80 complex (27). Structural analyses of the S. cerevisiae INO80 complex also demonstrate that the Arp5-les6 module is within close proximity of the Ino80 ATPase domain and Rvb1-Rvb2 subunits (16), thus linking Arp5-les6 to critical enzymatic components of the INO80 complex. *In vivo*, Arp5-les6 are important for nucleosome positioning (9), DNA damage responses (28–31), replication (32), transcriptional regulation (33, 34), and mitotic stability (35). *In vitro*, this subunit module is critical for INO80-mediated ATP hydrolysis, nucleosome sliding, and histone exchange that reconstructs nucleosomes by removing the Htz1 variant (H2A.Z in mammals) (16, 17, 19).

In this study we delineate the assembly of the Arp5-les6 module within the *S. cerevisiae* INO80 complex and its resulting influence on INO80 function. Through biochemical analysis, individual INO80 subunits and domains are identified as necessary for Arp5-les6 association with the INO80 complex. Furthermore, we identify critical domains within Arp5 that couple ATP hydrolysis to nucleosome sliding. Additionally, important differences between the requirements for Arp5-les6 assembly with the INO80 complex in different species are discussed.

**Experimental Procedures**

**Yeast Strains**—The strain list is shown in Table 1. Strain construction was in S288C background using standard techniques. All FLAG epitopes were chromosomally integrated to ensure endogenous expression of protein. Arp5 mutants were also integrated at chromosomal locus. Plasmids encoding *IES6* consist of endogenous promoter (−500 bp) and terminator (+500 bp) sequences. 5-Fluoro-orotic acid was used in synthetic media at 1 mg/ml.

**FLAG Affinity Purifications**—Protein complexes were purified using anti-FLAG affinity beads as previously described (22, 28, 36). Briefly, cells were frozen in liquid nitrogen then broken

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**Table 1** Strains used in this study

| Name          | Genotype                                   | Name          | Genotype                                   |
|---------------|--------------------------------------------|---------------|--------------------------------------------|
| BY4741*       | S288C MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0   | TZY001        | BY4741 ARPS:arp5 (Tyr-257–Lys-315)Δ-NATMX6 |
| BY4743*       | S288C MATa/α his3Δ1/α his3Δ1 leu2Δ0/leu2Δ0| TZY002        | BY4741 ARPS:arp5 (Lys-414–Asn-526)Δ-NATMX6 |
| Ino80-FLAG**  | S288C MATa his3Δ200 leu2Δ0 met15Δ0         | TZY003        | BY4741 ARPS:arp5 (Asp-545–Glu-581)Δ-NATMX6 |
| WYY0019       | Ino80-FLAG ies6::URA3                   | TZY004        | BY4741 ARPS:arp5 (Arg-596–Lys-656)Δ-NATMX6 |
| WYY0018       | Ino80-FLAG ies2::URA3                   | WYY0200       | BY4741 ARPS:2xFLAG-URA3                    |
| WYY0017       | Ino80-FLAG arps5::URA3                   | TZY005        | BY4741 ARPS:arp5 (Tyr-257–Lys-315)Δ-2xFLAG-KANMX4 |
| WYY0001       | Ino80-FLAG ies1::URA3                   | TZY006        | BY4741 ARPS:arp5 (Lys-414–Asn-526)Δ-2xFLAG-KANMX4 |
| SLBY047       | BY4743 derived haploid MATa INO80:ino80   | TZY007        | BY4741 ARPS:arp5 (Asp-545–Glu-581)Δ-2xFLAG-KANMX4 |
|               | (Glu-1018–Ala-1299)Δ-2xFLAG-KANMX4        |               |                                            |
| WYY0021       | BY4741 ies2::LEU2                       | TZY008        | BY4741 ARPS:arp5 (Arg-596–Lys-656)Δ-2xFLAG-KANMX4 |
| WYY0022       | BY4741 ies6::LEU2                       | TZY013        | BY4701 IES6–2xFLAG-URA3                    |
| WYY0023       | WYY0204 ies2::LEU2                      | TZY014        | BY4701 IES6–2xFLAG-URA3                    |
| WYY0024       | BY4741 IES2–2xFLAG-URA3              | TZY015        | BY4701 IES6–2xFLAG-URA3                    |
| ALBY001       | WYY0198 [pRS413–IES6]                  | TZY016        | BY4701 IES6–2xFLAG-URA3                    |
| ALBY002       | WYY0198 [pRS413–IES6–YLI-Cα]            | TZY009        | BY4701 INO80–2xFLAG-KANMX4                 |
| ALBY003       | WYY0202 [pRS413–IES6]                  | TZY010        | BY4701 INO80–2xFLAG-KANMX4                 |
| ALBY004       | WYY0202 [pRS413–IES6–YLI-Cα]            | TZY011        | BY4701 INO80–2xFLAG-KANMX4                 |
| WYY0195       | BY4743 derived haploid MATa ies6::LEU2  | TZY012        | BY4701 INO80–2xFLAG-KANMX4                 |
| WYY0005       | BY4743 [pRS413–2xFLAG-IES6–YLI-Cα]     | WYY0201       | BY4701 INO80–2xFLAG-KANMX4                 |
| ALBY007       | WYY0039 [pRS413]                        | WYY0202       | BY4701 ies2::LEU2                          |
| ALBY008       | WYY0039 [pRS413–IES6]                  | WYY0217       | BY4701 ies2::LEU2                          |
| ALBY009       | WYY0039 [pRS413–IES6–YLI-Cα]            | WYY0017       | BY4701 ies2::LEU2                          |
| WYY0014       | BY4741 arps5::LEU2                      | WYY0219       | BY4701 ies2::LEU2                          |
|               |                                            | WYY0220       | BY4701 ies2::LEU2                          |

5 The abbreviations used are: Arp, actin-related protein; MNase, micrococcal nuclease; YL1-C, YL1 nuclear protein C-terminal; SANT, switching-defective protein 3 (Swi3), adaptor 2 (Ada2), nuclear receptor co-repressor (N-CoR), transcription factor (TF) IIb.

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using a commercial blender. Cell lysates were resuspended in HEGN buffer (25 mM HEPES-KOH pH 7.6, 1 mM EDTA, 10% glycerol, 0.02% Nonidet P-40, 2.5 mM DTT, 2 mM MgCl₂, 1× protease inhibitors) with either 0.5 mM KCl or 0.2 mM KCl to retain histone association. Soluble lysate was obtained by ultracentrifugation and incubated with FLAG affinity-agarose (Sigma; catalog no. A2220). Beads were washed with HEGN buffer and eluted with FLAG peptide.

**Western Analysis and Chromatin Fractionation**—Protein from whole cell extracts were precipitated with 10% trichloroacetic acid. Proteins were detected by Western blot using anti-FLAG M2 antibody (Sigma; catalog no. F1804), anti-Arp5 (Abcam; catalog no. ab12099), and anti-hexokinase (Novus; catalog no. NB120-20547).

Chromatin fractionations were performed as previously described (37) with some modification. 50 ml (A660 ~0.5) of spheroplasted cells were resuspended in lysis buffer. For micrococcal nuclease (MNase) digestion, MgCl₂ was replaced by CaCl₂. 150 units of MNase or 10 units of DNase I was added to 150 µl of lysate for 30 min at 30 °C before centrifugation at 13,500 rpm for 15 min to separate soluble and chromatin pellets. Percent solubilization was calculated by quantifying the Arp5 chemiluminescence signal relative to the H3 signal before and after digestion with the following calculation: Arp5 [DNase I- or MNase-treated (S2) fraction/insoluble chromatin (C) fraction]/H3 [DNase I- or MNase-treated (S2) fraction/insoluble chromatin (C) fraction]. Non-saturating chemiluminescence signal was obtained using Bio-Rad ChemiDoc imager.

**In Vitro Biochemical Assays**—For nucleosome sliding assays, Cy5-tagged mononucleosomes with a 601 DNA sequence and 60 bp of linker DNA were prepared as previously described (38). Remodeling reactions contained 2 nM INO80 or (INO80-deficient, lacking Arp5-Ies6) INO80Δ and Arp5-Ies6 complexes purified by Arp5-FLAG and 2 nM mononucleosomes in reaction buffer (25 mM HEPES, pH 7.6, 70 mM KCl, 3.6 mM MgCl₂, 0.37 mM EDTA, 0.37 mM EGTA, 0.017% Nonidet P-40, 1 mM DTT, 100 µg/ml BSA, 10% glycerol, and protease inhibitors). After incubation at 30 °C for 30 min, reactions were initiated by the addition of 2 mM ATP-Mg⁺/2 for an additional 60 min. All reactions were stopped with 2× stop buffer (40 mM ADP, 20% glycerol, and 0.2 mg/ml nonspecific plasmid DNA). Samples were electrophoresed on native 6% PAGE gel in 0.5× Tris borate EDTA and visualized on a Typhoon 9210 (GE Healthcare).

ATPase reactions contained 2 nM INO80Δ and Arp5-Ies6 complexes and 1 nM mononucleosomes in reaction buffer (25 mM HEPES, pH 7.4, 75 mM KCl, 0.37 mM EDTA, 0.35 mM EGTA, 0.02% Nonidet P-40, 1 mM DTT, 100 µg/ml BSA, 10% glycerol). After incubation at 30 °C for 30 min, reactions were initiated by the addition of 5 mM MgCl₂ and 100 mM ATP. All reactions were stopped by the addition of 85 mM EDTA after 60 min. Reactions were added to Malachite Green solution (0.375 mM Malachite Green oxalate, 8.5 mM ammonium molybdate, 1 mM HCl); color development was allowed to proceed for 3 min before the addition of sodium citrate to 5.6% and measurement at 620 nm. ATPase activity was calculated via conversion of free phosphates using a sodium phosphate standard curve.

**Results**

**Association of the Arp5-Ies6 Subunit Module with the INO80 Complex**—Arp5 and Ies6 comprise a structurally distinct module of the INO80 complex important for INO80 catalytic activity and chromatin remodeling (16, 19). Accordingly, deletion of either Arp5 or Ies6 resulted in a loss of both subunits with the purified INO80 complex, whereas the association of other subunits with the INO80 complex was unchanged (Fig. 1A). To identify other subunits involved in Arp5-Ies6 association with the INO80 complex, we performed additional Ino80 purifications in wild-type and INO80 subunit deletion strains. Interestingly, Ies2 was needed for Arp5-Ies6 association, as deletion resulted in the loss of the Arp5-Ies6 module within the INO80 complex (Fig. 1A). Importantly, the total protein levels of Ies2 and Arp5 were not decreased in these deletion strains. However, deletion of ARPS resulted in an undetectable amount of Ies6 protein in whole cell extracts, whereas no substantial change in Ies6 was observed in ies2Δ cells (Fig. 1B). Although the precise reason for decreased Ies6 protein is currently unknown, it may be that Arp5 influences Ies6 protein stability. Deletion of other subunits, such as Ies1, did not influence the association of other subunits or subunit modules with the INO80 complex.

Biochemically, Ies2 is a relatively uncharacterized component of the INO80 complex, although structural studies place it within 30 Å of the ATPase domain (16). As mentioned, the Ino80 ATPase domain is separated by a large insertion region, a characteristic that is distinct from other chromatin remodelers including Snf2 of the SWI/SNF complex (Fig. 1C). Deletion of this insertion region of Ino80 resulted in loss of Arp5, Ies6, Ies2, and Rvb1-Rvb2 association with the complex (Fig. 1D). Notably, Arp5 or Ies6 deletion did not alter Ies2 or Rvb1-Rvb2 association of the INO80 complex (Fig. 1A). Likewise, Ies2 deletion did not dramatically alter association of Rvb1-Rvb2 with INO80 complex (Fig. 1A). These results demonstrate that Ies2 confers association of Arp5-Ies6 with a region of the INO80 complex critical for enzymatic activity.

As Ies6 is needed for Arp5 assembly with the INO80 complex (Fig. 1A), we sought to further examine this interaction. Ies6 is a relatively uncharacterized subunit with one annotated domain, the evolutionarily conserved YL1 nuclear protein C-terminal (YL1-C) domain (Fig. 2A). This domain is found in the YL1 family of proteins that exhibit DNA binding and is implicated in transcriptional regulation (39). In our protein purifications, deletion of the YL1-C domain resulted in loss of the Arp5-Ies6 module with INO80 complex (Fig. 2B). However, co-purification of Ies6 and Arp5 was maintained. In addition, deletion of the YL1-C domain resulted in fitness defects similar to that of complete IES6 deletion (Fig. 2C). Therefore, the YL1-C domain needed for Arp5-Ies6 association with the INO80 complex is critical for Ies6 cellular function.

Collectively, these results and those previously published (27) demonstrate that Ies2, Rvb1-Rvb2, the insertion region of Ino80, and the YL1-C domain of Ies6 are all involved in the association of the Arp5-Ies6 module with the Ino80 ATPase subunit and implicate the coordinated activity of these subunits in INO80 chromatin remodeling.
Distinct Domains of Arp5 Are Required for Association with Ies6 and the INO80 Complex—As mentioned, the INO80 complex contains Arps 4, 5, and 8, of which Arps 5 and 8 are specific to the INO80 complex. All Arps have significant identity to actin within the actin fold domains. S. cerevisiae Arp4, Arp5, and Arp8 are 30% identical (53% similar), 26% identical (51% similar), and 21% identical (44% similar) to actin, respectively (18). Previous research demonstrates that Arp4 and Arp8 contain unique insertion regions that facilitate subunit specific interactions, namely actin binding and nucleosome association (21, 40). To investigate Arp5-specific function, we biochemically characterized the conserved insertion regions unique to Arp5.

We identified four insertion regions within Arp5 that are distinct from actin and Arp4 (Fig. 3A) yet are shared with other species (Table 2) and thus may represent conserved Arp5-specific functional domains. Genomic deletions were made encompassing these insertion domains, referred to as arp5D1/H9004 to D4/H9004. Replacement of wild-type ARP5 with these arp5 insertion mutants resulted in reduced fitness (Fig. 3B). arp5D1/H9004 and -D4/H9004 exhibit fitness similar to that of complete ARP5 deletion, whereas arp5D2/H9004 and -D3/H9004 have intermediate fitness defects (Fig. 3B). These arp5 insertion mutants remain under the control of the endogenous promoter; however, Western analysis identifies reductions in protein abundance, which may be indicative of altered protein stability and contribute to decreased cellular fitness (Fig. 3C).

Purification of these Arp5 insertion mutants reveals reduced association with INO80 complex subunits. In particular, Arp5D1, -D3, and -D4 have decreased association of Ies6, INO80 ATPase, Rvb1, Rvb2, and Arp8 subunits (Fig. 3D). However, Arp5D2 co-purified with Ies6, yet had reduced association of INO80 complex subunits. These results demonstrate that deletion of any of the four insertion domains reduced Arp5 protein levels and INO80 complex association, whereas Arp5D1 and -D4 have reduced Ies6 association.

Purification of FLAG-tagged Ies6 from strains expressing these Arp5 insertion mutants confirmed that Arp5D2 associates with Ies6 (Fig. 4A). In addition, in these purifications where Ies6 protein is enriched, co-purification of Arp5D3 is detectable. However, in agreement with the purifications of the Arp5 insertion mutants (Fig. 3D), abundance of co-purifying subunits of the INO80 complex was greatly reduced. Furthermore, no detectable amount of Ies6 was found in purifications from arp5D1 and -D4 strains. Western blotting of whole cell extracts revealed a dramatic reduction in both Ies6-FLAG and Arp5D1/H9004 and -D4/H9004 protein (Fig. 4B). This suggests that the decrease of co-purifying Ies6 with Arp5D1 and -D4 may be a consequence of reduced Ies6 protein in the cell (Figs. 3D and 4A) rather than a loss of Arp5-Ies6 interaction.
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les6-FLAG was also reduced in the Arp5D3\(\Delta\) mutant (Fig. 4B); thus, the decrease in co-purifying les6 with Arp5D3\(\Delta\) (Fig. 3D) was also likely a consequence of total protein amounts in the cell. However, when les6-FLAG is purified and subsequently enriched, approximately stoichiometric amounts of co-purifying Arp5D3\(\Delta\) can be observed (Fig. 4A).

As in Fig. 1B, these results also point to a mechanism by which les6 may be stabilized by the presence of Arp5. Collectively, these assays indicate that Arp5D2\(\Delta\) and -D3\(\Delta\) have reduced INO80 complex association while retaining les6 interaction at levels corresponding to abundance of les6 cellular protein.

Purification of FLAG-tagged Ino80 from cells expressing Arp5D1\(\Delta\) to -D3\(\Delta\) demonstrates that the mutant Arp5-les6 module has decreased association with the complex (Fig. 4C). Although Arp5 mutants and les6 was not detected in silver-stained gels, ArpD2\(\Delta\) and D3\(\Delta\) was observed in Western analysis using an Arp5 specific antibody. Collectively, these results demonstrate that deletion of Arp5-specific insertion regions 2 and 3 result in disrupted association with the INO80 complex, whereas deletion of insertion domains 1 and 4 have greatly reduced Arp5 and les6 protein levels, thereby precluding examination of their interaction with the INO80 complex.

Association of the Arp5-les6 Subunit Module with Chromatin—Cell lysate fractionation was performed to identify the determinants of Arp5-les6 association with chromatin (Fig. 5A). In these assays, histone H3 and cytosolic hexokinase demarcate the chromatin and soluble fraction, respectively. Deletion of INO80, IES2, and IES6, but not IES1, resulted in a prominent reduction in Arp5 association with chromatin concomitant with an increase in the soluble fraction (Fig. 5B). These results suggest that the Arp5-les6 module is recruited to chromatin through association with the INO80 complex, as deletion of IES2 resulted in loss of the Arp5-les6 interaction with the complex (Fig. 1A). Interestingly, all Arp5 insertion region mutants retain significant association with chromatin (Fig. 5C). This result is unexpected, as Arp5D1\(\Delta\) and -D4\(\Delta\) have no measurable association with purified INO80 complex in our assays. To more directly assess the chromatin association of these Arp5 mutants, nucleases were added to the insoluble fraction to solubilize chromatin associated proteins while leaving non-chromatin associated proteins insoluble (Fig. 5A). As can be seen in Fig. 5C and D, MNase and Dnase I efficiently solubilize histone H3 and wild-type Arp5. In addition, Arp5D2\(\Delta\) is solubilized at a level comparable with wild-type. However, Arp5D3\(\Delta\) is solubilized less relative to wild-type Arp5, and Arp5D1\(\Delta\) and -D4\(\Delta\) were not solubilized with either Dnase I or MNase digestion (Fig. 5C and D). Thus, the deficiencies in Arp5D1\(\Delta\) and -D4\(\Delta\) to associate with other INO80 complex subunits and chromatin may be indicative of these mutants forming insoluble aggregates.

Influence of the Arp5-les6 Subunit Module in Chromatin Remodeling—In vitro biochemical assays were performed to determine the ability of Arp5D2\(\Delta\) and -D3\(\Delta\) to stimulate INO80-mediated ATP hydrolysis and nucleosome sliding. As Arp5D2\(\Delta\) and -D3\(\Delta\) had reduced association with the INO80 complex (Fig. 4C), we purified wild-type and mutant Arp5-les6 from ies2\(\Delta\) cells and normalized the amount of soluble Arp5-les6 module used in in vitro assays. The module was then added to the INO80 complex lacking Arp5-les6 (INO80\(^4\)), which was purified via Ino80-FLAG from ies6\(\Delta\) cells (Fig. 1A). INO80\(^4\) had minimal ATPase activity (Fig. 6A) in agreement with other studies demonstrating reduced nucleosome-stimulated ATPase activity of the S. cerevisiae INO80 complex purified from arp5\(\Delta\) cells (16, 19). The Arp5-les6 module alone, either mutant or wild type, also did not exhibit ATPase activity. However, the addition of the wild-type Arp5-les6 module to the INO80\(^4\) complex stimulated ATPase activity in vitro. Mutant Arp5D2\(\Delta\)-les6 and D3\(\Delta\)-les6 also significantly stimulate INO80-mediated ATPase activity, albeit to a reduced level from that of wild-type Arp5-les6. However, only the Arp5-les6 module from wild-type cells, and not from arp5D2\(\Delta\) or D3\(\Delta\) cells, was able to stimulate INO80-mediated nucleosome sliding in vitro (Fig. 6B).

The lack of nucleosome sliding activity of INO80\(^4\) complexes in the presence of Arp5-les6 modules lacking insertion regions 2 and 3 may be due to the inability of Arp5D2\(\Delta\)-les6 and D3\(\Delta\)-
les6 to stimulate enough INO80-mediated ATP hydrolysis to facilitate nucleosome sliding or that insertion regions 2 and 3 are required for sliding activity but dispensable for minimal ATP hydrolysis. However, it should be noted that previous studies have detected a >50% reduction in ATPase activity mediated by the INO80 complex lacking specific subunits; yet nucleosome sliding could still be detected (19). As such, Arp5 insertion regions 2 and 3 may “uncouple” different INO80-mediated activities, such as ATP hydrolysis and nucleosome sliding.

Discussion

Results presented in this study delineate the assembly of the S. cerevisiae Arp5-les6 module, which is required for INO80 chromatin remodeling. Biochemical purifications reveal particular regions within the catalytic ATPase domain of the INO80 complex required for its association with Arp5-les6. Specifically, Arp5-les6, les2, and Rvb1-Rvb2 require the Ino80 insertion domain to associate with the INO80 complex. As previously reported, Rvb1-Rvb2 are required for Arp5 association with the INO80 complex (27), although les2 and les6 were not discussed. Additionally, our results demonstrate that Arp5-les6, but not Rvb1-Rvb2, is dependent on les2 for assembly within the INO80 complex. Thus, a hierarchy of subunit assembly is proposed on the Ino80 ATPase as follows: 1) the Ino80 insertion domain is required for les2 and Rvb1-Rvb2 assembly; 2) les2 is needed for Arp5-les6 association; 3) Rvb1-Rvb2 is needed for Arp5-les6 assembly; 4) les6 YL1-C domain is needed for Arp5-les6 association.

It is not yet known if les2 association with the INO80 complex is dependent on the Rvb1-Rvb2 module or if they indepen-
FIGURE 4. Arp5 insertion regions influence association with Ies6 and the INO80 complex. A, Ies6-FLAG purifications from wild-type (ARP5) and the indicated insertion mutants (D1Δ-D4Δ) electrophoresed on 6% (top) and 15% (bottom) SDS-PAGE gels. Proteins were visualized via silver staining and show loss of Ies6 and INO80 complex association with D1Δ and D4Δ insertion mutants. * indicates wild-type and mutant D1Δ-D4Δ Arp5. Selected subunits of the INO80 complex are labeled on the right, molecular mass (KDa) is labeled on the left. B, Western analysis of wild-type and arpΔΔ strains without Ies6-FLAG and strains purified in A and labeled with Ies6-FLAG. Anti-Arp5 and FLAG antibodies detect Arp5 and Ies6, respectively. Hexokinase is a loading control. C, Ino80-FLAG purifications from wild-type (ARP5) and insertion mutants (D1Δ-D4Δ) were electrophoresed on 6% (top) and 15% (middle) SDS-PAGE gels as in A. Bottom, Western analysis of purified proteins using anti-Arp5 and FLAG antibodies shows loss of Arp5 insertion mutants D1Δ and D4Δ with the INO80 complex.

FIGURE 5. The Arp5-les6 module is chromatin-associated in an INO80-dependent manner. A, schematic of chromatin fractionation protocol. Total (T), soluble (S/S1), and insoluble chromatin (C) fractions are shown. Chromatin (C) fraction is solubilized (S2) with DNase I or MNase treatment. B, chromatin fractionation of Arp5-FLAG (WT) and the indicated deletion strains. Fractions were monitored with anti-FLAG antibody to determine Arp5 subcellular location. Hexokinase and histone H3 (H3) Western assays were used to confirm cytoplasmic and chromatin fractions, respectively. Arp5 is present in the chromatin fraction in WT and les1Δ strains but is in the soluble fraction in ino80Δ, les6Δ, and les1Δ strains (marked by *). C, chromatin fractionation of Arp5-FLAG wild-type (ARP5) and insertion domain mutant (D1Δ-D4Δ) strains show that Arp5 D1Δ and D4Δ (marked by *) are not solubilized after DNase I treatment (S2 fraction) as is histone H3. D, quantifications of Western blots shown in C and MNase-treated chromatin fractions (not shown). Percent solubilized was calculated as the amount Arp5 in the S2 fraction relative to total Arp5 and the amount of H3 solubilized (details are shown under “Experimental Procedures”).
**Subunit Organization of the INO80 Chromatin Remodeler**

A.

nucleosome-stimulated ATP hydrolysis of INO80 deficient complexes without Arp5-Ies6 (INO80Δ) stimulated with either wild-type Arp5-Ies6, mutant Arp5D2Δ-Ies6, or D3Δ-Ies6 module. Arp5-Ies6 module is needed for nucleosome sliding (24) and the large Rvb1-Rvb2 head module contains the ATPase subunit module that is situated at the neck of the INO80 complex that contains the ATPase domain, located between the body module and Rvb1-Rvb2 head module. Indeed, Ies6 is within 30 Å of the ATPase domain and may reflect the complexity of Arp5 folding into mature soluble protein. Interestingly, wild-type Arp5-Ies6 stimulates INO80-mediated chromatin remodeling in vitro and may be indicative of dynamic chromatin remodeling assemblies in vivo. Arp5 insertion regions 2 and 3 are dispensable for nucleosome-stimulated ATPase activity yet are required for nucleosome sliding. Although uncoupling of ATP hydrolysis and nucleosome sliding has not previously been reported for the INO80 complex, it has been observed for the Chd1 and ISWI remodelers, which are functionally related (15, 42). Thus, the Arp5-Ies6 subunit module bridges different enzymatic activities within the same complex (42). Indeed, Arp5 insertion domain 2 contains a site that can be cross-linked to H2B, suggesting that this domain is involved in H2A-H2B remodeling.

The SWR1 complex, another member of the INO80 chromatin remodeling subfamily, is also differentiated by distinctive insertion regions that split the RecA ATPase domain (24, 43). The SWR1 complex exchanges canonical H2A in the nucleosome for the histone variant Htz1 (43), whereas INO80 has been implicated in the removal of Htz1 (41). INO80 histone exchange function is dependent on Ies6 (17), whereas SWR1 activity is dependent on Swc2 (44), both of which require the insertion region of the ATPase subunit for association with their respective complexes (Ref. 44 and Fig. 1). Similar to Ies6, Swc2 also contains a YL1-C domain. Results presented in this study demonstrate that the Ies6 YL1-C domain is needed for association of the Arp5-Ies6 module with the INO80 complex. Thus, it may be that Swc2 YL1-C facilitates Swc2 and, consequently, Htz1 association with the SWR1 complex. Again, these results bolster common modes of subunit assembly for INO80 and SWR1 as well as chromatin remodeling of H2A-H2B by YL1-C-containing subunits.

However, the results presented here reveal important distinctions between the functional assembly of the mammalian and yeast INO80 complex. It was recently reported that siRNA-mediated depletion of INO80B (yeast Ies2) in HEK293T cells results in minimal loss of ACTR5-INO80C (yeast Arp5-Ies6) with the INO80 complex (25). Furthermore, INO80B is required for nucleosome and DNA-stimulated ATPase activity, whereas ACTR5 and INO80C are not. These results are in contrast to others from *S. cerevisiae* demonstrating loss of ATPase activity in INO80 complexes purified from cells with *Arp5* or *Ies6* deletion (16, 17, 19). Different methodologies of purifica-

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tions and depletion of subunits (siRNA knockdowns versus genomic deletions) may influence these conflicting results.

However, it may reflect different mechanisms of INO80 complex function in different species. Indeed, the insertion domain of Ino80, which is required for Ies2 and Arp5-Ies6 assembly, is less conserved (14% identical; 24% similar) than the full protein (27% identical; 35% similar) or functional domains such as the helicase-SANT-associated domain (37% identical; 54% similar) and the ATPase domain (67% identical; 75% similar). In particular, the two regions of human INO80 that were reported as crucial for Arp5-Ies6 binding (25) are relatively less conserved (9 and 14% identical; 9 and 22% similar) and may provide a binding platform for Arp5-Ies6 assembly with human INO80 that is distinct from yeast. Likewise, *S. cerevisiae* Arp5 insertion regions 2 and 3 (Table 2), which are critical for INO80-mediated nucleosome sliding, are relatively less conserved among mammals than other species and may also reflect divergent modes of chromatin remodeling that have evolved in higher eukaryotes.

It is also noteworthy that although both yeast Ies2 and mammalian INO80B have a conserved PAPA-1-like domain, they are not considered orthologs due to a lack of a common ancestor. Indeed, phylogenetic analysis distinguishes these two proteins as being on different branches, indicative of distinct functional evolutionarily lineages (data not shown). In contrast Arp5, Ies6, and Ino80 have mammalian orthologs. Thus, Ies2 and INO80B may have evolved differently to regulate INO80 chromatin remodeling in disparate species. Interestingly, Ies2 function differs even within the fungal kingdom as Ies2 (but not Arp5, Ies6, or Ino80) is one of 20 genes essential for anaerobic growth in *Saccharomyces* yet is absent from other fungi that are unable to grow in anaerobic conditions (45). Thus, it may be that INO80-mediated chromatin remodeling is important for expression of transcriptional programs necessary for growth regulation in response to nutrient and oxygen availability. Ies2 may provide additional regulation for the association of the critical Arp5-Ies6 module that facilitates chromatin remodeling. Although these speculations require additional examination, it is evident that the regulated association of the Arp5-Ies6 module is important for the function of the INO80 complex in different species.

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