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Investigating the assembly of INO80 and its mechanism of hexasome sliding

by
Elise Muñoz

DISSERTATION

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in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by

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Contributions

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Reorientation of INO80 on Hexasomes Reveals Basis for Mechanistic Versatility. *Science* **2023**, 381 (6655), 319–324. The authors are Hao Wu*; **Elise N. Muñoz***, Laura J. Hsieh, Un Seng Chio; Muryam A. Gourdet, Geeta J. Narlikar, and Yifan Cheng.

Elise N. Muñoz is a co-first author on this manuscript having contributed equally to Hao Wu. In this work Elise made the key observation that DNA gaps at the SHL-2 location inhibit the remodeling activity of INO80 on both nucleosomes and hexasomes, strongly suggesting that on both substrates, the ATPase subunit of INO80 acts from the SHL-2 location. These findings have substantially changed our understanding of INO80 mechanism. She performed and quantified all the biochemical experiments. She also made major contributions to the overall conceptualization of the project, along with H.W, M.A.G., G.J.N., and Y.C, to the

purification of INO80 with M.A.G. and L.J.H., to the generation of hexasomes and nucleosomes with M.A.G. and L.J.H. and to the interpretation and discussion of the results and writing of the manuscript with all the authors. The work in this paper is of the technical quality and scientific impact required for a thesis in the Tetrad graduate program.

Investigating the assembly of INO80 and its mechanism of hexasome sliding

Elise Noelle Muñoz

Abstract

The eukaryotic genome is organized and regulated in the form of nucleosomes - the fundamental unit of chromatin composed of ~147 base pairs of DNA wrapped around a histone octamer core. Accessing the DNA wrapped around nucleosomes is central to all DNA-based processes. A class of enzymes known as ATP-dependent chromatin remodelers uses the energy from ATP hydrolysis to catalyze transformations to the nucleosome that alter DNA accessibility. This thesis explores the unique mechanisms of INO80, a 15-subunit remodeler that transforms the nucleosome by sliding a histone octamer core along DNA. INO80 has been linked to various biological processes including DNA damage repair, replication, and transcription. How INO80 uses its biochemical activity to influence nucleosome organization at these various genomic loci remains an open question. This thesis will use biochemical and biophysical techniques to explore INO80's mechanism, specifically its (1) hexasome sliding activity and (2) assembly. In Chapter 2, we explore INO80's activity on hexasomes, subnucleosomal particles missing an H2A-H2B dimer that can form, for example in the wake of transcription. Prior to this work, our lab showed that INO80 prefers to slide hexasomes over nucleosomes. Here, we explore this mechanism further by using a combination of cryo-EM and biochemistry using (sub)nucleosomal substrates with DNA containing single base gaps. We solved high-resolution structures of *S. cerevisiae* INO80 bound to a hexasome and nucleosome. Surprisingly, we found that INO80 reorients to position its

core Ino80 ATPase subunit at superhelical location (SHL) -2 on a hexasome compared to engaging with the nucleosome at SHL-6. This nearly 180° rotation positions INO80 with dramatically different interactions between its core subunits and the nucleosomal DNA and histones. Interestingly, we show that single base gaps, which block DNA translocation, near SHL-2 inhibit INO80's activity on both hexasomes and nucleosomes. We propose a model where INO80 slides all (sub)nucleosomal substrates most efficiently from SHL-2 with INO80 first rotating on a nucleosome before sliding. This model could explain why INO80 slides hexasomes faster than nucleosomes. In Chapter 3, we explored INO80's assembly mechanism. Prior work from our lab showed that Rvb1 and Rvb2, two AAA+ ATPase subunits of INO80, display chaperone-like activity where they recognize a unique insertion domain of the core Ino80 ATPase, Ino80INS, as a protein client. This suggested that the Rvbs may play a role in INO80 assembly. Here, we explored this role further by developing a co-expression system of Rvb1, Rvb2, and a truncated form of Ino80 containing its core ATPase and insertion domains. We show that this complex is functional for ATP hydrolysis, yet the core ATPase domain of Ino80 appears to inhibit the stimulatory effect of Ino80INS alone. In addition, we show that other INO80 core subunits do not stimulate Rvb hydrolysis and we explore conformational changes of the Rvbs upon interactions with clients. Overall, this thesis explores two aspects of INO80's mechanism that add to the overall understanding of a versatile chromatin remodeler.

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Chapter 1: Introduction

1.1 Gene Regulation and ATP-Dependent Chromatin Remodelers

Eukaryotic genetic information is packaged in the form of chromatin. The basic unit of chromatin is a nucleosome composed of ~147 base pairs (bps) of DNA wrapped around a histone octamer core with DNA locations with respect to the octamer described as superhelical locations (SHLs) (Kornberg, 1974; Luger et al., 1997) (**Fig 1.1A**). While nucleosomes package DNA, they also occlude access to the nucleosomal DNA wrapped around the octamer core (Kornberg and Lorch, 1992). Accessing specific regions of DNA at the correct time and location is vital to all DNA-based processes, including DNA replication, DNA damage repair, and transcription.

DNA accessibility can be altered through nucleosome transformations, including DNA unwrapping, histone exchange, nucleosome sliding, and octamer eviction (**Fig 1.1B**). Nucleosomes can naturally undergo these transformations, yet at nonphysiological timescales. These transformations can be catalyzed through the action of ATP-dependent chromatin remodelers which use chemical energy from ATP hydrolysis to transform the nucleosome (Bartholomew, 2014; Narlikar et al., 2013).

Chromatin remodelers are classified into four families on the basis of homology within their core Snf2-like ATPase subunits: ISWI, CHD1, INO80, and SWI-SNF (Clapier and Cairns, 2009) (**Fig 1.2B**). These complexes act at overlapping genomic regions yet

catalyze distinct nucleosome transformations that contribute to higher order nucleosome organization (**Fig 1.2C**).

While chromatin remodelers have evolved to do different things to the nucleosome, the Snf2-like core ATPases of these remodelers all evolved from an ancestral SF2 (superfamily) NTPase,, likely a helicase or nucleic acid translocase (Zhou et al., 2016). They all contain two RecA-like lobes, RecA1 and RecA2, that form a two-lobed structure including an amino-terminus N-lobe and carboxy-terminus C-lobe (**Fig 1.2A**). These lobes interact with DNA and change from an open to closed conformation in coordination with the ATPase cycle, which drives DNA translocation. On nucleosomes, this translocation alters and breaks DNA-histone contacts which catalyzes the variety of nucleosome transformations performed by chromatin remodelers.

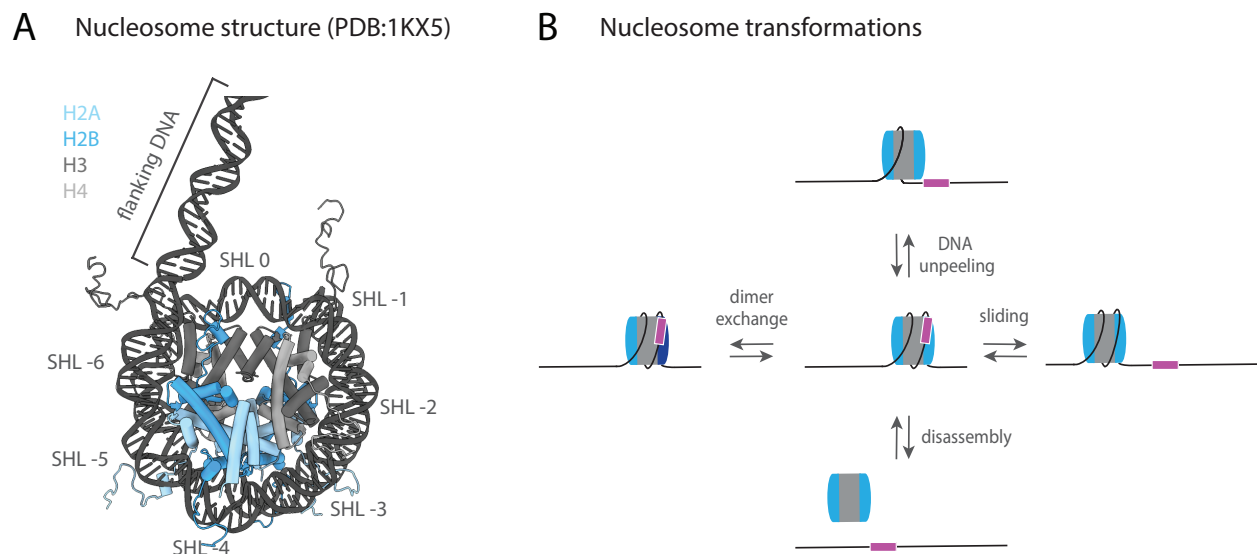


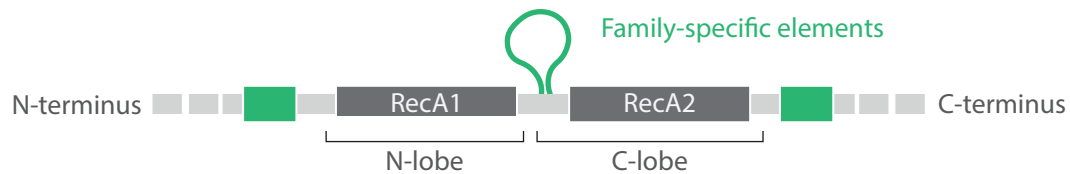
Figure 1.1 Nucleosome composition and transformations. (A) Crystal structure of a nucleosome from PDB: 1KX5 with histones and super helical locations (SHLs) labeled. Flanking DNA has been modeled in. (B) Cartoon of possible nucleosome transformations.

Through transforming nucleosomes in various ways, remodelers not only change DNA accessibility but also overall nucleosome organization on a larger scale. Nucleosome organization within the genome, including nucleosome positioning and spacing, can regulate many DNA-based processes, including replication, DNA-damage, and transcription. Specific nucleosome organizations can be either conducive or unconducive to different processes (Chacin et al., 2023; Garcia et al., 2010; Lai and Pugh, 2017; Venter et al., 1994). The role of chromatin remodelers in nucleosome organization is best understood from studies in *S. cerevisiae*, including genome-wide in vitro reconstitution studies and in vivo studies (Eustermann et al., 2023). Most of these studies have focused on exploring nucleosome organization at transcription start sites.

Transcription start sites (TSSs) in *S. cerevisiae* are characterized by a well-positioned +1 nucleosome, preceded by a nucleosome-free region (NFR) of ~150 bp, and followed by downstream nucleosomes that are regularly spaced by ~20 bps of linker DNA (Yuan et al., 2005) (**Fig 1.2C**). Chromatin remodelers from various families have been shown to influence the organization of nucleosomes at TSSs (**Fig 1.2C**). For example, the SWI/SNF remodeler RSC is involved at the promoter region and contributes to form the NFR. INO80 plays a role in positioning the +1 nucleosome and SWR1 is linked to exchanging the canonical H2A-H2B dimer of the +1 nucleosome for the H2A.Z-H2B variant dimer. ISWI1a, CHD, and INO80 all are involved in regularly spacing nucleosomes within the gene body. This array of nucleosomes is disturbed by processes like transcription, which generates irregular spacing and subnucleosomes including hexasomes that lack an H2A-H2B dimer (discussed further in section 1.3).

While all remodelers play important roles in organizing and regulating the genome, this work will focus on the INO80 chromatin remodeler.

A Snf2-like ATPase



B ATP-Dependent Chromatin Remodeler Families



C Chromatin Remodelers at Transcription Start Sites

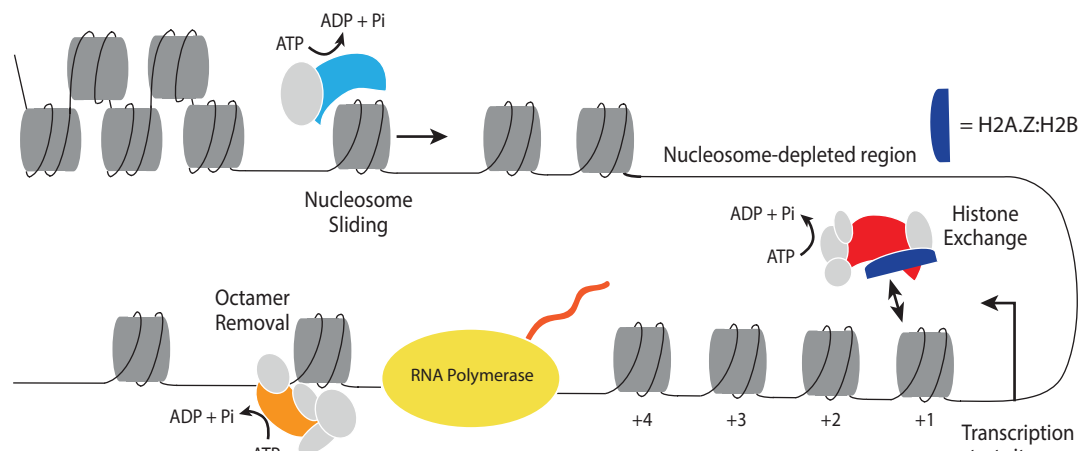


Figure 1.2 ATP-dependent chromatin remodelers transform the nucleosome. (A) Domain architecture of a Snf2-like ATPase motor with family-specific elements highlighted. **(B)** Cartoon model of the four families of ATP-dependent chromatin remodelers. **(C)** Cartoon model of chromatin remodelers acting at a *S. cerevisiae* transcription start site (adapted from Zhou et al., 2017).

1.2 The INO80 ATP-dependent Chromatin Remodeler

The INO80 family of chromatin remodelers consists of INO80 and SWR1 in *S. cerevisiae*. In this thesis, the mechanism of the INO80 chromatin remodeler will be explored. INO80 is ~1 MDa complex composed of 15 subunits, including a core Snf2-like ATPase, Ino80, which contains the two characteristic Snf2 RecA-like lobes separated by an INO80-family unique ~300 amino acid insertion domain (Clapier and Cairns, 2009; Willhoft and Wigley, 2020) and a modular architecture of other subunits. INO80 contains a Rvb1/Rvb2 AAA+ heterohexamer, found only in INO80 family remodelers, that interacts with the Ino80 ATPase insertion domain (**Fig 1.3A**).

INO80 was first discovered as a coactivator of genes in inositol metabolism, which gave it its name (Ebbert et al., 1999) and was further linked to expression of a broad set of genes in *S. cerevisiae* (Jónsson et al., 2001). It was first purified and characterized as a 15-subunit complex with DNA- and nucleosome- stimulated ATPase and chromatin remodeling activities (Shen et al., 2000). *S.c* INO80 consists of the core Ino80 ATPase, Rvb1, Rvb2, les2, the Arp5 module (Arp5, les6), the Arp8 module (Arp8, Arp4, actin, Taf14, les4), and the species-specific Nhp10-module (Nhp10, les1, les3, les5) (**Fig 1.3A**). INO80 is highly conserved across eukaryotes with human INO80 exhibiting similar activities and containing orthologs of many of the core subunits (Jin et al., 2005).

Biochemically, INO80 was initially shown to alter DNA accessibility of a model *Drosophila* chromatin template (Shen et al., 2000). It later was then shown to mobilize reconstituted mononucleosomes in an ATP-dependent manner and to equally space a

tri-nucleosome array (Shen et al., 2003; Udugama et al., 2011), suggesting that INO80 may be involved with organizing nucleosomes in vivo. In line with this, INO80 has been linked to transcription, DNA damage repair, and DNA replication (Morrison, 2017; Poli et al., 2017), processes that occur at genomic locations where nucleosome organization is constantly altered.

In terms of transcription, INO80 has been linked to the regulation of a broad set of genes under normal and stress conditions, both upregulating and downregulating genes. For example, INO80 was shown to associate with >90% of yeast gene promoters (Yen et al., 2013) and an RNA-sequencing study showed that INO80 was involved in regulating 15% of the yeast genome under normal conditions. This correlated with Ino80 and other core subunits localizing to the +1 nucleosome of TSSs of affected genes (Yao et al., 2016). RNAi of human Ino80 showed similar broad changes to gene regulation in human cells (Cao et al., 2015). INO80 also appears to be involved in preventing cryptic transcription of antisense transcripts and long non-coding RNAs (Alcid and Tsukiyama, 2014; Marquardt et al., 2014).

In an in vitro reconstituted system with yeast genomic sequences, nucleosomes can assemble at transcription start sites yet proper positioning relies on addition of purified factors (Krietenstein et al., 2016). INO80 alone was shown to be the sole remodeler that could fully reconstitute the NFR and +1 nucleosomes in this system, highlighting its importance and uniqueness in regulating nucleosome organization.

While INO80's role is more understood at sites of transcription, its activity has also been tied heavily to DNA damage repair and DNA replication. Ino80 mutant yeast strains are more sensitive to DNA damage stress (Shen et al., 2003, 2000). INO80 subunits have been shown to be recruited to double-strand break sites in vivo and shown to associate with H2A.X, a histone variant enriched at sites of DNA damage (Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004). A strain lacking arp8, an INO80 subunit, shows reduced production of ssDNA at sites of DNA damage, a step important for repair (van Attikum et al., 2004). INO80 is also important for the progression of stalled replication forks, is recruited to stalled forks in vivo, and plays a role in normal S phase progression (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008; Vincent et al., 2008). While INO80 is involved with many nuclear processes, how it is recruited to specific sites, how it acts at these sites, and whether its mechanism is different at these various genomic sites are open questions being explored.

To gain a better understanding of INO80's biological roles, many studies have focused on first understanding INO80's biochemical activities on a mononucleosome substrate.

INO80 can center a mononucleosome positioned on the end of a short fragment of DNA and regularly space an array of nucleosomes in an ATP-dependent manner (Shen et al., 2003; Udugama et al., 2011). It is sensitive to the length of flanking DNA, preferring to slide nucleosomes towards longer flanking DNA and preferring flanking DNA >40 bps with a switch-like behavior, compared to a gradual preference seen with ISWI and CHD family remodelers (Zhou et al., 2018). Recent foot printing and structural studies have

shown that INO80 is also unique in the way that it engages with its nucleosome substrate (Ayala et al., 2018; Brahma et al., 2017; Eustermann et al., 2018) (**Fig 1.3B**). At the start of this thesis (June 2018), all published structures of INO80 bound to a nucleosome showed core Ino80 ATPase subunit engaged with the nucleosome at SHL-6 to -7 and its Arp5 module engaged at SHL-2 to -3 (Aramayo et al., 2018; Eustermann et al., 2018) (**Fig 1.3B**). All other remodelers including the other INO80-family member SWR1 are shown to engage with their core ATPase subunits at SHL2 (Markert and Luger, 2021) (**Fig 1.3C**). Only one other study shows that the Snf2 ATPase alone can

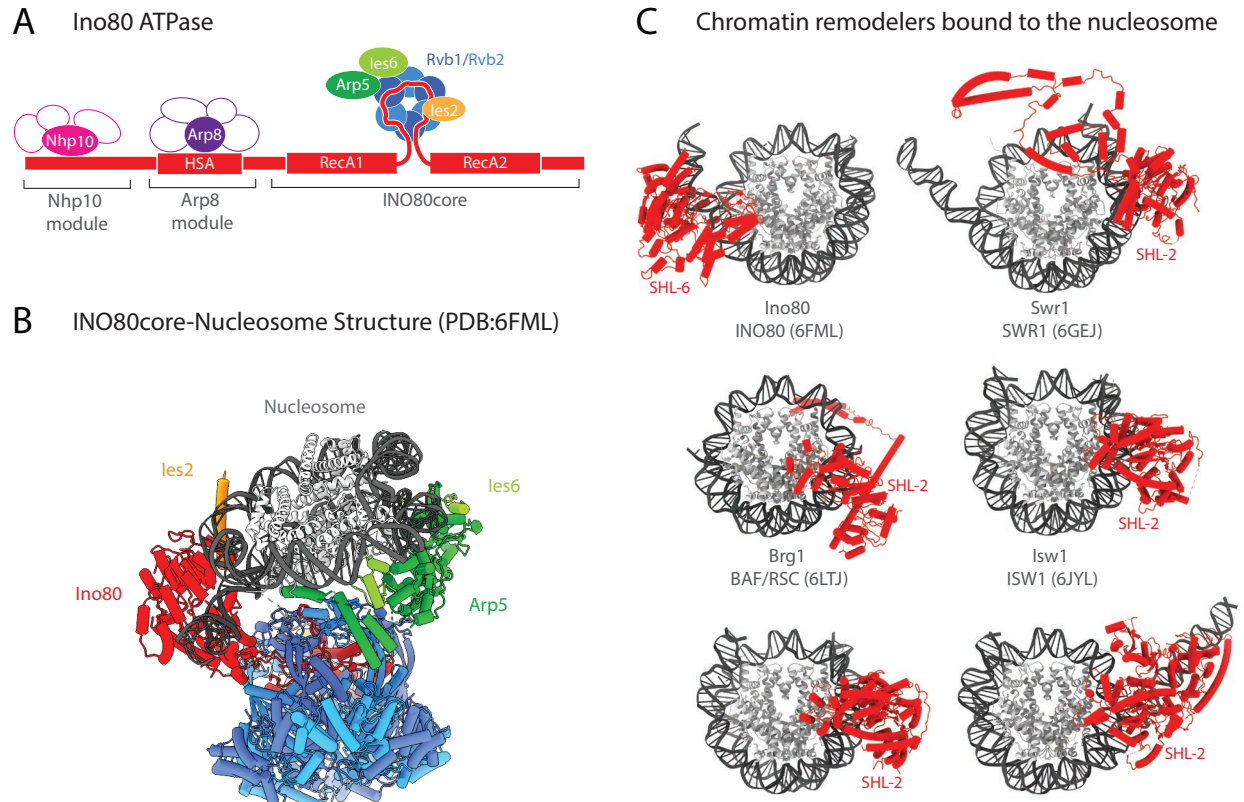


Figure 1.3 INO80 is a unique ATP-dependent chromatin remodeler. (A) Domain architecture of the core Ino80 ATPase showing where accessory subunits and modules associate. **(B)** Cryo-EM structure of *C. thermophilum* INO80_{core} bound to a nucleosome from PDB: 6FML. **(C)** Structures of remodelers bound to the nucleosome showing only the core ATPase subunits in red. Note that all other remodelers, besides INO80, associate with the nucleosome at SHL2.

also engage at SHL6 but this is thought to be only because it is missing its other accessory subunits (Liu et al., 2017).

SHL2 appears to be a hotspot for remodelers, suggesting a functional role in the mechanism of remodeling. Even in the absence of other factors, the nucleosomal DNA near SHL2 is more dynamic than other locations (Bilokapic et al., 2018; Davey et al., 2002; Edayathumangalam et al., 2005; Luger et al., 1997; Suto et al., 2003). Canonically, two SHLs are connected by 10 bps of DNA, however SHL2 and SHL5 can accommodate an extra base pair (Bilokapic et al., 2018; Luger et al., 1997; Ong et al., 2007; Suto et al., 2003; Tan and Davey, 2011). All remodelers share a homologous core ATPase that is a DNA translocase. The model in the field is that through DNA translocation at a site along the nucleosomal DNA, remodelers can disrupt DNA-histone contacts and promote transformations to the nucleosomes that I described above. It has been proposed that SHL2 may be the preferred location for translocation by remodelers because they can take advantage of being able to pull one more base pair into the more dynamic region near SHL2, like a 'sink', allowing for net DNA translocation (Markert and Luger, 2021). With Ino80 engaging at SHL-6 instead of SHL2, INO80 may utilize different nucleosome dynamics to act at SHL-6, however this remains unclear.

Single-molecule studies with INO80 have highlighted other differences in its mechanism. It was shown that INO80 took large step sizes when sliding a nucleosome, while other remodelers appear to take 1-2 bp steps (Zhou et al., 2018). Also seen in these studies is a long ATP-dependent pause that precedes nucleosome sliding by

INO80 that is not seen in other remodeler sliding reactions. These differences in INO80's mechanism observed biochemically and structurally, suggest that it acts in a unique way on nucleosomes.

In line with this, our lab recently showed that INO80 is unique in that it can recognize hexasomes, a subnucleosome missing an H2A-H2B dimer, and actually prefers hexasomes to nucleosomes in terms of ATP hydrolysis and sliding (Hsieh et al., 2022). We also show that INO80 plays a role in positioning nucleosomes and hexasomes in vivo supporting the idea that hexasomes are bona-fide substrates and play key regulatory roles in vivo. Other remodelers including yeast Chd1, yeast RSC, and human cBAF can act on hexasomes, but do not show the same preference as INO80 does (Dao et al., 2022; Ghassabi Kondalaji and Bowman, 2022; Levendosky et al., 2016).

1.2.1 INO80's accessory subunits

Strikingly, INO80 is a complex made up of many different subunits that have been shown to play different roles in INO80's mechanism. The Arp5 module is a key component that contributes to recognizing the acidic patch, of the nucleosome, a patch of acidic residues on the H2A-H2B dimer shown to be a hotspot for nucleosome interactions (Eustermann et al., 2018; Hsieh et al., 2022). While INO80 can still manage to slide nucleosomes without the Arp5 module, it does so very slowly, highlighting its importance as a key subunit (Hsieh et al., 2022; Jónsson et al., 2004; Tosi et al., 2013; Yao et al., 2016). Upon loss of the *les2* subunit, INO80 however cannot slide nucleosomes and loses its nucleosome-stimulated ATPase activity. It is hard to tease out the specific role of *les2* in the context of *S. cerevisiae* INO80 because deletion of

ies2 also results in loss of the Arp5 module with INO80 (Yao et al., 2015). The Arp8 module, containing nuclear actin, Arp8, Arp4, Ies4, and Taf14, interacts with the HSA domain of the core Ino80 ATPase and binds the flanking DNA of the nucleosome. Recent high-resolution cryo-EM structures of complexes of the Arp8 module, Ino80 HSA domain, and DNA from a variety of species (human, *S. cerevisiae*, and *C. thermophilum*), suggest that the module is very well conserved and show how the module interacts with DNA through the Ino80 HSA domain (Knoll et al., 2018; Kunert et al., 2022). The Arp8 module is not essential for INO80's sliding activity, however loss of the whole module or specific mutations in key subunits of the module inhibit sliding in all conditions explored to date (Brahma et al., 2018; Kunert et al., 2022; Oberbeckmann et al., 2021a; Tosi et al., 2013). A specific mutation in the INO80 HSA domain can lead to increased INO80 ATPase and sliding activity, suggesting the Arp8 module may potentially play both activating and autoinhibitory roles (Kunert et al., 2022). The Nhp10 *S. cerevisiae*-specific module interacts with the N-terminus of Ino80. It is not required for INO80 sliding however it is essential for its flanking DNA length-sensing mechanism (Zhou et al., 2018). The different species-specific modules that interact with the Ino80 N-terminal domain in human and *C. thermophilum* are also not required for INO80 sliding activity, but it is unclear how they may contribute to regulation of INO80's mechanism 5/31/24 8:26:00 AM.

1.2.2 Rvb1 and Rvb2

Core INO80 subunits, Rvb1 and Rvb2, are AAA+ ATPases that form a heterohexamer (Rvb1/Rvb2) that intricately interacts with the Ino80 insertion domain. They are essential in yeast making their role in INO80 remodeling hard to study. An earlier study showed

that Rvb2 is essential to recruitment of core INO80 subunits, including Rvb1 and the Arp5 module, and required for INO80 remodeling activity (Jónsson et al., 2004). Recent structural studies highlight the Rvb1/Rvb2 hexamer as a scaffold interface for the core INO80 subunits, including the Arp5 module and Ies2 (Aramayo et al., 2018; Ayala et al., 2018; Eustermann et al., 2018). While the Rvb1 and Rvb2 are ATPases, whether their activity plays a role in INO80's remodeling activity remains unknown. Work from our lab has shown that Rvb1/Rvb2 has chaperone-like activity, recognizing the Ino80 insertion domain as a client that stimulates Rvb1/Rvb2 ATPase activity 16-fold (Zhou et al., 2017). This work suggested that Rvb1/Rvb2 may play an active role in assembling the INO80 complex, which is further explored in Chapter 3 of this thesis.

1.3 Hexasomal Particles: Consequence or also Consequential?

1.3.1 Subnucleosomal particles in biology

The packaging of eukaryotic DNA into chromatin allows differential compartmentalization of the same genome into transcriptionally active (euchromatin) and heritably repressed (heterochromatin) states. Such compartmentalization is essential to enable specific transcriptional programs for stabilizing cell identity. Correspondingly, mis-regulation of chromatin states has been linked to developmental defects and diseases like cancer. Chromatin states are regulated by the combined action of chromatin binding proteins, chromatin remodeling enzymes and sequence specific transcription factors. Many of these regulators act at the level of the smallest unit of chromatin, a nucleosome, which contains ~147 bp of DNA wrapped around an octamer of histone proteins (**Fig 1.4**). In these cases, it is often assumed that the

substrates are intact nucleosomes. However, recent studies have provided evidence for the formation of subnucleosomal particles called hexasomes which lack one H2A-H2B dimer (**Fig 1.4**), primarily due to the action of RNA polymerase on a nucleosome (Angelov et al., 2006; Belotserkovskaya et al., 2003; Kireeva et al., 2002;

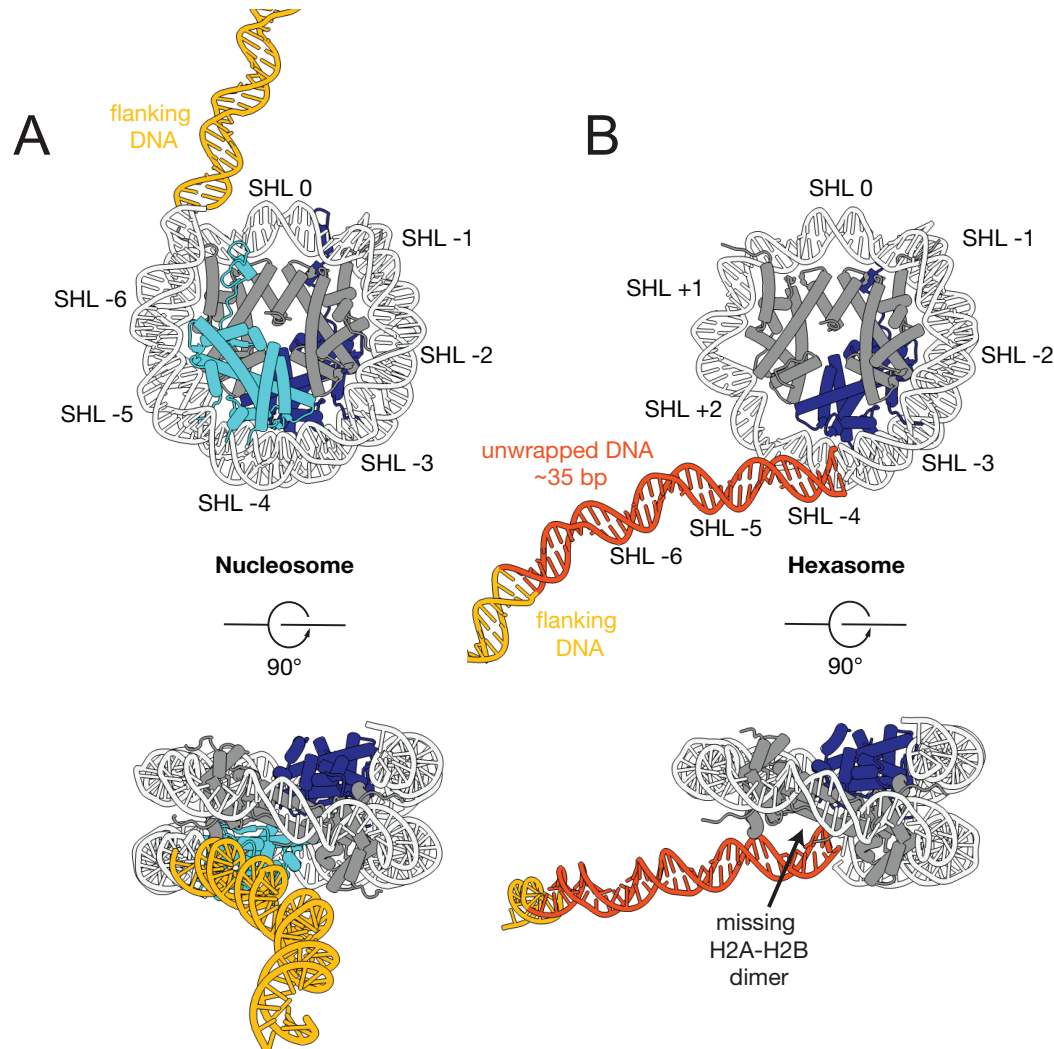


Figure 1.4 Comparison of nucleosome and hexasome structures. (A) Nucleosome structure (PDB: 1KX5) consisting of ~147 bp of DNA wrapped around an octamer core containing one H3-H4 tetramer (grey) and two H2A-H2B dimers (blue or cyan). The flanking DNA is shown in gold. (B) Hexasome structure (PDB: 6ZHY) consisting of ~112 bp of DNA wrapped around a hexamer core containing an H3-H4 tetramer and one H2A-H2B dimer. The DNA wrapped around a nucleosome can be divided into waypoints at superhelical locations (SHLs) separated by 10 base-pairs across the histone octamer surface, which are mirrored on the opposite face. The dyad is defined by SHL0. Note that here we show the promoter-proximal dimer (blue) as that closest to the end of the DNA away from the flanking DNA. The flanking (gold) and unwrapped (orange) DNA has been modeled in.

Ramachandran et al., 2017).

The identification of hexasomes ties back to a rich, almost 50-year-old history of observations about non-canonical nucleosomal particles. In these older studies, nucleosome-like particles were identified that showed greater accessibility of DNA and internal histone regions (Baer and Rhodes, 1983; Johnson et al., 1987, 1978; Rhee et al., 2014). Now, with advancements in high-resolution structure in vitro and high-resolution foot printing in cells, the field has an opportunity to revisit the significance of these older observations. A major question raised by the presence of hexasomal particles is whether these are simply collateral damage from the action of machines like RNA polymerase or whether they play regulatory roles.

An obvious consequence of forming a hexasome is breakage of the symmetry present in the nucleosomal structure (**Fig 1.4**). Here we explore how the asymmetry caused by hexasome formation may control the action of RNA Polymerases and ATP-dependent chromatin remodeling enzymes and impact higher-order chromatin structure. To do so, we integrate older and recent circumstantial evidence primarily within the context of transcription for hexasome generation. In addition to hexasomes, there is evidence for formation of tetrasomes in cells, where both H2A- H2B dimers are lost (Ramachandran et al., 2017; Rhee et al., 2014). We do not discuss tetrasomes here as less is currently known about their impact on chromatin regulators.

1.3.2 Formation of hexasomes and their consequence for transcription

Nucleosomes present a large barrier to transcription by RNA Polymerase II (RNAPII). Interestingly, biochemical studies have shown that RNAPII directly influences

nucleosome structure during transcription, resulting in the formation of hexasomes (Angelov et al., 2006; Belotserkovskaya et al., 2003; Bintu et al., 2011; Kireeva et al., 2002). Additional biochemical studies have shown that hexasomes lacking the promoter proximal H2A-H2B dimer are poor substrates for RNAPII and result in a higher rate of RNAPII stalling [10]. In contrast, loss of the promoter-distal dimer facilitates nucleosome passage of RNAPII in vitro and is associated with transcription elongation in vivo (Kulaeva et al., 2010, 2009; Ramachandran et al., 2017) (**Fig 1.5A**). These observations raise the interesting possibility that one round of transcription can change the chromatin landscape in a manner that either promotes or inhibits the next round of transcription. The promoter-proximal H2A-H2B dimer is the first one encountered by RNAPII (**Fig 1.5B**). However, which of the two H2A-H2B dimers is lost upon RNAPII action appears context dependent as described below.

Recent cryo-EM structures shed some light on what types of hexasomes are generated after passage of RNAPII through a nucleosome. The cryo-EM structure of the mammalian RNAPII elongation complex (RNAPII EC) on a nucleosome in the presence of elongation factors revealed a backtracked hexasomal intermediate (Farnung et al., 2022). The backtracked structure shows loss of the promoter proximal H2A-H2B dimer. The authors propose that RNAPII passage through a nucleosome leads to unwrapping of nucleosomal DNA and loss of the proximal H2A-H2B dimer (**Fig 1.5B**). However, in cells, RNAPII passage is often accompanied by the action of the histone chaperone FACT. It is therefore possible that upon RNAPII passage and disruption of histone contacts, FACT binds to the exposed H2A-H2B dimer and regulates its fate. Indeed, cryo-EM structures of RNAPII EC in the presence of FACT suggest that FACT initially

prevents the loss of the promoter-proximal dimer as the RNAPII EC passes through the nucleosome (Ehara et al., 2022) (**Fig 1.5C**). Instead, the promoter-distal dimer is lost possibly due to DNA unwrapping and dimer destabilization. Additional structures of the RNAP II EC stalled at multiple sites throughout the nucleosome suggest that FACT can subsequently (i) switch the orientation of the hexamer such that now the promoter proximal dimer is missing and (ii) deposit an H2A-H2B dimer to assemble an intact nucleosome shifted towards the promoter relative to its original position (**Fig 1.5C**). The mechanism for this switch in hexamer orientation remains unclear. However, consistent with FACT playing a role in determining the orientation of the hexamer, previous studies in *Drosophila* have shown that deleting specific FACT subunits led to either the loss of the promoter proximal (SPT16 subunit knockdown) or distal dimer (SSRP subunit knockdown) at a range of loci (Ramachandran et al., 2017).

Based on the collection of structures discussed above, we speculate that the speed of FACT action relative to the speed with which the next RNAPII EC reaches the hexasome determines what substrate the next RNAPII EC encounters. If RNAPII EC reaches the hexasome before FACT reorients the hexamer, this would result in facilitated transcription. If the RNAPII EC reaches the hexasome after FACT reorients the hexamer, this could result in stalling of the RNAPII. Finally, if FACT redeposits the H2A-H2B dimer before arrival of the next RNAPII EC this would result in the same rate of RNAPII passage as for the previous RNAPII EC (**Fig 1.5C**).

Previous biochemical studies have also shown that passage of multiple closely spaced RNAPII molecules can result in eviction of histones to generate a largely unbound DNA

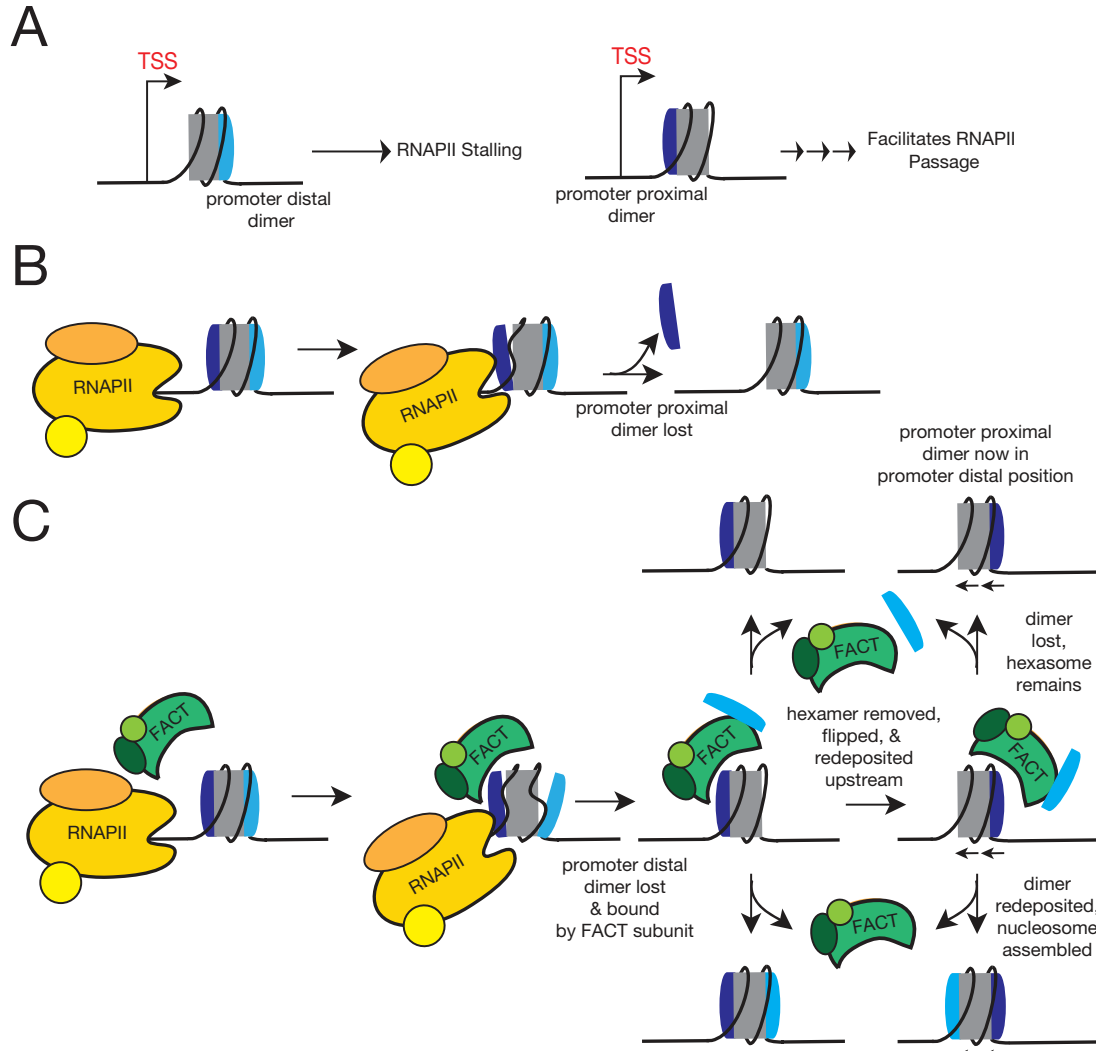


Figure 1.5 Models for the interplay between hexasomes and RNAPII transcription. (A) A hexasome missing either the promoter proximal or promoter distal dimer results in stalling of RNAPII or facilitates passage of RNAPII, respectively (Kulaeva et al., 2009). TSS refers to transcription start site. Notably, RNAPII transcribes faster through a hexasome missing the promoter distal dimer compared to a nucleosome. (B) When RNAPII and elongation factors (RNAPII Elongation Complex, RNAPIIEC), encounter a nucleosome, DNA is unwrapped from the promoter proximal dimer, which may destabilize the dimer and lead to its loss and formation of a hexasome that would lead to RNAPII stalling. Model based on (Farnung et al., 2022). (C) When RNAPII EC and a histone chaperone FACT encounter a nucleosome, DNA is unwrapped from the promoter proximal dimer but FACT stabilizes and prevents loss of the dimer. Downstream distal DNA is simultaneously unwrapped which destabilizes the promoter distal dimer, leading to its loss. A FACT subunit may interact with the dissociated free dimer. Multiple kinetically partitioned pathways can be imagined at this stage, that include the hexasome orientation remaining the same, FACT flipping the orientation of the hexamer and FACT redepositing the dimer to regenerate a nucleosome. Each pathway has a different consequence for the next transcribing RNAPIIEC. Model based on (Bintu et al., 2011, Ehara et al., 2022, Kulaeva et al., 2009, Kulaeva et al., 2010). For all panels, H3-H4 tetramer is in grey and H2A-H2B dimers are in blue or cyan.

template (Kulaeva et al., 2010). The specific orientation of hexasomes generated by RNAPII then provides opportunities to fine tune the speed of transcription and the level of positive feedback between successive RNAPII molecules. For example, a series of hexasomes missing the promoter distal dimer would speed up transcription of successive RNAPII ECs whereas the presence of a few hexasomes missing the promoter proximal dimer would stall the next RNAPII ECs. Such stalling could allow time for downstream hexasomes to be converted to nucleosomes by chaperones such as FACT, making the template less conducive to transcription by successive RNAPII molecules. Finally, formation of hexasomes may also help impose directionality where bidirectional transcription is possible, by promoting transcription in one direction while simultaneously inhibiting transcription in the other direction.

1.3.4 Impact of hexasomes on chromatin accessibility and folding

At a primary structural level, loss of an H2A-H2B dimer should increase the accessibility of (i) the underlying DNA, (ii) histone tails such as the H3 tail that interacts with DNA wrapped by the H2A- H2B dimer, and (iii) the H3-H4 interface that interacts with the H2A-H2B dimer. Indeed, small angle X-ray scattering (SAXS) data has shown that the hexasome stably wraps ~115 bp of DNA, exposing 30-40 bp of additional DNA compared to a nucleosome (Arimura et al., 2012). NMR studies have shown that the histone H3 tail becomes more dynamic in an asymmetric manner in a hexasome (Morrison et al., 2021). Other biophysical studies indicate that the DNA that unwraps upon loss of an H2A-H2B dimer becomes as accessible as naked DNA. Interestingly these studies also found that DNA wrapped around the remaining H2A-H2B dimer is 2-

fold less accessible compared to nucleosomes (Brehove et al., 2019) suggesting some anti-cooperativity between the two bound H2A-H2B dimers.

The findings described above show how loss of an H2A-H2B dimer generates an asymmetrically accessible substrate for DNA binding factors and histone modifying enzymes. An interesting example of hexasomes acting as substrates comes from recent data identifying a new type of modification on H3. The SULT1B1 histone sulfotransferase adds a sulfate group to H3Y99, which is usually buried inside an octamer (Yu et al., 2023). However, in the context of a hexasome or a tetrasome, a sulfated H3Y99 becomes accessible to the arginine methyl transferase PRMT1, which is recruited by the modification and upon recruitment generates H4R3me2a, a mark that promotes transcription (Yu et al., 2023).

The breaking of symmetry caused by hexasome formation should in principle, also affect higher-order chromatin organization. There are currently two qualitatively different chromatin folding models in the field. In a small proportion of cases, such as in some terminally differentiated cells, chromatin has been shown to adopt regular 30 nm-like structures, where inter-nucleosomal interactions show a repeated pattern (Xu et al., 2021). In most other cases including in highly condensed mitotic chromosomes, chromatin folding does not appear to show a regular pattern (Boopathi et al., 2020; Cai et al., 2018; Grigoryev, 2018; Maeshima et al., 2016). Further, biochemical data suggest that folded chromatin can adopt phase-separated states (Gibson et al., 2019). In these less regular structural contexts, the inter-nucleosomal interactions are still thought to use some of the interactions observed in the 30 nm fibers. These interactions include

contacts between histone tails of one nucleosome and the DNA or the H2A-H2B acidic patch of another nucleosome. The loss of an H2A-H2B dimer from a nucleosome substantially changes the path of the DNA while also resulting in the loss of an acidic patch interface (**Fig 1.4**). Therefore, a small proportion of hexasomes in the midst of several nucleosomes has the potential to disrupt and reorganize folded chromatin. We imagine that in the context of a 30 nm structure, introduction of hexasomes would disrupt the regularity of inter-nucleosomal interactions breaking the continuity of the 30 nm structure. In the context of less regularly folded chromatin states, the presence of hexasomes could have a few different mutually compatible effects such as (i) increasing the dynamics of inter-nucleosomal interactions, (ii) driving new interactions with the exposed H3-H4 interface and (iii) changing the material properties of phase-separated states.

1.3.5 Regulation of sub-nucleosomal particles

Consistent with hexasomes having functional consequences, their regulation by other enzymes is becoming more evident. One example is the male specific lethal (MSL) complex, which ubiquitylates H2B K34 and also promotes high RNAPII processivity (Krajewski et al., 2018). Interestingly, it has been shown that the H2B K34 mark increases the probability of forming hexasomes, possibly because the bulky ubiquitin group interferes with H2A-H2B dimer-DNA interactions. These findings suggest that one way the MSL complex promotes RNAP II processivity is by enabling formation of hexasomes (Krajewski et al., 2018). Similar to chemical modifications, incorporation of histone variants can also change the propensity of nucleosomes to be converted to hexasomes. H2A.Z is a histone variant that has been implicated in gene transcription

including RNAPII pausing and elongation. In vitro data suggests that H2A.Z nucleosomes are less stable than H2A nucleosomes (Horikoshi et al., 2016; Sato et al., 2020). Furthermore, structures of H2A.Z containing nucleosomes display more flexible DNA ends that could lead to H2A-H2B dimer loss. Interestingly, H2A.Z is commonly found with the H3.3 histone variant, which creates labile nucleosomes that more readily lose H2A.Z-H2B dimers at active promoters (Jin et al., 2009). In some cases, at the +1 nucleosome (defined as in **Fig 1.6**), there is enrichment of ubiquitylated H2B on the promoter proximal side, while H2A.Z is placed at the promoter-distal side of the nucleosome. The authors speculate that this composition could promote the passage of RNAPII (Rhee et al., 2014). Overall asymmetric placement of chemical modifications or histone variants in nucleosomes can further regulate transcription initiation such that RNAPII is either stalled or accelerated based on whether the promoter proximal or distal dimer is preferentially lost.

Biophysical and biochemical work has shown that on sufficiently long stretches of DNA, one outcome of RNA polymerases transcribing through a nucleosome is the transfer of the octamer upstream towards the promoter (Ehara et al., 2022; Hodges et al., 2009; Studitsky et al., 1997, 1994). This is also seen in vivo (Radman-Livaja et al., 2011; Weiner et al., 2010) (**Fig 1.6A,B**). Such a shift may be enabled by FACT as described above (Ehara et al., 2022) or by other RNAPII intrinsic mechanisms (Bintu et al., 2011). In cells, a shift in nucleosome position can potentially expose cryptic transcription sites. ATP- dependent chromatin remodeling enzymes have been implicated in resetting nucleosome positions in the wake of RNAPII action, thereby reducing cryptic transcription (Whitehouse et al., 2007; Yu et al., 2023). In the past few years

hexasomes have also been shown to be substrates for three different classes of ATP-dependent chromatin remodelers (**Fig 1.6B**). In the CHD class, the *S.cerevisiae* Chd1 chromatin remodeler slides hexasomes unidirectionally in the direction of the existing dimer (Levendosky et al., 2016). In the SWI/SNF class, the mammalian cBAF complex and the *S.cerevisiae* RSC complex can both slide hexasomes (Dao et al., 2022; Ghassabi Kondalaji and Bowman, 2022). Interestingly incorporation of site-specific DNA binding factors drives both Chd1 and RSC to slide the hexasome away from the bound factor, suggesting a mechanism to further regulate the directional movement of hexasomal substrates (Ghassabi Kondalaji and Bowman, 2022) (**Fig 1.6C**). In the INO80 class, the INO80 complex was recently implicated in sliding hexasomes. Interestingly, INO80 appears to prefer hexasomes over nucleosomes (Hsieh et al., 2022). While there are many cryo-EM studies highlighting how chromatin remodelers engage nucleosomes, to date INO80 is the only remodeler for which structures with hexasomes have been published. Two recent structural studies highlight how INO80 recognizes key features of a hexasome, including an exposed H3-H4 interface and an additional ~35 bp of unwrapped DNA, to make unique interactions and position itself in a completely different orientation on a hexasome compared to a nucleosome (Wu et al., 2023; Zhang et al., 2023). This reorientation corresponds with INO80's enhanced activity on a hexasome substrate. The identification of such a hexasome-specific mechanism strongly suggests that hexasomes play significant roles in genome regulation.

Finally, histone chaperones, are well known to reduce the life-time of hexasomes by driving the formation of complete nucleosomes (Torres-Arciga et al., 2022). Thus, we

imagine that in the crowded context of a nucleus, the steady level of hexasomes at any given locus will be a consequence of the competing effects of histone chaperones on one side versus the action of RNA Polymerase, histone variants and histone

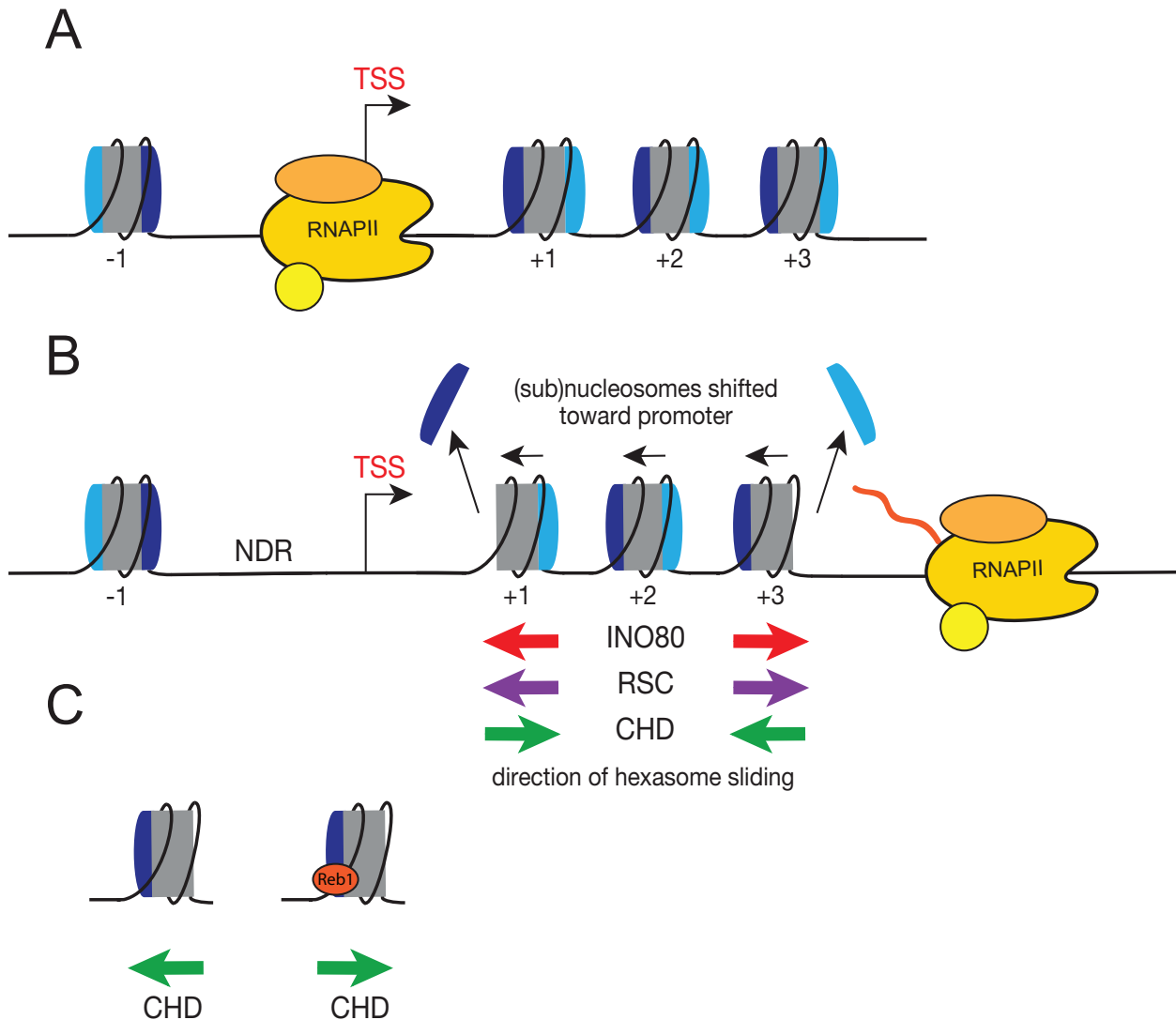


Figure 1.6 Model for chromatin remodelers acting on hexasomes in the wake of transcription. The context for the model is a promoter as found in *S. cerevisiae*. The promoter contains a Nucleosome Depleted Region (NDR) that contains the transcription start site (TSS). Well positioned nucleosomes flank the NDR. Nucleosomes within the gene body are shown numbered with “+” signs and the nucleosome upstream of the NDR is shown numbered with a “-” sign **(A)** RNAPII at the TSS. **(B)** As RNAPII moves through the gene body, hexasomes may form with either the promoter proximal or promoter distal dimer missing. All sub(nucleosomes) are shifted toward the promoter in the process (black arrows). Chromatin remodelers can slide the hexasomes in a directional manner depending on which dimer is lost (Dao et al., 2022, Ghassabi et al., 2022, Levendosky et al., 2016, Whitehouse et al., 2007). How these remodelers coordinate to reposition these particles correctly remains an open question. **(C)** Binding of Reb1 and other factors has been shown to change the directionality of sliding by Chd1 (Levendosky et al., 2016). Binding of additional factors may also play a role in regulating the process modeled in (B).

modifications on the other side.

1.3.6 Broader role for hexasomes?

The examples above suggest that the asymmetry induced by hexasome formation can provide new ways to regulate factor binding and transcription in a chromatin landscape. It is possible that the asymmetry also helps regulate other DNA-based processes such as repair and replication. For example, the H3.3 histone variant is deposited by histone chaperones at sites of DNA damage and recently, hexasomes with H3.3 were shown to facilitate DNA repair via base excision repair glycosylases (Li et al., 2022). To better understand how and in which contexts hexasomes regulate DNA- based processes, hexasome formation and dynamics need to be further studied in cells. Existing mapping technologies such as chromatin immunoprecipitation-exonuclease (ChIP-exo) and micrococcal nuclease sequencing (MNase-seq) have helped identify subnucleosomal particles in cells (Ramachandran et al., 2017; Rhee et al., 2014). These studies have highlighted that subnucleosomal particles exist in cells and can be formed due to transcription. However, most methods that rely on measuring DNA accessibility in cells cannot readily distinguish subnucleosomal particles from nucleosome conformations that are more susceptible to increased DNA breathing. What is needed are antibodies that specifically recognize hexasomes. Until such antibodies are available, it is possible that existing nucleosome mapping technologies could be further adapted to study the quantitative distribution and orientation of hexasomes and potential asymmetrically accessible nucleosomes formed during transcription and other DNA-based processes. We anticipate that once researchers start looking in more depth at the roles of

hexasomes in cells, additional roles for these subnucleosomal particles will be discovered.

Chapter 2: Reorientation of INO80 on Hexasomes Reveals Basis for Mechanistic Versatility

2.1 Preface

Unlike other chromatin remodelers, INO80 preferentially mobilizes hexasomes, which can form during transcription. Why INO80 prefers hexasomes over nucleosomes remains unclear. Here, we report structures of *S. cerevisiae* INO80 bound to a hexasome or a nucleosome. INO80 binds the two substrates in substantially different orientations. On a hexasome, INO80 places its ATPase subunit, Ino80, at superhelical location (SHL)-2, across from SHL-6/-7 as previously seen on nucleosomes. Our results suggest that INO80 action on hexasomes resembles action by other remodelers on nucleosomes, such that Ino80 is maximally active near SHL-2. The SHL-2 position also plays a critical role for nucleosome remodeling by INO80. Overall, the mechanistic adaptations used by INO80 for preferential hexasome sliding imply that sub-nucleosomal particles play considerable regulatory roles.

2.2 Introduction

In eukaryotes, central nuclear processes such as gene expression, DNA replication and DNA repair are coordinated with dynamic changes in chromatin states (Bar-Ziv et al., 2016; Ehrenhofer-Murray, 2004; Hübner and Spector, 2010). ATP-dependent chromatin remodeling enzymes play essential roles in catalyzing such changes. These enzymes are broadly categorized into four major families: SWI/SNF, ISWI, CHD, and INO80 (Clapier and Cairns, 2009; Zhou et al., 2016). Each of them contains a core remodeling ATPase subunit and several auxiliary subunits that regulate the core ATPase. It has typically been presumed that the preferred substrate of these enzymes is a

nucleosome, the smallest unit of chromatin, containing ~147 bp of DNA wrapped around an octamer of histone proteins (Luger et al., 1997). Consistent with this assumption, between them, these four classes slide the histone octamer, exchange histone variants, and transfer entire octamers (Clapier et al., 2017; Zhou et al., 2016).

The INO80 complex has been shown to play roles in regulating transcription, DNA replication and DNA repair (Bao and Shen, 2007; Morrison and Shen, 2009; Poli et al., 2017). How INO80's biochemical activities relate to its diverse biological roles is not well understood. Unlike remodelers from other families, whose core ATPase subunits bind the nucleosome near superhelical location (SHL)-2, Ino80, the core ATPase subunit of the INO80 complex, binds nucleosomes near SHL-6/-7 (Ayala et al., 2018; Brahma et al., 2017; Eustermann et al., 2018; Markert and Luger, 2021) (**Fig S2.1A**). It has been speculated that this key difference in nucleosome engagement reflects a fundamentally different remodeling mechanism (Markert and Luger, 2021; Yan and Chen, 2020). Indeed, we showed that the preferred substrate of the *S. cerevisiae* INO80 complex is not a nucleosome but a hexasome, which is a sub-nucleosomal particle that lacks a histone H2A-H2B dimer (Hsieh et al., 2022). Hexasomes are generated during transcription and may also be formed during DNA replication and repair (Henikoff, 2016; Kireeva et al., 2002; Kulaeva et al., 2010; Ramachandran et al., 2017). Further, INO80's activity on nucleosomes is more dependent on flanking DNA length than on hexasomes (Hsieh et al., 2022; Zhou et al., 2018). These results suggested that INO80 has the versatility to act on hexasomes or nucleosomes based on the density of nucleosomes and hexasomes at a given locus. Yet, fundamental mechanistic questions remain. It is

not clear how INO80 can act on both nucleosomes and hexasomes, which differ substantially in their structures. Additionally, why INO80 has different flanking DNA length dependencies on hexasomes versus nucleosomes is unclear.

Here, we report cryogenic-electron microscopy (cryo-EM) structures of endogenously purified *S. cerevisiae* INO80 bound to a hexasome and a nucleosome. We find that INO80 binds hexasomes and nucleosomes in opposite orientations, with Ino80 binding near SHL-2 on hexasomes and near -6/-7 on nucleosomes. The location of the Arp8 module suggests how flanking DNA length differentially regulates nucleosome and hexasome sliding. DNA gaps near SHL-2 inhibit sliding of both substrates by INO80. Together, our findings provide mechanistic insights into how INO80 slides both hexasomes and nucleosomes.

2.3 Results

2.3.1 Structures of the INO80-hexasome and -nucleosome complexes

To visualize how INO80 binds to a hexasome or a nucleosome, we prepared hexasomes and nucleosomes on the same DNA templates containing the 147 bp 601 nucleosome positioning sequence with 80 bp of additional DNA as described previously (+80H and +80N, with definition explained in **Fig. 2.1A** and **Fig S2.1A-B**, and Supplementary Text) (Hsieh et al., 2022; Levendosky et al., 2016; Levendosky and Bowman, 2019). Complexes were formed by mixing hexasomes or nucleosomes with endogenously purified *S. cerevisiae* INO80 without adding nucleotide (**Fig S2.1C-H**).

We determined cryo-EM structures of the INO80-hexasome complex in three different conformational snapshots (**Fig 2.1B,C** and **Fig S2.2 – Fig S2.6**). The overall shape of

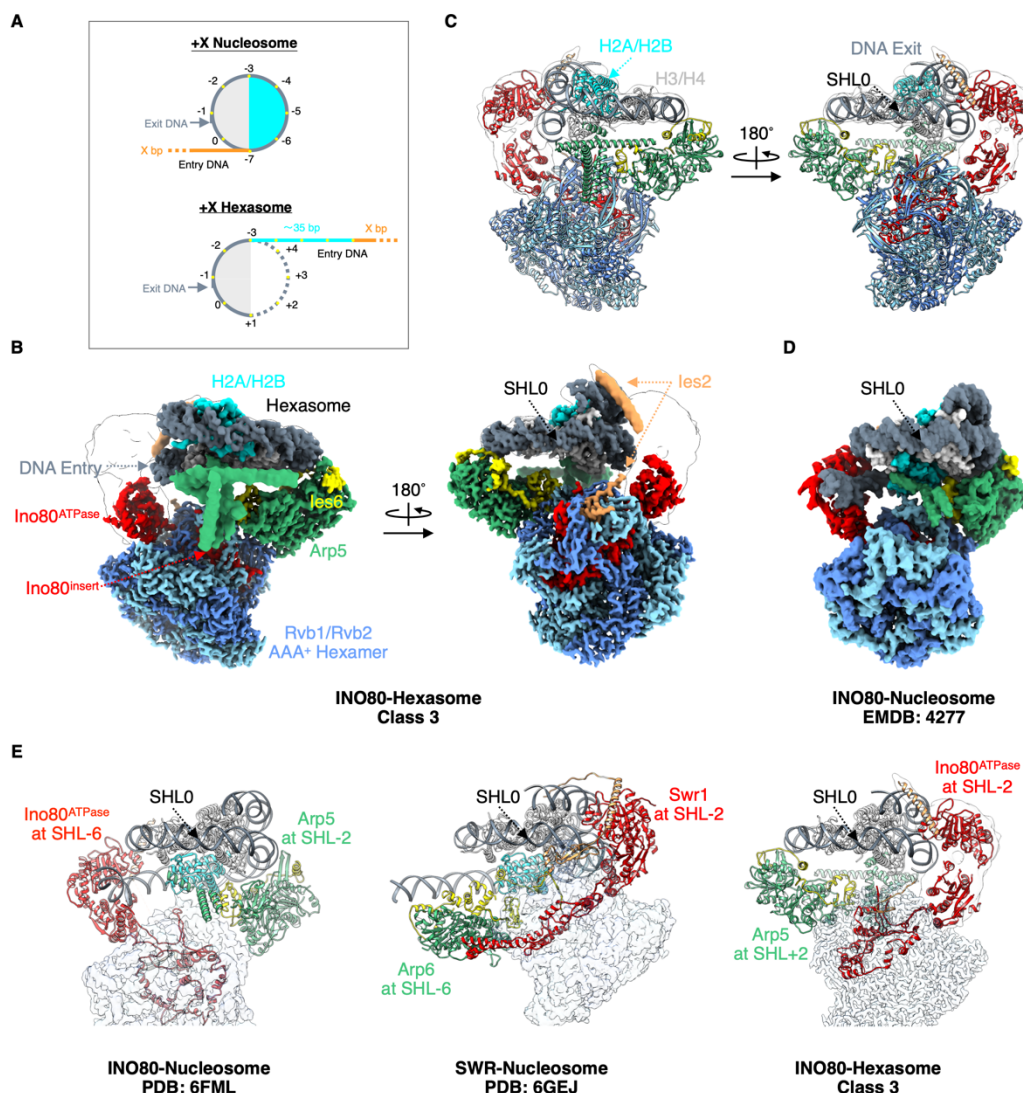


Figure 2.1 Structure of the INO80-hexasome complex reveals large rotation. **(A)** Cartoon illustration of a +X Nucleosome and a +X Hexasome. H2A-H2B dimer proximal to the flanking DNA (entry side dimer): cyan; H3-H4: light gray; 601 DNA: dark gray; flanking DNA: orange; additional free (unwrapped) DNA: cyan; super helical locations: yellow dots; DNA from the bottom gyre: dotted line. **(B)** Two different views of cryo-EM density map of the INO80-hexasome complex (class 3). **(C)** Atomic model of the INO80-hexasome complex (class 3), viewed in the same orientation as the map is viewed in (B). **(D)** Cryo-EM density map of *Chaetomium thermophilum* INO80-nucleosome complex (EMDB: 4277 (Eustermann et al., 2018)) displayed with its nucleosome dyad and H3-H4 tetramer aligned with that of the hexasome in the right panel of (B). Note that INO80 on a hexasome rotates ~180° from where it sits on a nucleosome when keeping the nucleosome/hexasome dyad and H3-H4 aligned. **(E)** Structural comparisons of INO80-nucleosome complex (left), SWR-nucleosome complex (middle) and INO80-hexasome complex (right), with nucleosome/hexasome dyad and H3-H4 aligned.

INO80 is similar within these structures and also to previously published structures of the nucleosome in complex with human (Ayala et al., 2018) and *Chaetomium thermophilum* (Eustermann et al., 2018) INO80. We group subunits of the INO80 complex into four modules: Rvb module (Rvb1/Rvb2), Arp8 module (Arp8/Arp4/Actin/Ies4 and Taf14), Ino80 module (Ino80/Ies2) and Arp5 module (Arp5/Ies6). The Ino80 protein consists of three major regions: the N-terminal domain (NTD), the HSA region (Ino80^{HSA}) and the ATPase domain (Ino80^{ATPase}). Detailed descriptions of these modules in our structures are in the Supplementary Text.

While the INO80 architecture appears similar to that in the INO80-nucleosome structures, a major difference is that it is rotated $\sim 180^\circ$ on a hexasome compared to a nucleosome (**Fig 2.1B-E**). We identified two primary interactions between INO80 and the hexasome: Ino80^{ATPase} binds the hexasome near SHL-3 (class 1), -2.5 (class 2) and -2 (class 3), and the Arp5/Ies6 module binds near SHL+1, +1.5 and +2 (**Fig S2.6A-B**) respectively. Class 3 is the predominant INO80-hexasome class. All Ino80^{ATPase} locations on hexasomes are different than on nucleosomes, which are near SHL-6 or SHL-7 (Ayala et al., 2018; Brahma et al., 2017; Eustermann et al., 2018). However, the Ino80 orientation on hexasomes is consistent with structures of other major chromatin remodelers on nucleosomes such as *S. cerevisiae* ISW1 (Armache et al., 2019; Chittori et al., 2019; Yan et al., 2019), Chd1 (Farnung et al., 2017; Nodelman et al., 2022; Sundaramoorthy et al., 2018), RSC (Patel et al., 2019; Wagner et al., 2019; Ye et al., 2019), Snf2 (Liu et al., 2017) and in particular the SWR1 complex (Willhoft et al., 2018),

For comparison, we also determined structures of *S. cerevisiae* INO80 bound to a nucleosome and captured two conformational snapshots (class 1 and 2) from the same dataset (**Fig S2.7 – Fig S2.9** and Supplementary Text). Ino80^{ATPase} in class 1 is located near SHL-7, similar to its location in the human INO80-nucleosome structure (Ayala et al., 2018), while in class 2, it binds near SHL-6, similar to the *C. thermophilum* structure (Eustermann et al., 2018) (**Fig S2.9A,C**). The Arp5/les6 module interacts with the nucleosome near SHL-3 and SHL-2 (**Fig S2.9D**), respectively. These observations are also consistent with previous findings showing that nucleosomal DNA between SHL-7 and -6 is protected by INO80 (Brahma et al., 2017).

2.3.2 The SHL-2 position plays a critical role in nucleosome and hexasome sliding

We observe that Ino80^{ATPase} engages the hexasome predominantly near SHL -2. These results raise the possibility that Ino80^{ATPase} acts near SHL-2 when sliding hexasomes. In contrast, consistent with prior findings (Ayala et al., 2018; Eustermann et al., 2018), we observe that Ino80^{ATPase} engages the nucleosome near two positions, SHL-7 and -6. Also as previously proposed, our findings are consistent with the possibility that Ino80^{ATPase} acts near SHL-6 when sliding nucleosomes (Brahma et al., 2017). A commonly used assay to identify the DNA location from where the ATPase domain of a remodeler acts to translocate DNA is to place a single nucleotide gap at the proposed site of action and test if the gap inhibits DNA translocation (Saha et al., 2005; Schwanbeck et al., 2004; Zofall et al., 2006). Therefore, to directly test the importance of the SHL-6 and SHL-2 locations, we assembled nucleosomes and hexasomes with

single base gaps near SHL-2 or SHL-6 and measured INO80 activity using a gel-based sliding assay (**Fig 2.3A**).

We found that a gap at SHL-6 inhibits INO80's sliding activity on nucleosomes by ~ 200-fold but so did a gap at SHL-2 (**Fig 2.3B-G**). In contrast, a gap at SHL-6 did not inhibit INO80's sliding activity on hexasomes, but a gap at SHL-2 slowed hexasomes sliding

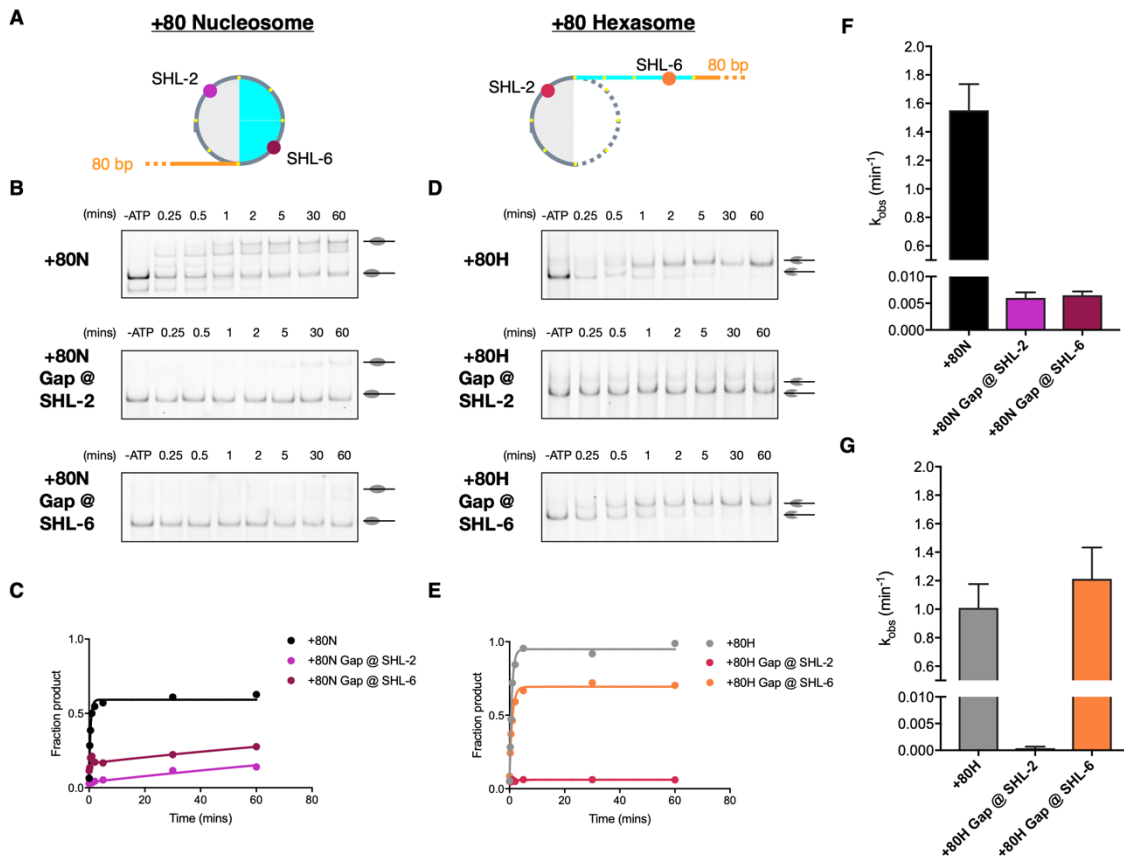


Figure 2.3 Inhibition of DNA translocation at specific SHL sites influence nucleosome and hexasome sliding by INO80. (A) Cartoon illustration of a +80 Nucleosome (left) and a +80 Hexasome (right) with approximate locations of site-specific single base gaps indicated. Colors are the same as in Fig. 1A. (B-C) Example gels and time courses of native gel-based remodeling assays of WT INO80 on +80 nucleosomes with no gap, gap near SHL-2, and gap near SHL-6. (D-E) Example gels and time courses of native gel-based remodeling assays of WT INO80 on +80 hexasomes with no gap, gap near SHL-2, and gap near SHL-6. (F-G) Average observed rate constants of INO80 sliding activity. k_{obs} (min^{-1}): +80N: 1.551 ± 0.1846 ; +80N Gap @ SHL-2: 0.005995 ± 0.001054 ; +80N Gap @ SHL-6: 0.006497 ± 0.0007117 ; +80H: 1.01 ± 0.1668 ; +80H Gap @ SHL-2: 0.000379 ± 0.0002849 ; +80H Gap @ SHL-6: 1.213 ± 0.2209 . Data represent the mean $\pm$ SEM for three technical replicates performed under single-turnover conditions with saturating enzyme and ATP.

by ~ 2000-fold (**Fig 2.3B-G**). These results are consistent with Ino80^{ATPase} acting near SHL-2 when sliding hexasomes and raise new questions about why both the SHL-2 and SHL-6 locations are critical for nucleosome sliding by INO80. We describe possible explanations in the Discussion.

2.3.3 The role of the Arp8 module in flanking DNA length dependence

S. cerevisiae INO80 slides +40 nucleosomes ~100-fold slower than +80 nucleosomes (Hsieh et al., 2022; Zhou et al., 2018). However, sliding hexasomes is less flanking DNA dependent. Our structures suggest that the Arp8 module requires ~40 bp of DNA for appropriate engagement. In class 1 of the INO80-hexasome structure, Arp8 engages with the ~35 bp of DNA unwrapped from removal of the H2A-H2B dimer and an additional ~5 bp of flanking DNA. In class 3 of the INO80-hexasome structure, the Arp8 module engages entirely with ~40 bp of unwrapped DNA that no includes additional DNA unwrapped relative to the unbound hexasome

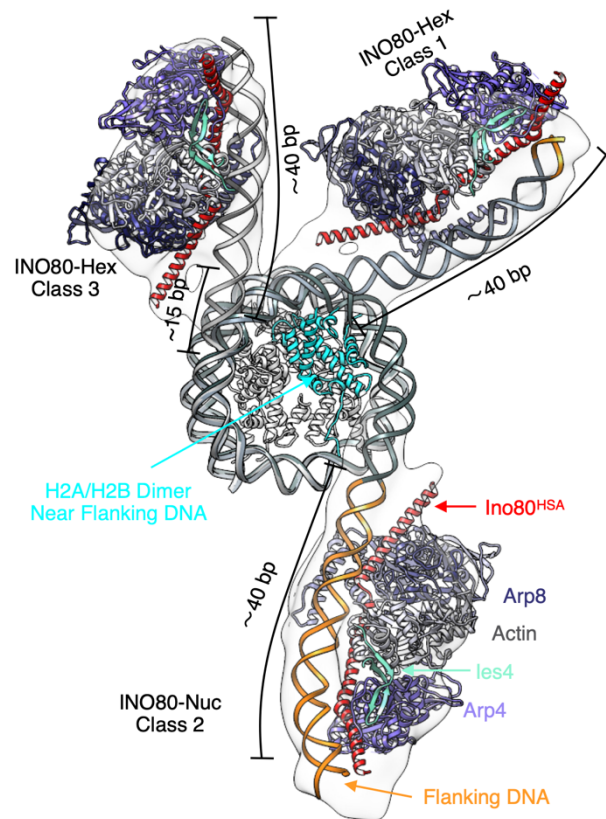


Figure 2.4 The Arp8 module engages different regions of DNA in nucleosomes versus hexasomes. Overlay of atomic models of the hexasome (class 1 and class 3) and the nucleosome (class 2) with the Arp8 module (PDB: 8A50), aligned by the H3-H4 tetramer.

(**Fig 2.4**). In contrast, in class 2 of the INO80-nucleosome structure, the Arp8 module engages entirely with flanking DNA consistent with previous findings (Kunert et al., 2022) (**Fig 2.4**). Our structural data with hexasomes along with the previous data with nucleosomes suggest that 40 bp may be the minimum amount of DNA needed for the Arp8 module to bind and that proper Arp8 module engagement is essential for maximal remodeling activity (Kunert et al., 2022).

2.3.4 Altered interactions by the Arp5 module

To understand why Ino80 may not bind a nucleosome directly near SHL-2, we compared interactions made by Arp5/les6 in hexasomes versus nucleosomes (Supplementary Text). When INO80 binds to a hexasome, the Arp5/les6 regions used in the context of a nucleosome are repurposed for different interactions. Modeling the missing H2A-H2B dimer into our INO80-hexasome structure reveals steric clashes of the Arp5 module with the entry side proximal H2A-H2B dimer and with part of the DNA that wraps around the H2A-H2B dimer near SHL-2 (**Fig S2.11**). These clashes could be avoided if the H2A-H2B dimer is sufficiently dislodged. To test for this possibility, we inhibited dimer dislodgement by introducing a site-specific disulfide crosslink between the two H2A molecules (N38C) (Frouws et al., 2018) or promoted dimer dislodgement by using an H2A mutant (R81A) that destabilizes the H2A-H2B/H3-H4 interface (Lehmann et al., 2017) (**Fig S2.12A,B,H**). The disulfide crosslink did not inhibit nucleosome sliding while the H2A mutant did not promote nucleosome sliding (**Fig S2.12C-G**), indicating that complete dimer dislodgement is not necessary for INO80-mediated nucleosome sliding. In the absence of dimer dislodgement, another way to

avoid these clashes could be by substantial rearrangement of the Arp5 module together with subtle rearrangements of the H2A-H2B dimer (**Fig S2.9E**).

2.4 Discussion

2.4.1 Implications of the INO80-hexasome structure for nucleosome sliding by INO80

The major conformation of the INO80-hexasome complex (class 3) has Ino80^{ATPase} near SHL-2 and approximately ~15bp of unwrapped DNA from the entry site in addition to the ~35bp of DNA that is unwrapped from removal of an H2A-H2B dimer. The placement of Ino80^{ATPase} near SHL-2 is consistent with how the ATPase subunits of other remodelers bind the nucleosome. Together with our prior finding that hexasomes are remodeled faster than nucleosomes, these results suggest that the class 3 structure represents the sliding-competent conformation of INO80 on hexasomes (**Fig 2.5A** and **Fig S2.13A**). In contrast, the states of INO80 bound to a nucleosome have Ino80^{ATPase} bound near either SHL-6 or -7 consistent with previous findings. These differences raise the question of whether the INO80-nucleosome structures represent sliding-competent conformations or whether a rearrangement of Ino80^{ATPase} to SHL-2 is necessary to achieve efficient nucleosome sliding.

Previous crosslinking studies have shown that detachment of nucleosomal DNA from H2A-H2B close to the entry site occurs during INO80 remodeling (Brahma et al., 2017). Our data show that progressively more DNA is unwrapped as Ino80^{ATPase} binds closer to SHL-2 on hexasomes (**Fig 2.2** and **Fig S2.61C**). Together these results suggest that

DNA unwrapping is coupled to Ino80^{ATPase} accessing its most sliding-competent state. Foot-printing studies have shown that while binding of INO80 to nucleosomes mainly protects nucleosomal DNA from SHL-5 to SHL-6 and near SHL-3, there is modest but detectable protection near SHL-2 (Brahma et al., 2017). Nicks and gaps between SHL-7 and SHL -2 have been shown to inhibit nucleosome sliding to different extents (Brahma et al., 2017; Mueller-Planitz et al., 2013). Here we show that site-specific gaps near SHL-2 or SHL-6 substantially inhibit INO80's sliding of nucleosomes (by ~200 fold). DNA gaps are commonly used to identify the site of action of the ATPase domain of remodelers (Saha et al., 2005; Schwanbeck et al., 2004; Zofall et al., 2006). We therefore speculate that INO80 initially binds the nucleosome with Ino80^{ATPase} near SHL-6/-7, and this is followed by an ATP-dependent rotation around the nucleosome to position Ino80^{ATPase} near SHL-2 from where Ino80^{ATPase} then translocates nucleosomal DNA (**Fig 2.5B** and **Fig S2.14A**). A gap at SHL-6 would then inhibit ATP-dependent movement of Ino80^{ATPase} on the nucleosome while the gap at SHL-2 would inhibit translocation of nucleosomal DNA by INO80 relative to the histone octamer (**Fig S2.14**). Single-molecule FRET studies have identified an ATP-dependent pause phase prior to ATP-dependent nucleosome sliding (Zhou et al., 2018). The pause could represent the reorientation of Ino80^{ATPase} from SHL-6/-7 towards SHL-2 and add a step that slows remodeling of nucleosomes compared to hexasomes. Simply placing the INO80 complex as is on nucleosomes with the Ino80^{ATPase} near SHL-2 results in steric clashes of the Arp5 module with the nucleosome (**Fig S2.11**). While partial H2A-H2B dimer dislodgment, as previously proposed, could avoid such clashes (Hsieh et al., 2022), our biochemical data here indicate that dimer dislodgement is not essential for nucleosome

sliding by INO80 (**Fig S2.12**). Thus more structural studies are needed to understand how INO80 might rotate around a nucleosome.

Alternatively, a gap near SHL-2 may affect the action of the Arp5 module. For such a scenario we speculate that Ino80^{ATPase} translocates DNA near SHL-6 and effective translocation also requires action of the Arp5 module near SHL-2 as previously proposed (Ayala et al., 2018; Eustermann et al., 2018). A gap at SHL-6 would then inhibit translocation of nucleosomal DNA by Ino80^{ATPase} and a gap at SHL-2 would inhibit productive engagement of the Arp5 module (**Fig S2.15**).

Clearly distinguishing between these two models will require substantial additional structural analysis of INO80 remodeling intermediates on nucleosomes.

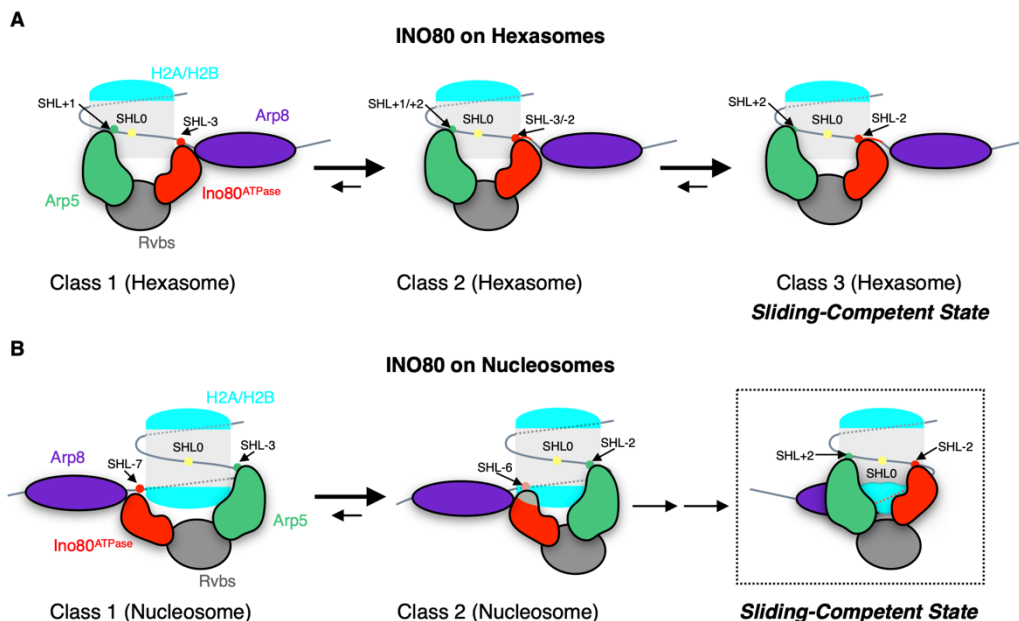


Figure 2.5 Model of INO80-induced hexasome and nucleosome sliding. (A) Hexasome sliding: the Ino80^{ATPase} samples different positions between SHL-3 and SHL-2 but binds predominantly near SHL-2. The INO80 complex becomes sliding-competent when Ino80^{ATPase} engages near SHL-2. **(B)** Nucleosome sliding: INO80 initially binds with Ino80^{ATPase} at SHL-7 or -6. Upon ATP-hydrolysis, Ino80^{ATPase} moves toward SHL-2 where INO80 becomes sliding-competent.

2.4.2 Implications for hexasome sliding by INO80

Our structures provide a view into how INO80 engages a hexasome. In the predominant INO80-hexasome structure, Ino80^{ATPase} binds near SHL-2. A site-specific gap at SHL-2 substantially inhibits INO80's sliding of hexasomes (~2000 fold) while a gap near SHL-6 does not have a major effect. We therefore hypothesize that Ino80^{ATPase} bound at SHL-2 on a hexasome represents the active structure. Compared to the subtle changes at SHL-2 observed when other remodelers bind nucleosomes (Yan and Chen, 2020), the 15 bp of unwrapped DNA (up to SHL-2.5) in class 3 substantially loosens histone DNA interactions and thus may allow more ready translocation from SHL-2. We further propose that the new contacts made by the Arp5/les6 module with the exposed H3-H4 surface provide an anchor allowing the Ino80 motor to efficiently pump DNA through the hexasome. These findings also explain the differential effects of the Arp5/les6 module on hexasome versus nucleosome sliding (Hsieh et al., 2022). The location of the Arp8 module is also different on hexasomes than on nucleosomes. On nucleosomes the Arp8 module binds ~ 40 bp entirely on the flanking DNA (**Fig 2.4**). In the most prevalent INO80-hexasome state (class 3), the Arp8 module is bound entirely to the unwrapped DNA, substantially reducing the need to bind flanking DNA (**Fig 2.4**). These different binding modes of the Arp8 module could explain why hexasome sliding by INO80 is less dependent on flanking DNA length compared to nucleosome sliding.

2.5 Supplemental Text

2.5.1 Nomenclature definition of +80H hexasome and +80N nucleosome

+80H Hexasome and +80N nucleosome are assembled on the same DNA templates containing the 147 bp 601 nucleosome positioning sequence with 80 bp of additional DNA. Hexasomes are asymmetric and lack the entry-site proximal H2A-H2B. We define the entry and exit DNA sites as the sites closest and farthest from the flanking DNA, respectively. Further, given the additional DNA that is unwrapped in a hexasome, we define flanking DNA as the additional DNA beyond the 147 bp of the 601 sequence, and free DNA as the DNA that is not bound to histones. Thus, +80N has 80 bp of free DNA while +80H has ~ 115 bp of free DNA because of the additional ~35 bp of DNA that is unwrapped from removal of an H2A-H2B dimer (**Fig 2.1A, Fig S2.1A,B**).

2.5.2 Cryo-EM structures of the INO80-hexasome complex

We determined cryo-EM structures of INO80-hexasome by single particle cryo-EM and captured three major conformational snapshots of the complex. The major conformation (class 3), which contains the largest portion of particles in our dataset, has a global resolution of 2.6Å and a local resolution for the hexasome at 2.9Å, allowing accurate model building for most of the complex (**Fig 2.1B-C, Fig S2.2 – Fig S2.5**). Two other conformational snapshots were isolated from the same dataset by focused classification centered on the hexasome (**Fig S2.2, Fig S2.6A-B**). The overall resolutions of these two reconstructions are 3.0Å (class 1) and 2.8Å (class 2), with the local resolution of the hexasome at 6.7Å and 3.2Å, respectively, sufficient to accurately position the atomic model of a hexasome (**Fig S2.3A-D**).

As described in the main text, the whole INO80 complex is grouped into four modules: Rvb module, Arp8 module, Ino80 module and Arp5 module. Common features that are resolved in all three conformational snapshots are described in the following: The Rvb module is well-resolved in all three classes with an almost identical conformation. It contains the heterohexamer formed by the Rvb1 and Rvb2 that serves as a scaffold to assemble other subunits (**Fig 2.1B**, **Fig S2.4A**). Although no nucleotide was added during sample preparation, clear density for ADP is seen in all nucleotide binding pockets of Rvb1/Rvb2 (**Fig S2.4C**). The two RecA lobes in the ATPase domain (N-lobe and C-lobe) are interrupted by a large insertion, which can clearly be seen threaded through the Rvb1/Rvb2 hexamer similar to previous INO80-nucleosome structures (Ayala et al., 2018; Eustermann et al., 2018). The Arp8 module which is known to interact with Ino80 through its HSA domain is resolved at a lower resolution and insufficient for model building but sufficient for docking the atomic model (PDB: 8A5O). Note that the Taf14 subunit of Arp8 module is not part of the atomic model, nor it is seen in our cryo-EM density map, even at a lower density counter level, suggesting that may be intrinsically flexible. For the Ino80 module, Ino80^{NTD} is not visible, Ino80^{HSA} is only partially resolved. For the Ino80^{ATPase}, the C-lobe of is clearly resolved but its N-lobe is more flexible, resolved only sufficiently for docking the atomic model (PDB: 6FML, (Eustermann et al., 2018)). The flexibility of the N-lobe of the Ino80^{ATPase} may be a consequence of nucleotide not being added to our sample. The core of the Arp5 subunit is well resolved with its DNA binding domain (Arp5^{DBD}) interacting with hexasomal DNA at SHL+2 (**Fig S2.5C,D**) and with density for an ATP molecule in the

nucleotide binding pocket (**Fig S2.4D**). Furthermore, similar to the previously published INO80-nucleosome structure (Ayala et al., 2018; Zhang et al., 2023), the C-terminal HIT-like domain of Ies6 forms a stable contact with Rvb1/2, while the rest of Ies6 wraps around Arp5 (**Fig S2.5E**). Nhp10 module is another module that is also present in the complex but is not resolved, suggesting their flexibility.

One available atomic model of a hexasome was determined with a short peptide bound (PDB: 6ZHY, (Lehmann et al., 2020)), which we approximate as representing an unbound hexasome. The hexasome bound by INO80 is well defined in all three structures (**Fig S2.3B,D,F** and **Fig S2.4B**), retaining the canonical structural features of a hexasome seen in previous structures (Farnung et al., 2022; Lehmann et al., 2020; Mayanagi et al., 2019).

2.5.3 Cryo-EM structures of the INO80-nucleosome complex

The prior INO80-nucleosome structures were determined with human and *C. thermophilum* INO80, while we use *S. cerevisiae* INO80. To control for any species related differences, we also determined structures of *S. cerevisiae* INO80 bound to a nucleosome without added nucleotide. 3D classification reveals two major conformations, with overall resolutions of 3.5Å for class 1 and 3.4Å for class 2, and the local resolutions for the nucleosomal region of 3.5Å and 3.3Å, respectively (**Fig S2.7, Fig S2.8, Fig S2.9A,C**). The INO80 structure is almost identical between these two states, with an rmsd of 0.4Å. The overall architecture of *S. cerevisiae* INO80 on a nucleosome is also similar to that in previous INO80-nucleosome structures (Ayala et al., 2018; Eustermann et al., 2018). The C-terminal tail of Ies6 is better resolved in class

2, and makes a close interaction with H2B, correlating with a shift of H2B towards les6 by $\sim 1\text{\AA}$, as measured from the position of Y118 in H2B (**Fig S2.9E**). We see also much less DNA unwrapping for each INO80-nucleosome class.

2.5.4 Engagement of Arp8 module with flanking DNA

Prior studies have suggested that the Arp8 and Nhp10 modules bind flanking DNA (Brahma et al., 2018; Knoll et al., 2018; Oberbeckmann et al., 2021b). To understand the role of these modules in hexasome and nucleosome sliding we compared their locations in our structures. In both the INO80-hexasome and INO80-nucleosome structures, we can resolve the Arp8 module after focused classification but with lower resolution (**Fig S2.2B, Fig S2.5A,B, Fig S2.7B, Fig S2.9B**). Nonetheless, the quality of the density maps allows us to place the available atomic model of the Arp8 module (PDB: 8A5O (Kunert et al., 2022)) into the cryo-EM density maps and to build an atomic model together with the rest of INO80. However, we did not resolve density of Nhp10 in our cryo-EM density maps, likely because it binds to flanking DNA further beyond where Arp8 module binds and is more flexible. Furthermore, similar to a previous report, we did not resolve Taf14, a subunit in the Arp8 module, likely because of its conformational flexibility (Kunert et al., 2022).

2.5.5 Differences in the interactions between Arp5 module with a hexasome and a nucleosome

Additionally, we observe that the Arp5/les6 module makes substantially different contacts in the hexasome compared to a nucleosome. In prior INO80-nucleosome structures and the structures obtained here, the Arp5 module makes interactions with

the acidic patch of the entry-site proximal H2A-H2B dimer using its grappler domain (**Fig S2.10A,D**) and with nucleosomal DNA between SHL-2 and -3 using its DNA binding domain (**Fig S2.10B**) (Ayala et al., 2018; Eustermann et al., 2018). In Class 1 of the INO80-nucleosome complex, the grappler is not as well resolved as in Class 2, suggesting that the grappler may be positioned too far away to fully engage the acidic patch. Ies6 wraps around Arp5 and interacts with the H2A-H2B dimer and DNA at SHL-2 (**Fig S2.10C**). In contrast, on a hexasome, the Arp5^{DBD} binds DNA between SHL+1 and +3 (**Fig S2.5C,D**) and the Arp5^{grappler} appears to interact with the exposed H3-H4 tetramer and flanking DNA at the entry site (**Fig S2.5F**). In addition, on a nucleosome, we observe an Arp5 interaction with flanking DNA as seen previously (Kunert et al., 2022) (**Fig S2.10E**). In comparison, on a hexasome, Arp5 interacts with DNA unwrapped from the histone core (**Fig S2.5G**). Ies6 maintains similar interactions with Arp5, but no longer interacts with the H2A-H2B dimer as it is missing in a hexasome. Its interaction with DNA changes to the SHL+1/+2 location. Thus, unlike in a nucleosome, where the Arp5 grappler contacts the H2A-H2B acidic patch, in a hexasome, the grappler contacts the exposed H3-H4 surface.

2.6 Materials and Methods

Expression and purification of INO80

A yeast strain containing FLAG-tagged Ino80 (s288c INO80-FLAG) (Shen et al., 2000) was grown in YPD at 30 °C to saturation. INO80 was then purified by FLAG immunoprecipitation based on previously published methods (Hsieh et al., 2022; Shen, 2003; Zhou et al., 2018) with minor modifications. A second 30-minute elution step was added to increase yield.

Preparation of nucleosomes and hexasomes

Recombinant *Xenopus laevis* histones were expressed in *E. coli* and purified as previously described (Dyer et al., 2003; Luger et al., 1999). DNA was amplified from a plasmid containing a Widom 601 sequence and labeled with a Cy3 fluorophore modified primer (IDT). Large scale PCR was performed, and products were separated on an 8% polyacrylamide gel and cut out. The gel slice containing the DNA was crushed, soaked in 1X TE overnight, and filtered through a 0.45 micron filter. The DNA was ethanol precipitated and dissolved in 1X TE.

Cy3-labeled DNA containing a single base gap near SHL-2 (25 base pairs from the dyad towards the flanking DNA) and near SHL-6 (58 base pairs from the dyad towards the flanking DNA) were made through annealing and ligating a set of overlapping complementary oligonucleotides, as described previously (Deindl et al., 2013). Briefly, single-stranded oligonucleotides spanning the Widom 601 sequence plus 80 base pairs of flanking DNA were designed to have ~20-30 base pairs of overlap with oligonucleotides of the opposite strand, with one designed to be one base short to incorporate the site-specific gap (oligonucleotide sequences listed below). 50 μ M oligonucleotides were mixed together in 10 mM Tris-HCl, pH 7.5-8, 50 mM NaCl, 1 mM EDTA and then heated and cooled slowly to anneal. DNA was then ligated together using T4 DNA ligase. All oligonucleotides except for the one directly following the site of the desired missing base contained a 5' phosphate group to enable ligation. Annealing and ligation was checked through non-denaturing and denaturing PAGE analysis on 5%

acrylamide and 10% acrylamide, 8M urea gels respectively. DNA was then separated on an 8% acrylamide gel, extracted, and precipitated as described above.

Oligonucleotides used to make DNA with gap near SHL-2 (-25):

T1: 5'-Cy3-ctggagaatcccgggtgccgaggccgctcaa-3'

T2: 5'-Phos-ttggtcgtagacagctctagcaccgcttaaacgcacgtacgcgctgtcc-3'

T3: 5'-Phos-cccgcgttttaaccgccaaggggattactccctagtctccaggcacgtg-3'

T4: 5'-Phos-tcagatatatacatcctgtgcatgtattgaacagcgaccttg-3'

T5: 5'-Phos-ccgggtgccagtcggatagtggtccgagctcccactctagaggatccccgggtaccga-3'

B1: 5'-tcggtacccggggatcctctagagtg-3'

B2: 5'-Phos-ggagctcggaacactatccgactggcaccggcaaggctcgctgttcaataca-3'

B3 G-2: 5'-Phos-tgcacaggatgtatatatctgacacgtgcctggagactaggagtaatcccc-3'

B4 G-2: 5'-tggcgggttaaaacgcgggggacagcgctacgtgcgtttaag-3'

B5: 5'-Phos-cggtgctagagctgtctacgaccaattgagcggcctcggcaccgggattctccag-3'

Oligonucleotides used to make DNA with gap near SHL-6 (-56):

T1: 5'-Cy3-ctggagaatcccgggtgccgaggccgctcaa-3'

T2': 5'-Phos-ttggtcgtagacagctctagcaccgcttaaacgcacgtacgcgct-3'

T3': 5'-Phos-gtccccgcgttttaaccgccaaggggattactccct-3'

T4': 5'-Phos-agtctccaggcacgtgtcagatatatacatcctgtgcatgtattgaacagcgaccttg-3'

T5: 5'-Phos-ccgggtgccagtcggatagtggtccgagctcccactctagaggatccccgggtaccga-3'

B1': 5'-tcggtacccggggatcctctagagtgaggagctcggaac-3'

B2 G-6: 5'-Phos-actatccgactggcaccggcaaggctcgctgttcaatacatgcacaggatgtatatatctg -3'

B3 G-6: 5'-cacgtgcctggagactagggagtaatccccttgg-3'

B4: 5'-Phos-cggttaaaacgcgggggacagcgcgtacgtgcgtttaag-3'

B5: 5'-Phos-cggtgctagagctgtctacgaccaattgagcggcctcggcaccgggattctccag-3'

Refolding of histone octamers was performed as described previously (Gamarra and Narlikar, 2021; Gkikopoulos et al., 2011; Rhee et al., 2014) . Octamer containing H2A N38C was refolded in the presence of DTT to prevent disulfide formation. Oxidation and reduction were performed using CuPhe as described previously (Gamarra and Narlikar, 2021). Briefly, octamers were treated with freshly made 25 μ M Cu(II)SO₄ and 100 μ M o-phenanthroline, followed by quenching with EDTA. The same octamer was reduced by dialyzing into buffer containing 100 mM DTT. Crosslinking efficiency was determined by quenching aliquots of the oxidized and reduced reactions with 50 mM iodoacetamide and running them on a non-reducing SDS-PAGE gel. Quantification of crosslinking was done by taking the ratio of the intensity of the crosslinked H2A band, which runs at a higher molecular weight, and the total of the crosslinked H2A band and the H2A/H2B band (**Fig S2.12H**). Because uncrosslinked H2A and H2B run at the same size, we assume that 100% crosslinking would result in a ratio of 0.5.

Nucleosomes and hexasomes were assembled using salt gradient dialysis (Dyer et al., 2003; Levendosky and Bowman, 2019). Oxidized nucleosomes were assembled using buffers without reducing agent while reduced nucleosomes were assembled in buffers containing 3 mM TCEP. Nucleosomes were purified by ultracentrifugation with a 10-30% glycerol gradient. Hexasomes were purified using a Mini PrepCell (BioRad) with a

7% acrylamide gel based on the previous published method. Briefly, asymmetry of the 601 DNA sequence yields weaker affinity of one side of the DNA sequence for the H2A/H2B dimer versus the other side. Placing the flanking DNA adjacent to the weaker side allows the assembly of hexasomes with the H2A/H2B dimer missing proximal to the longer flanking DNA. The hexasomes are further purified away from nucleosomes through a prep-cell as described by Levendosky and Bowman (Levendosky et al., 2016; Levendosky and Bowman, 2019).

Native gel-based remodeling assay

All remodeling reactions were done under single turnover conditions (enzyme in excess of nucleosomes) with saturating INO80 and ATP. The reactions were carried out at 30 °C. Briefly, 60 nM INO80 WT was incubated with 15 nM nucleosomes or hexasomes in reaction buffer (40 mM Tris-HCl, pH7.5, 50 mM KCl, 1.1mM free MgCl₂, 0.02% NP-40, and 7% glycerol) for 10 minutes at 30 °C before adding 1mM ATP· MgCl₂ to start the reaction. The no ATP control was taken at the last time point of the reaction. The reaction samples taken at specific time points were quenched with excess plasmid DNA and ADP. Samples were resolved on a native PAGE gel (6% acrylamide, 0.5X TBE) ran for 3 hours at 125V. Gels were scanned on a Typhoon Imager (GE Life Sciences) and quantified by densitometry using ImageJ. The fraction of nucleosome products was determined by the ratio of slower-migrating nucleosomes (everything above unremodeled nucleosomes) to the total nucleosome intensity. Using Prism 7 (GraphPad), data were fit to a single-phase exponential decay model (Equation 1),

$$y = (y_0 - p)e^{-k_{obs}t} + p,$$

where y_0 is the initial fraction product, k_{obs} is the observed rate constant, and p is the fraction product at the plateau. Due to the much slower rates of reactions containing +80N Gap @ SHL-2, +80N Gap @ SHL-6, and +80H Gap @ SHL-2, the plateau, p , was not empirically reached in the timeframe used. Therefore, to fit these data, we used a single-phase exponential decay that constrained p to a constant equal to the average fraction remodeled at the plateau of +80N or +80H reactions from fitting with no constraints. The assumption behind our approach is that when these very slow constructs are fully remodeled, the proportion remodeled will resemble that of our +80N and +80H constructs.

Amine functionalized GO grids preparation

Preparations and functionalization of Graphene Oxide (GO) grids were performed following the previously described protocol (Palovcak et al., 2018; Wang et al., 2020). Briefly, in a glass petri dish (60 mm in diameter, 15 mm in height) an epoxy coated stainless steel mesh stand was placed at the bottom and DI water was filled to the top. 300 Mesh, R1.2/1.3 Au Quantifoil grids were placed on the mesh stand with carbon side facing upward. Using a syringe, the GO solution (230 μ l in total volume) was spread onto the water surface. After draining the water, the GO coated grids were dried at room temperature for use. GO covered grids were then submerged in 10 mM ethylenediamine (Sigma-Aldrich E26266) solution diluted in dimethyl sulfoxide (DMSO) and incubated for 5 h at room temperature. The grids were washed thoroughly twice with DMSO without ethylenediamine, twice with autoclaved water, twice with ethanol,

and dried under ambient conditions. Amino modified grids were stored dry at -20°C until use.

Electron microscopy sample preparation and data collection

Freshly prepared INO80 was mixed with either hexasome or nucleosome in 1:1 molar ratio, and then incubated at 30°C for 30 min, after which the buffer was exchanged to EM buffer (25mM HEPES, pH 7.5, 100mM KCl, 2mM MgCl_2 , 2mM DTT, 1% glycerol). The final concentration of the complex was $\sim 0.05\text{--}0.1\ \mu\text{M}$ and no nucleotide was added. All cryo-EM grids were prepared using functionalized GO-amine grids.

Negative staining of the complex was performed with 0.75% uranum formate, following an established protocol (Ohi et al., 2004). Grids were examined using an FEI T12 microscope operated at 120 kV, and images were recorded using a 4k x 4k charge-coupled device (CCD) camera (UltraScan 4000, Gatan).

To prepare cryo-grids, samples (3 μl at $0.2\ \mu\text{M}$) were loaded onto the amine modified GO grids, and then blotted for 4 s before being plunge-frozen in liquid ethane cooled by liquid nitrogen using a FEI Vitrobot IV with the sample chamber set at 8°C and 100% humidity. The blotting force was 0, using $\varnothing$ 55/20 mm blotting filter paper from TED PELLA. Grids were examined and screened using an FEI Tecnai Arctica operated at 200 kV and equipped with a Gatan K3 camera. All cryo-EM datasets were collected on a Titan Krios at the UCSF Cryo-EM Center for Structural Biology operated at an

acceleration voltage of 300 kV and equipped with a BioQuantum energy filter (slit width set to 20 eV) and a K3 direct electron detector (Gatan).

All cryo-EM datasets were collected using SerialEM (Mastronarde, 2005). Multishot collection (3×3 arrays) was performed with using beam-tilt compensation and the maximum image shift is 3.5 microns. All images were acquired with a nominal magnification of 105 K, resulting in a pixel size of 0.4175 Å. Defocus range was set from -1.0 µm to -2.0 µm. For the INO80-hexasome sample, 18,991 images were collected, each was dose-fractionated to 117 movie frames with a total exposure time of 5.9 s, resulting in a total fluence of ~67 electrons per Å². For the INO80-nucleosome sample, 8,653 images were collected, each was dose-fractionated into 80 movie frames with a total exposure time of 2.024 s, resulting in a total fluence of ~43 electrons per Å².

Image processing

For the dataset of the INO80-hexasome sample, a total of 18,991 movie stacks were motion corrected and dose weighted using MotionCor2 (Zheng et al., 2017). The CTF parameters were estimated and all subsequent 2D and 3D classification were performed using cryoSPARC (Punjani et al., 2017). 500 micrographs were randomly selected to generate a template. In brief, 624,536 particles were picked by cryoSPARC blob picker and were subject to ab-initio reconstruction and multi-round heterogenous refinement. From one good class showing clear features of INO80 bound to the hexasome, we generated 16 different projection images for template picking, yielding 7,831,514 particles from all micrographs. After multiple rounds of heterogenous

refinement, a final 1,220,910 particles were selected to calculate a reconstruction of the INO80-hexasome complex with a global resolution of 2.8 Å. This reconstruction showed clear structural features of INO80, but the hexasome was not well resolved.

This particle stack was then exported to RELION (Scheres, 2012). 3D refinement (initial low-pass filter: 10 Å; mask diameter: 360 Å; reference mask: no) was applied to get the new reconstruction, after which segment map function in Chimera (Pettersen et al., 2004) was used to split this reconstruction and generate a map only containing the Arp5-les6 module and hexasome. A mask containing the Arp5-les6 module and hexasome was then generated from this map (initial threshold: 0.0001; extend_inimask: 4; width_soft_edge: 6). Particle subtraction function in RELION was applied to create the particle stacks that only contain the Arp5-les6 module and hexasome in theory (do center subtracted images on mask: yes; new box size: 240), followed by a relion reconstruction to obtain a reference map (maxres: 10; ctf: yes). A mask was then generated for the following procedure (initial threshold: 0.001; extend_inimask: 4; width_soft_edge: 6). 3D classification of background subtracted particles (reference mask: generated last step; initial low-pass filter: 10 Å; mask diameter: 200 Å; regularisation parameter T: 3; number of iterations: 50; number of classes: 5; angular sampling interval: 1.8°; perform local angular searches: yes; local search range: 18) producing a new map of 560,912 particles with clear Arp5-les6 and hexasome features. Then, a 3D reconstruction of the entire INO80-hexasome complex (initial low-pass filter: 10 Å; mask diameter: 360 Å; reference mask: no) was calculated from this same subset of particles and further refined, in which the densities of both INO80 and hexasome are

improved compared with the previous round. We then performed another round of background subtraction with a mask on the hexasome (initial threshold: 0.001; extend_inimask: 4; width_soft_edge: 6), followed by classification with local alignment focused entirely on the hexasome (reference mask: generated last step; initial low-pass filter: 10 Å; mask diameter: 200 Å; regularisation parameter T: 3; number of iteration: 50; number of classes: 5; angular sampling interval: 1.8°; perform local angular searches: yes; local search range: 18) . Three major classes were identified, among which the main difference is the hexasome orientation, mainly reflecting different binding positions of INO80 on the hexasome.

For each class, we then further refined the whole complex and hexasome to higher resolutions by using either cryoSPARC or cisTEM (Grant et al., 2018). In cryoSPARC, non-uniform refinement with default setting was performed. In cisTEM, generate 3D function with default setting was applied. The final 3D maps were sharpened by DeepEMhancer (Sanchez-Garcia et al., 2021), except for the hexasome of class 1. Lastly, reconstructions of INO80 and the hexasome were assembled into a composite map for model building and figure generating. For both class 1 and class 3, we also generated a mask around the flanking DNA for further focused 3D classification, producing a clear density that allows docking of the crystal structure of Arp8-N-actin-Arp4 into the density map (**Fig S2.2B**).

For the dataset of the INO80-nucleosome sample, a total of 8,653 movie stacks were motion corrected and dose weighted using MotionCor2. The CTF parameters were

estimated and all subsequent 2D and 3D classification were performed using cryoSPARC. 3,625,796 particles were picked by cryoSPARC template picker. The remaining parts of image processing were performed following the procedure described above.

In all reconstructions, no CTF refinement was performed. For each reconstruction, the numeric resolution was determined from Fourier Shell Correction (FSC) using criterion of FSC = 0.143 (Rosenthal and Henderson, 2003). In addition, directional FSC (dFSC) (Dang et al., 2017) was used to evaluate the directional uniformity of all reconstructions, as reported in **Fig S2.3** and **Fig S2.8**.

Model Building

For the model building, the initial model was generated by fitting the available coordinates into our cryo-EM density maps by using Chimera (58). These coordinates include the INO80 core (with its sequence changed to that of *S. cerevisiae* by Alphafold (Jumper et al., 2021) and ccp4em), the crystal structure of Arp8-N-actin-Arp4 and the model of a hexasome (PDB: 6FML, 8A5O, 6ZHY (Eustermann et al., 2018; Kunert et al., 2022; Lehmann et al., 2020)). The inconsistent parts were then manually built and refined in coot (Emsley and Cowtan, 2004). The structures were refined using Phenix (Afonine et al., 2012) with secondary structure constraints. Model building of INO80-nucleosome complexes was performed following the same procedure, except that the atomic model of a nucleosome (PDB: 1KX5 (Davey et al., 2002)) was used.

Summary of parameters used in data collection and model building are in **Table S2.1** (INO80-hexasome) and **Table S2.2** (INO80-nucleosome).

2.7 Acknowledgements

Cryo-EM facility at UCSF is managed by Dr. David Bulkley and Mr. Glenn Gilbert. Computation at Cheng laboratory is supported by Mr. Matthiew Harrington and Dr. Junrui Li. We thank Dr. Lvqin Zheng for advice on model building, Dr. Zanlin Yu for providing GO grids, Julia Tretyakova for expressing and purifying histones, Upneet Kaur for providing INO80 WT enzyme, and members of Narlikar and Cheng laboratories for helpful discussions. We thank Dr. Carl Wu and Dr. Anand Ranjan for sharing unpublished data showing inhibition of INO80 activity on nucleosomes containing a gap near SHL-2. We also thank Dr. Sebastian Diendl and Dr. Anton Sabantsev for detailed advice on generating DNAs with site-specific base gaps.

2.8 Data and Materials Availability

For the core INO80 of the INO80-Hexasome complex (class 1, class 2 and class 3) and the core INO80 of the INO80-Nucleosome complex (class 1 and class 2), the coordinates are deposited in the Protein Data Bank with the accession codes 8ETS, 8ETU, 8ETW, 8EU9, and 8EUF; the cryo-EM density maps are deposited in the Electron Microscopy Data Bank (EMDB) with the accession codes EMD-28597, EMD-28599, EMD-28601, EMD-28609, and EMD-28613. For the hexasome of the INO80-Hexasome complex (class 1, class 2 and class 3) and the nucleosome of the INO80-Nucleosome complex (class 1 and class 2), the coordinates are deposited in the Protein Data Bank with the accession codes 8ETT, 8ETV, 8EU2, 8EUE, and 8EUJ; the cryo-

EM density maps are deposited in the Electron Microscopy Data Bank (EMDB) with the accession codes EMD-28598, EMD-28600, EMD-28602, EMD-28612, and EMD-28614.

2.9 Supplemental Figures and Tables

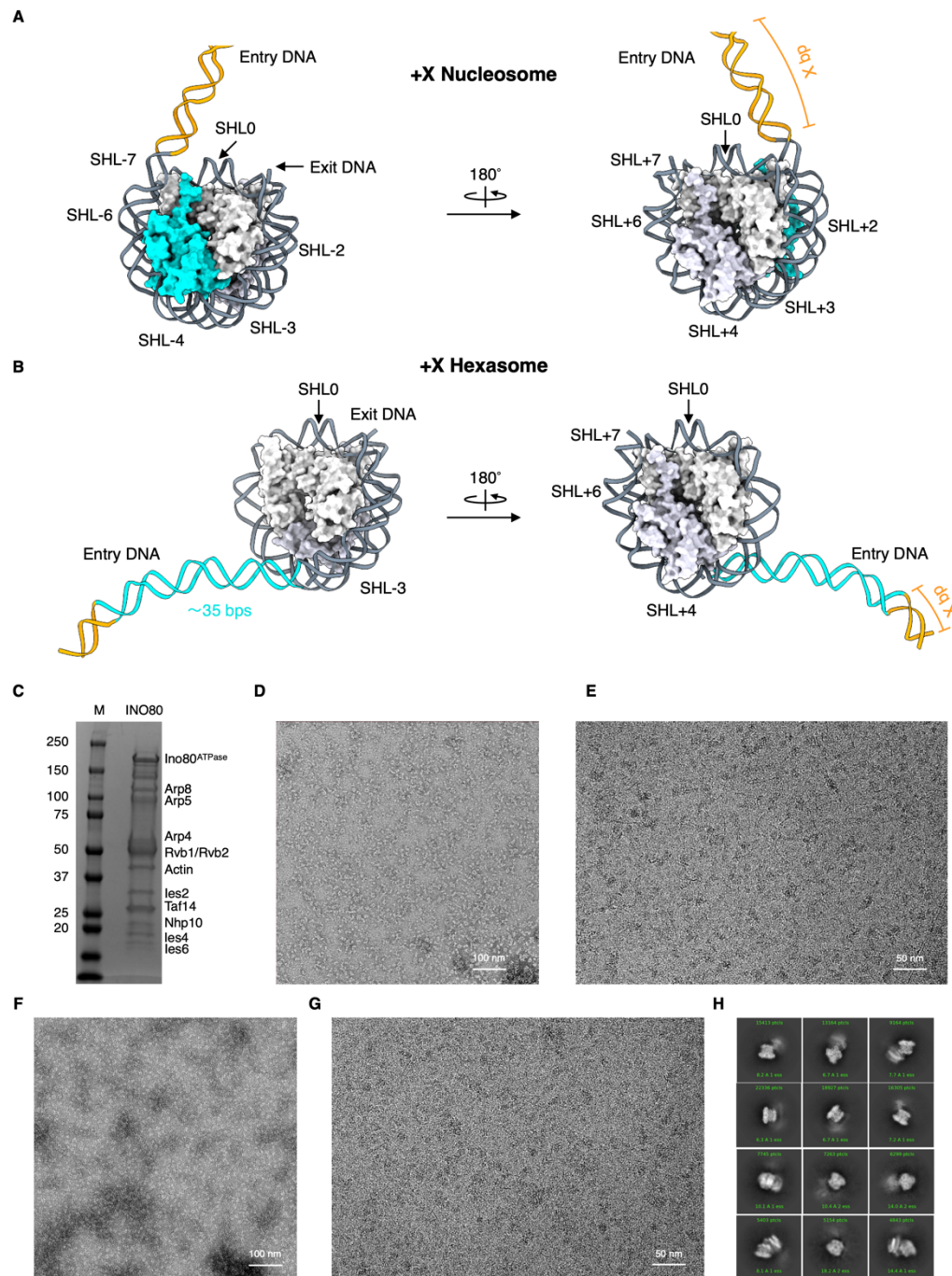


Figure S2.1 Preparation of INO80-nucleosome and INO80-hexasome samples.

- (A-B) Models of +X Nucleosome (A) and +X Hexasome (B).
 (C) SDS-PAGE gel of purified *S. cerevisiae* INO80 complex.
 (D) Negative staining of the INO80-hexasome sample.
 (E) Representative cryo-EM micrograph of the INO80-hexasome complex.
 (F) Negative staining of the INO80-nucleosome complex.
 (G) Representative cryo-EM micrograph of the INO80-nucleosome complex.
 (H) Representative 2D average image of the INO80-nucleosome complex.

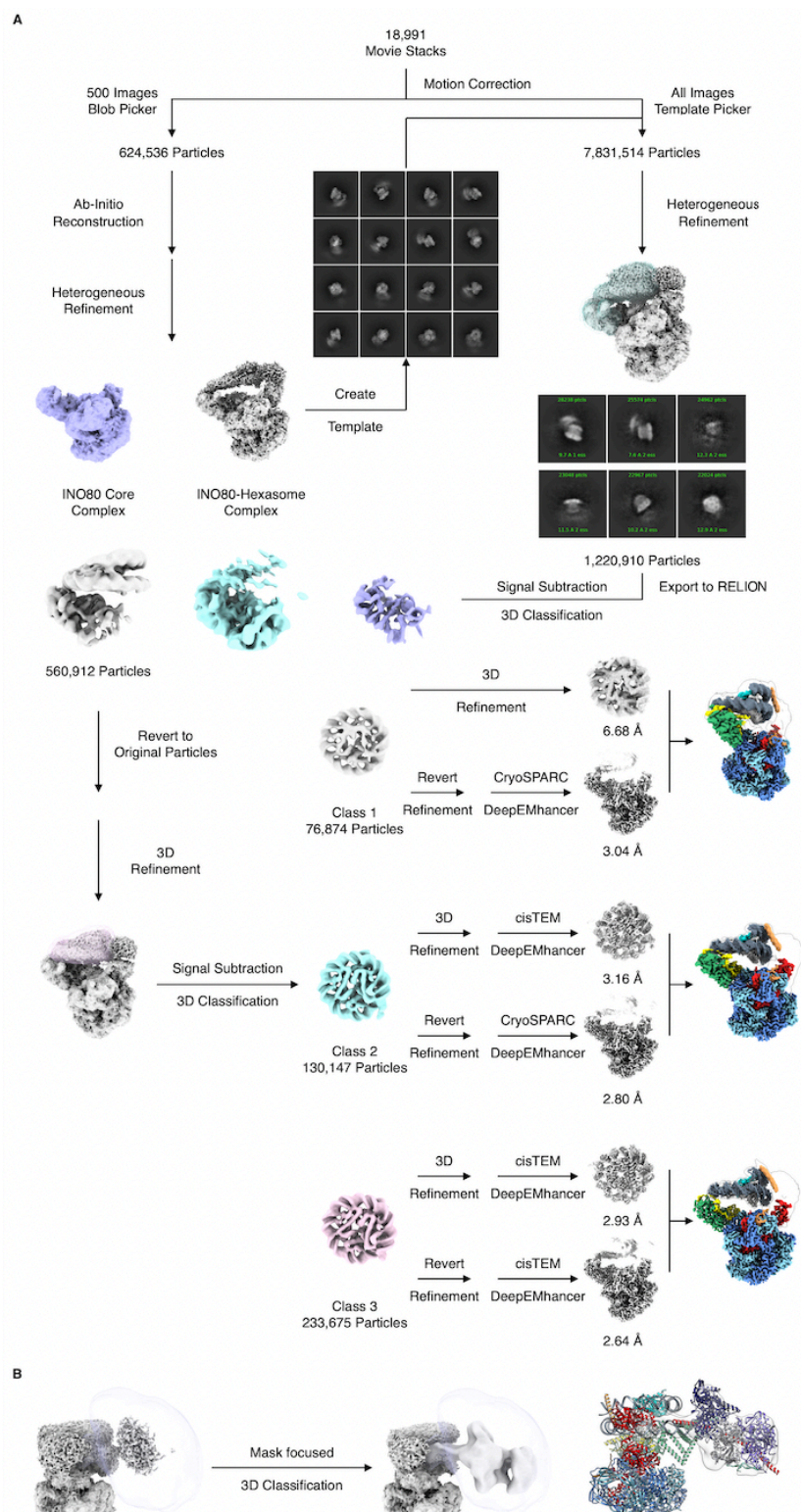


Figure S2.2 Image processing of the INO80-hexasome dataset.

(A) A flow-chart outlining the processing of the INO80-hexasome cryo-EM dataset.
 (B) Focused classification near flanking DNA produced density of the Arp8 module.

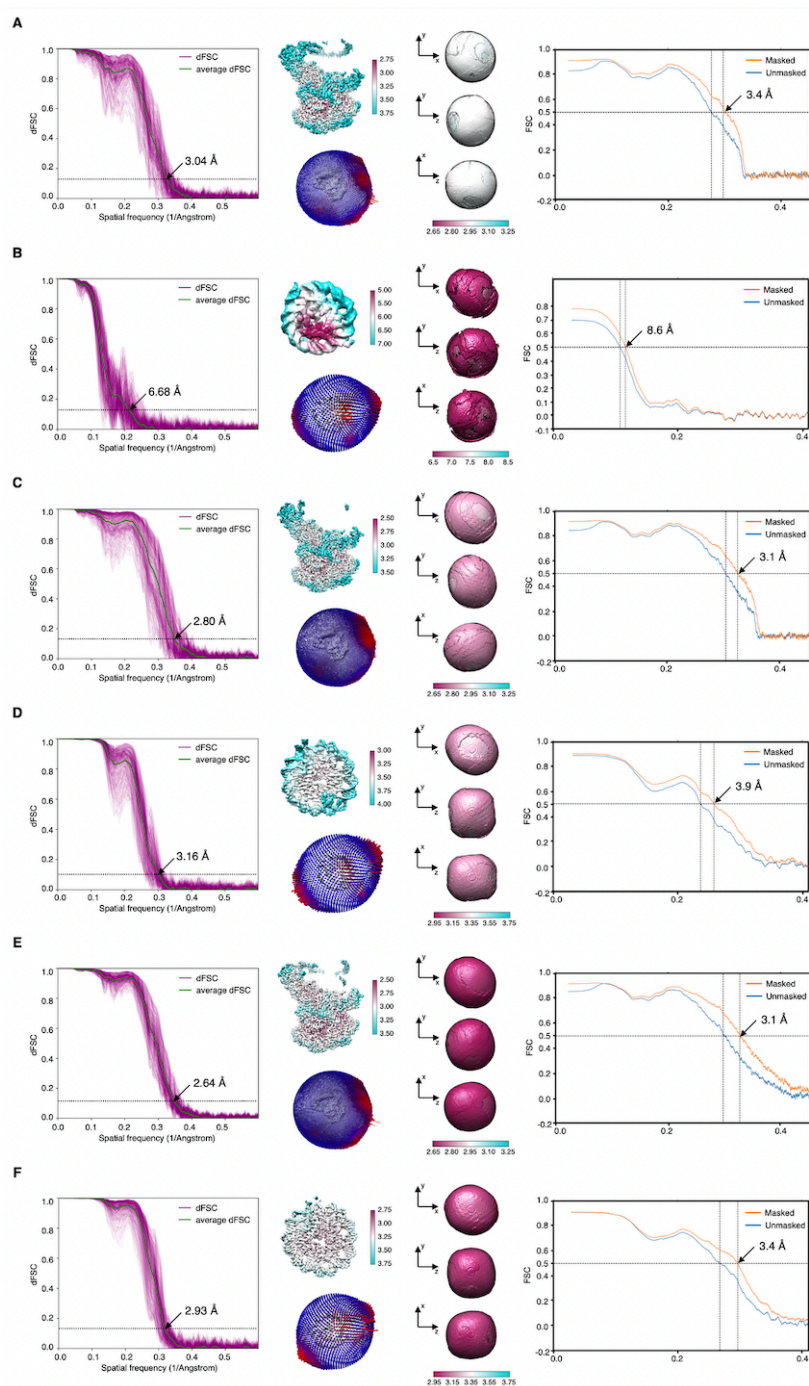


Figure S2.3 Resolution estimation of the INO80-hexasome structures.

All panels contain, from left to right, directional Fourier shell correlation (dFSC) curves of final maps with resolution determined by the FSC criterion of 0.143, cryo-EM map colored by local resolution with resolution scale bar, angular distributions of particles, three-dimensional representations of the dFSC, and model-map FSC plots calculated by Phenix between the map and the model.

(A-B) Class 1 of INO80-hexasome complex (A) and the corresponding hexasome (B).

(C-D) Class 2 of INO80-hexasome complex (C) and the corresponding hexasome (D).

(E-F) Class 3 of INO80-hexasome complex (E) and the corresponding hexasome (F).

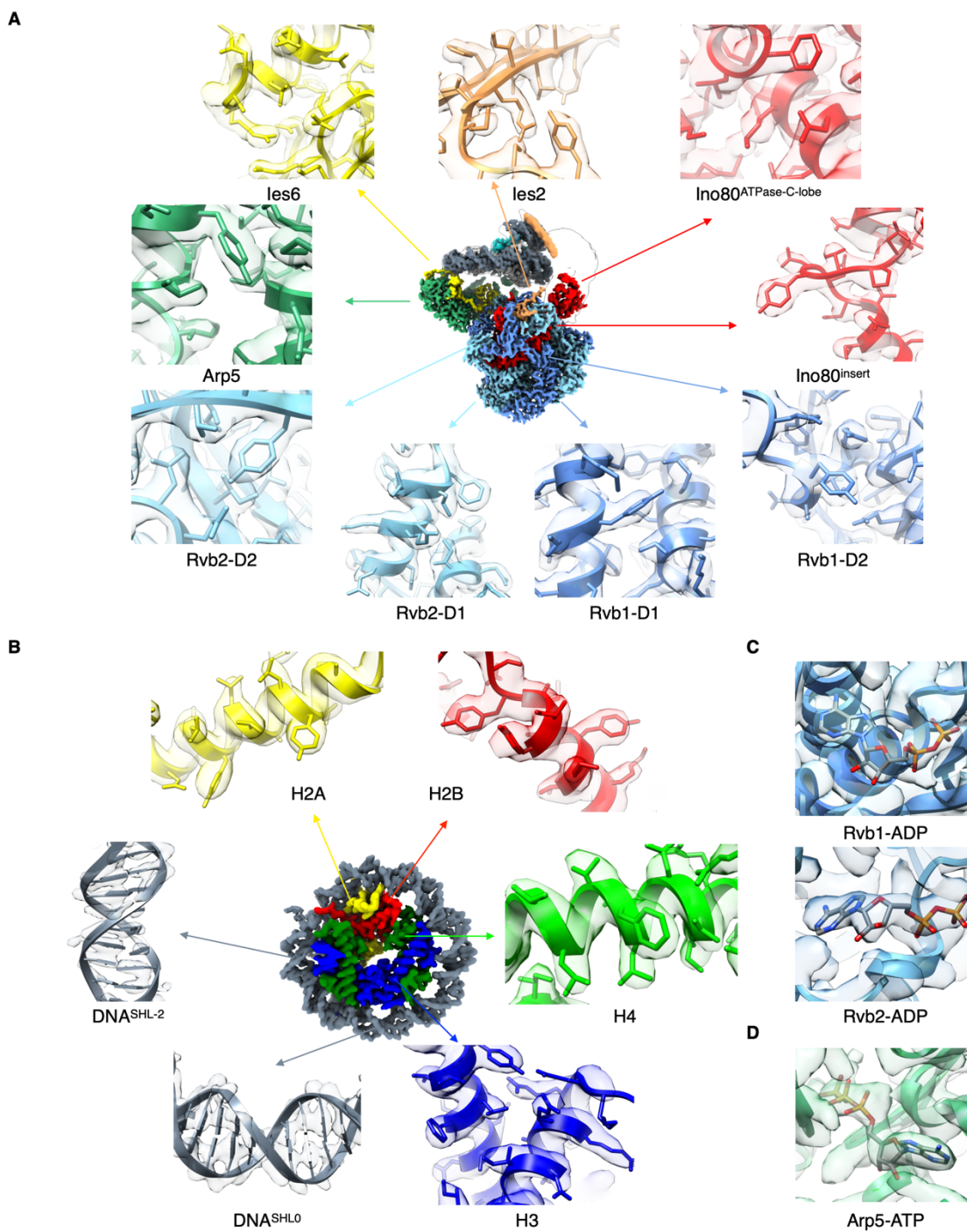


Figure S2.4 Representative densities of the INO80-hexasome complex.

(A) Representative densities of INO80 from class 3 of the INO80-hexasome complex.

(B) Representative densities of the hexasome region from class 3 of the INO80-hexasome complex.

(C) Nucleotide densities seen in all six nucleotide binding pockets of Rvb1/Rvb2 heterohexamer are modeled as ADP.

(D) Nucleotide density seen in the Arp5 nucleotide binding pocket is modeled as ATP.

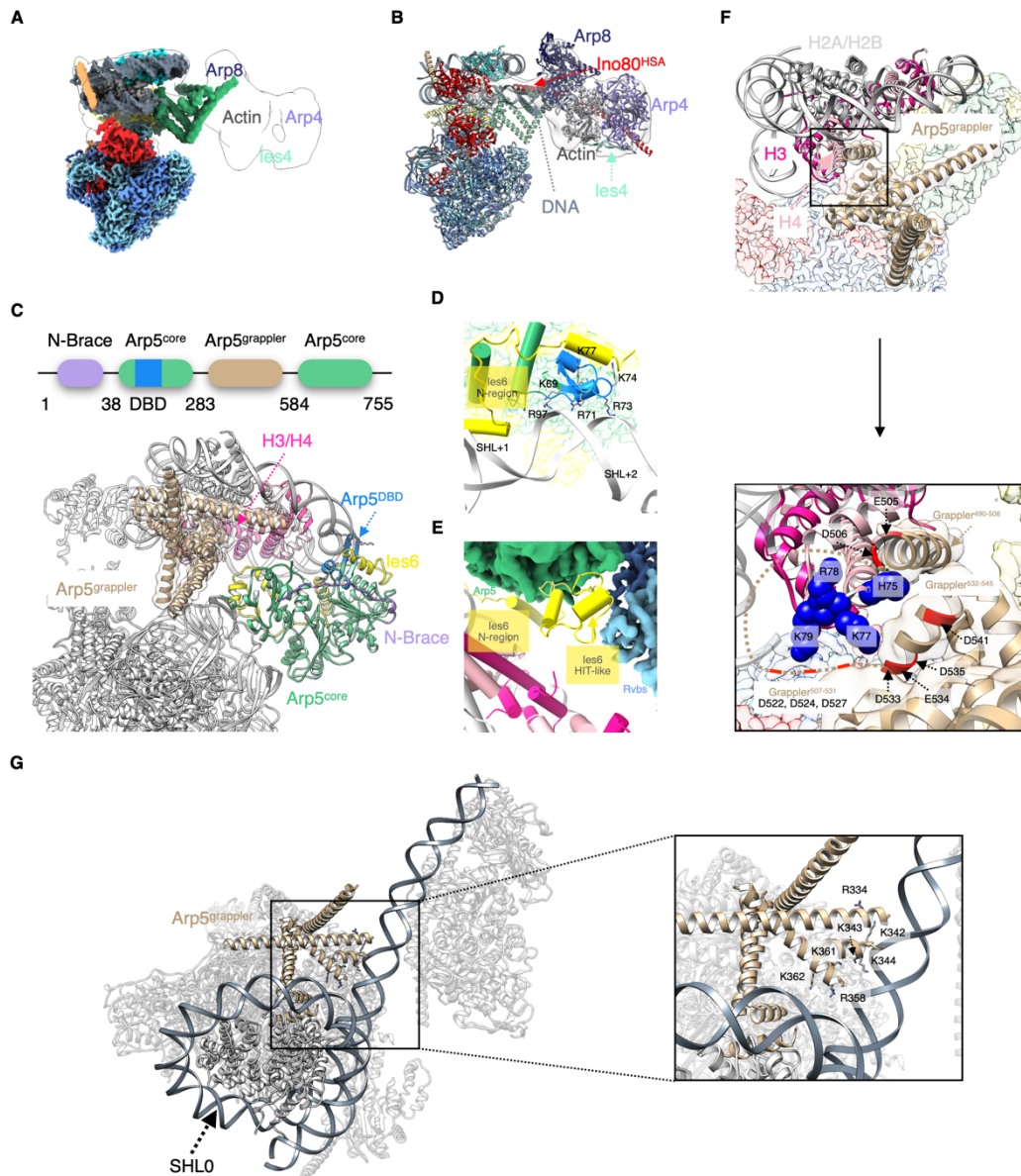


Figure S2.5 Arp5 and Arp8 modules in the INO80-hexasome structure.

(A) Cryo-EM map of the INO80-hexasome complex (class 3) with density of the Arp8 module shown as transparent.

(B) Atomic model from the INO80-hexasome complex (class 3) shown in the same view as (A), with a model of the Arp8 module (PDB: 8A5O) docked into the density map.

(C) Domain arrangement (upper) and atomic model (bottom) of the Arp5 module.

(D) An enlarged view showing interactions between Arp5^{DBD} and DNA.

(E) An enlarged view showing interactions between les6, Arp5 and Rvb1/Rvb2.

(F) Two enlarged views of the Arp5^{grapppler} and H3-H4 tetramer highlighting residues that may form contacts. Acidic residues: red; basic residues: blue, sphere.

(G) Two enlarged views of the Arp5^{grapppler} and DNA unwrapped from the histone core highlighting Arp5 residues that may form contacts with the unwrapped DNA.

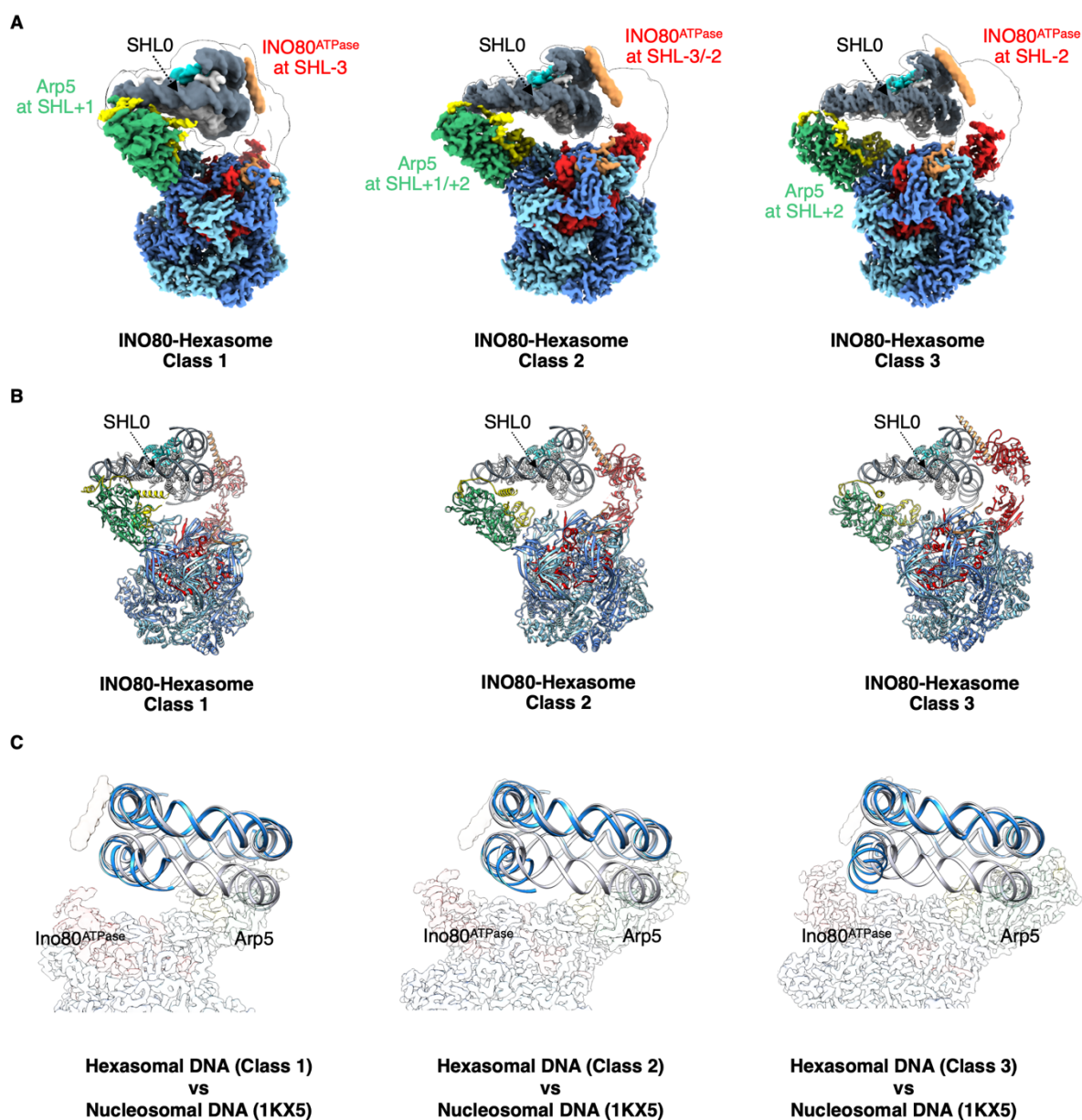


Figure S2.6 Comparison of three INO80-hexasome conformational snapshots.

(A) Cryo-EM density maps of the INO80-hexasome complex in three different conformations. Maps are aligned by the hexasome orientation. Ino80^{ATPase} and Arp5 binding sites are labeled.

(B) Comparison of atomic models of the three classes of the INO80-hexasome complex, where the hexasome dyads are aligned using the H3-H4 tetramers.

(C) Comparison of the hexasomes from the three classes of the INO80-hexasome complex with the crystal structure of a free nucleosome aligned by the histone core. Hexasomal DNA: blue; Nucleosomal DNA: gray.

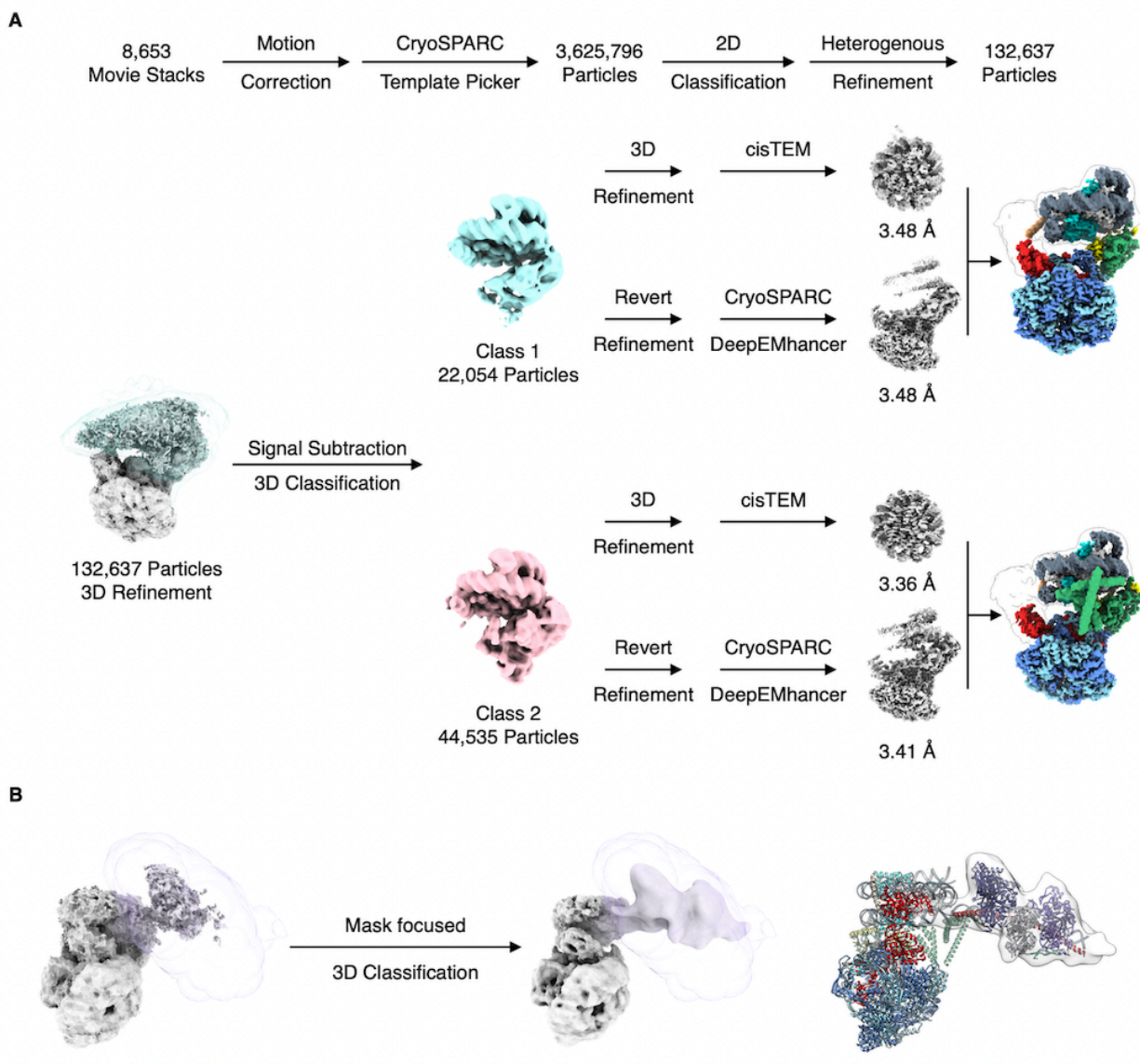


Figure S2.7 Image processing of the INO80-nucleosome.

(A) A flow-chart outlining the processing of the INO80-nucleosome cryo-EM dataset.

(B) Focused classification near flanking DNA produced density of the Arp8 module.

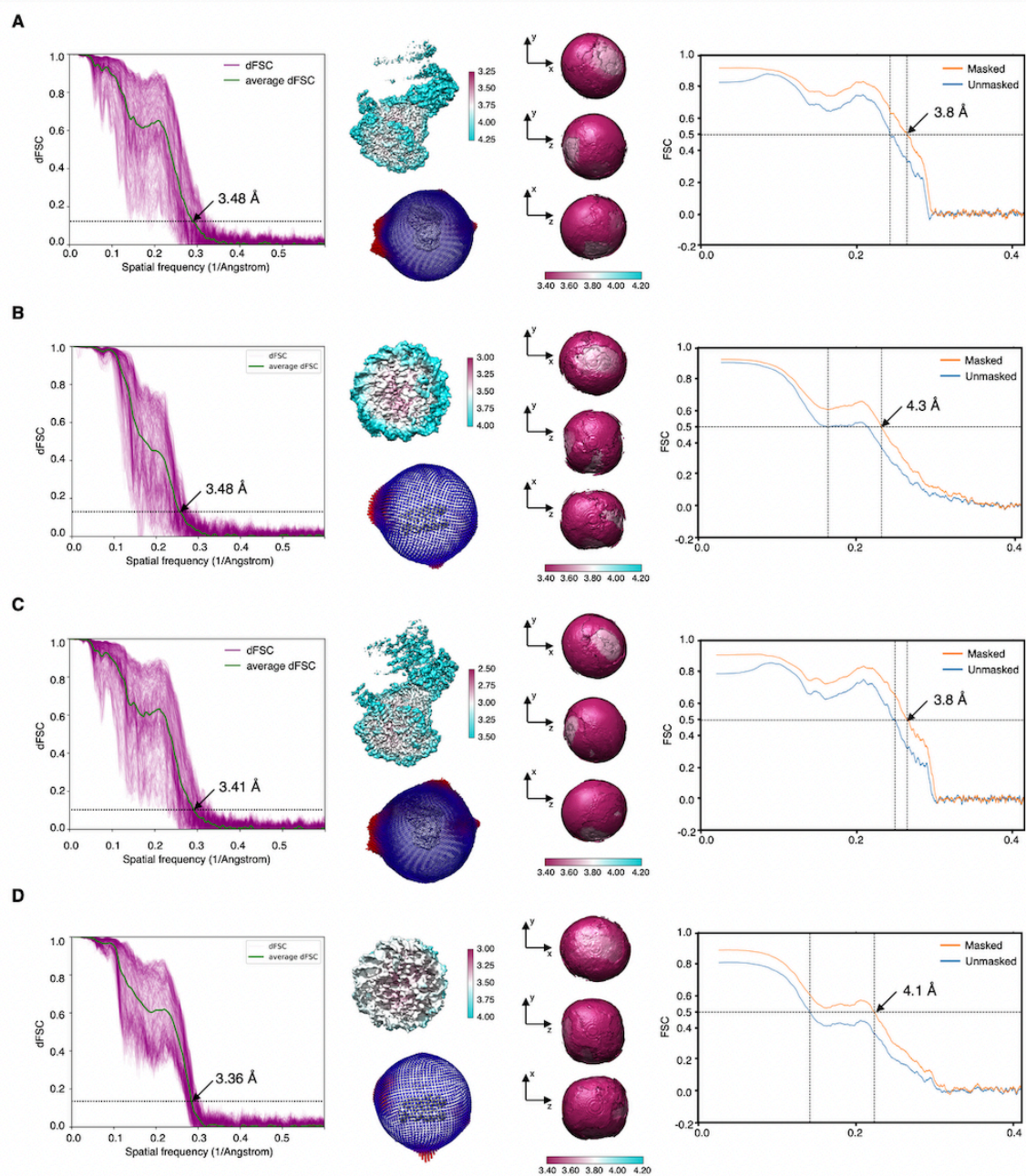


Figure S2.8 Resolution estimation of the INO80-nucleosome structures.

All panels contain, from left to right, directional Fourier shell correlation (dFSC) curves of final maps with resolution determined by the FSC criterion of 0.143, cryo-EM map colored by local resolution with resolution scale bar, angular distributions of particles, three-dimensional representations of the dFSC, and model-map FSC plots calculated by Phenix between the map and the model.

(A-B) Class 1 of INO80-nucleosome complex (A) and the corresponding nucleosome (B).

(C-D) Class 2 of INO80-nucleosome complex (C) and the nucleosome (D).

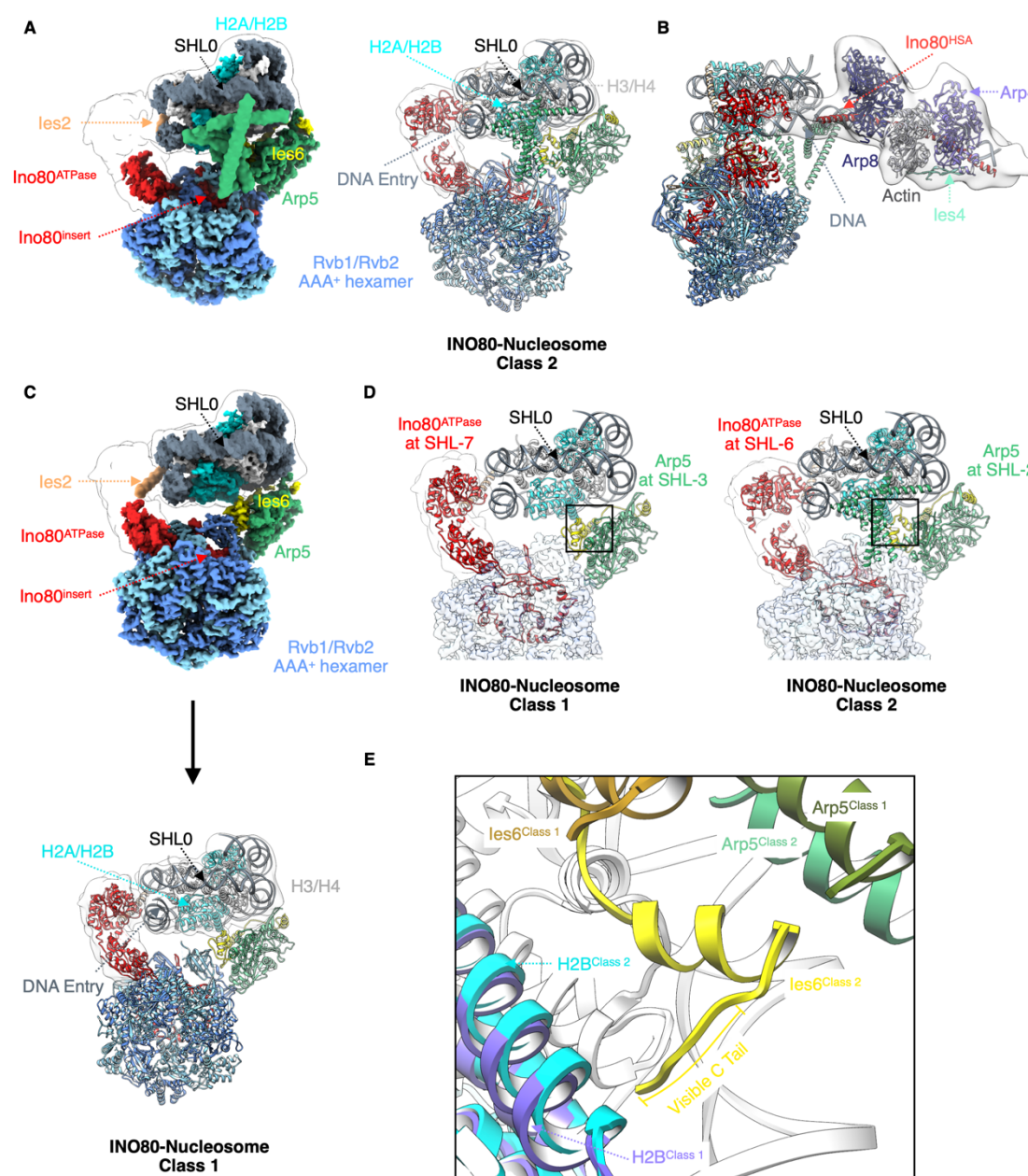


Figure S2.9 Structure of the INO80-nucleosome.

(A) Cryo-EM map and atomic model of the *S. cerevisiae* INO80-nucleosome complex (class 2).

(B) Atomic model of the INO80-nucleosome complex (class 2) with a model of the Arp8 module (PDB: 8A5O) docked into the density map.

(C) Cryo-EM map and atomic model of the *S. cerevisiae* INO80-nucleosome complex (class 1).

(D) Two conformational snapshots of INO80-nucleosome complex aligned by the nucleosome. Binding location of Ino80^{ATPase} and Arp5 are labeled.

(E) Enlarged view of the boxed areas in (D) shows overlay of nucleosome atomic models in the two conformations. In class 2, H2B near flanking DNA is shifted slightly and the C terminal tail of les6 becomes visible.

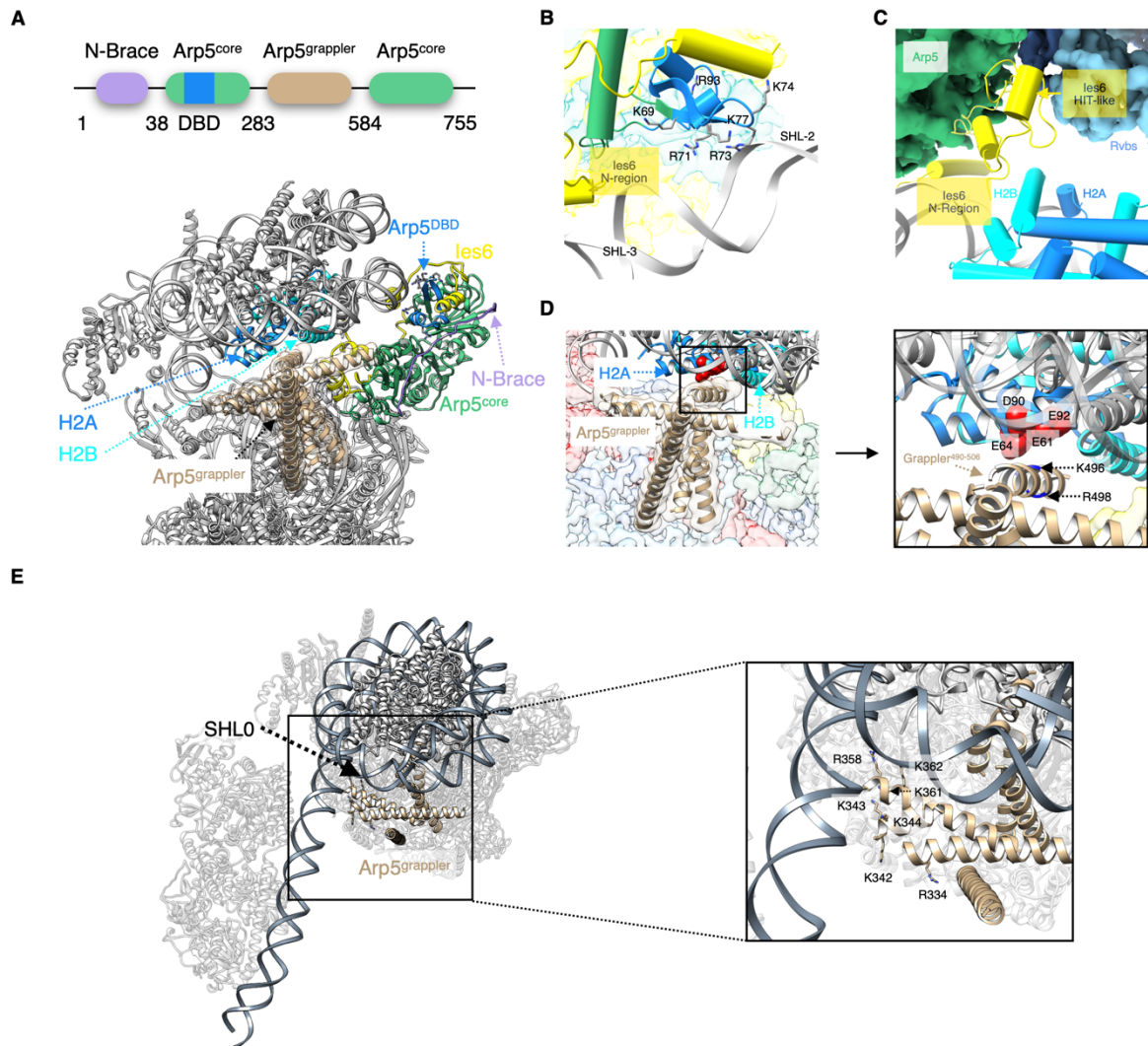


Figure S2.10 Arp5 interactions in the INO80-nucleosome structure.

(A) Domain arrangement (upper) and atomic model (bottom) of the Arp5 module.

(B) An enlarged view showing interactions between Arp5^{DBD} and DNA.

(C) An enlarged view showing interactions between Ies6, Arp5 and Rvb1/Rvb2.

(D) Two enlarged views of the Arp5^{grappler} and the H2A-H2B dimer proximal to the flanking DNA highlighting residues that may form contacts. Acidic residues: red, sphere; basic residues: blue.

(E) Two enlarged views of the Arp5^{grappler} and flanking DNA of the nucleosome highlighting Arp5 residues that may form contacts with the flanking DNA.

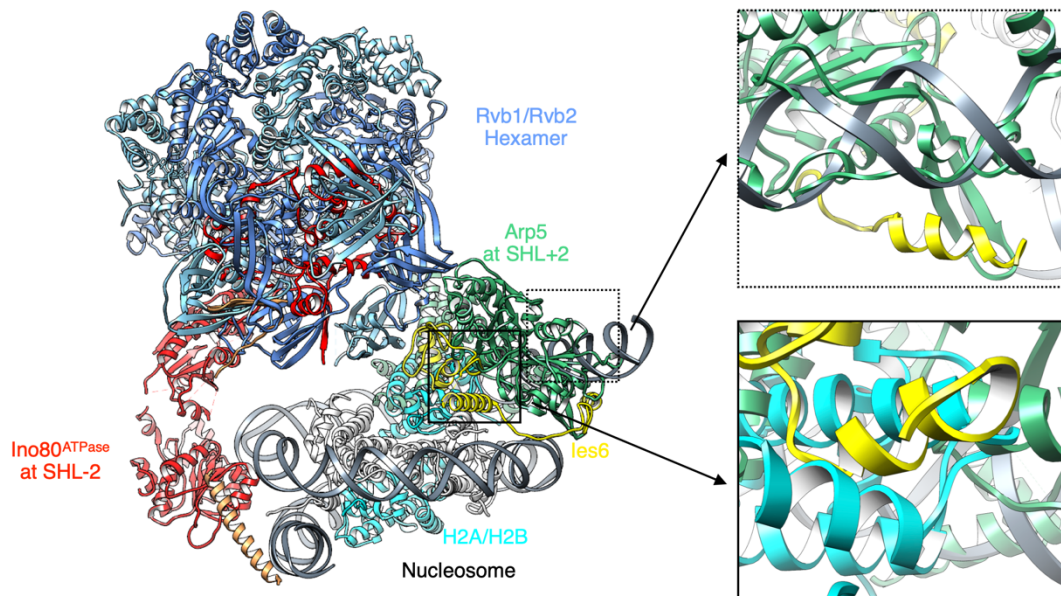


Figure S2.11 Modeling INO80 on a nucleosome with Ino80^{ATPase} at SHL-2 results in clashes.

A hypothetical model of INO80 on a nucleosome made by directly positioning Ino80^{ATPase} at SHL-2 reveals clashes between the Arp5 module and the entry-side H2A-H2B dimer (bottom panel) and between the Arp5 module and DNA that wraps around entry-side H2A-H2B dimer (top panel).

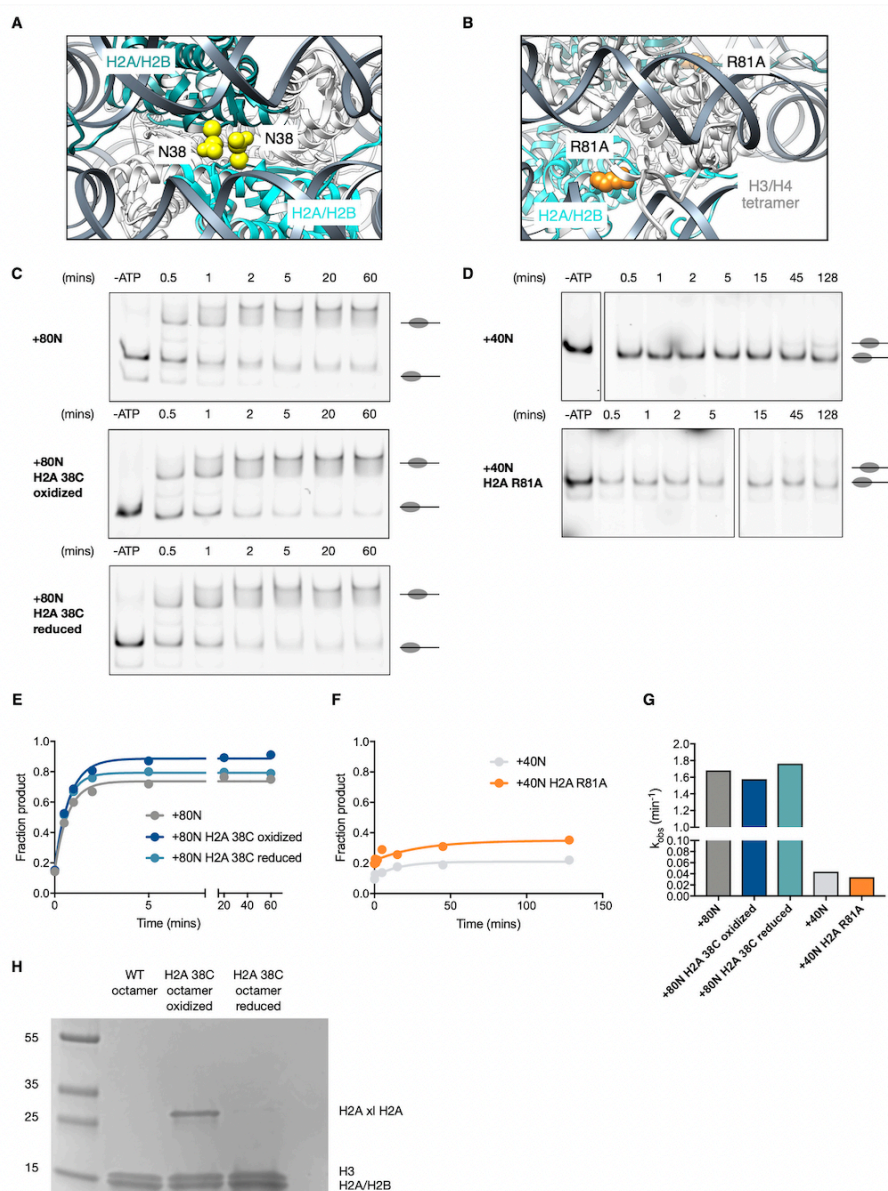


Figure S2.12 Crosslinking H2A or destabilizing dimer-tetramer interface does not significantly affect INO80 remodeling activity.

(A) Location of H2A N38C mutation used for crosslinking experiments (PDB: 1KX5).

(B) Location of H2A R81A mutation, which was shown to have destabilizing effects on the nucleosome (PDB: 1KX5).

(C-D) Example gels of native gel-based remodeling assays of (C) WT INO80 on wildtype +80N or +80N containing oxidized or reduced octamers containing H2A 38C; (D) WT INO80 on +40N or +40N containing H2A 81A (+40N R81A).

(E) Example time courses of native gel-based remodeling assays shown in (C).

(F) Example time courses of native gel-based remodeling assays shown in (D).

(G) Average observed rate constants determined from fitting a single-phase exponential decay model to two technical replicates. All assays were performed under single-turnover conditions with saturating enzyme and ATP.

(H) SDS-PAGE gel with wild-type, H2A 38C oxidized, and H2A 38C reduced octamer stained with SYPRO Red. Crosslinking efficiency shown below gel.

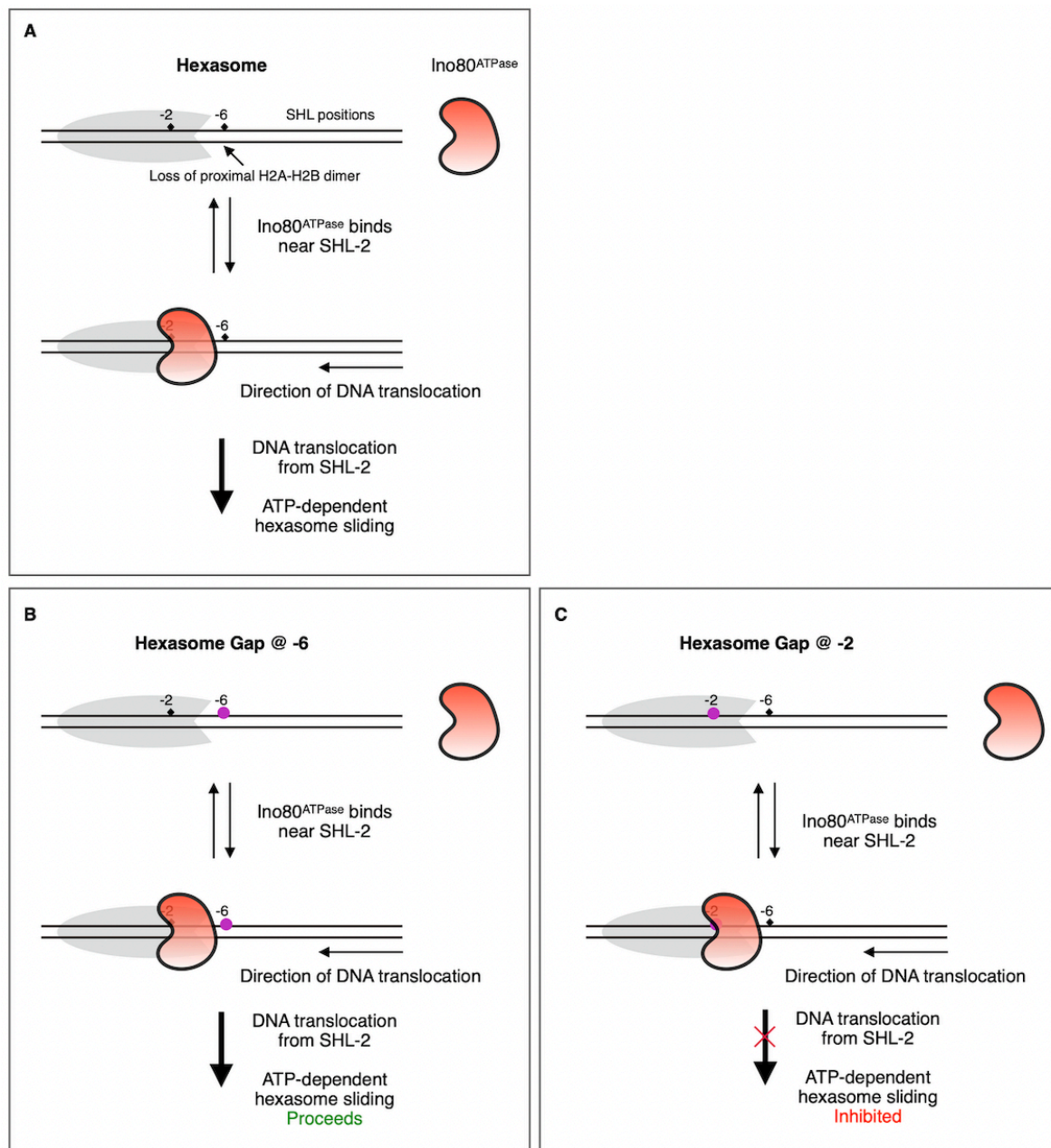


Figure S2.13 Model of INO80-hexasome sliding and predictions of effects of gaps near SHL-6 or -2.

(A) Model of INO80^{ATPase} interactions during hexasome sliding by INO80. Only the INO80^{ATPase} domain of INO80 is shown for clarity. During sliding of a hexasome, INO80^{ATPase} first binds near SHL-2. Upon ATP hydrolysis, INO80^{ATPase} then efficiently translocates DNA from SHL-2 to slide the hexasome.

(B) On a hexasome containing a single base gap near SHL-6, INO80^{ATPase} binds near SHL-2 and proceeds with hexasome sliding as described in (A).

(C) On a hexasome containing a single base gap near SHL-2, INO80^{ATPase} binds near SHL-2. The gap near SHL-2 prevents DNA translocation by INO80^{ATPase} and hexasome sliding is inhibited.

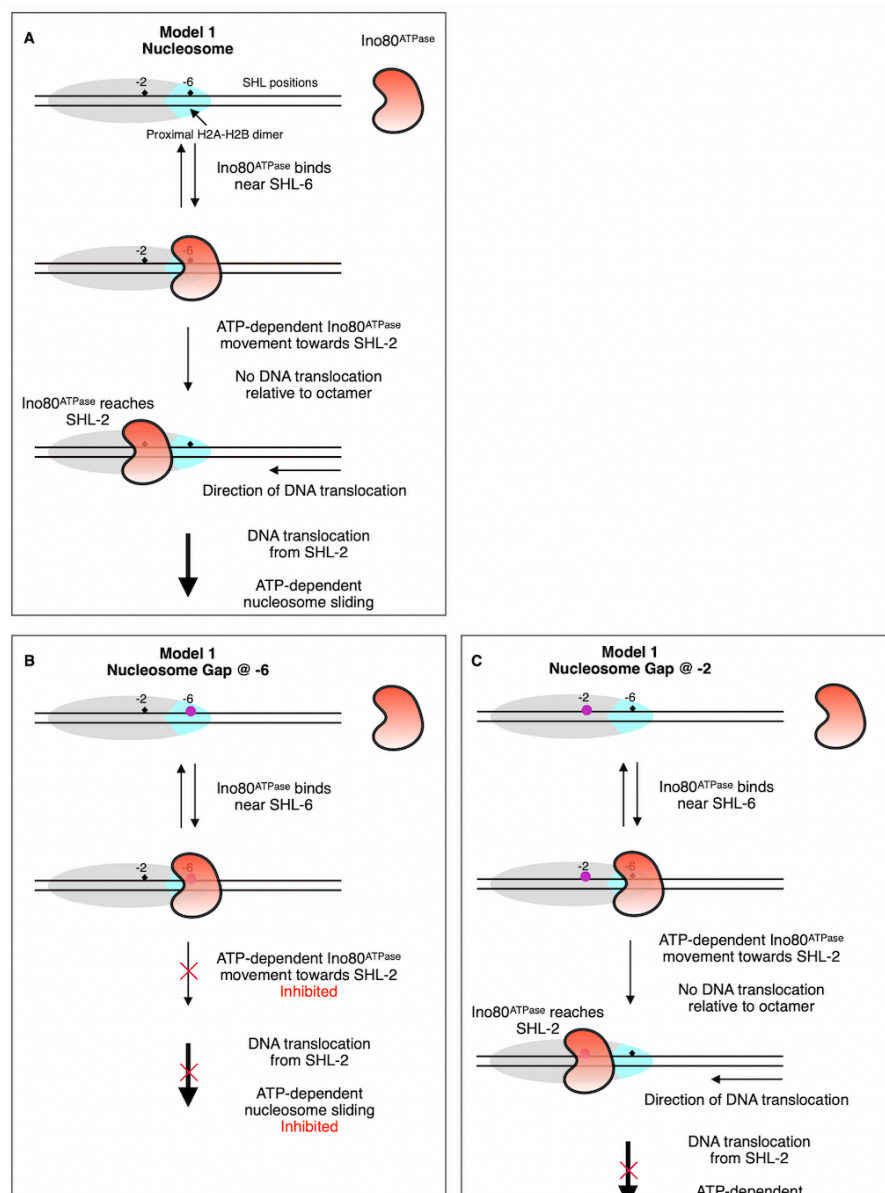


Figure S2.14 Model 1 of INO80-nucleosome sliding and predictions of effects of gaps near SHL-6 or -2.

(A) Model 1 of Ino80^{ATPase} interactions during nucleosome sliding by INO80. Only the Ino80^{ATPase} domain of Ino80 is shown for clarity. During sliding of a nucleosome, Ino80^{ATPase} first binds near SHL-6. Upon ATP hydrolysis, Ino80^{ATPase} then moves towards SHL-2 without translocating DNA relative to the octamer. Once near SHL-2, Ino80^{ATPase} couples ATP hydrolysis to DNA translocation to slide the nucleosome.

(B) On a nucleosome containing a single base gap near SHL-6, Ino80^{ATPase} binds near SHL-6. The gap near SHL-6 prevents Ino80^{ATPase} from moving towards SHL-2 and therefore downstream nucleosome sliding is inhibited.

(C) On a nucleosome containing a single base gap near SHL-2, Ino80^{ATPase} binds near SHL-6. Upon ATP hydrolysis, Ino80^{ATPase} then moves towards SHL-2 without translocating DNA relative to the octamer. Once the Ino80^{ATPase} is near SHL-2, the gap near SHL-2 prevents DNA translocation by Ino80^{ATPase} and nucleosome sliding is inhibited.

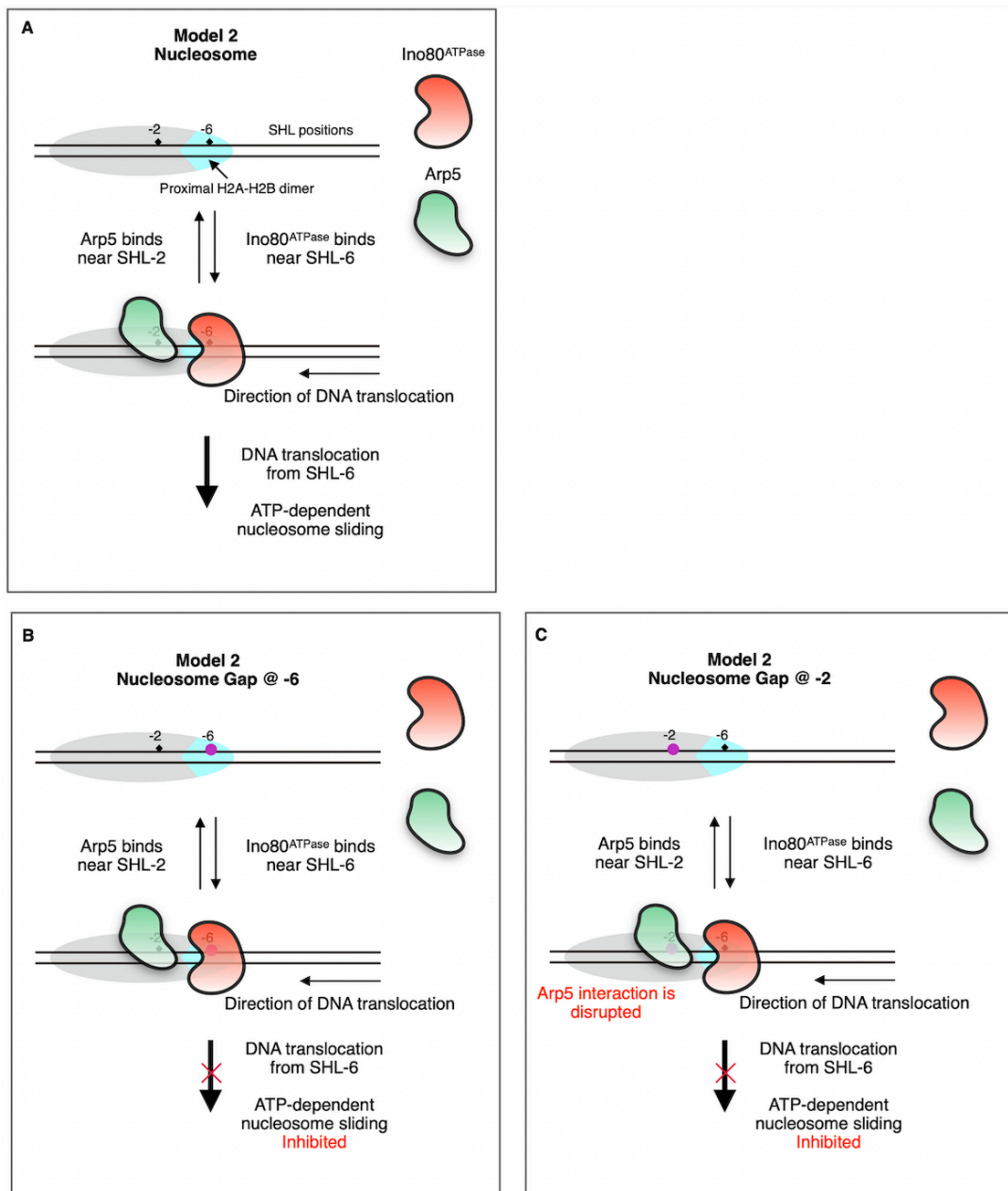


Figure S2.15 Model 2 of INO80-nucleosome sliding and predictions of effects of gaps near SHL-6 or -2.

(A) Model 2 of Ino80^{ATPase} interactions during nucleosome sliding by INO80. Only the Ino80^{ATPase} domain of Ino80 and the Arp5 subunit are shown for clarity. Ino80^{ATPase} binds near SHL-6 and Arp5 binds near SHL-2. Ino80^{ATPase} translocates DNA from SHL-6 resulting in nucleosome sliding.

(B) On a nucleosome containing a single base gap near SHL-6, Ino80^{ATPase} binds near SHL-6. The gap near SHL-6 prevents DNA translocation by Ino80^{ATPase} and nucleosome sliding is inhibited.

(C) On a nucleosome containing a single base gap near SHL-2, Ino80^{ATPase} binds near SHL-6. The gap near SHL-2 should not inhibit translocation by Ino80^{ATPase} near SHL-6, however, it may inhibit sliding of a nucleosome through disrupting Arp5 module interactions near SHL-2.

Table S2.1 Cryo-EM data collection, refinement, and validation statistics of the INO80-Hexasome sample

	Sample	INO80-Hexasome					
	Class	Class1		Class2		Class3	
	Region	INO80 ^{core}	Hexasome	INO80 ^{core}	Hexasome	INO80 ^{core}	Hexasome
	EMDB	28597	28598	28599	28600	28601	28602
	PDB	8ETS	8ETT	8ETU	8ETV	8ETW	8EU2
Data collection and Processing							
Microscope		Titan Krios					
Voltage (keV)		300					
Camera		Gatan K3 with Gatan Bioquantum energy filter					
Nominal/Calibrated Magnification		105,000					
Calibrated Magnification		119,760					
Pixel size at detector (Å/pixel)		0.4175					
Total electron exposure (e ⁻ /Å ²)		67					
Exposure rate (e ⁻ /pixel/sec)		16					
Number of frames		117					
Defocus range (µm)		(-0.8) - (-1.8)					
Automation software		SerialEM					
Energy filter slit width (eV)		20					
Micrographs used (no.)		18,991					
Total extracted/refined particles (no.)		7,831,514/560,912					
Reconstruction							
Final particles		76,874		130,147		233,675	
Symmetry		C1					
Resolution (global, Å)		3.04	6.68	2.80	3.16	2.64	2.93
Resolution (global, Å)							
FSC 0.5 (unmasked/masked)		3.6/3.4	9.3/8.6	3.3/3.1	4.2/3.9	3.4/3.1	3.7/3.4
FSC 0.143 (unmasked/masked)		3.0/3.0	7.2/6.8	2.8/2.8	3.3/3.1	2.7/2.5	3.1/2.9
Resolution range (local, Å)		2.75-3.75	5.00-7.00	2.50-3.50	3.00-4.00	2.50-3.50	2.75-3.75
Map sharpening <i>B</i> factor (Å ²)		81.6	139.66	80.9	145.7	58.42	114.13
3DFSC Sphericity value		0.91	0.94	0.95	0.93	0.96	0.97
Model composition							
Protein		3,664	524	3,664	524	3,664	524
Ligands		6	0	6	0	6	0
DNA		0	220	0	220	0	220
Model Refinement							
Refinement package							
- real or reciprocal space		Real space	Real space	Real space	Real space	Real space	Real space
- resolution cutoff		0.143	0.143	0.143	0.143	0.143	0.143
Model-Map scores							
-CC		0.87	0.75	0.88	0.78	0.87	0.82
<i>B</i> factors (Å ²)							
Protein residues		147.72	309.34	131.11	90.61	58.45	75.28
Ligands		125.68	N/A	109.77	N/A	43.77	N/A
DNA		N/A	429.67	N/A	130.97	N/A	118.55
R.m.s. deviations from ideal values							
Bond lengths (Å)		0.005	0.006	0.006	0.006	0.006	0.006
Bond angles (°)		1.136	1.123	1.207	1.006	1.203	0.960
Validation							
MolProbity score		1.34	1.38	1.53	1.13	1.54	1.07
CaBLAM outliers		2.04	0.60	2.23	1.00	2.26	1.00
Clashscore		3.62	4.35	5.71	2.67	5.64	1.98
Poor rotamers (%)		0.00	0.00	0.00	0.00	0.00	0.00
C-beta deviations		0.00	0.00	0.06	0.00	0.06	0.00
EMRinger score		2.13	N/A	2.08	1.34	2.90	1.87
Ramachandran plot							
Favored (%)		96.88	97.07	96.63	97.66	96.44	97.46
Allowed (%)		3.12	2.93	3.37	2.34	3.56	2.54
Outliers (%)		0.00	0.00	0.00	0.00	0.00	0.00

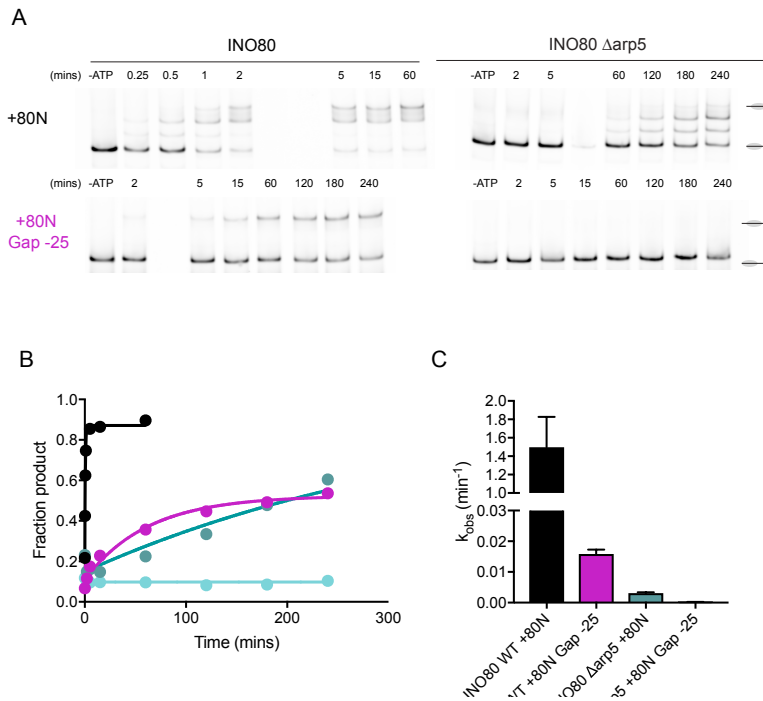
Table S2.2 Cryo-EM data collection, refinement, and validation statistics of the INO80-Nucleosome sample

	Sample Class	INO80-Nucleosome			
	Class	Class1		Class2	
	Region	INO80 ^{core}	Nucleosome	INO80 ^{core}	Nucleosome
	EMDB	28609	28612	28613	28614
	PDB	8EU9	8EUE	8EUF	8EUJ
Data collection and Processing					
Microscope		Titan Krios			
Voltage (keV)		300			
Camera		Gatan K3 with Gatan Bioquantum energy filter			
Nominal Magnification		105,000			
Calibrated Magnification		117232			
Pixel size at detector (Å/pixel)		0.417			
Total electron exposure (e ⁻ /Å ²)		43			
Exposure rate (e ⁻ /pixel/sec)		15			
Number of frames		80			
Defocus range (µm)		(-0.8) - (-1.8)			
Automation software		SerialEM			
Energy filter slit width (eV)		20			
Micrographs used (no.)		8,653			
Total extracted/refined particles (no.)		3,625,796/132,637			
Reconstruction					
Final particles		22,054		44,535	
Symmetry			C1		
Resolution (global, Å)		3.48	3.48	3.41	3.36
Resolution (global, Å)					
FSC 0.5 (unmasked/masked)		4.1/3.8	6.1/4.3	4.0/3.8	6.5/4.1
FSC 0.143 (unmasked/masked)		3.5/3.4	3.7/3.5	3.4/3.4	3.7/3.4
Resolution range (local, Å)		3.25-4.25	3.00-4.00	3.25-4.25	3.00-4.00
Map sharpening <i>B</i> factor (Å ²)		57.9	92.4	57.7	35.9
3DFSC Sphericity value		0.94	0.91	0.93	0.95
Model composition					
Protein		3,660	751	3,664	747
Ligands		6	0	6	0
DNA		0	294	0	294
Model Refinement					
Refinement package					
- real or reciprocal space		Real space	Real space	Real space	Real space
- resolution cutoff		0.143	0.143	0.143	0.143
Model-Map scores					
-CC		0.85	0.80	0.84	0.76
<i>B</i> factors (Å ²)					
Protein residues		158.73	117.30	127.57	120.31
Ligands		128.40	N/A	104.07	N/A
DNA		N/A	192.80	N/A	196.19
R.m.s. deviations from ideal values					
Bond lengths (Å)		0.007	0.007	0.007	0.006
Bond angles (°)		1.278	1.331	1.265	1.321
Validation					
MolProbity score		1.57	1.58	1.59	1.39
CaBLAM outliers		2.35	1.11	2.40	1.26
Clashscore		5.93	5.91	5.84	5.14
Poor rotamers (%)		0.00	0.00	0.00	0.00
C-beta deviations		0.00	0.00	0.03	0.00
EMRinger score		1.51	0.64	1.57	0.82
Ramachandran plot					
Favored (%)		96.38	96.19	96.03	97.40
Allowed (%)		3.62	3.81	3.97	2.60
Outliers (%)		0.00	0.00	0.00	0.00

2.10 Appendix: Unpublished data

In the preceding discussion, we present two models outlining how INO80 may interact with a nucleosome to facilitate nucleosome sliding: (1) INO80 slides a nucleosome with its Ino80 ATPase positioned at SHL-6 or (2) INO80 slides a nucleosome after it undergoes a conformational change, repositioning its Ino80 ATPase from SHL-6 to SHL-2.

Model 1 suggests that INO80 slides nucleosomes from the orientation observed in published INO80-nucleosome structures, with its Ino80 ATPase near SHL-6 and its Arp5 module near SHL-2. In Chapter 2, we showed that a single base gap in the nucleosomal DNA at position -25 relative to the dyad (near SHL-2) inhibits INO80 nucleosome sliding. By model 1, the Ino80 ATPase would be translocating DNA from SHL-6 and never encounter the gap near SHL-2, suggesting the gap does not inhibit sliding through stalling the ATPase. One possibility is that the gap inhibits INO80 nucleosome sliding instead through disrupting the action of the Arp5 module which appears to interact near SHL-2 in published INO80-nucleosome structures. To test this possibility, we asked how a gap near SHL-2 affects the sliding activity of a mutant INO80 complex lacking the Arp5 module (INO80 Δ arp5). If the gap only inhibits nucleosome sliding through disrupting Arp5 contacts, the prediction would be that a gap near SHL-2 would not inhibit nucleosome sliding by INO80 Δ arp5. Using a native gel-based nucleosome sliding assay, we observed that a gap near SHL-2 significantly slowed nucleosome sliding by INO80 Δ arp5 (**Appendix Figure 1A-C**). This result rules out the model that a gap near SHL-2 inhibits INO80 nucleosome sliding solely through disrupting the action



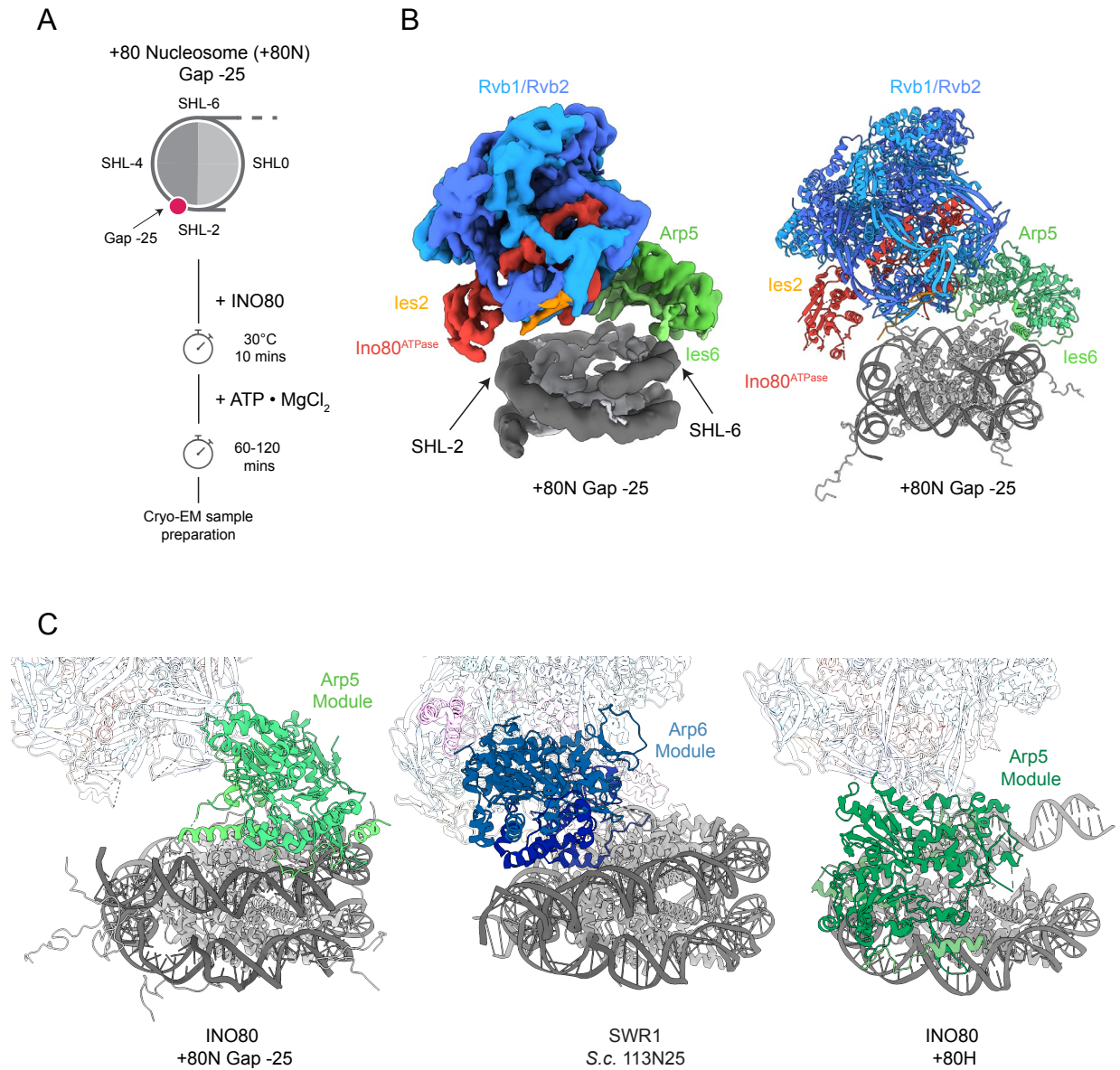
Appendix Figure 1 DNA gap near SHL-2 has an Arp5-independent inhibitory effect on INO80 nucleosome sliding.

(A) Example gel of native gel-based sliding assay. (B) Example time courses of native gel-based remodeling assays. (C) Average observed rate constants of INO80 sliding activity. Data represent the mean $\pm$ SEM for three technical replicates performed under single-turnover conditions with saturating enzyme and ATP. All assays were performed under single-turnover conditions with saturating enzyme and ATP.

of the Arp5 module. Instead, the data implies that a large part of the gap's inhibitory effect is independent of the Arp5 module. If INO80 is acting by model 1, it remains unclear how the gap near SHL-2 inhibits its nucleosome sliding activity.

Model 2 suggests that INO80 instead undergoes a

conformational change and reorients to position its core Ino80 ATPase near SHL-2 to effectively slide a nucleosome. The DNA gap near SHL-2 could then inhibit INO80 nucleosome sliding through directly stalling translocation of DNA relative to the octamer by the Ino80 ATPase. Model 2 would predict that on a nucleosome containing a gap near SHL-2, INO80 would reorient to position its ATPase near SHL-2 upon ATP hydrolysis but translocation of the DNA relative to the octamer would be stalled. To test this prediction, we asked if we could capture INO80 sliding intermediates using cryo-EM. Under conditions favorable for nucleosome sliding, we prepared a sample of INO80



Appendix Figure 2 INO80 reorients on a gap-containing nucleosome in the presence of ATP

(A) Flow chart describing the setup of cryo-EM samples containing INO80, +80 nucleosomes with a DNA gap at -25, and AT-MgCl₂. (B) Cryo-EM density map of INO80-NucGap-2.5+ATP (left) and roughly docked model of INO80 on a canonical nucleosome (1KX5) before accurate modeling. (C) Models of INO80 or SWR on various substrates highlighting the position of the Arp5 module or Arp6 module.

and +80 nucleosomes with a gap near SHL-2 and added saturating ATP-MgCl₂ (**Appendix Fig 2A**). We obtained a high-resolution structure of INO80 in complex with the gap-containing nucleosome, which we refer to as INO80-NucGap-2.5+ATP. In this structure, the Ino80 ATPase subunit is engaged near SHL-2 and the Arp5 module is

engaged near SHL-6 (**Appendix Fig 2B**), which suggests that the complex has reorientated.

Further, the whole INO80 complex is drastically repositioned relative to its orientation in previously published structures of INO80 on a nucleosome. The positioning of the Ino80 ATPase in our INO80-NucGap-2.5+ATP structure is similar to the Ino80 ATPase position in our INO80-hexasome structures discussed in Chapter 2. Notably, the positioning of the Ino80 ATPase near SHL-2 also resembles that of core ATPase subunits in all published structures of chromatin remodelers bound to nucleosomes. This data is consistent with model 2 and generally suggests that SHL-2 may be the key region of the nucleosome where all remodelers effectively act to transform the nucleosome.

Initially, based on our INO80-hexasome structures, we predicted clashes between the Arp5 module and the H2A-H2B dimer if INO80 were to adopt a similar conformation on a nucleosome as on a hexasome (**Appendix Fig 2C**). However, in our INO80-NucGap-2.5+ATP structure, we observed that the Arp5 module instead accommodates the potential clash by adopting a conformation that engages with the nucleosomal DNA near SHL-6, akin to the homologous Arp6 module in SWR1 (**Appendix Fig 2C**).

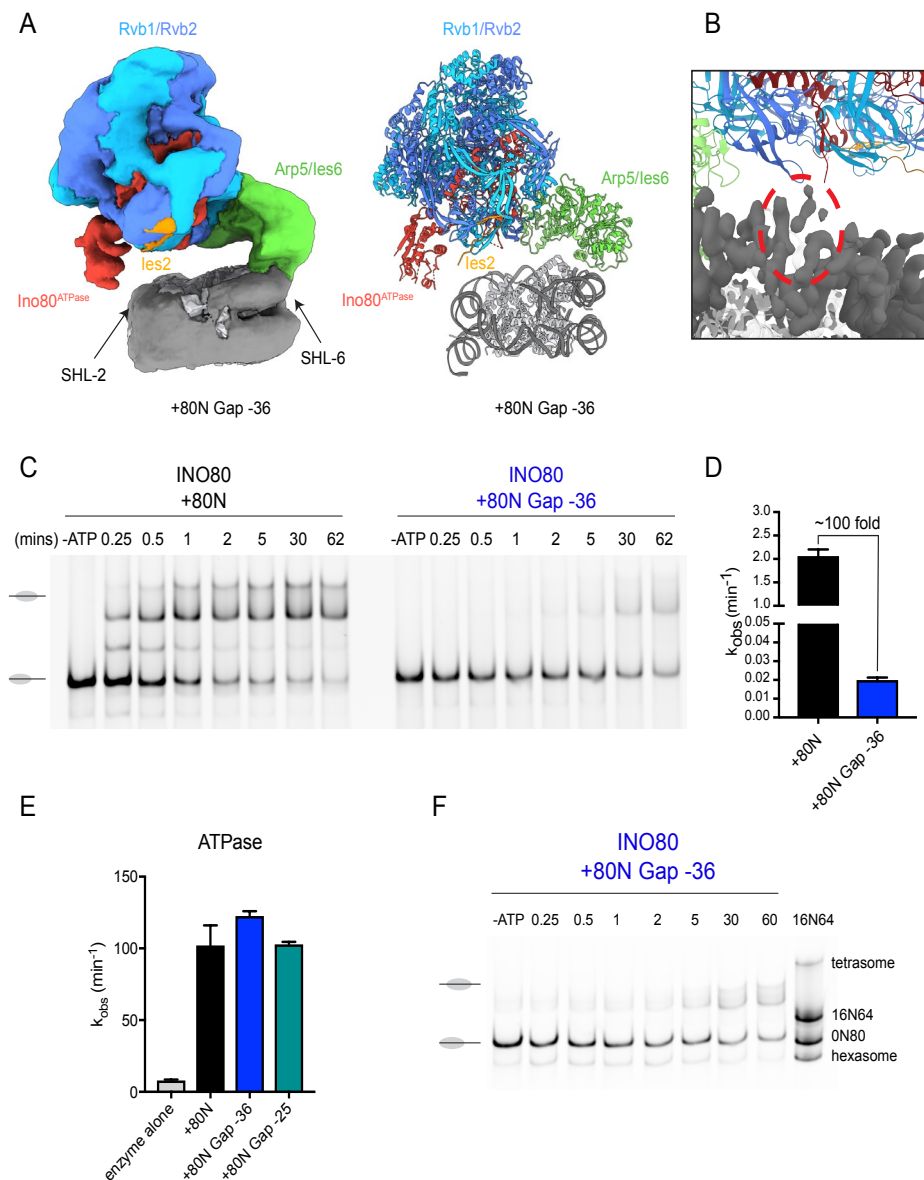
We also note that there is a subset of particles in which INO80 is oriented on the nucleosome with its ATPase positioned at SHL-6 after incubation with ATP. This population suggests that either INO80 does not reorient on a subset of nucleosomes

under the conditions tested or that INO80 may reorient, fall off, and rebind nucleosomes with its ATPase positioned near SHL-6.

Our INO80-NucGap-2.5+ATP structure suggests that INO80 undergoes an ATP-dependent reorientation to position its Ino80 ATPase near SHL-2 before nucleosome sliding. This model is consistent with single-molecule data which showed that INO80 undergoes an initial ATP-dependent pause phase prior to sliding a nucleosome (Zhou et al., 2018). Yet, how the reorientation of INO80 could occur remains unclear. Structural and foot printing data strongly support that INO80 initially binds a nucleosome with its Ino80 ATPase engaged at SHL-6. Previous data suggests that INO80 sliding is processive with a single INO80 molecule centering a nucleosome without falling off (Zhou et al., 2018). The proposed reorientation of INO80 must then also occur without the complex completely unbinding and rebinding. One possible way INO80 may accomplish this reorientation is through translocation of its Ino80 ATPase along nucleosomal DNA from SHL-6 to SHL-2 without translocation of DNA across the octamer. Previous data has shown that single base DNA gaps spanning from SHL-6 to SHL-2 inhibit INO80 nucleosome sliding (Brahma et al., 2017; Wu et al., 2023). This data is consistent with the possibility that the gaps inhibit sliding through blocking the Ino80 ATPase from translocating along the DNA from SHL-6 to SHL-2.

To test whether the Ino80 ATPase translocates along DNA from SHL-6 to SHL-2 before sliding a nucleosome, we introduced a single base gap in between SHL-6 and SHL-2 at position -36 relative to the dyad (near SHL-3.5) and determined the effect on INO80

reorientation by cryo-EM. If the gap inhibits Ino80 from reaching SHL-2, the prediction is that a gap near SHL-3.5 would trap the Ino80 ATPase near SHL-3.5 before it reaches



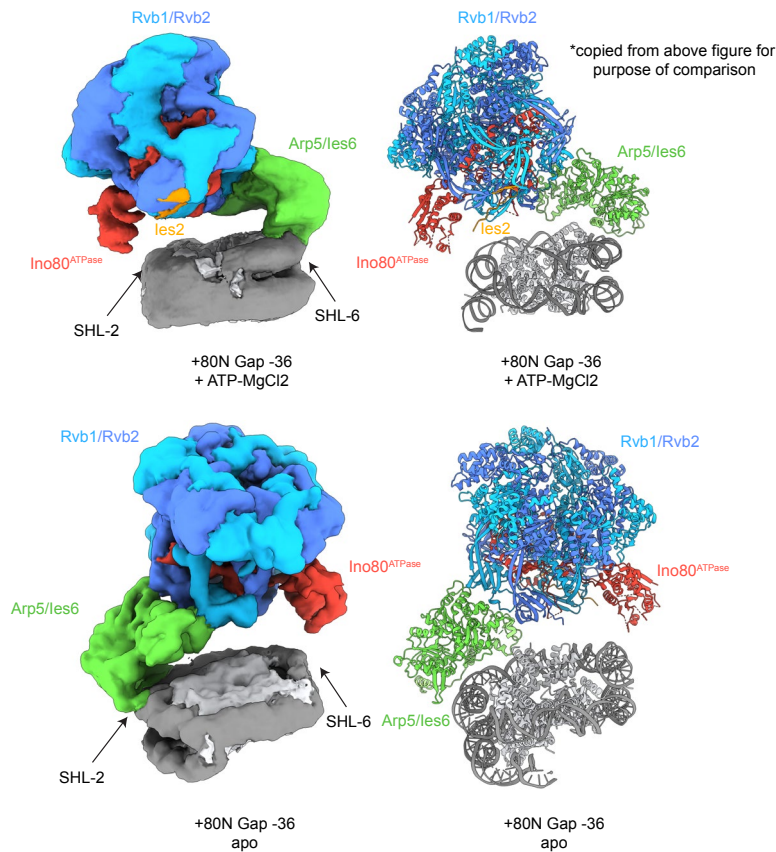
Appendix Figure 3 INO80's activity on nucleosomes with a gap at -36

(A) Cryo-EM density map of INO80-NucGap-3.5+ATP (left) and roughly docked model of INO80 on a canonical nucleosome (1KX5) before accurate modeling. (B) Zoom in on gap in the nucleosomal DNA at position -20. (C) Native gel-remodeling assay with 15 nM nucleosomes and 60 nM INO80. (D) Average observed rate constants of INO80 sliding activity. Data represent the mean $\pm$ SEM for three technical replicates performed under single-turnover conditions with saturating enzyme and ATP. (E) Average observed rate constants of INO80 ATPase activity measured by radioactive-based ATPase assay. Assays were performed with 10 nM INO80, 100 nM nucleosomes (saturating for nucleosomes), and 160 μM ATP-MgCl₂ with trace amounts of radioactive ATP. Data represent the mean $\pm$ SEM for three technical replicates. (F) Native gel-remodeling assay similar to right panel of (C) with a lane containing 16N64 nucleosomes.

SHL-2 and before any DNA translocation relative to the octamer occurs. We prepared a sample as described above, mixing INO80 and gap-containing nucleosomes in the presence of saturating ATP-MgCl₂, and obtained a structure which we refer to as INO80-NucGap-3.5+ATP (**Appendix Fig 3A**). In this structure, INO80 adopts an orientation with its ATPase near SHL-2 and its Arp5 module near SHL-6, similar to what we see in our INO80-NucGap-2.5+ATP structure. Strikingly, in a focused map of the nucleosome in our INO80-NucGap-3.5+ATP structure, we see the gap that was originally positioned -36 bases relative to the dyad near position -20 (**Appendix Fig 3B**). This indicated that ~16 bp were translocated out from the exit site of the nucleosome. Of these ~16 bp, we observe density near the exit site that corresponds to at least ~5 bp of DNA protruding from the nucleosome.

Importantly, we also acquired a cryo-EM structure of INO80 bound to +80 nucleosomes containing a single base gap at -36 (near SHL-3.5) without ATP, termed INO80-NucGap-3.5. In this structure, the Ino80 ATPase is engaged near SHL-6 and the Arp5 module is engaged near SHL-2 (**Appendix Fig 4**). This structure is similar to all previous INO80-Nucleosome structures, indicating that the DNA gap does not influence the interaction between INO80 and nucleosomes and that the reorientation of INO80 we observe in INO80-NucGap-2.5+ATP is ATP-dependent. Additionally, the gap in the DNA does not appear to change the structure and wrapping of the nucleosomal DNA.

Our INO80-NucGap-3.5+ATP structure suggested that a DNA gap near SHL-3.5 does not prevent reorientation of INO80, but because of the time scale of our cryo-EM



Appendix Figure 4 INO80's reorientation is ATP-dependent

Cryo-EM density map of INO80-NucGap-3.5+ATP (top left) and roughly docked model of INO80 on a canonical nucleosome (1KX5) before accurate modeling (top right). Cryo-EM density map of INO80-NucGap-3.5 (bottom left) and roughly docked model of INO80 on a canonical nucleosome (1KX5) before accurate modeling (bottom right).

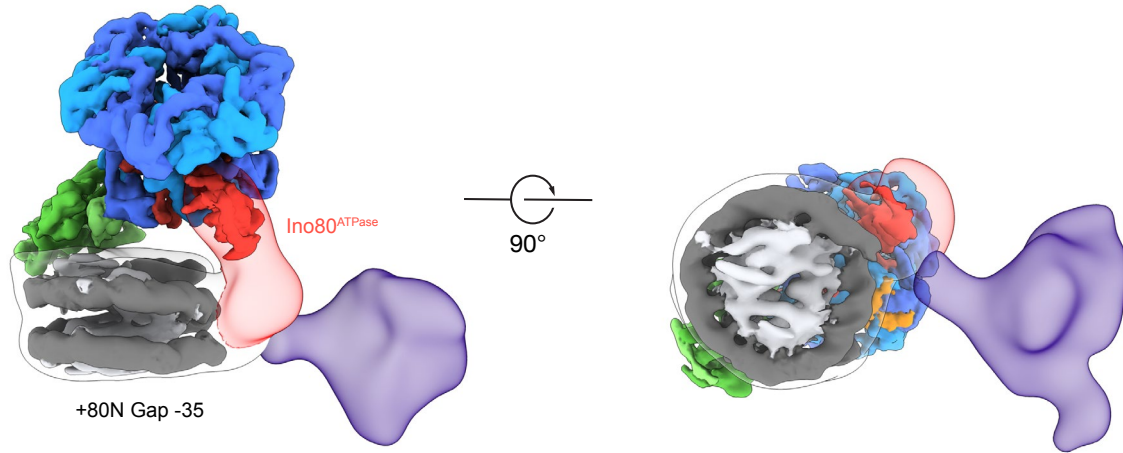
sample preparation it is unclear if the rate of reorientation of INO80 is at all affected by the gap. If we imagine the simple model that reorientation is unaffected and the DNA gap near SHL-3.5 is solely inhibiting DNA translocation across the octamer by the INO80 ATPase, then we would predict that INO80 would slide nucleosomes

with a DNA gap at position -36 quickly until the DNA gap reached position -20 (SHL-+80 nucleosomes containing a DNA gap at position -36 by native gel-based nucleosome sliding. The predicted end product would be a nucleosome with 16 base pairs of DNA protruding from the exit site and 64 base pairs of flanking DNA remaining on the entry site (16N64). Upon addition of INO80 and ATP to nucleosomes containing a DNA gap at position -36, we did not observe an accumulation of the predicted product; however, we did observe that INO80 slid the gapped nucleosomes ~100 fold slower than wildtype nucleosomes (**Appendix**

Fig 3C-D,F). Notably, gap-containing nucleosomes stimulated INO80 ATPase activity to a similar extent as wildtype nucleosomes, suggesting that the gaps do not interfere with INO80's ability to recognize the nucleosome and instead affect the coupling of INO80's ATPase activity to its nucleosome sliding activity (**Appendix Figure 3E**). Overall, our biochemical data implies that a gap at SHL-3.5 inhibits nucleosome sliding by INO80. However, which step of the reaction is inhibited remains unclear.

By our native gel-based sliding assay, we were unable to capture the partially slid nucleosome product that we were able to capture in our INO80-NucGap-3.5+ATP structure in which the DNA was translocated across the octamer by ~16 base pairs. One main difference between the two experiments is that in our gel-based sliding assay, we compete off INO80 with excess DNA to run the nucleosome products through a native gel. This competition step is skipped for our cryo-EM samples. One possibility for why we did not see partially slid nucleosome product by our gel-based assay is that it may not be stable without INO80 bound. There is evidence that unstable nucleosomes may collapse back into more stably positioned nucleosomes when INO80 is competed off (Partensky and Narlikar, 2009). INO80's potential to stabilize partially slid nucleosome products may reconcile the differences in the sliding products we observed by cryo-EM and our native gel-based sliding assay.

In addition to the ATP-dependent reorientation of INO80 we observe on gap-containing nucleosomes, we observed other significant conformational changes in INO80 as well, particularly with the Arp8 module. The Arp8 module interacts with the HSA domain of



Appendix Figure 5 The Arp8 module adopts a DNA-unbound conformation with Ino80ATPase is positioned at SHL-2 on a nucleosome.

Two views of the cryo-EM density map of INO80-NucGap-3.5+ATP (top). Focused maps of the Arp8 module and N-lobe of the Ino80 ATPase show lower resolution density.

the Ino80 ATPase and is composed of Arp8, Arp4, actin, les4, and Taf14. In INO80-nucleosome structures with flanking DNA longer than 40 base pairs, the Arp8 module and the Ino80 HSA interact with flanking DNA while in INO80-hexasome structures, they interact with free DNA that is unwrapped due to the lack of a dimer proximal to the flanking DNA ((Ayala et al., 2018; Eustermann et al., 2018; Zhang et al., 2023). Based on this data, we predicted that upon repositioning of the Ino80 ATPase to SHL-2 the Arp8 module may engage with nucleosomal DNA unwrapped from the entry-proximal H2A-H2B dimer. In our INO80-NucGap-3.5+ATP structure, we instead see that the Arp8 module adopts a novel conformation unbound to DNA and there is no unwrapping of the nucleosomal DNA from the entry-proximal dimer (**Appendix Fig 5**). This observation suggests that when INO80 engages the nucleosome with its Ino80 ATPase positioned near SHL-2, the Arp8 module adopts a DNA-unbound conformation. In this orientation with Ino80 ATPase near SHL-2, the DNA-dimer interactions in the vicinity of the Arp8

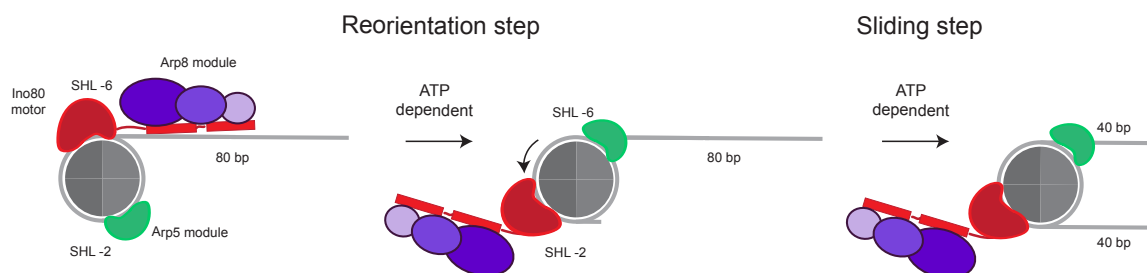
module may be stronger than the Arp8 module-DNA interactions promoting a DNA-unbound conformation of Arp8. We speculate that the conformation of the Arp8 module may be dynamic between the DNA-unbound and DNA-bound states during nucleosome sliding when DNA-histone contacts are loosened. Overall, our structures suggest that reorientation of INO80 on a nucleosome is coupled to large conformational changes in its various modules, which all may play a role in facilitating nucleosome sliding.

Discussion

INO80 acts at genomic loci undergoing various processes, including transcription, DNA replication, and DNA damage repair, each necessitating remodeling of the underlying nucleosome structure. How INO80's mechanism is regulated to adapt to the diverse remodeling requirements at these loci remains unclear. Here, we present a model that suggests an additional regulatory step in INO80's nucleosome sliding mechanism that may be influenced by different chromatin contexts.

Here, we present the first structures of INO80 nucleosome sliding intermediates. We show that upon addition of ATP, INO80 undergoes a major conformational change on a nucleosome, completely reorientating to position its ATPase subunit from SHL-6 to SHL-2. We captured this reaction intermediate using cryo-EM and nucleosome substrates containing single base DNA gaps that stall INO80 nucleosome sliding. Based on the positioning of the Ino80 ATPase subunit near SHL-2 in our two structures presented here, INO80-NucGap-2.5+ATP and INO80-NucGap-3.5+ATP, and our previously

published INO80-Hexasome structures, we propose that the Ino80 ATPase acts near SHL-2 to effectively translocate DNA both on a hexasome and nucleosome. This model suggests that previous foot printing studies and INO80-Nucleosome structures, which show INO80 binding with its ATPase near SHL-6, capture only the initial nucleosome-binding state of INO80.



Appendix Figure 6 Working model of nucleosome sliding by INO80

Cartoon model of INO80's two step nucleosome sliding mechanism. This includes an ATP-dependent reorientation step where the INO80 ATPase repositions to SHL-2 and the Arp8 module changes conformation to a DNA-unbound state and an ATP-dependent step where the Ino80 ATPase translocates DNA along the octamer facilitating nucleosome sliding.

We propose a model where INO80 reorients to position its ATPase from SHL-6 to SHL-2 before sliding a nucleosome (**Appendix Fig 6**). This initial reorientation step is consistent with single molecule data that shows INO80 undergoes a long ATP-dependent pause before sliding a nucleosome ((Zhou et al., 2018). This single molecule data also showed that a single INO80 molecule centers a nucleosome in a processive manner without falling off and rebinding. Therefore, we imagine two ways that INO80 may reorient on the nucleosome: (1) the Ino80 ATPase translocates along nucleosomal DNA from SHL-6 to SHL-2 or (2) INO80 undergoes a large conformational change that repositions the Ino80 ATPase without dissociation. Our INO80-NucGap-3.5+ATP structure suggests that a single base gap in the DNA between SHL-6 to SHL-2 does not

prevent reorientation of INO80. However, we currently do not have a quantitative assay way to test whether or not this gap slows down the reorientation process. Our cryo-EM sample was captured at 60-120 minutes, which may have been sufficient time for INO80 to reorient even if the gap slowed down the process.

Overall, we propose that INO80 initially engages a nucleosome with its Ino80 ATPase near SHL-6 which allows the complex to sense substrate cues, including flanking DNA, the dimer acidic patch, and DNA unwrapping. Upon sensing the proper cues, we think INO80 undergoes a reorientation to position its ATPase near SHL-2, coupled to a conformational change in its Arp8 and Arp5 modules, where it can effectively facilitate nucleosome sliding (**Appendix Fig 5**).

This model is consistent with previous data that suggests that the acidic patch in the proximal histone dimer is essential for INO80 nucleosome remodeling (Hsieh et al., 2022). If INO80 is not able to sense a functional acidic patch in the proximal dimer in its initial binding-state on the nucleosome, then based on the model we propose, Ino80 may not be able to successfully reposition to SHL-2 to facilitate nucleosome sliding. The same is true for a nucleosome with a gap near SHL-6, where INO80 may not be able to reorient to slide efficiently if an initial substrate cue is the DNA properties near SHL-6. This is consistent with previous data that suggests that rigid DNA only between SHL-6 and SHL-7 inhibits INO80 nucleosome sliding, although rigid DNA stretches that are positioned further into the flanking DNA do not (Kunert et al., 2022).

Additionally, this model is consistent with INO80's flanking DNA length-sensing ability. While INO80 can quickly slide nucleosomes with long flanking DNA, its sliding activity is ~300-fold slower on nucleosomes with ≤ 40 base pairs of flanking DNA (Zhou et al., 2018). The initial orientation of INO80 with its ATPase positioned near SHL-6 may enable Ino80's HSA domain and Arp8 module to interact with the flanking DNA of the nucleosome. If there are sufficient flanking DNA contacts, this may influence the change in conformation of the HSA and Arp8 module that appears to be coupled to the reorientation of INO80 we observed in our structures.

Understanding how cues in the chromatin substrate are linked to conformational changes in the Arp8 and Arp5 modules and INO80's reorientation will be key to further our understanding of INO80's mechanism and how it is regulated at different genomic loci.

Chapter 3: Dissecting the unique chaperone mechanism of the Rvb1/Rvb2 AAA+ ATPase complex

3.1 Preface

INO80's (sub)nucleosome-sliding activity is regulated in various ways. For example, INO80's activity is regulated by its substrate; INO80 slides a hexasome faster than a nucleosome and a nucleosome with >40 bps of flanking DNA faster than a nucleosome with <40 bps of flanking DNA. INO80's activity is also regulated by its various protein subunits, for example without the Arp5 module INO80's sliding activity is greatly inhibited while without the Nhp10 module INO80 loses its flanking DNA length-sensing ability. Another important level of regulation is whether INO80 is spatially and temporally available and functional in the cell. INO80, being a large complex composed of 15 subunits, like many other large multisubunit complexes, requires proper assembly and coordination of its components to be functional. One way the cell typically handles this issue is with chaperones, which assist client proteins with proper folding and with making proper protein-protein contacts. Our lab has previously shown that the Rvb1/Rvb2 AAA+ ATPase may serve as a chaperone in the context of INO80, where it can recognize a specific domain in the core ATPase of INO80 as a unique client. Here we explore the chaperone role of Rvb1/Rvb2 further by building on our reconstituted in vitro system of INO80 assembly.

3.2 Introduction

Rvb1 and Rvb2 are two AAA+ ATPases that are conserved from yeast to humans and essential for viability (Nano and Houry, 2013). They contain the canonical AAA+ Walker A and Walker B domains for ATP binding and hydrolysis, respectively, and ~170 amino

acid extension domains referred to as DII domains (**Fig 3.1A**). Rvb1 and Rvb2 have been linked to many important cellular processes including transcription, mitosis, cell signaling, snoRNP assembly, and DNA-damage repair (Nano and Houry, 2013). Although they are involved in many key biological processes and there is a plethora of structural information about the Rvbs, their biochemical mechanism remains under characterized.

The Rvbs exhibit two different stoichiometries, a hexamer and a dodecamer, that can be modulated by the presence of a tags (Gorynia et al., 2011; Niewiarowski et al., 2010; Puri et al., 2007). *S. cerevisiae* Rvb1/Rvb2 typically exists as a heterohexamer, yet a small proportion exist in the dodecameric state (Ewens et al., 2016; Jeganathan et al., 2015). *C. thermophilum* and human Rvb1/Rvb2 have a greater proportion in the stable dodecameric state (Lakomek et al., 2015; Niewiarowski et al., 2010). It remains unclear what role the different stoichiometric states play in the activity of Rvb1/Rvb2. Most available structures of Rvb1/Rvb2 alone show a dodecameric state, most likely because samples were prepared with N-terminally tagged proteins or with human or *C. thermophilum* proteins (Cheung et al., 2010; Ewens et al., 2016; Gribun et al., 2008; Lakomek et al., 2015; Puri et al., 2007; Torreira et al., 2008) (**Fig 3.1B**). However, in all existing structures of the Rvbs in complex with multisubunit complexes, including R2TP, INO80, and SWR1, Rvb1/Rvb2 form a heterohexameric complex with the DII domains forming an interaction interface with other subunits (Aramayo et al., 2018; Ayala et al., 2018; Eustermann et al., 2018; Kunert et al., 2022; Martino et al., 2018; Rivera-Calzada et al., 2017; Tian et al., 2017; Willhoft et al., 2018; Wu et al., 2023) (**Fig 3.1B**).

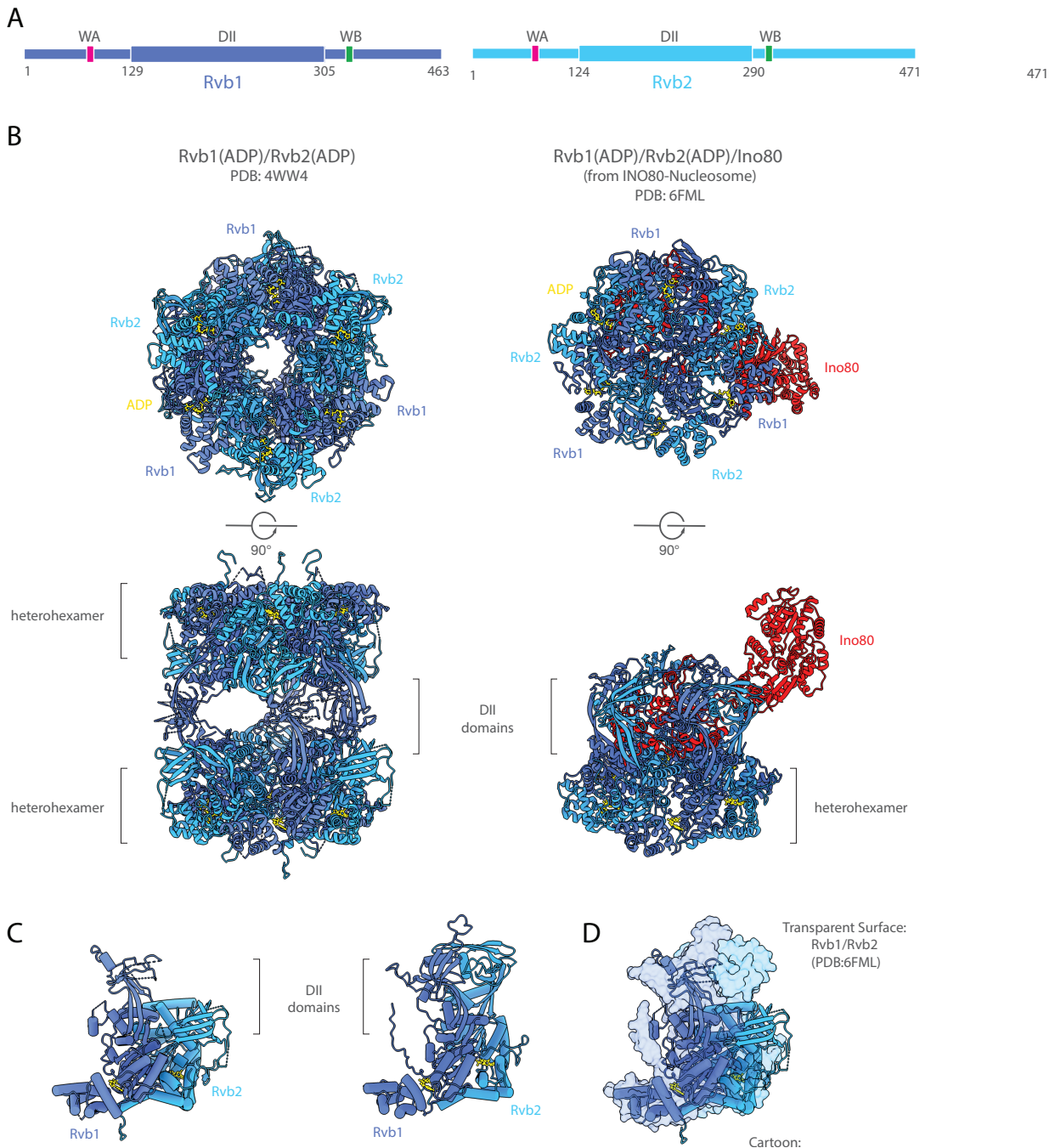


Figure 3.1 Rvb1 and Rvb2 domain architecture and structure. (A) Domain architecture of Rvb1 and Rvb2 highlighting their conserved Walker A (WA) and Walker B (WB) motifs and their unique DII extension domains. (B) Crystal structure of *C. thermophilum* Rvb1(ADP)/Rvb2(ADP). PDB: 4WW4. (C) Structure of *C. thermophilum* Rvb1(ADP)/Rvb2(ADP) hexamer and Ino80 from a structure of INO80-nucleosome. Other core subunits and the nucleosome not shown. PDB: 6FML. Note that the DII extension domains interact with Ino80. (D) Zoomed in view of a single Rvb1/Rvb2 dimer from (A) and (B) to showcase the DII conformational change. (E) Overlay of the two structures shown in (C) with PDB:4WW4 shown as a cartoon and PDB:6FML shown as a transparent surface.

The initial hypothesis that the Rvbs may serve as protein assembly chaperones came from the observation that the Rvbs co-purify with many multisubunit complexes (Nano and Houry, 2013). Molecular chaperones can be defined as proteins that assist in non-covalent folding and unfolding, and assembly and disassembly of macromolecular structures, but are not permanent components of these structures (Ellis, 2013). Some, but not all chaperones, including Hsp90, also exhibit stimulation of ATP hydrolysis by client proteins. Recently, work from our lab has shown chaperone-like activity of the Rvbs in the context of the INO80 chromatin remodeler (Zhou et al., 2017). Rvb1/Rvb2 co-purifies with the insertion domain of the core ATPase subunit of the INO80 chromatin remodeler, Ino80INS and in the presence of the Ino80INS, Rvb1/Rvb2 hydrolyzes ATP 16-fold times faster than Rvb1/Rvb2 alone. The Ino80INS domain also appears to stabilize the dodecameric state, which appears to be a potential intermediate in the Rvb1/Rvb2 ATPase cycle. This may explain why Rvb1/Rvb2 has increased ATPase activity in the presence of the Ino80INS.

Recent cryo-EM structures show that the intact functional INO80 complex contains an ADP-bound Rvb1/Rvb2 hexamer stably bound to Ino80 through interactions between the DII domains of Rvb1/Rvb2 and the Ino80INS (Aramayo et al., 2018; Ayala et al., 2018; Kunert et al., 2022). However, the dodecameric Rvb1/Rvb2/Ino80INS complex was shown to be metastable and upon ATP hydrolysis, the Rvb1/Rvb2 hexamers and Ino80INS fall apart. This led to the question of whether Ino80 domains which are not present in Ino80INS, such as the RecA-like lobes, or other core subunits of INO80 could potentially stabilize this interaction. This data led to a working model in which

Rvb1/Rvb2 normally cycle through a hexameric and dodecameric state as they hydrolyze ATP. In the presence of a protein client, like the Ino80 insertion domain, the cycling is increased leading to stimulated ATPase activity and multiple opportunities for other clients, such as core INO80 subunits, to interact with Rvb1/Rvb2 (**Fig 3.2A**).

This model led to a few open questions which we explored here: (1) do other subunits of INO80 act as Rvb1/Rvb2 clients, (2) do other domains of Ino80 influence Rvb1/Rvb2 activity and stoichiometry, and (3) do interactions with other Ino80 domains or subunits result in stable interaction of Rvb1/Rvb2 and Ino80 post ATP hydrolysis (**Fig 3.2B**)?

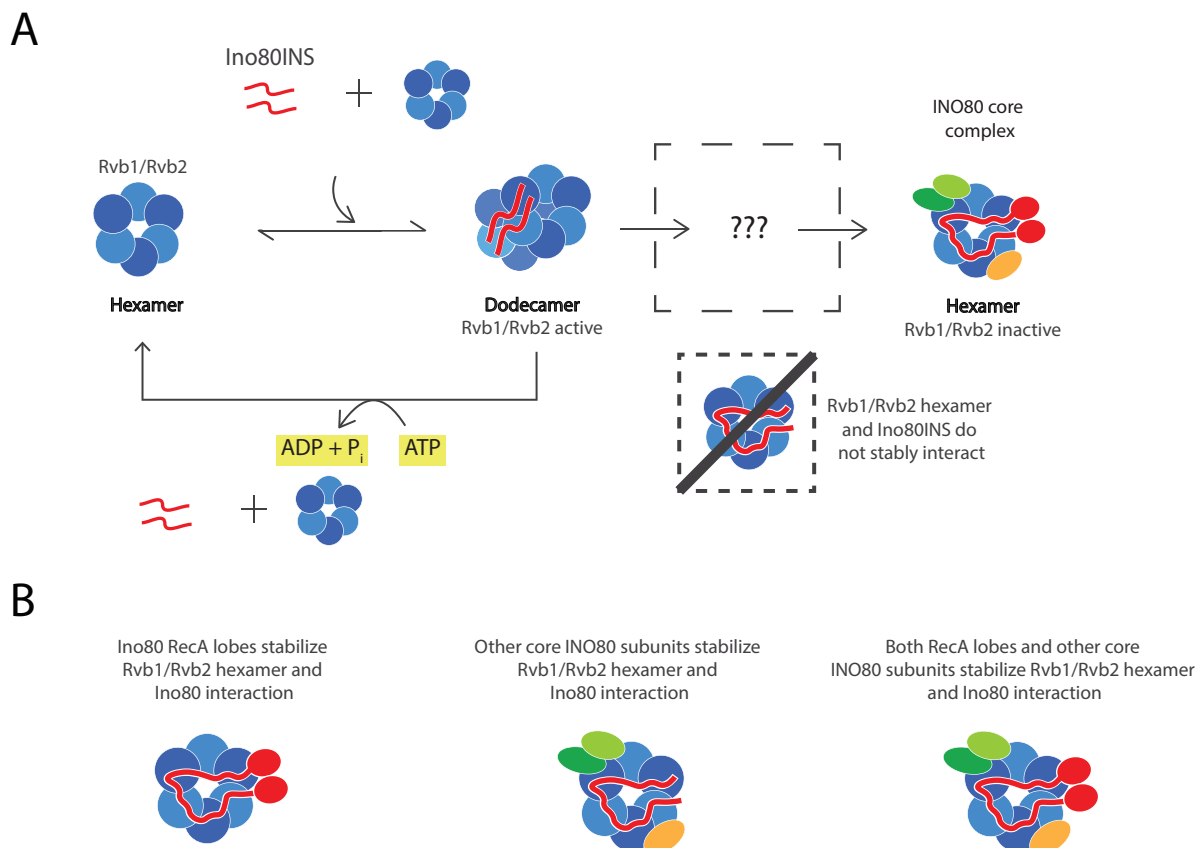


Figure 3.2 Model of Rvb1/Rvb2-mediated INO80 assembly based on previous work. (A) Cartoon model adapted from Zhou et al., 2017. **(B)** Cartoon depictions of three possible models for stabilizing the interaction between the Rvb1/Rvb2 hexamer and Ino80.

3.3 Results

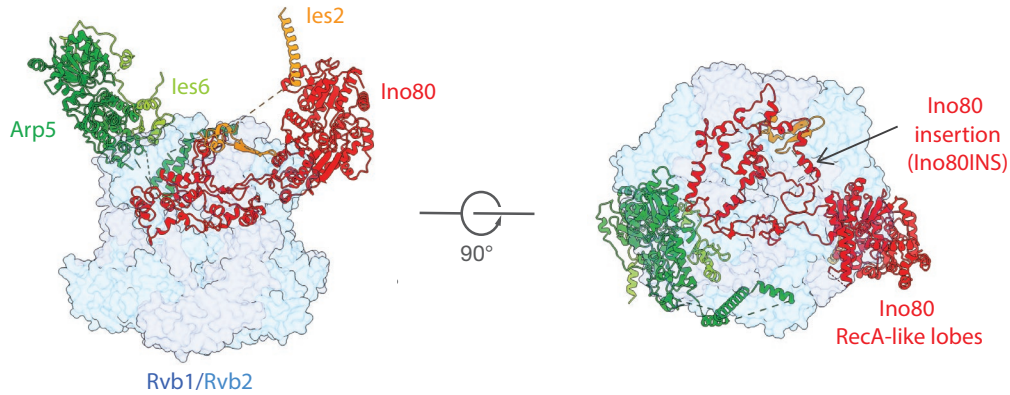
3.3.1 INO80 core components are not Rvb1/Rvb2 chaperone clients

Recently, our lab characterized the Ino80 and Swr1 insertion domains as the first protein clients of Rvb1/Rvb2 (Zhou et al., 2017). Currently, no additional protein clients have been identified. According to recent INO80 and INO80-nucleosome structures, core INO80 components, including the Arp5 module, consisting of Arp5 and Ies6, and the Ies2 subunit interact with the Rvb1/Rvb2 DII domains and Ino80 insertion domain (Aramayo et al., 2018; Ayala et al., 2018; Eustermann et al., 2018) (**Fig 3.3A**). This suggested that these subunits may also be chaperone clients for Rvb1/Rvb2. We asked this by assaying Rvb1/Rvb2 ATP hydrolysis in the absence and presence of these subunits. We found that under the conditions tested, the Arp5 module (Arp5/Ies6) or Ies2 did not stimulate Rvb1/Rvb2 activity (**Fig 3.3B**).

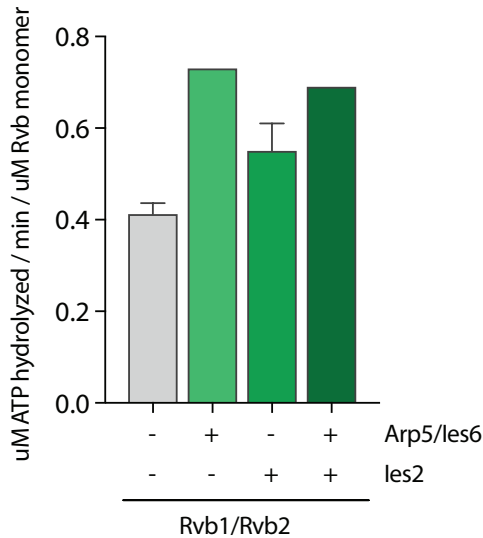
It was unclear whether these components properly interacted with Rvb1/Rvb2 alone as they appear to form contacts with the Rvb1/Rvb2 DII domains which adopt very different conformations in the full INO80 complex compared to Rvb1/Rvb2 alone (**Fig 3.1C, Fig 3.3A**). Additionally, our working model suggested that the interaction between Rvb1/Rvb2 and the Ino80 insertion domain promotes cycling between a dodecameric and hexameric state that may be a window for opportunity to interact with other clients, including other core INO80 subunits (**Fig 3.2**). Therefore, we asked whether the Arp5 module and Ies2 could stimulate Rvb1/Rvb2 in the context of Rvb1/Rvb2/Ino80INS. While we see the characteristic stimulation of ATP hydrolysis of Rvb1/Rvb2 in the presence of Ino80INS, we saw no change in ATP hydrolysis activity upon addition of the

other components (**Fig 3.3B,C**). These results support that while the insertion domains of INO80 chromatin remodelers, Ino80INS and SWR1INS, are unique clients of Rvb1/Rvb2, other core INO80 subunits are not.

A



B



C

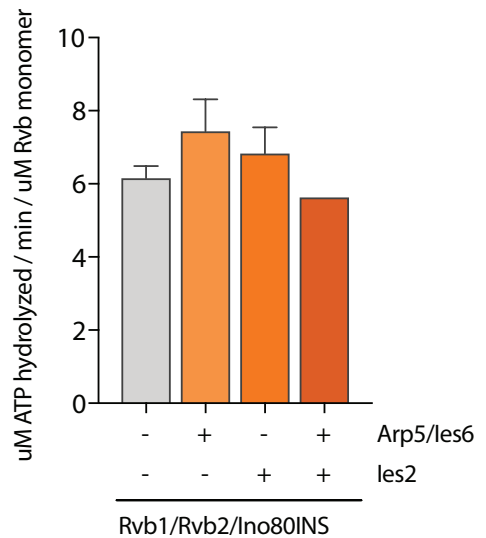


Figure 3.3 Core INO80 subunits, Arp5/les6 and les2, are not Rvb1/Rvb2 clients.

(A) Structure of INO80core from PDB:6FML highlighting the core subunits, Arp5/les6 and les2, that interact with Rvb1/Rvb2 and Ino80INS. (B) Comparison of k_{obs} values for ATP hydrolysis by Rvb1/Rvb2 in the presence of Arp5/les6 and/or les2 added in trans. (C) Comparison of k_{obs} values for ATP hydrolysis by Rvb1/Rvb2 in the presence of Arp5/les6 and/or les2 added in trans. Error bars represent the mean $\pm$ SEM of three replicates. Three conditions were performed in duplicate and averaged: Rvb1/Rvb2 + Arp5/les6, Rvb1/Rvb2 + Arp5/les6 + les2, and Rvb1/Rvb2/Ino80INS + Arp5/les6 + les2. Experiments contained 500 nM Rvb1/Rvb2 (determined by Rvb monomer concentration, ~83 nM hexamer) and 250 nM Arp5/les6 and/or 250 nM les2 when indicated.

Importantly, to validate that the Arp5 module and les2 components were folded properly, we utilized an endogenous INO80 complex purified from a yeast strain missing the *ies2* gene. This complex, Ino80 Δ ies2, does not contain the les2 and Arp5 module and exhibits inhibited nucleosome-remodeling activity (Yao et al., 2015) (**Fig 3.4A**). Upon addition of the Arp5 module and les2 in trans, we see that activity is partially rescued to that of wildtype INO80 activity (**Fig 3.4A**). This suggests that the components are at a minimum folded correctly and can interact with Rvb1/Rvb2 complexes. Adding the components in trans may not fully rescue INO80 activity due to the C-terminal FLAG tag on Arp5 and/or the N-terminal MBP tag and C-terminal His tag on les2. We do see

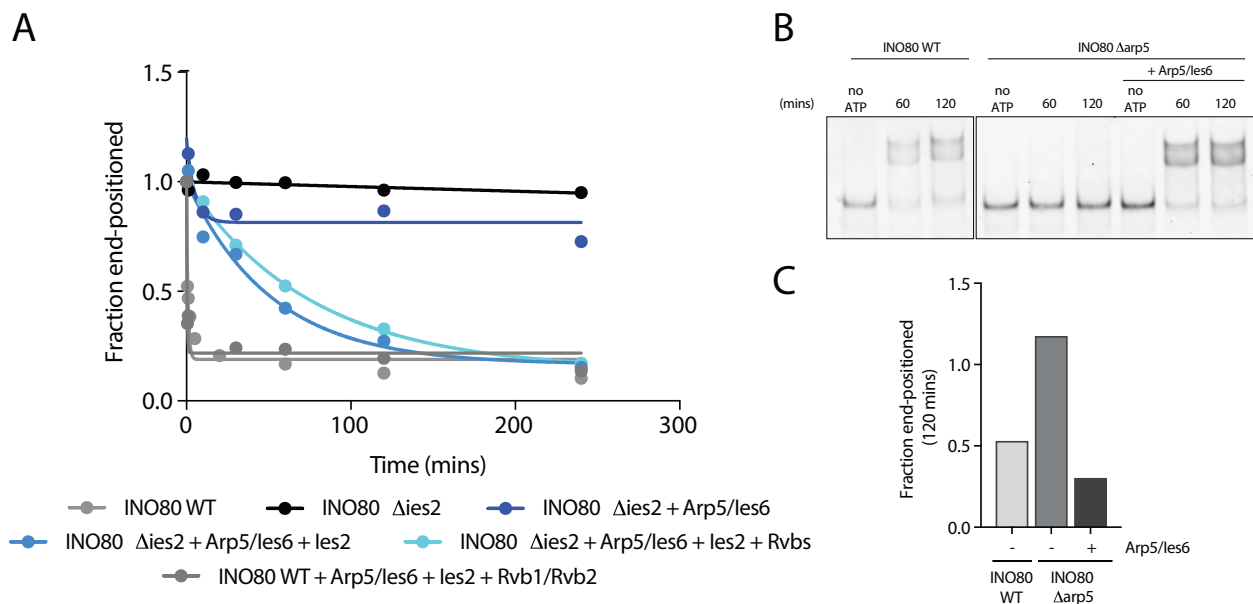


Figure 3.4 Arp5/les6 and les2 added in trans can rescue INO80 Δ ies2 and INO80 Δ arp5 remodeling activity. (A) Time course of native gel-based remodeling assays of INO80 WT or INO80 Δ ies2 on +80 nucleosomes with purified Arp5/les6, les2, and/or Rvb1/Rvb2 added in trans. (B) Native gel-based remodeling assay of INO80 WT or INO80 Δ arp5 on +80 nucleosomes with or without addition of Arp5/les6 in trans. (C) Quantification of end-positioned nucleosomes at 120 minutes for conditions shown in (B). Reactions were done with 10 nM nucleosomes and either 30 nM INO80 WT or 90 nM INO80 Δ ies2 or INO80 Δ arp5. 180 nM Arp5/les6, 180 nM les2, and 180 nM Rvb1/Rvb2 hexamer were added where indicated.

however that addition of FLAG-tagged Arp5/les6 in trans to an INO80 complex only missing Arp5/les6 (INO80 Δ arp5) shows a similar fraction of nucleosomes slid compared to wild-type INO80 after 120 minutes, however more timepoints are required to determine if there is effect on the overall rate of sliding. Additional experiments with untagged subunits would be ideal to explore this further.

3.3.2 Ino80 RecA-like lobes influence Rvb1/Rvb2 activity

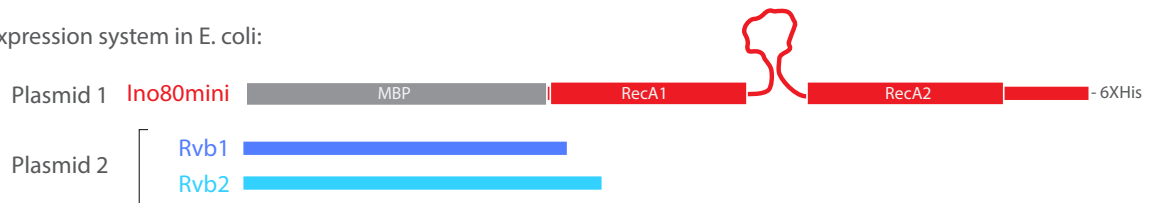
While the Ino80 insertion domain interacts with and modulates Rvb1/Rvb2 activity and conformation, the INO80 complex contains a full-length Ino80 protein which includes the Ino80 insertion domain flanked by two RecA-like lobes that form an ATPase active site and an N-terminus which includes a helicase-SANT-associated (HSA) domain (**Fig 1.3A**). To explore the mechanism of Rvb1/Rvb2 further, we built on our minimal system in *E. coli* to co-express Rvb1, Rvb2, and an MBP- and His-tagged truncated Ino80 (Ino80_{mini}), which included residues 698-1040 and consisted of the two RecA-like lobes and insertion domain (**Fig 3.5A**). All three components co-purified and remained in a stable complex over a size-exclusion column (**Fig 3.5B**). Attempts at purifying Ino80_{mini} alone with or without an MBP tag were not successful suggesting that Ino80_{mini}, like Ino80INS, requires Rvb1/Rvb2 for stability and solubility. We refer to the copurified complex as Rvb1/Rvb2/Ino80_{mini}.

We first asked if the complex had ATPase activity and how it compared to the activity of Rvb1/Rvb2 alone and Rvb1/Rvb2/Ino80INS. Rvb1/Rvb2/Ino80_{mini} showed ATP hydrolysis activity that was in between that of Rvb1/Rvb2 alone and Rvb1/Rvb2/Ino80INS showing ~10-fold stimulation in activity compared to Rvb1/Rvb2

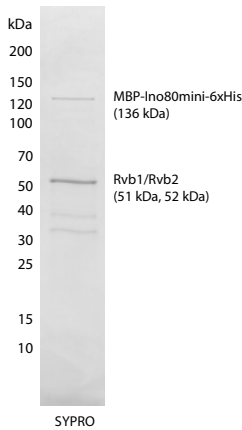
alone but a ~2-3-fold less than that of Rvb1/Rvb2/Ino80INS (**Fig 3.5C**). This suggested that the presence of the RecA-like lobes may inhibit the stimulatory effect of the Ino80 insertion domain on Rvb1/Rvb2 ATP hydrolysis activity. Interestingly, the ~10-fold stimulation is similar to the increase in activity of Rvb1/Rvb2 when their DII extension domains were truncated (Zhou et al., 2017), suggesting that Ino80_{mini} may promote a conformational change in the DII domains.

A

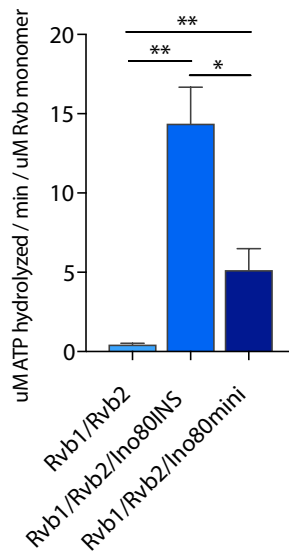
Co-expression system in *E. coli*:



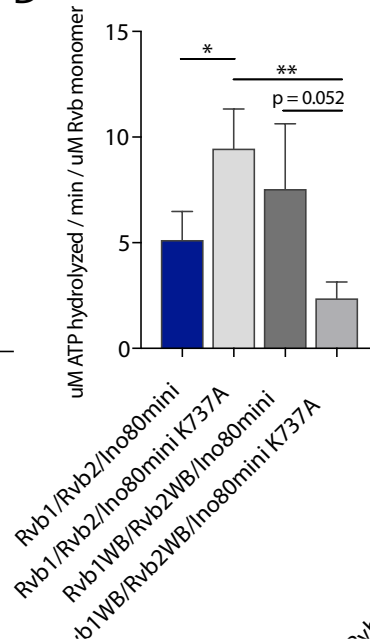
B



C



D



E

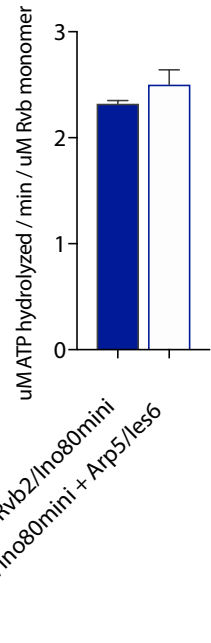


Figure 3.5 Rvb1/Rvb2 and Ino80_{mini} form a complex that is active. (A) Diagram of co-expression system of Rvb1/Rvb2 and Ino80_{mini} in *E. coli*. (B) SDS-PAGE gel of purified Rvb1/Rvb2/Ino80_{mini} complex. (C) Comparison of k_{obs} values for ATP hydrolysis by Rvb1/Rvb2, Rvb1/Rvb2/Ino80INS, and Rvb1/Rvb2/Ino80_{mini}. (D) Comparison of k_{obs} values for ATP hydrolysis by Rvb1/Rvb2/ Ino80_{mini}, Rvb1WB/Rvb2WB/ Ino80_{mini}, Rvb1/Rvb2/ Ino80_{mini}K737A, and Rvb1WB/Rvb2WB/ Ino80_{mini}K737A. (E) Comparison of k_{obs} values for ATP hydrolysis by Rvb1/Rvb2/ Ino80_{mini} with or without Arp5/les6 added in trans. Experiments contained 500 nM Rvb1/Rvb2 (determined by Rvb monomer concentration, ~83 nM hexamer) and 250 nM Arp5/les6 when indicated.

Error bars represent the mean $\pm$ SEM of three replicates.

* represents $p \leq 0.05$, ** represents $p \leq 0.01$.

A major question that arose in this system because of the presence of the Ino80 RecA-like lobes is whether the Ino80_{mini} component of the Rvb1/Rvb2/Ino80_{mini} complex could hydrolyze ATP. To get at this question, we made a complex containing ATPase-deficient WB mutants of Rvb1 and Rvb2, Rvb1WB/Rvb2WB/Ino80_{mini}. This complex exhibited ATPase activity, suggesting that Ino80_{mini} was functional (**Fig 3.5D**).

If the activity of the complex was solely coming from Ino80_{mini}, we hypothesized that a complex containing an ATPase-deficient mutant of Ino80_{mini} would show little to no ATP hydrolysis activity. To test this, we made a complex containing an ATPase-deficient mutant of Ino80_{mini}, Rvb1/Rvb2/Ino80_{mini}K737A. This complex showed a small but consistent ~2-fold increase in ATPase activity compared to the wild-type complex (**Fig 3.5D**). This suggested that a functional Ino80 active state may be important in regulating the Rvb1/Rvb2 ATPase activity. Importantly, a complex with all mutant components, Rvb1WB/Rvb2WB/Ino80_{mini}K737A, showed background ATPase activity significantly less than all complexes tested indicating that the main ATPase activities we were observing in the system were coming from our proteins of interest.

3.3.3 Rvb1/Rvb2 dodecameric state is stabilized by Ino80_{mini}

Previous work showed that an increase in ATPase activity of Rvb1/Rvb2 correlated with promoting a dodecameric state of Rvb1/Rvb2 composed of two heterohexamers (Zhou et al., 2017). With Rvb1/Rvb2/Ino80_{mini} showing an intermediate ATPase activity that is greater than that of Rvb1/Rvb2 alone but less than Rvb1/Rvb2/Ino80INS, we asked what the stoichiometry of the complex was. First, by comparing band intensity on an

SDS-PAGE gel, the ratio of Rvb proteins to Ino80_{mini} was ~13:1 (**Fig 3.5B**). We then attempted to measure the approximate mass of our species using SEC-MALS and AUC, which both proved difficult. By SEC-MALS, Rvb1/Rvb2 gave a clear peak and mass of ~345 kDa suggestive of a hexamer. However, Rvb1/Rvb2/Ino80INS and Rvb1/Rvb2/Ino80_{mini} showed much broader peaks with heterogenous masses ranging from ~1-3 Mda. By AUC Rvb1/Rvb2 appeared to exist mostly as a single well-defined species, while Rvb1/Rvb2/Ino80INS existed as various much larger species with sedimentation coefficients ranging from 20-100. Attempts at running AUC experiments with Rvb1/Rvb2/Ino80_{mini} were limited by the amount of protein available. These techniques required large quantities of homogenous protein that was difficult to obtain using this system, so we turned to cryo-EM which. We prepared various samples with different Rvb1/Rvb2/Ino80 complexes and nucleotides, yet a Rvb1WB/Rvb2WB/Ino80_{mini} sample prepared with ADP·MgCl₂ appeared to be the most stable sample tested (**Fig 3.6A**). Two 3D classes were obtained, class 1 which looked like a Rvb1/Rvb2 dodecamer and class 2 which looked like a possibly more flexible or bent Rvb1/Rvb2 dodecamer with additional density that could represent a very dynamic or flexible Ino80_{mini} (**Fig 3.6A**). At the current resolution, it is not possible to dock Ino80_{mini} into the density maps, but what we can see suggests that at least one copy of Ino80_{mini} may interact with a Rvb1/Rvb2 dodecamer in an asymmetric manner. This is interesting because it suggests that although the Rvb1/Rvb2 dodecamer conformation is stabilized in the context of Ino80_{mini}, the ATPase stimulation is not as great as that of the Rvb1/Rvb2 dodecameric complex containing only the Ino80 insertion domain. This suggests that there is something more nuanced occurring in the system due to the

presence of the RecA-like lobes that affects the ATPase activity of the complex apart from simply promoting the dodecameric state of the Rvbs. We speculate that these

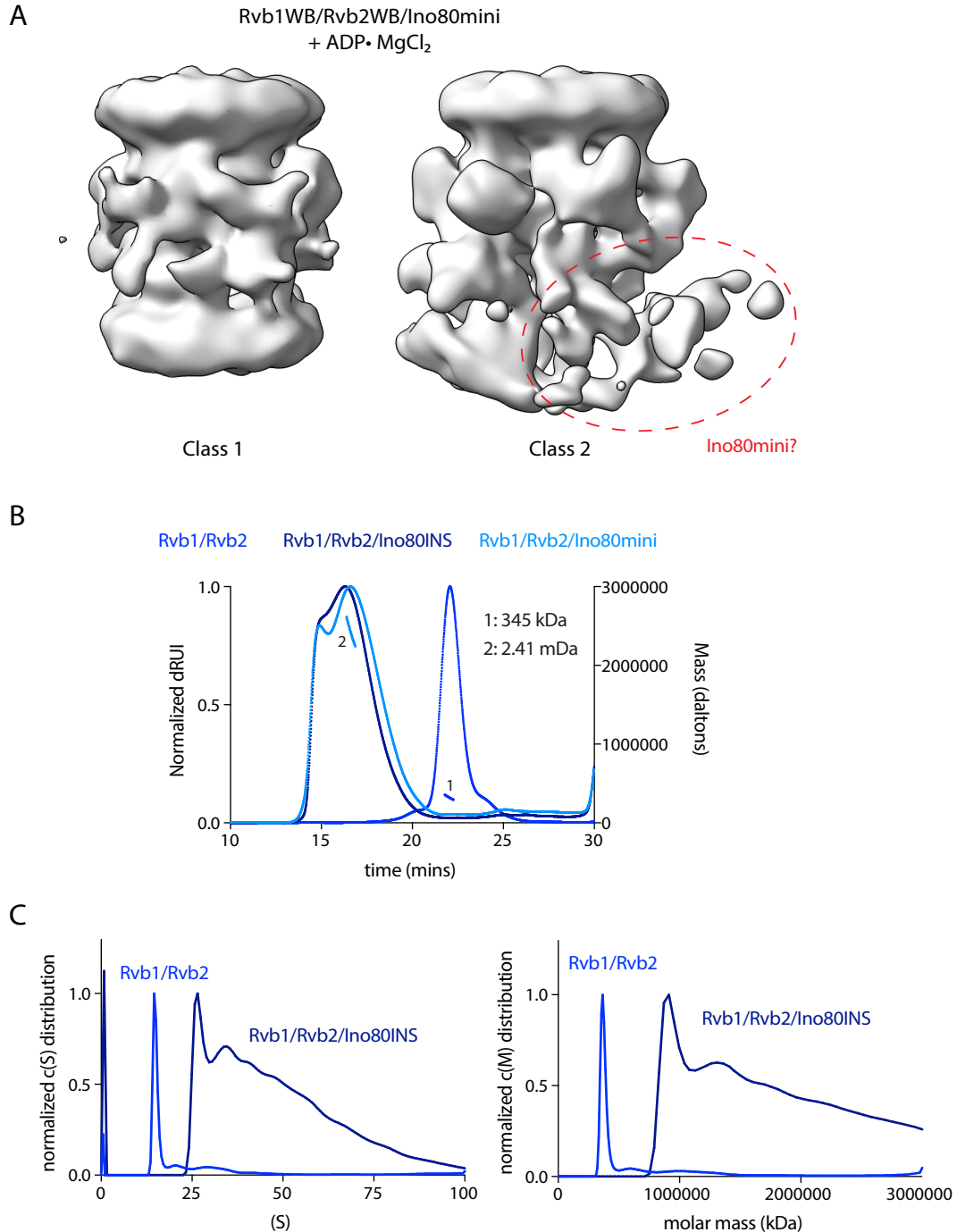


Figure 3.6 Understanding Rvb1/Rvb2/Ino80_{mini} stoichiometry. (A) Low-resolution cryo-EM maps for two classes of Rvb1/Rvb2/Ino80_{mini} in the presence of ADP•MgCl₂. (B) SEC-MALS of Rvb1/Rvb2, Rvb1/Rvb2/Ino80INS, and Rvb1/Rvb2/Ino80_{mini}. Normalized differential refractive index (left y axis) and molecular weight of the indicated peaks (right y axis) are plotted against time (mins) of elution. (C) Sedimentation velocity analytical ultracentrifugation of Rvb1/Rvb2 and Rvb1/Rvb2/Ino80INS.

effects on ATPase activity are due in part to conformational changes of the DII domains mediated by the different Ino80 clients.

3.3.4 *Arp5* module does not influence *Rvb1/Rvb2/Ino80_{mini}* activity

To test whether the presence of the Ino80 RecA-like lobes influences the interaction with other core INO80 components, we added Arp5/les6 in trans with Rvb1/Rvb2/Ino80_{mini} and assayed for ATP hydrolysis. We saw no influence on activity (**Fig 3.5E**), suggesting that if there is a physical interaction there is no influence on the ATP hydrolysis activity of the Rvbs or Ino80_{mini}. Alternatively, the concentration required for a physical interaction may be higher than what was assayed. It is important to note that other components like les2 may be necessary for proper interaction at this stage of complex assembly. It is also possible that the core components may need to all co-fold together to form stable interactions.

3.4 Discussion

The Rvbs are integral components of INO80. Our work suggests that the Rvbs ATPase activity plays an important role in the initial nucleation of complex assembly. The Rvbs ATPase activity is greatly stimulated by the insertion domains of Ino80 and Swr1 but not by other components of the core INO80 complex, including Arp5/les6 and les2. This suggests that Arp5/les6 and les2 are not clients of Rvb1/Rvb2. Alternatively, the Rvbs may need to cofold together with these other components to form the interactions necessary for ATPase stimulation. The Ino80 and Swr1 client insertion domains are unique to INO80 chromatin remodeling complexes and split the functional ATPase lobes of Ino80 and Swr1. Structures of INO80 and SWR show these insertion domains thread

through the Rvb DII domains to form an interaction platform for other core subunits. This data suggests a model where the initial nucleating step in assembly of the INO80-family remodelers is an interaction between the insert region of the core ATPase subunits, Ino80 or Swr1, and the Rvbs. This initial interaction may then lead to the threading of the insertion domains through the Rvb DII domains and the concomitant “closure” of the RecA-like lobes of the Ino80 or Swr1 ATPase (**Fig 3.7**).

Here, we also show that the Rvbs are stimulated by a mutant Ino80_{mini}K737A (**Fig 3.5D**), where the nucleotide binding site of Ino80 is disrupted. This suggests that Ino80 K737A may not be able to adopt its active, or properly “closed” conformation. At this stage, it is not clear from the literature whether K737A affects nucleotide binding or hydrolysis. Regardless, the two clients that stimulate the Rvbs ATPase activity the most are a partial domain of Ino80, Ino80INS, or an ATPase-deficient Ino80_{mini}, Ino80_{mini}K737A. We speculate that the Rvbs may go through cycles of ATP hydrolysis upon recognizing a misfolded or non-functional Ino80 client. In cases when the client is persistently misfolded or non-functional, for example due to a truncation or active site mutation, this would result in futile ATPase cycles. In this way, Rvb1/Rvb2 may serve as a quality control sensor for proper folding of a functional Ino80 client (**Fig 3.7**), and the cycling of ATPase activity could be a way to disassemble non-functional complexes.

This idea is supported by data showing that the Rvb1/Rvb2/Ino80INS complex falls apart upon ATP hydrolysis by the Rvbs (Zhou et al., 2017). Based on this model, we would hypothesize that the same may be true in the context of the Rvb1/Rvb2/Ino80_{mini}K737A complex, but this remains unexplored.

We speculate that an ATPase-competent Ino80 active site may inhibit ATP hydrolysis by the Rvbs, allowing for stable association of the Rvbs and the Ino80INS and providing a proper loading dock for the other INO80 core components (**Fig 3.7**). Interestingly, structures of all complexes containing the Rvbs always show the Rvbs in their ADP-bound state. This supports a model in which proper client interaction may block ADP release from the Rvb active sites and could explain how the proper client could inhibit the ATPase cycling and concomitant complex disassembly by the Rvbs. This idea of blocking ADP release by a conformational change in the N-terminus of Rvb2 has been discussed in two previous studies (López-Perrote et al., 2012; Muñoz-Hernández et al., 2019), suggesting it may be an avenue to further explore in the context of INO80 assembly by Rvb1/Rvb2.

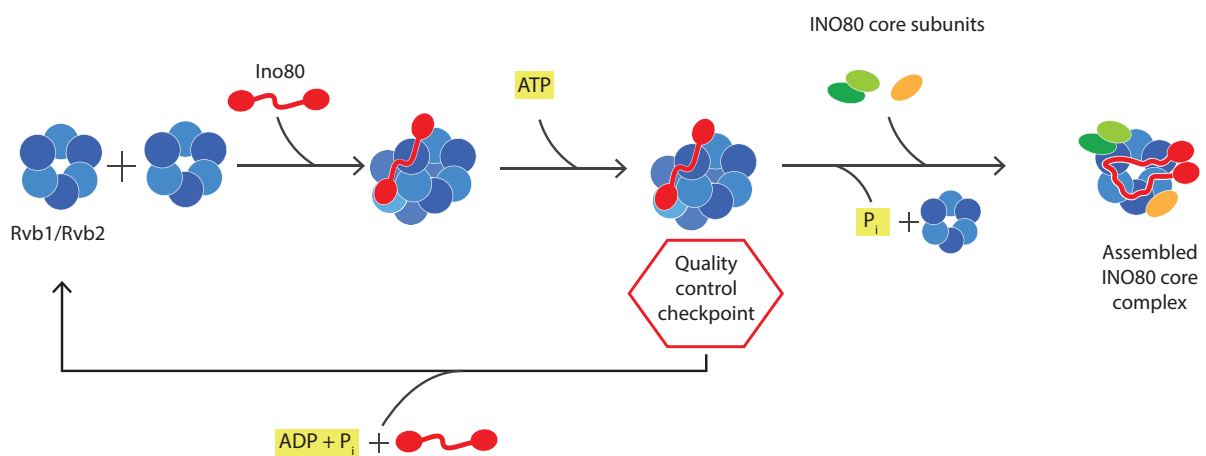


Figure 3.7 Speculative model of Rvb1/Rvb2-mediated assembly and quality control of INO80. Cartoon model showing how Rvb1/Rvb2 may act as a sensor for correctly folded and functional client, Ino80. If the client is not properly assembled, the complex may fall apart upon ATP hydrolysis by Rvb1/Rvb2. If the client is properly assembled, Rvb1/Rvb2 ATPase activity may instead be inhibited leading to a stable Rvb1/Rvb2/Ino80 interaction and association of other core INO80 subunits. This model is informed by data presented here.

This model is suggestive of a protein quality control sensor, which may be applicable to other systems as well. In the R2TP complex for instance the ATP hydrolysis of the Rvbs releases certain components from the snoRNPs that the Rvbs are helping chaperone. In the case of the Rvbs, ATP hydrolysis can be a form of altering interactions with clients which is the case with other chaperones like Hsp90. In addition, the Rvbs may use their nucleotide state as a readout for proper intermediate protein interactions and eventual functional complex assembly.

Further building on this minimal system would be very powerful, however the current *E. coli* coexpression system limits the amount of usable material to study. Building this system in a different expression system, such as Sf9 or mammalian cells, that would allow for more material and consistent expression would be ideal. While we did not have the time to explore this further, Sf9 expression plasmids for a variety of different complexes explored here have been constructed. Additionally, a recent study focusing on human INO80 showed that a subcomplex containing human Rvb1/Rvb2 and truncated Ino80, containing the RecA-like lobes, insertion domain, and HSA domain, could be co-expressed in Sf9 cells (Willhoft et al., 2016). A low-resolution cryo-EM structure of this subcomplex appears to show a Rvb1/Rvb2 hexamer, which may support our model discussed here, where proper client interactions may promote a stable interaction with a Rvb1/Rvb2 hexamer (Aramayo et al., 2018). Further building on an in vitro reconstituted system for INO80 assembly would provide an ideal system to explore the elusive mechanism of Rvb1/Rvb2.

3.5 Materials and Methods

Protein expression and purification

All proteins expressed and purified were from *S. cerevisiae*. The Rvb1/Rvb2 complex was made by co-expressing Rvb1 and 6XHis-Rvb2 on a pET28a-DUET plasmid, a gift from the Hopfner lab, in *Rosetta* (DE3) *E. coli*. Cells were grown in 2X LB (2x peptone, 2x yeast extract, 1X NaCl) to OD₆₀₀ ~0.3-0.4 at 37°C, transferred to 18°C, grown to OD₆₀₀ ~0.6-0.8, induced with 0.3 mM IPTG, and grown for ~18 hours. Cells were harvested and resuspended in lysis buffer (~10 mL/1L cells - 25 mM HEPES pH 7.5, 300 mM KCl, 7.5 mM imidazole, 10% glycerol, 2 µg/mL aprotinin, 1 µg/mL pepstatin, 3 µg/mL leupeptin, and 1 mM PMSF) and lysed using high-pressure on an Emulsiflex-C3 homogenizer for ~20 minutes. Lysates were cleared by spinning at 30,000 x g for 45 minutes and supernatant was incubated with TALON cobalt beads (2 mL slurry per 1L cells) for 1 hour with rotation at 4°C. The beads were then incubated in lysis buffer with 100 µM ATP-MgCl₂ (~25 mL/1L cells) for 30 minutes with rotation at 4°C and transferred to 20 mL disposable columns (Bio-Rad). Beads were washed in 10 column volumes lysis buffer. Protein was eluted in 4 column volumes elution buffer (25 mM HEPES pH 7.5, 300 mM KCl, 500 mM imidazole). TEV protease (homemade) was added to elution at a final concentration of ~0.1 mg/mL and sample was dialyzed overnight into size-exclusion buffer (SEC buffer - 25 mM HEPES pH 7.5, 300 mM KCl, 10% glycerol, 2 mM DTT). Protein was then purified by size-exclusion chromatography on a Superose 6 Increase 10/300 GL column (Cytiva) in SEC buffer. Sample was concentrated, flash-frozen in liquid nitrogen, and stored at -80°C.

Rvb1/Rvb2/Ino80INS and Rvb1/Rvb2/Ino80_{mini} was made by coexpressing untagged Rvb1 and Rvb2 from a pET28a plasmid and either MBP-Ino80 insertion domain (residues 1022-1294)-6XHis or MBP-Ino80mini (residues 699-1490)-6XHis from a pMAL plasmid. The complexes were purified as described above except for the ATP wash and TEV cleavage steps. The protein concentration was determined using a quantitative gel and a BSA protein standard curve. The concentration used for any Rvb-containing complex was the monomeric Rvb concentration (obtained using the average molecular weight of Rvb1 and Rvb2).

MBP-les2-6XHis was expressed from a pACYC plasmid and purified as described above for the Rvb1/Rvb2/Ino80INS complex.

Arp5/les6 was copurified from a *S. cerevisiae* strain in which *ies2* was deleted and Arp5 was endogenously 2X FLAG tagged, a gift from the Morrison lab. Arp5/les6 was purified by FLAG immunoprecipitation following the same protocol as endogenous INO80.

NADH enzyme-coupled ATPase assays

The reactions contained 10 U/mL pyruvate kinase (Sigma), 10 U/mL lactate dehydrogenase (Sigma), 2 mM phosphoenolpyruvate (Sigma), 0.18 mM NADH, 4 mM ATP, 4.5 mM MgCl₂, 150 mM KCl, 25 mM HEPES pH 7.5. The concentrations of Rvb complexes and additional components are described in figure legends. All components of the reaction except for the Rvb component were mixed and incubated at 30°C in a clear bottom 384-well plate (Corning) for 10 minutes. Rvb complex was mixed in and

absorbance was measured at 340 nm and background was simultaneously measured at 420 nm using a SpectraMax iD5 plate reader. Background was subtracted and the data was fit to a linear regression using Prism software. The slope was determined and divided by the Rvb concentration for the rate of ATP hydrolysis per Rvb enzyme (min^{-1}).

Native gel-based remodeling assays

All experiments followed the same protocol described in Chapter 2: Materials and Methods. Concentrations of components added to reactions are described in figure legends.

SEC-MALS

Samples were applied to SEC (using a Shodex PROTEIN KW-804 column) with an Ettan LC system (GE Healthcare) an in-line DAWN HELEOS MALS system and Optilab T-rEX differential refractive index detectors (Wyatt Technology). SEC was performed in 25 mM HEPES pH 7.5, 300 mM KCl, 1 mM DTT. Samples were at 70 μM Rvb1/Rvb2, ~20 μM Rvb1/Rvb2/Ino80INS, or ~10 μM Rvb1/Rvb2/Ino80mini (determined by Rvb monomer concentration). Data were analyzed by ASTRA VI software (Wyatt Technology). BSA standard was run previous to sample injections for internal calibration.

Sedimentation velocity analytical ultracentrifugation

Sedimentation velocity experiments were performed with ~20 μM Rvb1/Rvb2 or Rvb1/Rvb2/Ino80INS (determined by Rvb monomer concentration) in 25 mM HEPES

pH 7.5, 300 mM KCl, 1 mM DTT. Samples were dialyzed overnight at 4°C and loaded into cells containing 12 mm centerpieces (Beckman Coulter). Samples were equilibrated at temperature for one hour under vacuum. Runs were performed at 36,000 rpm for 8 hrs at 30°C in a Beckman XL/A analytical ultracentrifuge. Scans were collected at 280 nm with a radial step size of 0.003 cm at approximately 60-s intervals. Analysis was done using SEDFIT (NIH) software.

Cryo-EM sample preparation and data collection

Samples were prepared at ~5 μ M Rvb1WB/Rvb2WB/Ino80_{mini} (concentration based on Rvb monomer) in 25 mM HEPES pH 7.5, 300 mM KCl, 10% glycerol, 2 mM DTT with addition of 1 mM ADP-MgCl₂. Ideally, future samples should be dialyzed in buffer containing <2% glycerol. For cryo-EM grid preparation, ~3 μ l sample were applied to functionalized GO-amine grids. Preparation of GO-amine grids, grid preparation, sample screening, and data collection were done as described in Chapter 2.6 Materials and Methods (*Electron Microscopy sample preparation and data collection, Amine functionalized GO grids preparation*) with the following modifications. Grids were examined and screened using an FEI Tecnai Arctica operated at 200 kV and equipped with a Gatan K3 camera. The pixel size used was 1.44 Å and the total exposure time was 61.48 s. 1,737 micrographs were collected.

Cryo-EM data collection and processing

Stacks were motion corrected and dose weighted using MotionCor2 (Zheng et al., 2017). The CTF parameters were estimated and all subsequent 2D and 3D

classification were performed using cryoSPARC (Punjani et al., 2017). 3,341,968 initial particles were used for processing. 659,062 of these were used after 2D classification. In brief, particles were picked by cryoSPARC blob picker and were subject to ab-initio reconstruction and multi-round heterogenous refinement. Two classes with features resembling a Rvb1/Rvb2 dodecamer were observed. Class 1 contained 217,607 particles and class 2 contained 219,593 particles.

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