# Regulation of Chromatin Remodeling: Linker DNA and Histones

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ABSTRACT

ATP-dependent chromatin remodeling enzymes (remodelers) regulate access to genomic DNA by reading epigenetic marks such as histone modifications and using the energy of ATP hydrolysis to assemble, reposition, disassemble, and modify the composition of nucleosomes. The catalytic activity of remodeling enzymes is highly regulated by various substrate characteristics including extranucleosomal linker DNA, histone modifications, and linker histones. In this thesis, we developed and employed a synergistic combination of single-molecule biophysical techniques and biochemical approaches to elucidate the mechanisms underpinning the regulation of chromatin remodelers by these substrate features.

The imitation switch (ISWI)-family of remodelers promotes heterochromatin formation and transcriptional silencing by generating regularly-spaced nucleosome arrays. It was previously known that this nucleosome-spacing activity arises from the dependence of nucleosome translocation on the length of linker DNA, but the underlying mechanism remains unclear. We studied nucleosome remodeling by the human ATP-dependent chromatin assembly and remodeling factor (ACF), an ISWI enzyme composed of a catalytic subunit, Snf2h, and an accessory subunit, Acf1. The H4 tail bears significant sequence homology to an autoinhibitory domain, AutoN, present in the catalytic subunit. The presence of an unmodified H4 tail is thought to stimulate catalytic activity by competing with AutoN for a binding site on the ATPase. Our results suggest a mechanism for nucleosome spacing where linker DNA is sensed by the N-terminus of Acf1 and allosterically transmitted to Snf2h through the H4 tail of the nucleosome. For nucleosomes with short linker DNA, Acf1 preferentially binds to the H4 tail, allowing AutoN to inhibit the ATPase activity of Snf2h. As the linker DNA lengthens, Acf1 shifts
its binding preference to the linker DNA, freeing the H4 tail to compete AutoN off the ATPase and thereby activating ACF. This intricate signal transduction between the accessory and catalytic subunits coupled to two distinct substrate features may be a paradigm for this entire class of critical enzymes.

Most of our knowledge on the function of chromatin remodeling complexes has been gleaned from studies using nucleosomes with only core histones. In contrast, physiological chromatin is replete with linker histones, e.g., H1, at a prevalence of approximately one linker histone per nucleosome in differentiated eukaryotic cells. The complex formed by a core nucleosome and linker histone is known as a chromatosome. There has been significant disagreement among prior investigations on the effects of linker histones on remodeling activity, with some studies reporting varying degrees of general repression while others describe qualitative changes in remodeling outcomes. Using single-molecule fluorescence resonance transfer (smFRET), we provided the first direct observation of intact chromatosome remodeling by ACF. Furthermore, we discovered that the presence of linker histones changes the remodeling outcomes of the linker DNA-insensitive SWI/SNF enzymes by preventing nucleosome translocation past the DNA edge.
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seen before and I am so proud of her. I can’t imagine a better partner to go through
life’s journeys with.
CITATION TO PUBLISHED WORK

Portions of this thesis were adapted from the following publications:


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1 INTRODUCTION

1.1 Biology of chromatin remodeling

Eukaryotic genomes are packaged into chromatin, which condenses the roughly two meters of genomic DNA to fit in the micron-size nucleus and regulates access to DNA by cellular machinery that participate in gene regulatory processes such as DNA replication, transcription, and repair (1, 2). The fundamental unit of chromatin is the nucleosome, which is composed of approximately 147 base pairs (bps) of any DNA sequence wrapped 1.7 left-handed superhelical turns around a core octamer of histones (two copies each of H2A, H2B, H3, and H4) (3, 4). In the octamer, four dimers (two copies each of H2A-H2B and H3-H4) are arranged about a two-fold symmetry axis called the dyad, which also corresponds to the center of the nucleosomal DNA fragment (Figure 1). Each histone dimer binds three consecutive minor grooves of DNA with positively charged residues. Adjacent nucleosomes are separated by linker DNA of varying lengths (5).

Since multicellular organisms maintain a common genetic blueprint, the wide variety of cellular phenotypes observed requires dynamic partitioning of the genomic DNA into transcriptionally active and transcriptionally inactive chromatin compartments (6). To address this need, cells have evolved two groups of enzymes that synergistically control chromatin structure: enzymes that covalently modify the DNA or histones and chromatin remodeling complexes (remodelers), which interpret these epigenetic marks and use the energy of ATP hydrolysis to assemble, disassemble, reposition, and modify the composition of nucleosomes (2, 7).

All chromatin remodelers share four basic properties: (i) ATPase subunit that is homologous to the superfamily 2 helicases, (ii) specific affinity for nucleosomes over
naked DNA, (iii) domains that recognize histone modifications, and (iv) accessory domains and/or subunits that modulate the function of the ATPase domain, facilitate enzyme targeting, and/or interact with other DNA-binding proteins. Five families of chromatin remodeling complexes have been identified that are distinguished by unique domains residing in their catalytic ATPases (Figure 2) (2, 8, 9): (i) ISWI remodeling complexes facilitate chromatin assembly, optimize nucleosome spacing to repress transcription and promote higher-order chromatin structure, enable DNA replication through heterochromatin, and assist the DNA damage response (10-13); (ii) SWI/SNF remodelers have many activities, including nucleosome sliding and ejection to create nucleosome-free regions but lack roles in chromatin assembly (14, 15); (iii) the CHD family of remodelers is diverse, with some members that promote transcription and others that have repressive roles (16); (iv) INO80/SWR1 remodelers have been shown to promote transcriptional activation, DNA replication and repair, and replacement of canonical histones with histone variants (17-20); and (v) ATRX remodelers bind to DAXX and catalyze the deposition of H3.3-containing nucleosomes (21). Within each family, different members can be distinguished by their distinct combination of accessory subunits.

Chromatin remodelers are involved in virtually every process that requires access to genomic DNA, including but not limited to chromatin assembly, DNA repair, replication, recombination, transcription, embryonic development, chromosome segregation, and tumor suppression (2, 8, 22-30). Remodelers have programmatic roles during development and are essential for the establishment and maintenance of pluripotent and multipotent states in cells (31-33). Defects in chromatin remodeling are directly linked to many diseases (34, 35), including multisystem developmental
disorders such as Williams-Beuren syndrome and CHARGE syndrome (36-40) and a variety of malignances such as ovarian, breast, lung, pancreatic, and prostate cancers (41-64). Indeed, a meta-analysis of 24 whole-exome sequencing studies demonstrated that mutations in SWI/SNF genes are widespread across a broad range of cancers, with an overall frequency approaching that of TP53 (19% in SWI/SNF, 26% in TP53) and chromatin remodelers have both causal and enabling roles in the progression to advanced cancers (65).

To date, most of the attention in the potential pharmacological modulation of chromatin remodeling has been focused on enzymes that covalently modify the DNA or histones (66). However, it is increasingly recognized that ATP-dependent chromatin remodeling complexes are promising therapeutic targets (67). For example, while cancer cells typically express SNF2L, an ISWI catalytic subunit, at similar levels compared with non-malignant cells, SNF2L siRNA inhibition had a selective effect on the cancer cells in terms of significant growth inhibition, DNA damage, and apoptosis (68). Given that cancer cell lines often have reduced expression of other chromatin remodeling complexes compared with normal lines, cancer cells may be more sensitive to SNF2L knockdown because of insufficient compensation from other remodelers, which could be described as a form of synthetic lethality. Nevertheless, it is clear that developing novel therapeutics to combat diseases caused by remodeler dysfunction will require a better understanding of the biophysical and biochemical principles underlying the function and regulation of chromatin remodelers.
1.2 Single-molecule biophysics

Elucidating the mechanisms and regulation of chromatin remodeling is a significant challenge because chromatin remodelers are multi-protein complexes for which structural information is difficult to obtain and their interactions with chromatin are both biochemical and mechanical in nature. Hence, there is a need for supplementing traditional biochemistry and molecular biology methods with modern imaging techniques and single molecule approaches in a cross-disciplinary, synergistic effort to understand the mechanisms of chromatin remodeling (69).

Single-molecule techniques yield the distribution of values for a given property rather than just the mean value of the property averaged over a large molecular ensemble (70). These distributions provide direct access into the molecular heterogeneity that characterizes complex biomolecules. Ensemble studies are not ideal for multi-step reactions because even if all of the molecules are initially in the same state, the stochastic nature of individual steps rapidly results in complete asynchrony and prevents detailed kinetic analyses. On the other hand, single-molecule experiments can clearly identify and analyze each step because they involve tracking individual molecules in real-time. Moreover, single-molecule approaches can be used to identify unique structural states of a biomolecule, even if they are rare or short-lived. It is also possible to glean time-resolved, nanometer-scale structural information from single-molecule studies, which represents a powerful bridge between traditional biochemical experiments and X-ray crystallography.

Since the advent of the patch clamp and single ion channel recordings in 1976 (71), numerous single molecule techniques have been developed and adapted for application to biological samples. These methods can be broadly divided into two
categories: (1) force-based manipulation and detection, e.g., atomic force microscopy, optical and magnetic tweezers, and (2) fluorescence imaging and spectroscopy, e.g., single particle tracking, single molecule fluorescence resonance energy transfer (smFRET). Low signal-to-noise ratio has been the primary barrier to technical developments in the single-molecule field. In fluorescence imaging and spectroscopy approaches, reduction of the background signal to a suitable level has been achieved by either constraining the illumination to a small volume, e.g., confocal or two-photon microscopy, or a shallow depth, e.g., total internal reflection fluorescence (TIRF) microscopy (72). Technical advances in fluorophore photophysics, cooled charge-coupled devices (CCD), objective lenses, and laser technologies have also contributed to the successful adaptation of single-molecule techniques for biological systems.

Single-molecule approaches have made significant contributions to understanding protein folding (73), transcription (74), replication (75), translation (76), membrane proteins (77), viral biology (78), and molecular motors (79, 80). Within the past decade, the first single-molecule investigations of nucleosomes and chromatin remodeling motors appeared in the literature (81, 82). Using a single-molecule optical trap, Mihardja and colleagues found that a stretching force of only 2-3 pN can disrupt the outer wrap of DNA on the histone core whereas a force of > 20 pN is necessary to disrupt both the inner and outer wrap (83). Lia and colleagues used magnetic tweezers with a single tethered DNA molecule to demonstrate the generation of a confined, negatively supercoiled DNA loop by single RSC complexes (84). In another study, Zhang et al. applied optical tweezers for real-time study of SWI/SNF and RSC complexes on nucleosomes (85). Interestingly, both SWI/SNF and RSC were observed to cause DNA shortening events that were attributed to the formation of DNA loops on
the nucleosome surface. Single-molecule tracking of fluorescently-tagged Rad54 along DNA molecules was performed by two groups (86, 87). Using a single nucleosome unzipping technique, Shundrovsky and colleagues analyzed single nucleosome products after remodeling by SWI/SNF (88). Specifically, this method determines the distance the nucleosome moves with 3 bp precision and can also detect H2A-H2B dimer release, a remodeling activity attributed to SWI/SNF. However, the main drawback of the single nucleosome unzipping technique is that it provides a detailed analysis of an already remodeled nucleosome rather than a real-time measurement of the remodeling process. A study using high-resolution optical tweezers to study the real-time activity of a minimal RSC remodeler on naked DNA characterized the processivity, speed, and step size of the motor (89). They found that the minimal RSC exhibited ~2 bp translocation steps and was capable of moving against forces up to 30 pN, making it one of the most force-resistant motors known.

1.3 Single-molecule fluorescence resonance energy transfer remodeling assay

Prior single-molecule studies of chromatin remodeling have suffered from drawbacks such as limited resolution (> 3 bp) (84, 85, 88), incompatibility with real-time tracking (88), non-physiologic remodelers and non-nucleosomal substrates (89). We overcame these limitations by developing a single-molecule fluorescence resonance energy transfer (smFRET) assay to study the ATP-dependent remodeling of surface-tethered nucleosomes (90-92).

Fluorescence resonance energy transfer (FRET) is the nonradiative transmission of energy from an excited donor chromophore to an acceptor chromophore via long-range, induced dipole-dipole coupling (93, 94). Unlike many other single-molecule
methods, FRET measures molecular motion in the center of mass frame rather than the laboratory frame and therefore it is relatively straightforward to achieve nanometer sensitivity. The efficiency of FRET is inversely proportional to the sixth power of the distance separating the acceptor and donor such that FRET can be used as an effective spectroscopic ruler in the range of roughly 1-10 nm (Figure 3) (95):

\[ E = \frac{1}{1 + \left( \frac{R}{R_0} \right)^6} \]

where \( R \) is the distance between the donor and acceptor dyes and \( R_0 \) is the Förster radius, which is defined as the dye separation at which 50% of energy is transferred from the donor to the acceptor. For the commonly used FRET pair Cy3-Cy5, \( R_0 \) is approximately 6 nm (96). If the donor is directly excited and the donor and acceptor emissions are measured, then the apparent FRET efficiency can be computed as

\[ E_{app} = \frac{I_A}{I_A + I_D} \]

where \( I_A \) is the emission intensity of the acceptor and \( I_D \) is the emission intensity of the donor (97).

To harness smFRET for the study of nucleosome remodeling, we reconstituted mononucleosomes with a Widom 601 positioning sequence (98) such that the octamer was at a well-defined initial position on the DNA. By convention, we will refer to the linker DNA that lengthens during remodeling as the exit side (generally the shorter linker) and the linker DNA that shortens as the entry side (generally the longer linker). We labeled histone H2A of the histone octamer with the FRET donor Cy3, the 5’ end of the exit-side linker DNA with the FRET acceptor Cy5, and the 5’ end of the entry-side linker DNA with biotin (Figure 4a, Chapter 2). Nucleosome reconstitutions were
performed using equimolar amounts of Cy3-labeled and unlabeled H2A. Since each histone octamer contains two H2A subunits, there are three distinct populations of labeled nucleosomes that can be distinguished by their FRET values: (i) singly-labeled nucleosomes where the Cy3 dye is on the H2A proximal to the exit-side linker DNA, (ii) singly-labeled nucleosomes where the Cy3 dye is on the H2A distal to the exit-side linker DNA, and (iii) doubly-labeled nucleosomes (Figure 4b). We selectively analyzed the first population because it gives the highest starting FRET value and therefore the greatest dynamic range.

Reconstituted mononucleosomes were tethered to quartz slides via a streptavidin-biotin linkage (Figure 5, Chapter 2). Surface immobilization enables recording fluorescence time traces of sufficient duration to capture the entire remodeling process. The FRET donor Cy3 was excited with a 532 nm laser in a prism-type TIRF configuration and fluorescence emissions from both Cy3 and Cy5 were captured as described in Chapter 2. An oxygen scavenging system and triplet-state quencher were used to reduce photobleaching and photoblinking, respectively (99, 100).

1.4 Mechanisms of nucleosome translocation

The mechanism(s) by which remodelers use the free energy of ATP hydrolysis to disrupt histone-DNA contacts and reposition or restructure nucleosomes has been a major challenge in the field. As the remodeling mechanism may differ among different remodeler families, this thesis will focus on the ISWI family with a brief discussion of the SWI/SNF family in Chapter 3.

Previous studies have found that the catalytic subunits of remodelers are key determinants of remodeling outcomes while the accessory subunits regulate enzyme
activities (2, 11, 101). The catalytic subunits translocate along DNA at an intra-
nucleosomal location two superhelical turns (~20 bp) from the dyad referred to as the
SHL2 site (12, 102-105). Nucleosomal DNA is highly constrained in a protein-DNA
complex so translocation must be coupled to the disruption of histone-DNA contacts.
Hence, to effect nucleosome translocation, remodelers must transiently disrupt histone-
DNA contacts. In particular, remodelers need to push/pull DNA into the nucleosome at
one side (entry side), move the excess DNA across the nucleosome, and release DNA
at the opposite side (exit side). The coordination of these activities is not well
understood.

Numerous models have been proposed for how remodelers may translocate
nucleosomes along DNA (Figure 6) (2, 6, 7, 9, 28, 106-108). The three most prominent
models are based on (1) twist diffusion, (2) loop propagation, and (3) concerted
swiveling. The twist diffusion model posits that remodelers generate a twist defect at the
entry side of the nucleosome, which then propagates around the octamer and shifts the
nucleosome 1 bp at a time (Figure 6a) (3, 109, 110). Empirical evidence in support of
the twist diffusion model include the observations that nucleosomes can readily
accommodate overtwisted DNA (3, 111) and remodeling complexes can introduce
superhelical torsion into nucleosomal DNA (112). However, ATP-dependent
nucleosome repositioning is not inhibited by physical barriers such as DNA hairpins,
tethered magnetic beads, and biotin cross-links that would block rotation of the DNA
duplex during sliding (113, 114), suggesting that twist diffusion is unlikely used by
remodelers as the primary mechanism for ATP-dependent sliding of nucleosomes. In
contrast, the loop propagation model hypothesizes that remodelers scoop linker DNA in
from the entry side, creating a loop of DNA that propagates around the histone core to
the exit side (Figure 6b) (104, 105, 114-116). The translational position of the nucleosome would shift in accordance to the size of the DNA loop. The loop propagation model predicts that nucleosomal DNA will become accessible during remodeling. Consistent with this idea, SWI/SNF remodelers expose a substantial amount of intranucleosomal DNA to nuclease digestion during remodeling (113, 117-119). On the other hand, ISWI remodelers expose relatively little intranucleosomal DNA to nuclease digestion (120, 121) but do generate accessible DNA near SHL-2 based on ethidium bromide-dependent cleavage (114). Finally, the concerted swiveling model proposes that remodelers disrupt all of the major DNA-histone contacts concurrently, enabling the DNA to swivel relative to the core octamer in a concerted manner (Figure 6c) (7, 120). It is unclear whether one of these models, a combination of several models, or an entirely different model reflects the true remodeling mechanism.

Many ISWI remodelers, including human ACF and yeast ISW2, arrange nucleosomes in an array with regular spacing between them, which is thought to be a prerequisite for heterochromatin formation (11, 122-126). When acting on mononucleosomes, these remodelers slide nucleosomes to the center of the DNA fragment (121, 127-131).

We used the smFRET remodeling assay (Chapter 1.3) to study the remodeling mechanism of ISWI enzymes. Upon addition of ACF/ISW2 and ATP to end-positioned nucleosomes, a decrease in FRET is observed, consistent with the nucleosome centering activity of ISWI complexes (Figure 7a). The dye-labeling and surface immobilization does not perturb the remodeling reaction (Figure 8). The single-molecule FRET traces demonstrate that ISWI-mediated remodeling of individual nucleosomes consists of an initial waiting period (t\text{wait}) that likely involves enzyme binding and
formation of a template-committed complex, followed by a remodeling period (Figure 7a). The latter is characterized by incremental translocation along the DNA interrupted by kinetic pauses, which divides the remodeling period into alternating translocation phases ($t_{T1}$, $t_{T2}$), with a monotonic FRET decrease, and pause phases ($t_{P1}$, $t_{P2}$), with no appreciable FRET change (90-92).

To correlate the observed FRET values with nucleosome positions, we generated a FRET vs. exit-side linker length calibration curve using nucleosomes with different linker lengths on the exit side (Figure 7b). Conveniently, there is a linear relationship between FRET and nucleosome position for exit-side linker lengths of $m = 3$-$15$ bp. The deviation from the expected nonlinear distance dependence of FRET is likely because of the flexible linkers used to connect the dyes to the DNA or histone H2A, which significantly broadens the distance dependence of FRET.

Using the calibration curve, we found that the first pause took place after $\sim 7$ bp of translocation and the second pause took place after $\sim 3$ bp of translocation (Figure 7c). The dynamic range of the smFRET assay can be increased by shortening the exit-side linker length. In fact, with $m = -3$ bp (3 bp of the 601 positioning sequence removed), more than two steps can be observed (data not shown). The size of these subsequent steps are also $\sim 3$ bp, indicating that the step sizes on the exit-side are uniformly $\sim 3$ bp after an unique $\sim 7$ bp first step (90). These step sizes are independent of DNA sequence and conserved across a broad range of ISWI enzymes featuring distinct accessory subunits and remodeling outcomes (90, 91). In fact, the translocation step sizes are intrinsic to the conserved catalytic subunit harboring the ATPase domain (91).

Although most chromatin remodelers do not exhibit helicase DNA-unwinding activity, the ATPase domains of chromatin remodelers are homologous to that of SF2
helicases, which are known to translocate along DNA or RNA in 1 bp increments (132-134). When we decreased the remodeling rate by lowering the temperature or using low ATP concentration in combination with high concentrations of the slowly-hydrolyzed nucleotide analog, ATP-γ-S, we observed 1 bp translocation steps (Figure 9) (91). Hence, the larger ~7 bp and ~3 bp compound steps described previously are composed of 1 bp elementary steps. These fundamental 1 bp steps are likely mediated by the ATPase domain bound at the SHL2 site of the nucleosome.

To further elucidate the mechanism of nucleosome translocation by ISWI enzymes, we need to understand the dynamics at both sides of the nucleosome. With Cy3 on the histone octamer, exit-side dynamics can be monitored by placing the Cy5 on the exit-side linker DNA as described before (Figure 10a) while entry-side dynamics can be observed by moving the Cy5 to the entry-side linker DNA (Figure 10b) (91). When an entry-side labeled nucleosome is remodeled, smFRET time traces exhibit an initial waiting period followed by a FRET increase as the nucleosome is moved towards the center of the DNA fragment (Figure 10b). As translocation proceeds, the Cy5 acceptor dye enters the nucleosome and follows the spiral path of nucleosomal DNA, resulting in oscillatory FRET changes (Figure 10b).

The coordination between exit-side and entry-side remodeling activity can be investigated by comparing the waiting times, \( t_{\text{wait}} \), before remodeling on either side of the nucleosome (Figure 10c). Surprisingly, we discovered that the average wait time for entry-side dynamics was significantly longer than that for exit-side dynamics, which suggests that exit-side DNA translocation likely occurs prior to DNA movement on the entry side (Figure 10c) (91). Moreover, the delay between the exit- and entry-side movements increased as the ATP concentration decreased (Figure 10c). Therefore, the
delay between exit-side translocation and entry-side movement can be interpreted as DNA translocation at SHL2 by the ATPase prior to any movement of DNA into the nucleosome from the entry side.

Since \( t_{\text{wait}} \) was measured at the exit and entry side of nucleosomes with different labeling schemes, the time difference observed is only an indirect measure of the order of remodeling events. To directly determine whether DNA translocation by the ATPase from SHL2 towards the exit side is necessary for DNA movement at the entry side, we created a series of entry-side labeled nucleosomes with a 2 nt ssDNA gap placed at varying distances, \( x \), from the SHL+2 site (Figure 11a). Exit-side translocation is limited to \( x \) bp because remodeling ceases when the gap aligns with SHL+2 (105). Remarkably, no entry-side movement was observed for nucleosomes with \( x = 0-7 \) bp after the addition of ISW2 and ATP (Figure 11b,c). This suggests that entry-side DNA movement occurs only after the ATPase has translocated at least 7 bp of DNA towards the exit side. If exit-side translocation occurs without concomitant entry-side translocation, net strain accumulates on the nucleosome, perhaps in the form of DNA stretching (110) or transient conformational changes in the histone octamer (135). Furthermore, the post-remodeling entry-side FRET values were the same for nucleosomes with \( x = 8-10 \) bp, but distinct from those observed for \( x = 11-13 \) bp, which were also the same (Figure 11c). Taken together, these observations suggest that 7 bp of strain must accumulate on the DNA before entry-side movement is triggered. Entry-side steps are 3 bp in size, which enable the ATPase to translocate an additional 3 bp of DNA to the exit side. This explains why the first kinetic pause on the exit side occurs after a 7 bp step while subsequent pauses occur after only 3 bp of additional translocation (91).
Our data is inconsistent with the three previously proposed models for nucleosome translocation by chromatin remodeling enzymes (Figure 6). The twist diffusion model predicts that DNA translocation occurs at the entry side before the exit side, with 1 bp steps everywhere. The loop propagation model posits that entry-side movement precedes exit-side movement and the fundamental translocation step sizes should be larger than 1 bp, depending on the size of the loops formed. Finally, the concerted swiveling model envisages simultaneous DNA translocation on both sides of the nucleosome.

We put forth a novel “spring-loaded” mechanism that is supported by our experimental findings (Figure 12). Remodeling begins with the ATPase domain translocating DNA from the SHL2 site towards the exit side, 1 bp at a time. The entry-side DNA is initially immobile, perhaps through an interaction with the C-terminal DNA-binding module (Figure 2; HAND-SANT-SLIDE) (102, 136, 137). After 7 bp of DNA have been translocated towards the exit side, the strain accumulated is sufficient to trigger an action such as an enzyme conformational change that pulls a 3 bp equivalent of entry-side DNA into the nucleosome. This partial relaxation enables the ATPase to translocate another 3 bp of DNA to the exit side, at which point the strain is again sufficient to trigger another entry-side movement. This cycle continues to facilitate processive nucleosome translocation. Importantly, this strain-based mechanism would explain why there is a unique ~7 bp first compound step on the exit side followed by ~3 bp compound steps whereas all entry-side movements are ~3 bp in size.
1.5 Regulation of chromatin remodeling

The basic activities of chromatin remodelers are highly regulated in the physiological context (2). Noncatalytic accessory subunits can modify the activity of the catalytic subunit. For example, the addition of an Acf1 accessory subunit to an ISWI catalytic subunit changes the enzyme's requirement for extranucleosomal linker DNA and increases the processivity and efficiency of nucleosome translocation (128). Post-translational modifications of remodelers including phosphorylation, acetylation, and PARYlation are another mechanism by which remodeling enzymes are regulated (2). Finally, remodelers have differing sensitivities to various substrate features that are critical for their biological functions.

For example, many ISWI remodelers have the ability to create regularly-spaced nucleosome arrays at specific locations in the genome (11, 122, 124, 126), a requirement for transcriptional silencing and the generation of repressive heterochromatin (138). Underlying this phenotype is the regulation of ISWI catalytic activity by linker DNA length and the modification state of the H4 tail. Specifically, shortening the linker DNA reduces the catalytic activity of ISWI remodelers such that they preferentially translocate nucleosomes towards the longer linker DNA, thereby promoting regular spacing in a nucleosome array. An unmodified H4 tail is thought to stimulate ISWI remodeling by releasing the autoinhibitory effect of the AutoN domain within the catalytic subunit (139). Acetylation of the H4 tail is a common epigenetic mark associated with transcriptionally active chromatin (140-142). Since the primary role of ISWI remodelers is to produce repressive chromatin structures, ISWI enzymes may use the unmodified H4 tail as a signal to discriminate against transcriptionally active DNA regions (143-146). In support of this hypothesis, previous studies employing
mononucleosomes have shown that deletion or acetylation of the H4 tail reduces ISWI activity (127, 143-150), but the opposite effect was recently observed with reconstituted nucleosome arrays (151).

On the other hand, SWI/SNF remodelers do not require the presence of extranucleosomal linker DNA or unmodified H4 tails for efficient remodeling (103, 131, 148, 152). The former is not surprising given that SWI/SNF enzymes lack the extensive linker DNA interactions and nucleosome-spacing activity seen with ISWI remodelers (153, 154). Acetylation of the H3 tail is recognized by the bromodomains of SWI/SNF complexes and is known to stimulate remodeling activity (155, 156).

Most of our knowledge on the function of chromatin remodeling complexes has been gleaned from studies using nucleosomes with only core histones. In contrast, physiological chromatin is replete with linker histones, e.g., H1, at a prevalence of approximately one linker histone per nucleosome in differentiated eukaryotic cells (157). In living cells, nearly all H1 is bound to chromatin at any given time but its association with chromatin is much more dynamic than core histones (average residence time is 3 min for H1 vs. hours for core histones) (158). Linker histones constrain the path of linker DNA and are therefore believed to hinder dynamic detachment of DNA from the core octamer, which is necessary for both spontaneous sliding of histone octamers on DNA at high temperatures (159) and ATP-dependent nucleosome translocation (160). Linker histones also limit the amount of free linker DNA available, which is important for efficient ISWI remodeling. Furthermore, linker histones and remodeling complexes may compete for the same binding sites on the nucleosomal substrate. The higher-order folding and chromatin compaction promoted by linker histones may also limit access by remodeling complexes. Previous studies on the effects of linker histones on remodeling
activity have been conflicting, with some studies reporting varying degrees of general repression while others describe qualitative changes in remodeling outcomes (161-164).

The mechanism(s) by which critical features of the nucleosomal substrate including linker DNA, core histones, and linker histones regulate remodeling activity are not well understood. In this thesis, we investigate the regulation of ISWI chromatin remodeling by linker DNA and the histone H4 tail (Chapter 3.1) and study the effects of linker histone H1 on the remodeling activity of ISWI and SWI/SNF complexes (Chapter 3.2).
2 METHODS

**DNA constructs.** Double-stranded (ds) DNA constructs containing the 601 nucleosome positioning sequence (98), varying DNA linker lengths on the entry side of the nucleosome, 3 bp linker DNA on the exit side of the nucleosome, a FRET acceptor Cy5 attached to the 5' end of the exit-side linker DNA, and a biotin moiety attached to the 5' end of the entry-side linker DNA were generated by PCR and purified by PAGE. The dsDNA constructs with an additional 38 nucleotide (nt) single-stranded (ss) DNA spacer used for single-molecule mononucleosome remodeling experiments were created by annealing and ligating a set of overlapping, complementary oligonucleotides. The 38 nt ssDNA spacer was used to prevent surface perturbation in remodeling. These HPLC-purified oligonucleotides (Integrated DNA Technologies) were mixed at equimolar concentrations in 50 mM Tris pH 8.0, 100 mM KCl, 1 mM EDTA and annealed with a temperature ramp (95°C - 3°C), ligated with T4 DNA ligase (New England Biolabs), and purified by PAGE. Successful ligation was confirmed by denaturing PAGE. To make dinucleosomes, DNA constructs for each mononucleosome were first created using the above anneal-ligate approach. In this case, the 38 nt ssDNA spacer was not used since the nucleosome to be remodeled was the one distal to the surface anchoring site. To give a couple of example oligonucleotide sequences, the following oligonucleotides were used to assemble the dsDNA for the mononucleosome with 40 bp entry-side linker DNA:

**Top strand:**

5' /5Cy5/ gccctggagaatcccggtctgcaggccgctcaattg 3'

5' /5Phos/ gtcgtagacagcttagcaccgcttaaacgcacgtcgcctgtcccgcgtttaaccg 3'
The following oligonucleotides were used to assemble the dsDNA for the two nucleosomes that were subsequently ligated to obtain the dinucleosome with 40 bp internucleosome spacing:

**Nucleosome 1, top strand:**

5' /5Cy5/gccctggagaatcccggtctgcaggccgctcaattg 3'
5' /5Phos/gtcgtagacagctctagcaccgcttaaacgcacgtacgcgctgtcccc 3'
5' /5Phos/cgcgttttaaccgccaagggattactccctagtctccaggcac 3'
5' /5Phos/gtgtcagatatatacatcctgtgcttgtattgcccagcgaccttgcccgtgc 3'

**Nucleosome 1, bottom strand:**

5' /5Phos/caatacaagcacaggatgtatatatctgacacgtgcctggagactagggagtaatc 3'
5' /5Phos/cccttggcggttaaaacgcgggggacagcgcgtacgtgcgtttaag 3'
5' /5Phos/cggtgctagatatatccttgtgttattgccacagcgccttgccctgg 3'

5' /5Phos/ccaagggattactccctagtctccaggcacgtgctagatatacatc 3'
5' /5Phos/ctgtgagtgttccgagctccccactctagaggatccccgggtacc 3'

**Bottom strand**

5' /5Bioteg/cccgcccgccaaaaaaaaaaaaaaaaaaaaaaaaaaaggtacccggggatcctctagagtgg 3'
5' /5Phos/gagctcggaacactcacaggatgtatatatctgacacgtgcctg 3'
5' /5Phos/gagactagggagtaatccccttggcgttaaaacgcgggggacagcgcgtacgtg 3'
5' /5Phos/cgtttaaagcgtgctagcgtgctctacgaccaattgacgggctgcagacccgggttcctccagggc 3'
Nucleosome 2, top strand:
5' /5Phos/cagtcggatactggagaatcccggtctgaattgtcctgacagctctagca 3'
5' gcttaaacgcacgtacgcgctgtcccccgcgttttaaccgccaagggattactccctagtctccag 3'
5' /5Phos/gcacgtgtcagatatacatcctgtgcatgtattgaacagcgac 3'
5' /5Phos/cttgccggtgccagtgctggata/3alexf488n/ 3'

Nucleosome 2, bottom strand
5' /5Bioteg/tatccgactggcaccggcaagttgctgttatcatatgcacag 3'
5' /5Phos/gatgtatatatctgacacgtgcctggagactagggagtaatccccttg 3'
5' ggttaaacgcggggacagcgcgtctgtcaataagctgctagctgctacgacacagcggc 3'
5' /5Phos/ctgcagacccgggtctccagtatccgactggcactggcaaggtcgctgctg 3'

At the ssDNA gaps (2 nt) on the unlabeled nucleosome proximal to the surface-anchoring site, the corresponding oligonucleotides were not 5'-phosphorylated. The final, ligated DNA sequences of the various nucleosome constructs are provided in Figure 13.

Histone octamers. Recombinant X. laevis H2A, H2AK120C, H2B, H3, H3C110A, H4, H4K16A, H4Δ1-19, and H4NCys (with an N-terminal cysteine residue inserted) histones were expressed in BL21 (DE3) pLysS competent cells (Stratagene) and purified from inclusion bodies by gel filtration (Sephacryl S-200 column, GE Healthcare) and ion exchange (Resource-S column, GE Healthcare) chromatography under denaturing conditions (4, 165). The H4K16A, H4Δ1-19, and H4NCys mutants were created by site-directed mutagenesis using the following primers:
**H4K16A**

5'-cttaagaagagatacatatgaaagtctgcgtgacaacatccag-3'
5'-ctggatgtgtgcagcagaactttcatatgtatatctctcttaaag-3'

**H4Δ1-19**

5'-aaggtctggtaaaggtgtgcagtcaccgtaaag-3'
5'-ctttacggtcagtgaccacqacccacctttaccagacctt-3'

**H4NCys**

5'-gaaggagatatacatatcgcaccctggtcgtggtaaagg-3'
5'-cctttaccacgaccagcacatgtatctcttc-3'

For site-specific labeling of histone H2A, a cysteine substitution was introduced at residue 120 (K120C) as described previously (130). Lyophilized H2A was dissolved in labeling buffer (7 M guanidine-HCl, 20 mM Tris pH 7.0, 5 mM Na-EDTA, 1.25 mM TCEP) and incubated for 2 hr in the dark. Cy3-maleimide was dissolved in DMSO and added to the reaction to a final concentration of 2.5 mM. After incubating for 3 hr in the dark, the reaction was quenched by adding 80 mM β-mercaptoethanol. The labeled H2A was dialyzed three times against dialysis buffer (7 M guanidine HCl, 20 mM Tris pH 7.0, 1 mM DTT) in a 7K MWCO dialysis cassette (Pierce) and quantified.

To reconstitute histone octamer, each lyophilized core histone was dissolved in unfolding buffer (7 M guanidine-HCl, 20 mM Tris-HCl pH 7.5, 10 mM DTT), mixed in a molar ratio of 1.2:1.2:1:1 H2A:H2B:H3:H4, dialyzed against refolding buffer (2 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM Na-EDTA, 5 mM β-mercaptoethanol), and purified by gel
filtration chromatography (Superdex 200 10/100 GL column, GE Healthcare) as previously described (4). For making FRET-labeled nucleosomes, a ~1:1 ratio of Cy3-labeled and unlabeled H2A mixture was used. Because each histone octamer contains two H2A subunits, this reconstitution yielded three distinct populations of Cy3-labeled nucleosomes: (i) with Cy3 attached only to the H2A subunit proximal to the FRET acceptor Cy5 on the DNA, (ii) with Cy3 attached only to the H2A subunit distal to the Cy5 on the DNA, and (iii) with both H2A labeled by Cy3. These three labeling configurations yielded distinct FRET levels that could be clearly distinguished at the single-molecule level, as shown previously (90). To maximize the dynamic range for single-molecule FRET measurements, we selected the population with the highest FRET value, which corresponded to nucleosomes with a single Cy3 on the proximal H2A, for further analysis. For ensemble FRET measurements, because the overall remodeling rate was derived from the decay curve of the acceptor signal, distinguishing the three populations was unnecessary. For crosslinking experiments, only unlabeled H2A was used.

**Nucleosomes and chromatosomes.** Mononucleosomes were reconstituted from DNA and histone octamers at 4 °C (4). The nucleosome reconstitution reaction consisted of 2 M KCl, 20 mM Tris-HCl pH 7.5, 1 mM Na-EDTA, 1.2 μM histone octamer, 1 μM DNA construct, 10 mM DTT, and 0.5 mM benzamidine. The sample was injected into a pre-hydrated 7K MWCO dialysis cassette (Pierce) and subjected to salt gradient dialysis (2 M KCl to 250 mM KCl over 60 hours) followed by incubation in TCS buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT) for 8 hr at 4 °C. Assembled nucleosomes were purified on a 10-30% glycerol gradient in 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1%
Igepal CA-630. Fractions were analyzed by PAGE (5% 0.5× TBE) and fractions containing pure and correctly assembled nucleosomes were pooled and concentrated with a 100K MWCO Amicon Ultra-0.5 mL centrifugal filter (Millipore) to ~30 μL final volume and stored at 4°C. For the assembly of nucleosomes used in crosslinking experiments, reducing agent was omitted.

Monochromatosomes were reconstituted in the same way as mononucleosomes with the addition of recombinant human linker histone H10 (New England Biolabs) at a 2.5-fold molar excess to core histones, which was previously determined to be sufficient to achieve stoichiometric, saturating levels of linker histone (162). Alternatively, excess linker histone was added to previously reconstituted mononucleosomes as described further in Chapter 3.2.

Dinucleosomes were prepared by assembling each mononucleosome separately and then ligating these mononucleosomes (Figure 17a). The distal nucleosome farther away from the surface-anchoring site was FRET-labeled in the same way as the mononucleosome constructs. The proximal unlabeled nucleosome closer to the surface-anchoring site featured 2 nt gaps at the super-helical location ±2 (SHL±2) sites to prevent translocation and biotin at the DNA end for surface-anchoring. Each ligation reaction contained approximately 1 pmole of total nucleosomes with an excess of the distal FRET-labeled nucleosome over the proximal unlabeled nucleosome, 1x T4 DNA ligase buffer (New England Biolabs) supplemented with 10 mM KCl, 0.1% Igepal CA-630, and 2000 units of T4 DNA ligase (New England Biolabs) in a final volume of 20 μL. Ligation was allowed to proceed at room temperature for 1 hr followed by the addition of EDTA and glycerol to final concentrations of 1 mM and 20%, respectively, and storage at 4°C. Purification was unnecessary because only the constructs that contained both
distal FRET-labeled and proximal nucleosomes with a biotin moiety could be anchored to the surface and generate a FRET signal.

**ATP-dependent Chromatin assembly and remodeling Factor (ACF).** To produce isolated Snf2h-FLAG or isolated Acf1-FLAG, these proteins were overexpressed in Sf9 cells using a baculovirus expression system (Kinnakeet Biotechnology). To produce ACF complexes, Snf2h and Acf1-FLAG were co-expressed in Sf9 cells. Nuclear extraction was performed as described previously (128, 130). Nuclear extract was fortified with 0.5 mM benzamidine, 60 µg/mL TLCK, and 1x Roche Complete Protease Inhibitor Cocktail and then purified by M2-affinity chromatography (Anti-FLAG M2 beads and FLAG peptide, Sigma-Aldrich), as described previously (128, 130). Specifically, the FLAG-tagged subunit or complex was bound to M2 beads, and the beads were washed several times with wash buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 1 mM benzamidine, 1 mM DTT, Roche EDTA-free complete protease inhibitor, and various concentrations of KCl (300, 150, and 100 mM sequentially). Protein was subsequently eluted with a 1 mg/mL FLAG peptide solution in wash buffer with 100 mM KCl. Elution fractions were analyzed by PAGE and fractions containing the isolated subunits or the intact ACF complex were pooled and concentrated with a 50K MWCO Amicon Ultra-15 mL centrifugal filter (Millipore). Concentrated ACF complexes, Snf2h subunit, and Acf1 subunit were then aliquoted, flash frozen, and stored at -80°C in 20 mM HEPES, pH 7.9, 20% Glycerol, 0.2 mM EDTA, 100 mM KCl, and 1 mM DTT. To purify enzyme for crosslinking experiments, the complete protease inhibitor pills and DTT were omitted from the buffers during the affinity purification steps. Typical yields were ~50-100 µg, 20-40 µg, and 1-2 µg of ACF complexes, Snf2h subunit, and Acf1.
subunit, respectively, per liter of insect cell culture. Quantification was performed by SYPRO-red staining and comparison to BSA standards. The Snf2h and Acf1 mutants were created by site-directed mutagenesis using the following primers:

**AutoN-2RA Snf2h (R142A, R144A)**

5'-actatcgtggcgtttacgcatgctagctagctagctagctagctagctagctagctagctagctagctagc-3'
5'-ctcctctttgcttccttctcttgcttgccgtaatcggcttgccgtaatcggcttgccgtaatcggct-3'

**ΔNegC Snf2h (Δ669-700, insert SGSGS)**

5'-gtgtttgctttcagaaagttcagtctgtggtctctctgctctctgctctctgctctctg-3'
5'-ctctgtatcctttcagaaagttcagtctgtggtctctctgctctctgctctctg-3'

**ΔHSS Snf2h (ΔC743)**

5'-gagaaagaaagcaactaatgactataaaaggtgagctagctagctagctagctagctagctagc-3'
5'-cgtcatcgttccttctctctctctctctctctctctctctctctctctctctctctctctctc-3'

**ΔNegC,HSS Snf2h (ΔC669)**

5'-gcttcaagaaagttacgagctagctagctagctagctagctagctagctagctagctagc-3'
5'-gtcatcgttccttctctctctctctctctctctctctctctctctctctctctctctctctc-3'

**ΔC-term Acf1 (Δ1423-1556)**

5'-gcctgtgacactgggtgactataaaaggtgagctagctagctagctagctagctagctagc-3'
5'-catctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctc-3'
ΔN-term Acf1 (Δ1-371)

5'-gcagggtaccggagatggtagaaagagaaaagaaag-3'
5'-ctttccttttctctttctaccatctccggtaccctgc-3'

Remodel the Structure of Chromatin (RSC). RSC was purified from *S. cervisiae* strain BCY211, which expresses a TAP-tagged Rsc2 subunit, using tandem affinity purification as previously described (166). Protein concentration was quantified by SYPRO-red staining and comparison with a BSA standard ladder.

Electrophoretic mobility shift nucleosome remodeling assay. When end-positioned mononucleosomes are translocated by ISWI enzymes to a more central position, the electrophoretic mobility is decreased by an amount proportional to the translocation distance (128, 130). Each 10 μL reaction included 0.25 pmoles of nucleosomes, a varying amount of enzyme, and 2 mM ATP in reaction buffer (40 mM Tris pH 7.5, 12 mM HEPES pH 7.9, 60 mM KCl, 0.32 mM EDTA, 3 mM MgCl₂, 10% (v/v) glycerol, and 0.02% (v/v) Igepal CA-630) and was incubated for 30 min at 30°C. The reactions were quenched with the addition of excess ADP and unrelated plasmid DNA and analyzed by PAGE (5% TBE).

Ensemble FRET assay for nucleosome remodeling. The ensemble remodeling kinetics of nucleosomes were measured by monitoring the total acceptor (Cy5) signal (under a constant 532 nm illumination that excites the donor Cy3) of a solution of FRET-labeled nucleosomes after addition of ACF and ATP to initiate remodeling. As
nucleosomes were translocated towards the center of the DNA, the FRET efficiency decreased, which resulted in a decrease in the Cy5 signal.

The ensemble nucleosome remodeling assay was performed by diluting nucleosomes in remodeling buffer (40 mM Tris pH 7.5, 12 mM HEPES pH 7.9, 60 mM KCl, 0.32 mM EDTA, 3 mM MgCl$_2$, 100 µg/mL acetylated BSA, 10% (v/v) glycerol, 0.02% (v/v) Igepal CA-630, 10% glucose, 2 mM Trolox, and 2.5 mM protocatechuic acid) in a cuvette fitted for a Cary Eclipse Fluorescence Spectrophotometer (Varian). Immediately before starting a kinetic scan (ex = 532 ± 10 nm, em = 670 ± 20 nm, 1 Hz acquisition frequency, 20°C), ACF, ATP, and additional MgCl$_2$ equimolar to ATP were mixed in the remodeling buffer and added to the nucleosomes, achieving a final concentration of 2.5 nM nucleosomes and varying concentrations of enzyme and ATP, as reported. For remodeling experiments in the presence of the H4-tail peptide, synthetic peptide containing the H4-tail sequence (residues 1-21 of histone H4, SignalChem) was mixed together with ACF, ATP, and MgCl$_2$ in the remodeling buffer before being added to the nucleosomes. The final concentration of the H4-tail peptide was 20 nM.

**Single-molecule FRET assay for nucleosome remodeling.** Quartz slides were cleaned, functionalized with poly(ethylene glycol) (PEG, Laysan Bio), and assembled into flow chambers as previously described (90). Reconstituted nucleosomes were anchored to coated quartz slides via a streptavidin-biotin linkage. To eliminate potential perturbation of remodeling by surface anchoring, especially for mononucleosomes with relatively short entry-side linker DNA, we inserted a 38 nt ssDNA spacer between the biotin and dsDNA. With the ssDNA spacer, the remodeling kinetics of surface-anchored
nucleosomes with 40 bp or longer entry-side linker DNA were indistinguishable from those of freely diffusing nucleosomes measured using the ensemble FRET assay (Figure 8). We performed our single-molecule FRET studies on nucleosomes with at least 40 bp of entry-side linker DNA.

The FRET donor Cy3 was directly excited with a 532 nm Nd:YAG laser (CrystaLaser) in a total internal reflection (TIRF) configuration and fluorescence emission from Cy3 and Cy5 was captured with a 60x water immersion objective (Olympus), filtered with a 550 nm long-pass filter (Chroma Technology), spectrally split by a 630 nm dichroic mirror (Chroma Technology), and imaged onto two halves of a CCD camera (Andor iXon³EM+888) (Figure 5). The imaging conditions were 30 °C in the imaging buffer (40 mM Tris pH 7.5, 12 mM HEPES pH 7.9, 60 mM KCl, 0.32 mM EDTA, 3 mM MgCl₂, 100 µg/mL acetylated BSA, 10% (v/v) glycerol, 0.02% (v/v) Igepal CA-630), 10% (w/v) glucose, 800 µg/mL glucose oxidase, 50 µg/mL catalase, 2 mM Trolox, and 2.5 mM protocatechuic acid). In certain experiments, 20 nM H4-tail peptide was included. The enzymatic oxygen scavenging system and antioxidants (10% glucose, 800 µg/mL glucose oxidase, 50 µg/mL catalase, 2 mM Trolox, and 2.5 mM protocatechuic acid) were used to reduce fluorophore photobleaching and blinking (99, 100). Remodeling was initiated by infusing the flow chamber with a solution containing ACF and ATP in imaging buffer using a syringe pump (KD Scientific).

**Fluorescence anisotropy binding assay.** For binding studies, we used a synthetic peptide (SignalChem) that contained residues 1-21 of human histone H4 as well as an additional C-terminal cysteine to enable thiol-based labeling. Tetramethylrhodamine-5-iodoacetamide (TMRIA) was coupled to the C-terminus of the synthetic H4-tail peptide
to yield TMR-labeled H4 peptide (H4-tail-TMR). The coupling reaction was carried out by shaking 0.2 μmol peptide, ~2 fold molar excess of TMRIA, and 10 fold molar excess of TCEP in 1 mL of 20 mM Tris (pH 7.5) in the dark for 2 h at room temperature. The labeled peptide was purified by RP-HPLC and its identity confirmed by liquid chromatography–mass spectrometry analysis.

Fluorescence anisotropy measurements were carried out at 25°C using a Molecular Devices SpectraMax microplate reader. Binding curves were recorded in opaque low-volume 384-well assay plates. Individual wells contained 20 nM H4-tail-TMR and the indicated concentration of Acf1 in 20 mM Tris (pH 8.0), 100 mM KCl, and 20% (vol/vol) glycerol. The anisotropy of fluorescence was measured as a function of the Acf1 concentration. Dissociation constants were determined by fitting the fluorescence anisotropy data to the solution of a quadratic equation derived from the binding isotherm, which takes depletion of Acf1 protein into account:

\[
\frac{\text{r}}{\text{r}_0} = \frac{\alpha / (2[H4]_t)}{([P]_t + [H4]_t + K_d - (([P]_t + [H4]_t + K_d)^2 - 4[P]_t[H4]_t)^{0.5})} + \text{r}_0,
\]

where \(\text{r}\) is the fluorescence anisotropy, \(\text{r}_0\) represents an offset value, \(\alpha\) is a scaling factor, \(K_d\) is the dissociation constant, \([H4]_t\) represents the total concentration of the H4-tail-TMR peptide, and \([P]_t\) is the total concentration of titrated Acf1 protein. Errors in the determination of \(K_d\) were estimated by calculating the 95% confidence interval.

**Protein crosslinking.** Homobifunctional maleimide crosslinker BM(PEG)_3 (17.8 Å; Pierce) was added to concentrated H4NCys nucleosomes (cysteine residue added to N-terminal end of H4 tail) to achieve a final concentration of ~5 μM nucleosomes and 500 μM crosslinker. The H4NCys nucleosome contains a single labeling site in the form of a cysteine added to the N-terminus of histone H4, immediately following the initial
methionine. The other histone proteins used to prepare H4NCys nucleosomes were wt H2A, wt H2B, and H3C110A, and do not contain additional cysteine residues. The crosslinker-labeling reaction was performed at room temperature for 1.5 hr. Buffer exchange with wash buffer (20 mM HEPES pH 7.0, 1 mM EDTA, 10% glycerol, 10 mM KCl, 0.1% Igepal CA-630) was performed using a 100K MWCO Amicon Ultra-0.5 mL centrifugal filter (Millipore) five times to remove unreacted crosslinker. ACF was then added to the BM(PEG)₃-derivatised nucleosomes to achieve a final enzyme concentration of 200-400 nM and nucleosome concentration of 5 μM, and incubated for 2 hr. Crosslinking products were analyzed by SDS-PAGE (4-15%, 1x Tris/Glycine/SDS) and SYPRO-red staining. Under each condition, the Acf1-H4 crosslinked fraction was calculated as $I_{\text{Acf1-H4}} / [(I_{\text{Acf1-H4}} + I_{\text{Acf1}}) \times I_{\text{rel,histones}}]$, where $I_{\text{Acf1-H4}}$ and $I_{\text{Acf1}}$ are the integrated intensities of the Acf1-H4 and Acf1 bands, respectively, and $I_{\text{rel,histones}}$ is the relative histone amount obtained by dividing the histone band intensity in a given lane by the maximum histone band intensity in the gel. The Snf2h-H4 crosslinked fraction was obtained analogously. Normalization by the relative histone intensity in each lane compensates for any small variation in the nucleosome concentration used, even though efforts were made to maintain identical nucleosome concentrations for all conditions. For validation of the H4-crosslinking product, immunoblotting was performed using a primary rabbit polyclonal α-histone H4 antibody (Abcam ab10158, tested for Western Blotting applications by the “Abpromise” guarantee) and a HRP–conjugated secondary antibody, and H4 was detected by chemiluminescence (GE Healthcare ECL).
Yeast genetics experiments. Mutant *S. cerevisiae* strains were generated in the BY4741 background (ATCC 4006733, MATa; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*). This strain lacks the *ura3* gene that encodes an enzyme involved in uracil synthesis and can only grow in media supplemented with uracil (or uridine). The Δ*itc1* strain was derived from BY4741 by replacing (through yeast transformation and integration by homologous recombination) the *itc1* coding sequence with a *URA3* cassette that includes the *ura3* gene. Positive transformants were selected on media lacking uracil (uracil-dropout). The *URA3* cassette was generated by PCR from the pRS306 plasmid (primers used: taacaataggagaaagaaagccgtaataaaatcgtcggttatccacggc and atttgggacagtttcactgttcttgaggtttgccca). The Δ*itc1*-Nterm strain was derived from the Δ*itc1* strain by replacing the *URA3* cassette with the *itc1* coding sequence lacking the portion that encodes residues 2-374 at the N-terminus of Itc1. DNA for transformation was prepared by PCR from a plasmid encoding Itc1 lacking the N-terminal region (ΔN-term Itc1). Positive transformants were selected on uracil-containing media additionally supplemented with 5-Fluoroorotic acid (5-FOA) for counterselection against cells that still contained the *URA3* cassette and could therefore convert 5-FOA into a toxic suicide inhibitor causing cell death. The rescue-Δ*itc1* strain was derived from the Δ*itc1*-Nterm strain by replacing the inserted coding sequence for the ΔN-term Itc1 construct with a *URA3* cassette and selecting on uracil-dropout media. All strains were confirmed by PCR screening and sequencing at the *itc1* locus. For growth assays, liquid overnight cultures of the different strains were diluted to the same cell density, and 10-fold serial dilutions of each strain were spotted onto YPD (complete medium including uracil) plates and grown at 30°C.
**Data analysis.** Single-molecule time traces were analyzed using a custom-written Matlab code. Nucleosome translocation steps were identified manually or by fitting a staircase function to the FRET time traces using a step-finding algorithm (167) or hidden Markov modeling algorithm (168). Non-linear curve fitting of the fluorescence anisotropy data was carried out in Matlab, and 95% confidence intervals for fit parameters were obtained using the “confint” routine. Experimental sample sizes as indicated in the figure captions gave the reported s.e.m. (standard error of the mean) values that were sufficiently low to allow meaningful interpretation of the data.
3 RESULTS AND DISCUSSION

3.1 Regulation of chromatin remodeling by linker DNA and histone H4 tail

Many ISWI remodelers exhibit a nucleosome spacing activity (122, 126, 169, 170). Critical to this spacing activity are two features of the nucleosome that modulate the activity of ISWI remodelers: (1) the N-terminal tail of histone H4 (127, 143, 145, 146, 148), and (2) the length of the extranucleosomal linker DNA (127-130, 171). The unmodified H4 tail stimulates ISWI activity likely by relieving the autoinhibitory effect of the AutoN domain within the catalytic subunit (139). H4 tail acetylation associated with transcriptionally active chromatin is thought to help prevent ISWI-induced nucleosome spacing at actively transcribed genes (143, 145, 146). Regulation by the extranucleosomal linker DNA is responsible for generating the regularly-spaced nucleosome arrays important for heterochromatin formation. Shortening the linker DNA reduces the remodeling activity of nucleosome-spacing ISWI enzymes (127-130, 171). As a result, nucleosomes are preferentially moved towards longer linkers to promote uniform spacing on nucleosome arrays. Accordingly, the catalytic activity of many ISWI-family enzymes is sensitive to linker DNA lengths up to ~60-70 bp (127-130, 171), consistent with the inter-nucleosome spacing of heterochromatin observed in human cells (172). This linker-length sensing range substantially exceeds the binding footprint (20-30 bp) of the catalytic subunit (123, 137), whereas the accessory subunits of ISWI complexes can bind linker DNA as far as ~60 bp from the nucleosome edge (123). However, it is unknown how accessory subunits communicate linker length information to the catalytic subunit to regulate remodeling activity. In this section, we investigate the mechanism underlying DNA linker-length sensing by a prototypical ISWI-family enzyme,
human ACF, which is composed of a catalytic subunit, Snf2h, and an accessory subunit, Acf1 (122).

3.1.1 The linker DNA length and histone H4 tail regulate remodeling pauses

To examine how linker DNA regulates nucleosome translocation by ACF, we reconstituted mononucleosomes with varying linker lengths \( (n = 20-78 \text{ bp}) \) on the entry side but typically a constant exit-side linker length of 3 bp (Figure 14a). We also constructed mononucleosomes with wildtype (wt) histone H4 and two H4 mutants: (1) H4 tail deletion (H4\(\Delta\)1-19), and (2) H4 with K16A mutation (H4K16A). We refer to nucleosome constructs with the following nomenclature: [wt H4/H4\(\Delta\)1-19/H4K16A, \( n \) bp] for nucleosomes with \( n \) bp of DNA on the entry side and an octamer containing wt H4, H4\(\Delta\)1-19, or H4K16A. In instances where the exit-side linker length is \( m \neq 3 \text{ bp} \), we will use the notation: [\( m \) bp, wt H4/H4\(\Delta\)1-19/H4K16A, \( n \) bp]. We detected ACF-catalyzed nucleosome translocation using fluorescence resonance energy transfer (FRET) by labeling the end of the exit-side linker DNA with the FRET acceptor Cy5, and the histone H2A with the FRET donor Cy3 (Figure 14a) (90).

We first compared the remodeling kinetics of [wt H4, 78 bp], [wt H4, 40 bp], [wt H4, 20 bp], and [H4\(\Delta\)1-19, 78 bp] nucleosomes using an ensemble FRET assay (130). Upon addition of ACF and ATP, the FRET efficiency decreased as DNA was translocated towards the exit side (Figure 14b,c). As expected, the remodeling rate decreased as the linker DNA was shortened and deletion of the H4 tail drastically reduced the remodeling activity (Figure 14b).

To identify which step(s) of the remodeling process are regulated, we monitored the remodeling of individual nucleosomes using single-molecule FRET (90, 173).
Single-nucleosome remodeling traces featured incremental translocation of DNA to the exit side interrupted by kinetic pauses (Figure 14d, 15). The first pause occurred after ~7 bp of DNA translocation and the second pause occurred after an additional ~3 bp of translocation, consistent with previous findings (90, 91). Moreover, the step sizes did not change with linker DNA length or histone H4 modification (Figure 15). We divided the remodeling time trace into two translocation phases (T1, T2), during which the FRET efficiency decreased, and two pause phases (P1, P2) without appreciable FRET change (Figure 14d). Notably, the DNA translocation rates between pauses did not change, whereas the pause-phase exit rates decreased dramatically when the linker DNA was shortened (Figure 16a,b). Moreover, the dependence of remodeling kinetics on entry-side linker lengths of mononucleosomes was quantitatively similar to the dependence on inter-nucleosome linker lengths of dinucleosomes (Figure 17), validating the use of mononucleosomes as a model system to study linker-length sensitivity. Interestingly, the H4 tail appeared to regulate the same phase of the remodeling process as the linker DNA (Figure 16c). The H4K16A mutation and H4 tail deletion (H4Δ1-19) decreased the pause-phase exit rate by ~2 and ~20 fold, respectively (Figure 16c). In contrast, neither modification had any appreciable effect on the translocation rates between pauses (Figure 16c).

The above results indicate that both linker DNA and the H4 tail regulate the remodeling rate by changing the duration of pause phases, suggesting that these nucleosome features may act on an inhibitory mechanism that prevents the initiation of the DNA translocation phases. It has been shown that although the ISWI ATPase domain can translocate nucleosomes autonomously (174), the catalytic subunit contains two well-conserved autoregulatory domains, AutoN and NegC, which inhibit ATP
hydrolysis and its coupling to DNA translocation, respectively (139). The AutoN inhibition can be relieved by the H4 tail whereas the NegC inhibition can be relieved by binding of the HAND-SANT-SLIDE module to linker DNA (139). Could the regulation of remodeling by linker DNA length occur through these inhibitory domains?

3.1.2 Short-range linker length sensing: NegC and HAND-SANT-SLIDE

The isolated catalytic subunit, Snf2h, is only capable of centering nucleosomes with a short linker length (< 40 bp) whereas addition of the Acf1 accessory subunit significantly extends this range to > 60 bp (128). Furthermore, it was previously demonstrated that the catalytic and remodeling activity of Snf2h alone is sensitive to linker lengths between 0-40 bp while that of the full ACF complex is sensitive to linker lengths between 40-60 bp (130). Hence, the Snf2h catalytic subunit has an intrinsic short-range linker length sensitivity that is modulated by the Acf1 accessory subunit.

In order to determine the mechanism of short-range linker length sensing by Snf2h, we generated a series of human Snf2h mutants: AutoN-2RA, ΔNegC, ΔHAND-SANT-SLIDE (HSS), and ΔNegC,HSS (Figure 18a). Deletion of the HSS module alone (ΔHSS) abolished remodeling activity while the additional deletion of the NegC domain (ΔNegC,HSS) restored remodeling activity (Figure 18b). Thus, remodeling activity is preserved as long as NegC and HSS are either both present or both absent. This finding is consistent with previous results obtained using D. melanogaster ISWI suggesting that HSS interaction with linker DNA is required to relieve NegC inhibition of coupling (139). Furthermore, the isolated deletion of NegC (ΔNegC) or AutoN-2RA mutation both yielded faster remodeling kinetics than wt Snf2h and ΔNegC,HSS Snf2h (Figure 18b), which is consistent with the autoinhibitory roles of NegC and AutoN (139).
To determine which domain(s) are important for short-range linker length sensing, we studied the translocation of individual nucleosomes by each Snf2h mutant with the smFRET assay. Unfortunately, short-range linker length sensing cannot be directly measured by using nucleosomes with entry-side linker lengths between 0 and 40 bp because of the surface inhibition associated with such short linker lengths. Instead, we monitored translocation of [6 bp, wt H4, 78 bp] nucleosomes with 6 bp of exit-side linker DNA and 78 bp of entry-side linker DNA and determined the frequency of initial remodeling towards the short linker (6 bp) versus the long linker (78 bp) (Figure 19a). We used nucleosomes with a short linker of 6 bp instead of 3 bp to increase the dynamic range of nucleosome translocation towards the entry-side. There is evidence that Snf2h functions as a dimer, with each subunit interacting with opposing sides of the nucleosome and competing to determine the direction of remodeling (175). We reasoned that an enzyme with intact short-range linker length sensing would favor nucleosome translocation towards the long linker whereas a mutant that lacks this ability would show a diminished preference for the long linker. Consistent with this conjecture, wild-type (wt) Snf2h only remodeled towards the short linker 12% of the time (Figure 19b). Similarly, AutoN-2RA Snf2h remodeled towards the short linker in only 14% of traces (Figure 19b), indicating that the AutoN domain is not involved with short-range linker length sensing. Deletion of NegC and HSS (ΔNegC, HSS) increased the frequency of remodeling towards the short linker to 39%, suggesting that short-range linker length sensing is compromised and is consistent with the role of HSS in binding linker DNA (Figure 19b) (102, 136, 137, 176). Furthermore, deletion of NegC alone (ΔNegC) increased the frequency of remodeling towards the short linker to 34% (Figure 19b). To further confirm the role of NegC in short-range linker length sensing, we
examined ensemble remodeling of nucleosomes with varying linker lengths and found that ΔNegC Snf2h does indeed remodel nucleosomes with 20-78 bp of linker DNA at comparable rates (Figure 20).

These findings suggest that for short-range linker length sensing, HSS acts as the sensor of linker DNA length and communicates that information to the ATPase through the autoinhibitory NegC domain. Interestingly, we found that a stripped-down version of the ISWI catalytic subunit without autoinhibition or short-range linker length sensitivity (AutoN-2RA + ΔNegC,HSS Snf2h) behaves like SWI/SNF remodelers such as RSC in that it appears to be able to partially unravel DNA from mononucleosomes by pulling the DNA end inside the nucleosome (Figure 21) (177, 178). We also confirmed that both AutoN-2RA + ΔNegC,HSS Snf2h and RSC engage the SHL2 site for DNA translocation as a 2 nt gap at SHL-2 blocks entry-side movement whereas a gap at SHL+2 blocks exit-side movement (data not shown).

3.1.3 Long-range linker length sensing: AutoN, H4 tail, and Acf1 N-term

We then turned our attention to understanding the more physiologically-relevant long-range linker length sensitivity of human ACF, a prototypical ISWI complex that is composed of a catalytic subunit, Snf2h, and accessory subunit, Acf1. Given the importance of the NegC domain in the short-range linker length sensitivity of the isolated catalytic subunit, we first examined the role of the NegC domain in the context of the full ACF complex (Figure 22a and 23a). Surprisingly, deletion of the NegC domain in the ACF complex (ΔNegC ACF) did not substantially affect the dependence of remodeling kinetics on linker DNA lengths ranging from 20 to 78 bp (Figure 23b,c). Removing the H4 tail dramatically reduced the remodeling rates of both wt and ΔNegC
ACF (Figure 23b). These results suggest that the NegC domain does not play a substantial role in linker length sensing by the ACF complex.

Next, we mutated the AutoN domain with two point substitutions (R142A and R144A) in the ACF complex (AutoN-2RA ACF; Figure 22a and 24a). AutoN bears sequence homology to the H4 tail, which can compete the inhibitory AutoN domain off the ATPase, and the 2RA mutation in AutoN is expected to diminish the H4 tail dependence of remodeling by ISWI enzymes (139). Remarkably, this mutation not only increased the remodeling rate of nucleosomes lacking the H4 tail, but also completely abolished the linker-length dependence of remodeling by selectively increasing the remodeling rate of short-linker nucleosomes (Figure 24b,c and 25). These results suggest an essential role for AutoN in long-range linker length sensing by the ACF complex. Of note, AutoN-2RA ACF has significantly reduced but not a complete lack of dependence on the H4 tail, which suggests that the H4 tail has other role(s) in promoting efficient remodeling beyond its contributions to relief of AutoN inhibition and linker length sensing (Figure 24b,c).

Since AutoN competes with the H4 tail for binding to the ATPase (139), we considered the possibility that this competition is involved in sensing linker DNA length and hypothesized that the H4 tail is only available to compete AutoN off the ATPase when the linker DNA is sufficiently long. Consistent with this hypothesis, adding exogenous H4-tail peptide, which should help compete AutoN off the ATPase when the nucleosomal H4 tail is unavailable, specifically increased the remodeling rate of nucleosomes with short linker lengths (e.g., [wt H4, 40 bp]) by wt ACF (Figure 26a). Furthermore, deletion of the nucleosomal H4 tail, in addition to slowing down
remodeling, abolished the dependence of remodeling rate on linker DNA length (Figure 26b). These results indicate that the H4 tail is indeed involved in linker DNA sensing.

Because the catalytic subunits of ISWI-family enzymes only interact with ~20-30 bp of linker DNA, the linker-length sensitivity of ACF cannot be accounted for by the catalytic subunit alone. Our findings raise the intriguing possibility of a linker-length sensing mechanism where the accessory subunit Acf1 interacts with the H4 tail in a linker-length dependent manner, which modulates the H4 tail availability for competing with AutoN. To test this possibility, we generated two Acf1 mutants, ΔC-term Acf1 and ΔN-term Acf1, in which 134 residues at the C-terminus or 371 residues at the N-terminus were deleted, respectively (Figure 22b, 27a, and 28a). Because the central region of Acf1 required for Snf2h binding (179, 180) was preserved, both mutants were able to form complexes with Snf2h, which are referred to as ΔC-term and ΔN-term ACF (Figure 22b).

We first probed which region of Acf1 interacts with the H4 tail by comparing the binding affinities of wt, ΔC-term and ΔN-term Acf1 for the H4-tail peptide using a fluorescence anisotropy assay. Interestingly, wt Acf1 exhibited specific, nanomolar affinity for the H4-tail peptide (Figure 27b) that was not substantially altered upon deletion of the C-terminal region (Figure 28b), but was completely lost upon deletion of the N-terminal region (Figure 27b). These results indicate that Acf1 likely interacts with the H4 tail through its N-terminal region. Acf1 also bound double-stranded DNA and deletion of the N-terminal region abolished this interaction as well (Figure 29), consistent with the previous finding that the WAC motif within the N-terminal region is important for binding of ACF to the linker DNA (179). Given the distinct properties of
DNA and the H4 tail, their specific binding interfaces within Acf1 N-term are likely distinct.

Next, we investigated nucleosome remodeling by the ΔC-term and ΔN-term ACF complexes. Notably, the ΔC-term mutation did not substantially alter the dependence of remodeling kinetics on linker DNA length (Figure 28c), whereas the linker-length sensitivity was eliminated in the ΔN-term ACF complex (Figure 27c,d). This finding is consistent with the specific affinity of Acf1 N-term for the H4 tail (Figure 27b). Furthermore, if the loss of linker-length sensitivity was simply a result of losing the linker DNA binding affinity of Acf1, ΔN-term ACF should demonstrate inefficient remodeling for all linker DNA lengths. Instead, ΔN-term ACF remodeled both short- and long-linker nucleosomes at rates similar to the rate with which wt ACF remodeled long-linker nucleosomes (Figure 27c,d), suggesting that deletion of Acf1 N-term disabled a mechanism that inhibits remodeling at short linker lengths. Unlike AutoN-2RA ACF, ΔN-term ACF maintained the H4-tail requirement in remodeling (Figure 24b,c and 27c).

Since Acf1 has affinity for both DNA and the H4 tail, a plausible interpretation of the above observations is that nucleosomal linker DNA and H4 tail are in competition for binding to the N-terminal region of Acf1 and that this competition is modulated by the length of the linker DNA. Only when the linker is sufficiently short does Acf1 preferentially bind to the H4 tail, making it unavailable to compete with the inhibitory AutoN. Deletion of Acf1 N-term diminishes the Acf1-H4 tail interaction such that the H4 tail is equally available to activate the ATPase in the context of both short and long linker DNA lengths (Figure 27c,d). To further test this model, we probed the linker-length dependence of the proximity between Acf1 and the H4 tail in ACF-bound nucleosomes featuring a cysteine-reactive crosslinker (1.78 nm) attached to the N-
terminus of the H4 tail (Chapter 2). Specific Acf1-H4 crosslinking product was clearly observed as a band with reduced electrophoretic mobility compared to non-crosslinked Acf1 (Figure 30). Remarkably, the Acf1-H4 crosslinking efficiency decreased substantially with increasing linker DNA length (Figure 30a), supporting our model that the Acf1-H4 tail interaction is modulated by the linker DNA length. In contrast, the Snf2h-H4 crosslinking efficiency did not change substantially with linker DNA length, likely because Snf2h remains sufficiently close to the H4 tail regardless of the linker DNA length, which allows crosslinking even when the H4 tail was not specifically bound to its putative binding pocket on Snf2h (Figure 30a).

Finally, we tested the physiological importance of the N-terminal region of Acf1 by studying the role of its homologue in yeast (181). Yeast ISW2 is functionally similar to ACF. It is composed of a catalytic subunit (Isw2) that is homologous to Snf2h and three accessory subunits (Itc1, Dpb4 and Dls1), among which Itc1 is homologous to Acf1. We generated three mutant yeast strains: (1) deletion of the entire itc1 gene (Δitc1), (2) deletion of only the portion of itc1 that encodes the N-terminal region of Itc1 equivalent to Acf1 N-term (Δitc1-Nterm), and (3) a rescue strain that was derived from the Δitc1-Nterm strain by deleting the remainder of itc1 (rescue-Δitc1). Both Δitc1 and rescue-Δitc1 showed growth rates similar to that of the wt strain (Figure 31), consistent with previous observations (181). In contrast, the Δitc1-Nterm strain displayed dramatically slower growth (Figure 31), consistent with an aberrant chromatin-misregulation phenotype.

Taken together, our results suggest a nucleosome-spacing mechanism for ACF in which the linker DNA length is sensed by the Acf1 accessory subunit and allosterically transmitted to the Snf2h catalytic subunit through the H4 tail of the
nucleosome (Figure 32). Surprisingly, Acf1 and the AutoN domain of Snf2h function collectively in DNA linker-length sensing. When the linker DNA is short, Acf1 preferentially binds to and sequesters the H4 tail, making it unavailable to compete its sequence homologue, AutoN, off the ATPase. Hence, the ATPase activity is inhibited by AutoN. As the linker DNA length increases, Acf1 shifts its binding preference to the linker DNA and releases the H4 tail, allowing it to compete AutoN off the ATPase and activate ACF. This competition between the H4 tail and linker DNA for Acf1 binding likely involves the N-terminal region of Acf1. It is interesting to note that linker DNA sensing occurs during the pause phases when the ATPase domain is not actively translocating DNA, suggesting that AutoN engages the ATPase domain during the pauses. In order to exit the pauses, the H4 tail is required to relieve the inhibitory effect of AutoN. The re-engagement of AutoN with the ATPase domain after each translocation phase would give ACF an opportunity to periodically sense the linker DNA length. Such frequent sensing may enable more efficient nucleosome spacing, as previously hypothesized (182). The linker DNA and the H4 tail are two important substrate features that regulate nucleosome remodeling by ISWI-family enzymes, the former enabling uniform nucleosome spacing for heterochromatin formation and the latter specifying regions of chromatin marked for silencing. Our results now reveal an unexpected convergence of the regulatory pathways defined by these two distinct nucleosome features.

3.2 Regulation of chromatin remodeling by linker histone H1

Linker histones are vital for the formation and stabilization of higher-order chromatin structure (183). Linker histone H1 binds to two locations on the nucleosome:
(i) approximately 10 bp of nucleosomal DNA centered about the dyad, and (ii) ≥ 10 bp of linker DNA on one or possibly both sides of the nucleosome (Figure 33a) (184, 185). The complex formed by a core nucleosome and linker histone is known as a chromatosome. The amount of DNA (147 bp) wrapped around the core octamer (~1.7 turns) does not change in the presence of linker histones.

In the absence of linker histones, the two linker DNA segments on either side of a nucleosome typically diverge from each other due to their strong mutual repulsion, especially at low ionic strength (Figure 33b) (186-188). Linker histones induce a characteristic stem conformation in which the two linker DNA segments become closely apposed ~8 nm from the nucleosome center and remain in contact for approximately 3-5 nm before diverging (Figure 33b) (189). The linker histone-mediated stem motif is believed to direct the arrangement of nucleosomes and linker DNA within the chromatin fiber to establish a three-dimensional, zigzag folding pattern (core nucleosomes peripheral, linker DNA internal) that is conserved during higher-order compaction (Figure 33b). Although there have been numerous studies on the structure and position of the linker histone within the chromatosome (184, 189-192), the effect of linker histones on chromatin remodeling is not well understood (157). Indeed, much of the function of chromatin remodeling enzymes has been gleaned from studies using nucleosomal substrates that bear little resemblance to physiological chromatin, which is generally composed of chromatosomes as the fundamental unit (157). Among the handful of prior studies on the regulation of remodeling activity by the presence of linker histones, there is significant disagreement with some reporting that linker histones significantly hinder remodeling whereas others describe primarily changes in remodeling outcomes (161-164). In this section, we probe the effects of linker histone
H1 on ISWI and SWI/SNF remodeling activity at the single-molecule level to answer some outstanding questions in the field.

3.2.1 ISWI remodelers can slide intact chromatosomes

In one noteworthy study, Maier and colleagues subjected arrays of a dozen nucleosomes or chromatosomes to remodeling by ACF or CHD1 complexes (162). While the presence of linker histones prevented remodeling by CHD1, ACF was able to catalyze significant repositioning of chromatosomes with > 50% of the remodeling efficiency seen with nucleosomal substrates. This result was somewhat surprising because linker histones constrain the path of linker DNA, limit the amount of free linker available (but only the proximal 10-20 bp of linker DNA, which may not play an important role in long-range linker length sensing as discussed in Section 3.1), and compete with remodelers for some of the same binding sites on the nucleosomal substrate. Both ACF and CHD1 promote nucleosome assembly, but only ACF can catalyze in vitro assembly of H1-containing, periodic chromatosome arrays (193). Given the proficiency of ACF in catalyzing the assembly and remodeling of chromatosomes, it has been posited that the ability to modify chromatosome arrays may be an intrinsic characteristic of the ISWI family. Indeed, loss of ISWI function in vivo does not cause obvious defects in nucleosome assembly, but leads to a significant reduction in the level of H1 associated with chromatin (194). This finding suggests that ISWI may play a global role in chromatin compaction by facilitating the association of linker histones with chromatin.

Current evidence for ACF remodeling of chromatosomes is based solely on bulk assays assessing the susceptibility of chromatosomal DNA to nuclease digestion (162).
While useful, this information is both indirect and inadequate for a mechanistic understanding of chromatosome remodeling by ACF. For example, how does ACF accomplish chromatosome repositioning? Since ACF is known to promote the assembly of H1-containing chromatin (193), it may also catalyze the reverse reaction, namely the removal of H1. Does chromatosome translocation by ACF require eviction (active or spontaneous) of H1 or can ACF slide an intact chromatosome? To answer this question, we developed a smFRET assay to monitor chromatosome remodeling by ACF in real-time.

To simplify our initial experiments, we decided to first focus on monochromatosomes. However, past studies on the linker histone-induced stem motif were performed in the context of chromatin or reconstituted nucleosome arrays^{34,38-41}. Hence, we first needed to determine whether binding of a linker histone to a mononucleosome generates a stem conformation similar to that observed in arrays. Towards this end, we designed a [45 bp, wt, 45 bp] nucleosome construct with unlabeled octamer, Cy5 located 25 bp away from the exit-side nucleosome edge, and Cy3 located 25 bp away from the entry-side nucleosome edge (Figure 34a). The starting FRET of this construct was 0.29 (Figure 34b). After the addition of 1 μM recombinant human histone H1^0 (K_D ~ 7.4 nM) (195), essentially all of the nucleosomes shifted to a higher FRET of approximately 0.84 (Figure 34b). This finding confirms that linker histone binding to mononucleosomes causes the linker DNAs to become closely apposed—a defining feature of the chromatosome stem motif. Since there is evidence that linker histones may bind to linker DNA asymmetrically (i.e., bind to ≥ 10 bp of linker DNA on one side of the nucleosome), we may be able to control the side that H1^0 binds by only providing adequate linker DNA on one side of the nucleosome.
In order to monitor linker histone binding and chromatosome remodeling simultaneously, we labeled recombinant H1⁰ with Alexa Fluor 488 (A488)-TFP ester. There are eight solvent accessible lysine residues on H1⁰ and four of these have been shown to be important for nucleosome binding (K69,78,85,97) while the other four are not (K27,40,52,59) (Figure 33a) (184). The effect of labeling H1⁰ on its ability to bind nucleosomes was determined by varying the degree of labeling (DOL) and performing a binding gel shift assay (Figure 35a). Naked DNA did not bind H1⁰ as efficiently as nucleosomes (Figure 35a: lanes 2 and 3). Moreover, an increase in DOL led to reduced nucleosome binding (Figure 35a: lanes 4-6). To minimize the effects of labeling on binding, we used a labeled H1⁰ with a DOL of 0.5 for reconstituting chromatosomes.

Reconstitution of end-positioned chromatosomes ([3 bp, wt H1⁰-A488, 78 bp]) (Figure 35b) yielded a mixture of chromatosomes and nucleosomes, which could be separated at the single-molecule level based on the presence or absence of H1⁰-A488, respectively. A electrophoretic mobility shift assay showed remodeling, but the results are difficult to interpret (Figure 35c). Field FRET histograms demonstrated that surface-anchored chromatosomes/nucleosomes remodeled, but it is not clear whether the enzyme is remodeling bare nucleosomes exclusively or a combination of intact chromatosomes and bare nucleosomes (Figure 35d). To distinguish between these two possibilities by obtaining real-time movies of individual remodeling events, we modified the smFRET microscope and analysis software to accommodate alternating 488 nm (A488) and 532 nm (Cy3) laser lines and three-color detection of Cy3, Cy5, and A488 fluorescence emissions. After adding ACF and ATP to surface-anchored chromatosomes/nucleosomes, we observed remodeling of a combination of bare nucleosomes and intact chromatosomes (A488 signal plus stepwise reduction in FRET;
Figure 35e). To the best of our knowledge, this is the first direct observation of chromatosome remodeling by an ISWI remodeling complex.

Several additional lines of inquiry are ongoing. Given that the linker histone and ACF may compete for the same nucleosome binding sites on one side of the nucleosome, does the presence of a linker histone force ACF to act as a unidirectional, monomeric translocase instead of a bidirectional, dimeric translocase? To answer this question, we are investigating ACF-mediated remodeling of centered chromatosomes (90). Interestingly, we have evidence that linker histones convert SWI/SNF from a bidirectional remodeler into a unidirectional remodeler (Section 3.2.2). Moreover, we are interested in characterizing the chromatosome remodeling mechanism and kinetics (e.g., step size/pause positions, translocation and pause phase rates). Finally, non-specific labeling of H1\(^0\) has a small but appreciable effect on binding to the nucleosomal substrate (Figure 35a). To circumvent the potential effects secondary to non-specific labeling of lysine residues, we will also employ a specifically labeled H1\(^0\)CCys mutant (196).

3.2.2 Linker histone H1 regulates SWI/SNF remodeling directionality

Unlike ISWI remodelers, SWI/SNF enzymes such as RSC are not regulated by linker DNA length (2). Hence, rather than centering mononucleosomes on a DNA fragment, SWI/SNF remodelers partially unravel DNA from mononucleosomes by sliding nucleosomes ~50 bp beyond the DNA edge until the DNA end resides at SHL2 (178). Indeed, remodeling of FRET-labeled mononucleosomes with 6 bp and 78 bp of linker DNA by RSC generates two distinct products, depending on which side of the nucleosome the ATPase engages (Figure 21a). If RSC engages SHL-2, it translocates
DNA towards the long linker, thereby repositioning the histone octamer ~50 bp toward the short linker and generating a product in which the labeled DNA end is located at SHL-2 (entry-side translocation). Based on the nucleosome crystal structure, this final product should have a Cy3-Cy5 separation of ~6.5 nm, which should yield FRET < 0.4 (Figure 21a). If RSC engages SHL+2 instead, it translocates DNA towards the short linker, thereby repositioning the histone octamer ~130 bp toward the long linker (exit-side translocation). In this case, the final product should exhibit a Cy3-Cy5 separation of > 40 nm, resulting in zero FRET (Figure 21a).

Consistent with the fact that RSC lacks linker length sensitivity, we found that it remodels [6 bp, wt, 78 bp] mononucleosomes in both directions with equal frequency (“-CGCG” column in Figure 36). The Rsc3 accessory subunit of the RSC complex is essential for the maintenance of nucleosome-free regions in many yeast promoters and is known to act as a DNA-binding protein with specificity for the Gal4-like consensus sequence CGCG (197). When we inserted the Rsc3 binding site on the long linker, RSC showed a greater than 5-fold preference for engaging SHL-2 and promoting entry-side translocation (“+CGCG” column in Figure 36). Interestingly, when we added H1 link histones to the [6 bp, wt, 78 bp] nucleosomes and subjected them to remodeling by RSC, entry-side translocation was essentially precluded in favor of exit-side translocation, regardless of the presence or absence of the CGCG binding site (“-CGCG,H1” and “+CGCG,H1” columns in Figure 36). This is consistent with a previous biochemical study that showed that the presence of histone H1 caused human SWI/SNF to slide mono-chromatosomes to more central positions rather than off the ends of the DNA (164). This observation of linker histones modulating the outcome of SWI/SNF remodeling may have important physiological implications. It has been
suggested that the mechanism of SWI/SNF mediated octamer ejection is the translocation of one nucleosome in close proximity to a neighboring nucleosome and causing DNA unwrapping and destabilization of the latter (198). We hypothesize that the ability to slide mono-nucleosomes off the DNA edge may be equivalent to the ability to mediate octamer ejection in multi-nucleosome substrates. To confirm this hypothesis, we plan on studying octamer ejection in the context of dinucleosomes and nucleosome arrays with and without linker histones. To further characterize RSC remodeling of chromatosomes, we will employ labeled linker histones as described in Section 3.2.1. Nevertheless, the effect of linker histones on remodeling directionality cannot be explained by exclusive remodeling of a small subset of nucleosomes that lack linker histones.
5 CONCLUSIONS AND FUTURE OUTLOOK

The packaging of genomic DNA into chromatin provides a means to accommodate a large amount of genetic material in the small space provided by the nucleus as well as regulate access to DNA by cellular machinery that are involved in critical processes such as DNA replication, transcription, and repair (2, 6, 9). Chromatin remodeling enzymes are a diverse and pervasive group of ATP-dependent molecular motors that catalyze a wide range of structural transformations to dynamically modulate transitions between chromatin states. Given their fundamental epigenetic roles, it is not surprising that mutations in chromatin remodeling complexes have been linked to many cancers and developmental disorders (41-64). There is increasing interest and efforts in developing novel therapeutic agents to target chromatin remodelers, the success of which will rely on having a greater understanding of the mechanistic principles underlying the function and regulation of chromatin remodelers.

In this thesis, we describe the development and application of single-molecule biophysical techniques, namely single-molecule fluorescence resonance energy transfer (smFRET), to investigate the mechanisms and regulation of chromatin remodeling enzymes at an unprecedented level of detail.

First, we demonstrated that contrary to previous models of nucleosome translocation, ISWI remodelers use a spring-loaded mechanism in which the ATPase pumps DNA from inside the nucleosome towards the exit side in 1 bp increments and the accumulation of strain triggers periodic multi-bp movements of DNA into the nucleosome from the entry side (Section 1.4).

Next, we unraveled the mechanistic basis for the linker length sensing ability of ISWI remodelers such as human ACF, a property that enables creation of regularly-
spaced nucleosome arrays – a prerequisite for higher-order chromatin compaction and heterochromatin formation (Section 3.1). The short-range (0-40 bp) linker length sensing by the isolated catalytic subunit (Snf2h) relies on the HAND-SANT-SLIDE DNA-binding module interacting with linker DNA and modulating the autoinhibitory domain NegC. In contrast, the long-range (40-60 bp) linker length sensing exhibited by the full ACF complex depends on communication between the accessory subunit (Acf1) and the catalytic subunit (Snf2h). We discovered that the N-terminus of Acf1 (Acf1 N-term) binds linker DNA and the unmodified H4 tail with high affinity, likely utilizing two distinct domains that are regionally co-localized. The unmodified H4 tail is thought to relieve the catalytic autoinhibition conferred by the AutoN domain through competition for a common binding site on the ATPase (139). Crosslinking and remodeling studies support a model in which the linker DNA and H4 tail compete for binding with the Acf1 N-term. In the presence of long linker DNA, Acf1 N-term will preferentially bind to the DNA, leaving the H4 tail free to relieve AutoN inhibition. On the other hand, if there is inadequate linker DNA, Acf1 N-term will sequester the H4 tail instead and maintain AutoN inhibition.

Finally, we examined the effects of linker histones (chromatosomes) on chromatin remodeling, an understudied area of research with little agreement among prior investigations. Using labeled linker histones and a two-color alternating laser excitation/FRET scheme, we provided the first direct, real-time evidence that ISWI complexes are capable of translocating intact chromatosomes. Remodelers that lack linker length dependence such as the SWI/SNF family typically have no preference for remodeling direction and slide mono-nucleosomes off the edge of the DNA fragment. Interestingly, the presence of a linker histone modifies the remodeling outcome of
SWI/SNF enzymes such that chromatosomes are preferentially translocated to more central positions and prevented from being slid off the DNA end.

There are several exciting new directions of investigation we are exploring. First, we are extending our studies to other remodeler families to determine the similarities and differences in the mechanisms underlying their interactions with chromatin. To increase the physiological accuracy of our *in vitro* experiments, we are interested in further study of di-nucleosomes and nucleosome arrays. These complex substrates can enable investigation of remodeling activities such as SWI/SNF-mediated octamer ejection that seem to be dependent on the presence of more than one nucleosome (198). Furthermore, some regulatory mechanisms may only occur at the level of nucleosome arrays. For example, inter-nucleosomal H4 tail interactions are important for chromatin compaction (147) and there are conflicting reports on the effects of H4 tail acetylation on ISWI remodeling depending on whether the substrate is a mono-nucleosome or nucleosome array (146, 151).

H2A.Z is a H2A variant that localizes to approximately half of annotated promoter regions (199-203). Interestingly, the presence of H2A.Z nucleosomes stimulates ISWI remodeling (204). An acidic patch within the M6 region of H2A (D_{99}D_{100}E_{101}) is extended in the H2A.Z variant (D_{99}D_{100}E_{101}L_{102}D_{103}) (205). The M6 domain is essential for embryonic development in *Drosophila* and is the only region of H2A.Z that does not allow transgenic flies to survive to pupation when replaced with the analogous region in canonical H2A (206). Importantly, mutations in the extended M6 patch of H2A.Z obviate the H2A.Z-induced increase in ISWI activity (204). The basic patch of the H4 tail interacts with the acidic patch of H2A.Z, which is believed to play a role in chromatin fiber folding (143, 147). It has been conjectured that the extended acidic patch in H2A.Z
may stimulate ISWI activity through changes in the position or accessibility of the H4 tail (207). To test this hypothesis, we will compare ISWI-mediated remodeling of H2A.Z + H4, H2A.Z + H4Δ1-19, and H2A + H4Δ1-19 nucleosomes in bulk and smFRET remodeling assays. If augmentation of ISWI remodeling by H2A.Z is entirely dependent on the presence of the H4 tail, then we would expect that the remodeling kinetics of H2A.Z + H4Δ1-19 nucleosomes would be the same as that of H2A + H4Δ1-19 nucleosomes. If, however, the H2A.Z variant can at least partially stimulate ISWI remodeling independent of the H4 tail, then the remodeling rate of H2A.Z + H4Δ1-19 nucleosomes should be greater than that of H2A + H4Δ1-19 nucleosomes. Moreover, to determine whether the interaction between the H4 basic patch and H2A.Z extended acidic patch per se is recognized by ISWI, or whether the specific location of each domain on the nucleosomal core particle is important, we will use site-directed mutagenesis to swap the location of the two domains, i.e., basic patch moved to H2A.Z and acidic patch moved to H4.

While the majority of our experiments focus on observing real-time changes in nucleosome position, we have also developed complementary site-specific labeling strategies for ISWI remodelers. In particular, we have created and validated a “Cys-Light” Snf2h mutant that has all solvent accessible cysteines mutated to serines (208). At any desired labeling site, a single residue can be converted to cysteine and specific labeling is achieved with dye-maleimide conjugates. We are currently using Snf2h mutants with specific labels in the HAND, SANT, SLIDE, and/or ATPase domains and substrates labeled at different locations along the DNA or histones to harness smFRET for learning more about dynamic enzyme-substrate interactions and enzyme conformational changes during remodeling. It would be even more powerful if we could
combine this labeled enzyme-substrate smFRET assay with concurrent monitoring of nucleosome translocation so that we can observe enzyme-substrate interactions during nucleosome translocation. One strategy is to use multicolor FRET to capture both of these processes at the same time. Alternatively, we are developing new strategies to monitor nucleosome translocation without using smFRET. For example, as the remodeler ATPase translocates along one strand of DNA, torsion is generated and the free DNA end will rotate. By attaching a DNA origami propeller to the free end of the DNA and labeling the ends of the propeller, the rotation that occurs during remodeling can potentially serve as a readout of nucleosome sliding (209).

In summary, chromatin remodeling complexes are a diverse and ubiquitous class of molecular motors that play vital roles in the dynamic control of chromatin structure and accessibility. Single-molecule biophysical techniques such as single-molecule FRET are powerful complements to more traditional biochemical and molecular biology approaches and we have shown that this synergistic combination can yield significant insights into the mechanisms underlying the function and regulation of chromatin remodeling.
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Figure 1. Nucleosome structure and nomenclature. Crystal structure of the nucleosome demonstrating 147 bp of DNA wrapped around a histone octamer in a left-handed superhelix (3, 4). Additional B-form DNA is added to the entry side (longer) and exit side (shorter) to represent linker DNA. Positions around the nucleosome are named based on superhelical locations (SHL), which refer to the major and minor grooves of DNA as they alternately face the histone octamer. The dyad is denoted as SHL0 and moving away from the dyad, each inward facing major groove is denoted SHL±1, ±2, etc. while each inward facing minor groove is denoted SHL±0.5, ±1.5, etc.
Figure 2. Chromatin remodeler families. The five chromatin remodeler families are defined by unique flanking domains in their catalytic subunits. All families possess a SWI2/SNF2-family ATPase domain composed of two parts: DExx (purple) and HELICc (red). The SWR1-related complexes are typically included in the INO80 family.
Figure 3. FRET can be used as a nanoscopic ruler. Plot of theoretical FRET efficiency ($E$) as a function of dye separation ($R$) for a Förster radius ($R_0$) of 5 nm. Excitation of the donor dye results in a combination of donor fluorescence and energy transfer to the acceptor depending on the distance between the dyes.
**Figure 4. FRET-labeled nucleosomes.** (a) Crystal structure of a nucleosome from Figure 1 with labeling sites for the FRET donor Cy3 (H2A-K120C) and FRET acceptor Cy5 (exit-side 5’ DNA end) indicated by green and red stars, respectively. A biotin moiety (blue square) is attached to the entry-side 5’ DNA end. (b) FRET distributions of nucleosomes with 3 bp of exit-side linker DNA and 78 bp of entry-side linker DNA before (blue bars) and 10 minutes after (red bars) the addition of 5 nM ACF and 2 mM ATP. The three initial peaks centered at FRET = 0.87, 0.68, and 0.47 (Gaussian fit, black line) are the result of three distinct labeling configurations as shown: nucleosomes with a single Cy3 on the H2A proximal to the Cy5, nucleosomes with a single Cy3 on the H2A distal to the Cy5, and doubly-labeled nucleosomes. A substantial decrease in FRET after the addition of ACF and ATP indicates remodeling of the nucleosome substrate. No FRET change was observed when ACF was added in the absence of ATP.
Figure 5. Single-molecule FRET remodeling assay. Nucleosomes are anchored to the surface of a passivated quartz slide via a biotin-streptavidin linkage and imaged in a prism-type total internal reflection fluorescence (TIRF) configuration with a water-immersion objective lens.
Figure 6. Prior models of nucleosome translocation. Schematic diagrams of (a) twist diffusion, (b) loop/bulge propagation, and (c) concerted swiveling models for nucleosome translocation by ATP-dependent remodeling enzymes. See text for details. The DNA and histone octamer are shown in black/gray and yellow, respectively. The upper and lower DNA gyres are depicted as solid black and dotted gray lines, respectively.
Figure 7. ISWI remodelers catalyze nucleosome translocation with well-defined step sizes. (a) Representative Cy3 and Cy5 fluorescence (green and red, respectively) and FRET (blue) time traces during the remodeling of a single nucleosome with 3 bp of exit-side linker DNA and 78 bp of entry-side linker DNA. After a wait period \( (t_{\text{wait}}) \), gradual remodeling is interrupted by well-defined kinetic pauses that divide the remodeling process into alternating translocation phases \( (t_{T1}, t_{T2}) \) and pause phases \( (t_{P1}, t_{P2}) \). (b) FRET values for a series of nucleosome constructs with varying exit-side linker DNA lengths \( (m \text{ bp}) \). The dependence of FRET on nucleosome position is approximately linear in the range of 3-15 bp with a slope of \(-0.059 \pm 0.002 \text{ bp}^{-1}\). (c) Histogram of FRET levels for the initial position and subsequent translocation pauses constructed from many nucleosomes. The peak FRET values were obtained from a Gaussian fit (black line) and used to compute the indicated average translocation distances (step sizes) between the initial position and the first pause as well as between the first pause and the second pause.
Figure 8. Surface-anchoring does not perturb nucleosome remodeling. Comparison of the average remodeling kinetics for surface-anchored [wt H4, 40 bp] nucleosomes (measured by the single-molecule assay, > 250 nucleosomes) and freely diffusing [wt H4, 40 bp] nucleosomes in solution (measured by the ensemble assay). [ACF] = 5 nM and [ATP] = 20 μM.
Figure 9. Compound exit-side translocation steps are composed of single base pair elementary steps. (a) Representative FRET time trace, with (blue) and without (gray) applying a five-point median filter, of a single nucleosome with 3 bp of exit-side linker DNA and 78 bp of entry-side linker DNA after the addition of 6.2 nM ISW2, 2 µM ATP, and 2 mM ATP-γ-S at time zero. Traces were fit using a hidden Markov modeling algorithm (HMM, red line) (168). The dotted orange lines demarcate 1 bp intervals based on the calibration curve in Figure 7b. (b) Histograms of FRET plateaus derived from many remodeling traces using HMM analysis. We divided the FRET space into two regions (0.32–0.62 and 0.59–1) and allowed 10 initial states in each region. HMM analysis of the FRET traces converged to ~4-5 states in each region, indicating that state identification was unlikely affected by the initial parameter setting. The average translocation distances (bp) for each identified step is shown.
Figure 10. Exit-side translocation precedes entry-side DNA movement during ISWI remodeling. (a) Top: Schematic diagram illustrating the ISWI-mediated translocation of an exit-side labeled nucleosome. Bottom: Representative Cy3 (green) and Cy5 (red) fluorescence and FRET (blue) time traces showing the translocation of a single exit-side labeled nucleosome after the addition of ISW2 and ATP. (b) Top: Schematic diagram illustrating the ISWI-mediated translocation of an entry-side labeled nucleosome. The Cy5 dye is initially positioned 10 bp from the nucleosome edge. Bottom: Representative Cy3 (green) and Cy5 (red) fluorescence and FRET (blue) time traces showing the translocation of a single entry-side labeled nucleosome after the addition of ISW2 and ATP at time zero. (c) Comparison of $t_{\text{wait}}$ on the exit (blue bars) and entry sides (red bars) of the nucleosome under the same remodeling conditions. Data are displayed as mean ± s.e.m. ($N = 80 - 220$ events).
Figure 11. Entry-side DNA movement occurs after 7 bp of DNA translocation towards the exit side and proceeds in 3 bp steps. (a) Nucleosome constructs used to observe entry-side DNA movement when exit-side translocation is restricted by a 2 nt ssDNA gap positioned $x$ bp from the SHL+2 site. Translocation stops when the gap coincides with SHL+2 so only $x$ bp of exit-side translocation is allowed. (b) Representative FRET time traces of individual $x = 0$, 7, and 8 bp nucleosomes after addition of 12 nM ISW2 and 2 µM ATP at time zero. (c) FRET values before (red) or after (blue) remodeling by ISW2 as a function of the gap distance $x$ from the SHL2 site. Data is shown as the mean ± s.e.m. ($N = 80 - 150$ nucleosomes). Since the DNA path on the entry side may involve twisting and/or bending as a result of direct interaction with the remodeling enzyme, we do not anticipate a similar linear dependence of FRET on the linker DNA length as on the exit side where the linker DNA is mostly free of enzyme-induced distortion.
Figure 12. Model for nucleosome translocation by ISWI family remodelers. The histone octamer is represented as a yellow oval. The upper and lower DNA gyri are shown as solid black and dotted gray lines, respectively. Each base pair of DNA translocated to the exit side is depicted as a red dot. The remodeling enzyme is shown as a semi-transparent, amorphous shape that is either blue or green depending on its conformation. The linker DNA-binding domain and ATPase domain are highlighted as green and blue spheres, respectively. Remodeling begins with the ATPase domain translocating DNA towards the exit side in 1 bp increments. The entry-side linker DNA is immobilized by the linker DNA-binding domain of the enzyme and therefore accumulates strain (purple/pink coloring of the DNA). Once 7 bp of exit-side translocation has occurred, the strain is adequate to stall the ATPase and trigger an entry-side action (e.g., conformational change of the enzyme) that draws in a 3 bp equivalent of DNA into the nucleosome. The reduction in strain from 7 to 4 bp allows another 3 bp of DNA to be translocated to the exit side before the ATPase stalls again. This cycle then repeats to facilitate processive nucleosome translocation.
Figure 13. DNA constructs for mononucleosomes and dinucleosomes. The 601 nucleosome positioning sequence is shown in green (601\* represents the introduction of 2-nt gaps at nucleotides 53 and 54 in the 601 positioning sequence, respectively). For mononucleosome DNA constructs, the ssDNA spacer used to circumvent surface effects in single-molecule FRET measurements is underlined. Constructs referred to as “mononucleosome or dinucleosome with \( n \) bp linker DNA" were used in single-molecule and bulk remodeling experiments. For ensemble remodeling experiments with mononucleosomes, the ssDNA spacer was omitted without any appreciable change in the overall remodeling kinetics. Constructs referred to as “symmetric mononucleosomes with \( n \) bp linker DNA" were used in crosslinking experiments. Asymmetric constructs with the same linker length on one side of the nucleosome but only 3 bp of linker DNA on the other side displayed quantitatively similar crosslinking behavior.
Figure 14. The linker DNA length and histone H4 tail regulate the catalytic activity of ISWI remodelers. (a) Schematic of a FRET-labeled mononucleosome undergoing remodeling by ACF. (b) Ensemble remodeling time courses of [wt H4, 78 bp], [wt H4, 40 bp], [wt H4, 20 bp], and [H4Δ1-19, 78 bp] nucleosomes by 40 nM ACF at 5 µM ATP. Nucleosome translocation is monitored by the emission intensity of the FRET acceptor Cy5 under excitation of the FRET donor Cy3. (c) Ensemble remodeling time courses of [wt H4, 78 bp] nucleosomes by 10 nM ACF with 5 µM ATP (filled symbols) or without (open symbols) ATP. Nucleosome translocation is monitored by the emission intensity of the FRET acceptor Cy5 under constant 532 nm illumination that excites the FRET donor Cy3. (d) Cy3 and Cy5 fluorescence (top) and FRET (bottom) time traces during the remodeling of a single [wt H4, 78 bp] nucleosome with the translocation (T1, T2) and pause (P1, P2) phases indicated.
Figure 15. Translocation step sizes during nucleosome remodeling. (a) Histogram of FRET levels for the initial position and pause positions of [wt H4, 40 bp] nucleosomes upon remodeling by ACF. The histogram was fit by multiple Gaussian peaks (black line) and the peak values were used to compute the average translocation distances between pauses. The translocation distances can be quantified using a calibration curve of FRET efficiency versus exit-side linker DNA length (Figure 7b) (90, 91), yielding a 7.0 bp step size between the initial position and the first pause and a 3.4 bp step size between the first pause and the second pause. (b) Step sizes for various mononucleosomes and dinucleosomes. The dinucleosome constructs, [wt H4, 40 bp, wt H4] and [wt H4, 78 bp, wt H4], are each composed of one FRET-labeled nucleosome and one unlabeled nucleosome, spaced by 40 bp and 78 bp of internucleosomal linker DNA, respectively. The flanking linker DNA is 3 bp on the side of the FRET-labeled nucleosome and 40 bp on the side of the unlabeled nucleosome. The unlabeled nucleosome contains 2-nt ssDNA gaps at the SHL±2 sites to prevent translocation. Data are mean ± s.e.m derived from at least 100 remodeling traces derived from three independent experiments.
Figure 16. The linker DNA length and histone H4 tail regulate the remodeling pause phases but not the translocation phases. (a) Linker DNA length dependence of the translocation rates between pauses (left, defined as the average number of base pairs moved per second) and pause-phase exit rates (right, defined as the inverse of the average pause durations). (b) Dwell time distributions for the first translocation phase, $t_{T1}$, and the first pause phase, $t_{P1}$, for nucleosomes with different lengths of linker DNA. [ACF] = 10 nM and [ATP] = 20 μM. (c) Dependence of the translocation rates between pauses (left) and pause-phase exit rates (right) on the H4 variants. In (a) and (c), [ACF] = 10 nM and [ATP] = 2 mM. Data are mean ± s.e.m. derived from at least 100 (a) or at least 50 (c) individual nucleosome remodeling traces from three independent experiments.
Figure 17. DNA linker-length sensing by ACF is quantitatively similar for mononucleosomes and dinucleosomes. (a) Left: The dinucleosomes contain a distal FRET-labeled nucleosome and a proximal unlabeled nucleosome connected by \( n \) bp of inter-nucleosomal linker DNA. The flanking linker DNA is 3 bp and 40 bp on the side of the FRET-labeled and the unlabeled nucleosome, respectively. To facilitate the study of translocation of the FRET-labeled nucleosome, we placed 2-nt ssDNA gaps at the SHL±2 sites of the proximal nucleosome to prevent its repositioning. Right: Polyacrylamide gel electrophoresis showing the distal FRET-labeled nucleosome (lane 1, red band), the proximal unlabeled nucleosome (lane 2, green band: an Alexa 488 dye is attached for visualization), and the ligated dinucleosome product (lane 3, yellow band). Ligation reactions were performed with an excess of the distal FRET-labeled nucleosome (lane 3, red band). Only substrates with both nucleosomes could bind the streptavidin-coated surface and exhibit FRET. (b) Comparison of translocation and pause-phase exit rates for mononucleosomes (filled bars) and dinucleosomes (hashed bars) with 40 and 78 bp linker lengths. [ACF] = 10 nM and [ATP] = 20 μM. Data are mean ± s.e.m derived from at least 100 individual nucleosome remodeling traces from three independent experiments.
Figure 18. NegC exhibits autoinhibitory activity that is relieved by HAND-SANT-SLIDE interaction with linker DNA. (a) Left: The conserved domains present in ISWI catalytic subunits are depicted. The following Snf2h mutants were created: AutoN-2RA (R142A, R144A), ΔNegC (Δ669-700 replaced with SGSGS linker), ΔHSS (ΔC743), and ΔNegC,HSS (ΔC669). All constructs contained a C-terminal FLAG epitope for purification by anti-FLAG M2 affinity chromatography. Right: Purified Snf2h variants were analyzed by SDS-PAGE (4-15%). (b) Representative traces of ensemble nucleosome remodeling by wt Snf2h, ΔNegC Snf2h, ΔHSS Snf2h, and ΔNegC,HSS Snf2h using 2.5 nM [wt H4,78 bp] nucleosomes, 130 nM enzyme, and 2 mM ATP. Nucleosome translocation is monitored by the decrease in Cy5 intensity. A negative control (gray) with 2.5 nM [wt H4, 78 bp] nucleosomes only is provided.
Figure 19. Short-range linker length sensing by the Snf2h catalytic subunit requires the NegC domain – Part 1. (a) Representative Cy3 and Cy5 fluorescence (green and red, respectively) and FRET (blue) time traces during the remodeling of a single [6 bp, wt H4, 78 bp] nucleosome either towards the short linker (FRET increase) or long linker (FRET decrease). (b) Fraction of [6 bp, wt H4, 78 bp] nucleosomes initially remodeled towards the short linker in the presence of wt Snf2h, AutoN-2RA Snf2h, ΔNegC Snf2h, or ΔNegC,HSS Snf2h using 300 nM enzyme and 2 mM ATP. The fractions of nucleosomes remodeled towards the short linker for each mutant were as follows: wt Snf2h (15/126), AutoN-2RA Snf2h (23/159), ΔNegC Snf2h (53/154), and ΔNegC,HSS Snf2h (53/135).
Figure 20. Short-range linker length sensing by the Snf2h catalytic subunit requires the NegC domain – Part 2. Ensemble remodeling time courses of [wt H4, 78 bp], [wt H4, 40 bp] and [wt H4, 20 bp] nucleosomes by 130 nM of wt (a) or ΔNegC Snf2h (b) at 2 mM ATP.
Figure 21. The AutoN-2RA + ΔNegC,HSS Snf2h mutant exhibits remodeling behavior similar to SWI/SNF enzymes such as RSC. (a) Schematic diagrams depicting a [6 bp, wt, 78 bp] mononucleosome before (left) and after (middle) remodeling by RSC. Since RSC is not regulated by the linker DNA, it demonstrates exit-side translocation (top) and entry-side translocation (bottom) with equal frequency. Note that in both cases, the octamer can be remodeled ~50 bp beyond the edge of the DNA (remodeling terminates when the DNA end arrives at SHL±2). (b) Domain architecture of AutoN-2RA + ΔNegC,HSS Snf2h. Like RSC, this stripped down version of Snf2h exhibits exit-side translocation (left) and entry-side translocation (right) at approximately equal frequencies.
Figure 22. SDS-PAGE analysis of wt, ΔNegC, AutoN-2RA, ΔN-term, and ΔC-term ACF complexes. (a) wt Snf2h, ΔNegC Snf2h, or AutoN-2RA Snf2h were co-expressed with Acf1-FLAG in Sf9 insect cells and purified by affinity chromatography. (b) ΔN-term Acf1 or ΔC-term Acf1 was co-expressed with Snf2h-FLAG and purified by affinity chromatography. The presence of both Acf1 and Snf2h in each case indicated that wt SNF2h, ΔNegC SNF2h and AutoN-2RA SNF2h can all form complexes with Acf1 and that ΔN-term Acf1 and ΔC-term Acf1 can both form complexes with Snf2h.
Figure 23. Deletion of the NegC domain of the Snf2h catalytic subunit does not substantially affect linker DNA length sensing by the ACF complex. (a) Domain architecture of wt and ΔNegC Snf2h (residues 669-700 replaced with a SGSGS linker). (b) Ensemble remodeling time courses of [wt H4, 78 bp], [wt H4, 40 bp], [wt H4, 20 bp], and [H4Δ1-19, 78 bp] nucleosomes by 40 nM wt ACF (black/gray lines) and ΔNegC ACF (red/pink symbols) at 5 µM ATP. (c) Linker DNA length dependence of the pause-phase exit rate (P1 phase) measured for wt ACF (black) and ΔNegC ACF (red). [ACF] = 10 nM and [ATP] = 20 µM. Data are mean ± s.e.m. derived from at least 100 individual nucleosome remodeling traces from three independent experiments.
Figure 24: The AutoN domain of Snf2h is important for linker DNA length sensing by the ACF complex. (a) Domain architecture of wt and AutoN-2RA (R142A and R144A) Snf2h. (b) Ensemble remodeling time courses of [wt H4, 78 bp], [wt H4, 40 bp] and [H4Δ1-19, 78 bp] nucleosomes by 40 nM wt ACF (black/gray lines) and AutoN-2RA ACF (blue/cyan symbols) at 5 μM ATP. (c) Dependence of the pause-phase exit rate on the linker DNA length and H4 tail for wt (black) or AutoN-2RA ACF (blue/cyan). * too slow to be measured. [ACF] = 10 nM and [ATP] = 20 μM. Data are mean ± s.e.m from at least 100 individual nucleosome remodeling traces from three independent experiments.
Figure 25. Ensemble remodeling time courses of nucleosomes by wt and AutoN-2RA ACF at two different enzyme concentrations. (a) Remodeling of [wt H4, 78 bp] and [wt H4, 40 bp] nucleosomes by 40 nM (top) and 10 nM (bottom) wt ACF. (b) Remodeling of [wt H4, 78 bp] and [wt H4, 40 bp] nucleosomes by 40 nM (top) and 10 nM (bottom) AutoN-2RA ACF.
Figure 26. The nucleosomal H4 tail is important for linker DNA length sensing by the ACF complex. (a) Effect of the exogenously added H4-tail peptide on the pause-phase exit rates during remodeling by wt ACF. [ACF] = 10 nM, [ATP] = 20 μM. (b) Pause-phase exit rates of nucleosomes lacking the H4 tail during remodeling by wt ACF. [ACF] = 10 nM and [ATP] = 2 mM. Data are mean ± s.e.m from at least 100 (a) or at least 50 (b) individual nucleosome remodeling traces from three independent experiments.
Figure 27. The N-terminal region of the Acf1 accessory subunit is important for linker DNA length sensing by the ACF complex. (a) Domain architecture of wt and ΔN-term (residues 1-371 deleted) Acf1. (b) Fluorescence anisotropy of dye-labeled H4-tail peptide in the presence of varying amounts of wt (black symbols) or ΔN-term (green symbols) Acf1. Data are mean ± s.e.m. (n = 3 independent experiments). The K_d for wt Acf1 is 3 ± 9 nM (error represents the 95% confidence interval). (c) Ensemble remodeling time courses of [wt H4, 78 bp], [wt H4, 40 bp], and [H4Δ1-19, 78 bp] nucleosomes by 40 nM wt ACF (black/gray lines) and ΔN-term ACF (green/light green symbols) at 5 µM ATP. (d) Dependence of the pause-phase exit rate on the linker DNA length for wt ACF (black) or ΔN-term ACF (green). [ACF] = 10 nM and [ATP] = 20 µM. Data are mean ± s.e.m derived from at least 100 individual nucleosome remodeling traces from three independent experiments.
Figure 28. The C-terminal region of Acf1 is not required for specific binding to the H4 tail or for linker-length sensing by the ACF complex. (a) Domain architecture of wt and ΔC-term Acf1 (residues 1423-1556 deleted). (b) Fluorescence anisotropy of TMR-labeled H4-tail peptide in the presence of varying amounts of wt or ΔC-term Acf1. The measured $K_d$ for ΔC-term Acf1 is $2 \pm 7$ nM, which is similar to that for wt Acf1 ($3 \pm 9$ nM). Data are presented as mean ± s.e.m. (errors report the 95% confidence interval, $n = 3$ independent titration experiments). (c) Ensemble remodeling time courses of [wt H4, 78 bp], [wt H4, 40 bp], and [H4Δ1-19, 78 bp] nucleosomes by 40 nM wt (black/gray lines) and ΔC-term ACF (purple/light purple symbols) at 5 µM ATP.
Figure 29. Binding of dsDNA by Acf1 depends on its N-terminal region. Electrophoretic mobility of dsDNA (225 bp, 8 nM) in the presence or absence of 22 nM wt or ΔN-term Acf1. As a comparison, lanes 2 and 4 show Acf1 samples without the dsDNA.
Figure 30. Crosslinking of the H4 tail to Acf1 depends on the linker DNA length.
(a) The crosslinking products were analyzed by SDS-PAGE (left). The Acf1-H4 crosslinking band was absent for ACF without nucleosomes (lane “- nucleosomes”). (right) Quantification of the H4-crosslinked fractions of Acf1 and Snf2h as a function of linker DNA length. Data are mean ± s.e.m. (n = 3 independent crosslinking experiments). (b) Left: SDS-PAGE analysis of samples containing ACF alone or ACF with nucleosomes (20 bp linker DNA) that do not possess the cysteine-reactive crosslinker on the H4 tail. Both samples yield two distinct bands corresponding to the Acf1 and Snf2h subunits (180 kDa and 122 kDa, respectively). Additionally, histone bands at low molecular weights are present in the lane for the sample containing nucleosomes. Right: Corresponding immunoblot using α-H4 antibody. In the presence
of nucleosomes without crosslinker, a single H4 band is visible at ~11 kDa corresponding to the histone itself. (c) Top: Incubation of ACF and nucleosomes that contain a crosslinker at the H4 tail yield Acf1-H4 and Snf2h-H4 crosslinking bands. These bands are absent for ACF without addition of nucleosomes (“- nucleosomes”) or upon addition of nucleosomes without a crosslinker. Proteolytic degradation of Acf1 gave rise to a fainter band immediately below Acf1. Bottom: α-histone H4 immunoblotting reveals specific Acf1-H4 and Snf2h-H4 bands that are absent for ACF without addition of nucleosomes (“- nucleosomes”) or upon addition of nucleosomes without a crosslinker.
Figure 31. Effects of deletion of Itc1 (Acf1 homolog) and its N-terminal region on the growth of yeast cells. Top row: wt. Second row: the itc1 gene is deleted ($\Delta$itc1). Third row: the coding sequence of the N-terminal region of Itc1 is deleted ($\Delta$itc1-Nterm). Bottom row: The remaining portion of itc1 is deleted from $\Delta$itc1-Nterm (rescue-$\Delta$itc1). One representative of three independent growth experiments is shown.
Figure 32. Model for linker DNA length sensing by the ACF complex. DNA: gray lines, histone octamer: beige cylinders, Snf2h: blue/cyan, Acf1: green, Acf1 N-terminus: unlabeled green circle connected to Acf1 by a dark green line. The ATPase domain of Snf2h is depicted as a cyan sphere and labeled “ON” when active and “OFF” when inactive. The H4 tail and linker DNA compete for binding to the Acf1 N-terminus. The presence of long linker DNA (top) promotes binding of the Acf1 N-terminus to the linker DNA. The H4 tail is then free to compete with the autoinhibitory AutoN domain for a common binding site on the ATPase domain, which activates catalytic activity. On the other hand, short linker DNA (bottom) favors Acf1 N-terminus binding to the H4 tail, which prevents the latter from relieving AutoN inhibition and keeps the enzyme in an inactive state. Changes to the length of the linker DNA shift the dynamic equilibrium between Acf1 N-terminus binding to the linker DNA or H4 tail, thereby modulating the activity of the catalytic subunit to create regular nucleosome spacing.
Figure 33. The structure and function of linker histones. (a) Top: map of linker histone H1\(^0\) interaction surface (basic residues). Yellow residues comprise the first binding site (approximately 10 bp centered about the dyad), green residues form the second binding site (one of the linker DNA strands), and blue residues are non-binding and located on the opposite face relative to the yellow and green residues. Bottom: model depicting the location of H1\(^0\) within the chromatosome. Chromatosomal DNA is shown in blue, nucleosome dyad in yellow, and globular domain of H1\(^0\) in red. Adapted from Brown et al. (184). (b) Top: space-filling models of nucleosomes in the presence and absence of linker histones shown en face and from the side. Bottom: electron cryomicroscopy image of chromatin released from COS-7 cells and vitrified in 20 mM monovalent ions. While most nucleosomes exhibit the stem motif (arrows), a small number have a divergent linker DNA conformation (arrowhead) characteristic of linker histone-free nucleosomes. Scale bar is 30 nm. Adapted from Bednar et al. (189).
Figure 34. Chromatosome stem motif smFRET assay. (a) Schematic of [45 bp, wt, 45 bp] nucleosomes labeled with Cy3 and Cy5 on opposing linker DNA strands each 25 bp away from the nucleosome edge. FRET was measured before and after the addition of linker histone (purple oval). (b) Left: Characteristic smFRET trace derived from the chromatosome stem motif assay. Linker histone H10 (1 μM) was flowed onto surface-anchored [45 bp, wt, 45 bp] nucleosomes at $t = 20$ s. Right: Field histogram of FRET values before and after the addition of H10 (1 μM) to surface-anchored [45 bp, wt, 45 bp] nucleosomes. Gaussian fits demonstrated that an initial peak centered at FRET = 0.29 shifts to a final peak centered at FRET = 0.84, confirming close juxtaposition of the opposing linker DNA strands after linker histone binding that is characteristic of the stem motif.
Figure 35. ACF is capable of translocating intact chromatosomes. (a) Labeled H1\textsuperscript{0} -A488 binding gel shift assay. C = Cy5-labeled competitor dsDNA, N = Cy3- and Cy5-labeled [wt H4, 40 bp] nucleosomes, and H\textsubscript{x} = A488-labeled histone H1\textsuperscript{0} (DOL = x). Lane 1: C, Lane 2: 1 H\textsubscript{0.72} + 1 C, Lane 3: 1 H\textsubscript{0.72} + 1 N + 1 C, Lane 4: 2 H\textsubscript{0.72} + 1 N + 1 C, Lane 5: 2 H\textsubscript{0.90} + 1 N + 1 C, Lane 6: 2 H\textsubscript{1.01} + 1 N + 1 C. (b) Fractions from glycerol gradient purification of chromatosomes assembled by salt gradient dialysis using an input ratio of 1.2:1.2:1 H1\textsuperscript{0}-A488 (DOL = 0.5):core octamer:DNA. Fractions 28-31 containing a mixture of chromatosomes and nucleosome octamers were pooled and concentrated. (c) Chromatosome remodeling gel shift assay. Chromatosomes were incubated with 20 nM ACF and 7.5 mM ATP for 30 min at 30\textdegree C. The remodeling reaction was stopped with the addition of 60 mM ADP. Prior to remodeling (- lane), an upper band corresponding to chromatosomes and a lower band corresponding to nucleosomes can be distinguished. After remodeling (+ lane), the lower band is absent and the upper band increased in intensity and broadness. The nucleosome fraction clearly remodeled, but it is unclear whether the chromatosome fraction did not remodel or the electrophoretic mobilities of centered and end-positioned chromatosomes are similar. (d) Field histograms from chromatosome remodeling smFRET assay before and
after the addition of 10 nM ACF and 2 mM ATP. Remodeling is observed by the downward shift in FRET, but it cannot be determined at the bulk level whether this shift is attributed to nucleosome remodeling only or both nucleosome and chromatosome remodeling. (e) Two representative A488, Cy3, Cy5, and FRET time traces of ACF-mediated remodeling of intact chromatosomes. [ACF] = 10 nM ACF, [ATP] = 200 μM. Loss of A488 signal may reflect H1\(^0\) dissociation or A488 photobleaching.
Figure 36. The remodeling outcome of RSC is altered by linker histones. All nucleosomes used in this figure have a general [6 bp, wt, 78 bp] framework. The presence or absence of a Rsc3 consensus binding sequence “CGCG” and linker histone H1^0 (purple oval) is indicated in the diagrams below each column. The fraction of remodeling events towards the short linker (entry side) is depicted in orange. Data are mean ± s.e.m from three independent experiments.