Regulation of ISWI involves inhibitory modules antagonized by nucleosomal epitopes

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Chromatin-remodelling complexes (CRCs) mobilize nucleosomes to mediate the access of DNA-binding factors to their sites in vivo. These CRCs contain a catalytic subunit that bears an ATPase/DNA-translocase domain and flanking regions that bind nucleosomal epitopes5. A central question is whether and how these flanking regions regulate ATP hydrolysis or the coupling of hydrolysis to DNA translocation, to affect nucleosome-sliding efficiency. ISWI-family CRCs contain the protein ISWI5, which uses its ATPase/DNA-translocase domain to pump DNA around the histone octamer to enable sliding6. ISWI is positively regulated by two ‘activating’ nucleosomal epitopes: the ‘basic patch’ on the histone H4 tail, and extranucleosomal (linker) DNA8–11. Previous work defined the HAND-SANT-SLIDE (HSS) domain at the ISWI carboxy terminus that binds linker DNA, needed for ISWI activity12,13. Here we define two new, conserved and separate regulatory regions on Drosophila ISWI, termed AutoN and NegC, which negatively regulate ATP hydrolysis (AutoN) or the coupling of ATP hydrolysis to productive DNA translocation (NegC). The two aforementioned nucleosomal epitopes promote remodelling indirectly by preventing the negative regulation of AutoN and NegC. Notably, mutation or removal of AutoN and NegC enables marked nucleosome sliding without the H4 basic patch or extranucleosomal DNA, or the HSS domain, conferring on ISWI the biochemical attributes normally associated with SWI/SNF-family ATPases. Thus, the ISWI ATPase catalytic core is an intrinsically active DNA translocase that conducts nucleosome sliding, onto which selective ‘inhibition-of-inhibition’ modules are placed, to help ensure that remodelling occurs only in the presence of proper nucleosomal epitopes. This supports a general concept for the specialization of chromatin-remodelling ATPases, in which specific regulatory modules adapt an ancient active DNA translocase to conduct particular tasks only on the appropriate chromatin landscape.

ISWI is the catalytic subunit of a set of CRCs, with orthologues in all eukaryotes (Fig. 1). To define how ISWI is intrinsically regulated, we undertook an extensive structure–function analysis of the Drosophila ISWI ATPase, and its regulation by the histone H4 tail and extranucleosomal DNA. In the amino terminus of ISWI, we recognized a region similar to the ‘basic patch’ of the histone H4 tail (Fig. 1a) that is conserved in eukaryotes (Fig. 1b). To test function, we replaced either (R91A or R93A) or both (2RA) arginine residues with alanines. Nucleosome sliding by wild-type ISWI normally requires nucleosomes with intact H4 tails6,8 and the presence of extranucleosomal DNA11,12; however, wild-type ISWI ATPase activity can be elicited by combining DNA and H4 tail peptide bearing the basic patch (Fig. 1c)13. Notably, the ISWI(2RA) derivative showed ~threefold higher ATPase activity than wild-type ISWI and was independent of H4 epitopes (Fig. 1c). For extended nucleosomes (200 base pairs (bp), synthetic centrally positioned sequence (601)), loss of either the basic patch (Ala 17, Ala 19) or the H4 tail (globular H4 (g4)) markedly diminished (fivefold) wild-type ISWI activity, whereas ISWI(2RA) was only modestly attenuated, retaining ~63% activity and ~ninefold higher activity than wild-type ISWI (Fig. 1d). Notably, ISWI(R91A) and ISWI(R93A) resemble moderate and weak gain-of-function derivatives, respectively; as their ATPase activities increase, their dependence on the H4 tail decreases (Supplementary Fig. 1). Thus, ISWI(2RA) greatly activates ATPase activity, and renders the enzyme largely independent of the H4 tail, defining an N-terminal autoinhibitory region (AutoN) with similarity to the H4 tail.

Remodeller ATPases are SF2-family14 DNA translocases that couple DNA translocation to ATP hydrolysis3–7,17–19, wherein the two RecA-like family CRCs contain the protein ISWI2, which uses its ATPase/DNA-translocase domain to pump DNA around the histone octamer to enable sliding6. ISWI is positively regulated by two ‘activating’ nucleosomal epitopes: the ‘basic patch’ of the histone H4 tail, and extranucleosomal (linker) DNA8–11. Previous work defined the HAND-SANT-SLIDE (HSS) domain at the ISWI carboxy terminus that binds linker DNA, needed for ISWI activity12,13. Here we define two new, conserved and separate regulatory regions on Drosophila ISWI, termed AutoN and NegC, which negatively regulate ATP hydrolysis (AutoN) or the coupling of ATP hydrolysis to productive DNA translocation (NegC). The two aforementioned nucleosomal epitopes promote remodelling indirectly by preventing the negative regulation of AutoN and NegC. Notably, mutation or removal of AutoN and NegC enables marked nucleosome sliding without the H4 basic patch or extranucleosomal DNA, or the HSS domain, conferring on ISWI the biochemical attributes normally associated with SWI/SNF-family ATPases. Thus, the ISWI ATPase catalytic core is an intrinsically active DNA translocase that conducts nucleosome sliding, onto which selective ‘inhibition-of-inhibition’ modules are placed, to help ensure that remodelling occurs only in the presence of proper nucleosomal epitopes. This supports a general concept for the specialization of chromatin-remodelling ATPases, in which specific regulatory modules adapt an ancient active DNA translocase to conduct particular tasks only on the appropriate chromatin landscape.

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Remodeller ATPases are SF2-family14 DNA translocases that couple DNA translocation to ATP hydrolysis3–7,17–19, wherein the two RecA-like

Figure 1 | AutoN resembles the histone H4 tail basic patch and restricts ISWI ATPase activity. a. Alignment of Drosophila ISWI and the histone H4 N terminus, with the basic patch depicted. b. ISWI protein and domains/regions, including ATPase, HSS and new functional regions (AutoN and NegC), with zoom of AutoN alignments for ISWI orthologues in different species. c. Comparison of ISWI ATPase activity with mononucleosomes (nuc): native (WT), wild-type (2RA), ISWI(2RA)17, A19, and mutation of AutoN (2RA) mutation with intact H4 tails. Sliding (Fig. 2).
protein lobes of the ATPase domain sequentially grip and release the DNA backbone, typically moving a small number of base pairs per ATP hydrolysis\(^{20,21}\). ‘Coupling’ refers to how efficiently ATP hydrolysis results in productive DNA translocation. Uncoupling by mutation is well documented\(^{22,23}\), but of unknown relevance for regulation. The key question is whether and how coupling is regulated by nucleosomal epitopes. To monitor coupling, we used a ‘tethered translocation’ system, which uses plasmid supercoiling to quantify DNA translocation by ISWI (Supplementary Fig. 2). Notably, DNA translocation by wild-type ISWI was greatly enhanced by H4 tail peptide, whereas ISWI(2RA) showed robust DNA translocation without peptide (Fig. 2a), consistent with our ATPase results (Fig. 1c, d).

Nucleosome sliding was monitored by achieving an equilibrium between end- and centre-positioned octamers, from an initial centre-positioned population (Fig. 2b). ISWI(2RA) yielded an end→centre equilibrium much faster than wild-type ISWI; ISWI(2RA) shifted in 30 s the amount shifted in 30 min by wild-type ISWI (Fig. 2b). Wild-type ISWI required the full H4 tail for in vitro sliding (shown previously)\(^{9}\), and here we establish the importance of the basic patch (Fig. 2c, top panels). Notably, ISWI(2RA) partially bypassed mutations in the H4 basic patch, enabling sliding, but only weakly bypassed the full H4 tail (Fig. 2c, bottom panels). This suggested an additional H4 epitope (beyond the basic patch) needed for coupling high ATPase activity to efficient sliding, and an extensive set of experiments involving peptides and nucleosomes bearing mutations in the H4 tail revealed a major contribution from H4 lysine 12 (H4K12) in promoting coupling (Supplementary Fig. 3).

We next examined core nucleosomes (147 bp, 601 sequence). Supporting previous work\(^1\), wild-type ISWI activity required both extranucleosomal DNA and H4 tail epitopes (Figs 1d and 2d). Interestingly, with ISWI(2RA), both ATPase and sliding remained efficient on core nucleosomes (Figs 1d and 2d), establishing independence from extranucleosomal DNA. However, omitting the H4 tail greatly reduced sliding but not ATPase activity with ISWI(2RA) (Figs 1d and 2d). Taken together, AutoN functions as an autoinhibitory region that, when mutated (in ISWI(2RA)), largely bypasses the H4 tail for ATPase stimulation, but retains reliance on the H4 tail to ‘couple’ that ATP hydrolysis to productive DNA translocation. One simple model is that AutoN binds to and holds the ATPase domain in an inactive conformation, with the H4 tail competing for part of that surface; the binding of the H4 tail displaces AutoN and enables a conformational change that activates ISWI ATPase activity, an effect mimicked by the ISWI(2RA) mutation. Here, the ATPase activity of our derivative series (2RA>R91A>R93A) was the reciprocal of H4 tail stimulation (Supplementary Fig. 1), consistent with competition/antagonism for a region on ISWI involving the H4 tail and AutoN. Consistent with this model, a peptide from the AutoN region of wild-type ISWI (Lys 76–Glu 98) does not activate ISWI (Fig. 1c).

Previous work defined a HSS domain present in the C terminus of ISWI (and the related yeast Chd1 protein) that binds extranucleosomal DNA, needed for ISWI and Chd1 remodelling activity\(^{1,15,24}\). However, our results with AutoN prompted a search for an analogous unknown region that prevents ATPase action unless the HSS is bound to extranucleosomal DNA. Multiple approaches (alignments, protease mapping, structural modelling) yielded a candidate region (covering residues 617 to 648) immediately following the second of the two RecA-like ATPase lobes, which we term NegC (Fig. 3a, b). A structural model using Phyre2 (ref. 25; Fig. 3b) revealed NegC as a ‘C-terminal bridge’ traversing from ATPase lobe 2 back to the ATPase lobe 1, crossing the key functional ATPase cleft. Furthermore, this structural model of NegC is consistent with crosslinking experiments of ISWI\(^{26}\), and a recent crystal structure of Chd1 (ref. 27). Notably, NegC is conserved only in remodellers that bear an HSS domain and are regulated by extranucleosomal DNA (ISWI and Chd1, not SWI/SNF), although NegC function is entirely unknown.

To test NegC function, we isolated three ISWI C-terminal truncation derivatives: ΔC697 (omitting the HSS), ΔC648 (further deletion) and ΔC617 (omitting NegC), as well as combinations with AutoN mutations. First, omitting C-terminal regions from wild-type ISWI did not render them stimulatable by naked DNA (Fig. 3c). By

**Figure 2** | AutoN mutation (ISWI(2RA)) increases DNA translocation and nucleosome sliding. a, Comparative DNA-translocation activity (see Supplementary Fig. 2) of ISWI derivatives. Translocation generates supercoiled topoisomers (SC). b, c, Comparative sliding of extended nucleosomes by ISWI derivatives, as a time course (b), and their reliance on H4 tail epitopes, in titration series (c). d, Comparative sliding activity on core nucleosomes reveals a reliance on H4 tail epitopes. For b and d, enzyme:substrate molar ratio is 1:2.
remarkably, and in-keeping with our tethered translocation coupling of ATPase activity and DNA translocation above (Fig. 3c), high ATPase activity, but completely failed to slide either core (lanes 3, 4, 19, 20). Surprisingly, ISWI(2RA/ΔC697) failed to slide nucleosomes that either contain or lack the H4 tail (Fig. 3e). As expected, omitting the HSS domain in ISWI(ΔC697) greatly reduced sliding of extended nucleosomes (lanes 3, 4, 19, 20). Surprisingly, ISWI(2RA/ΔC697) and ISWI(2RA/ΔC648) displayed high ATPase activity, but completely failed to slide either core (lanes 23, 24) or extended (lanes 7, 8) nucleosomes, mirroring the uncoupling of ATPase activity and DNA translocation above (Fig. 3c, d). Remarkably, and in-keeping with our tethered translocation coupling of ATP hydrolysis to DNA translocation, and nucleosome sliding. a, NegC conservation, revealed in zoomed alignment of ISWI orthologues. b, Modellization of ISWI(ΔC697) showing NegC (orange) traversing the cleft (red dashed line) from ATPase lobe 2 (blue) to ATPase lobe 1 (green). Model generated using Phyre2; depicted with zoomed alignment of ISWI orthologues. c, The AutoN (2RA) mutation elicits robust DNA-dependent ATPase activity independent of the HSS. Values represent mean of three experiments, with 200-bp DNA fragment. Error bars denote ± s.e.m. d, Deletion of NegC through truncation restores DNA translocation. e, Impact of NegC region on sliding activities of core and extended nucleosomes (and H4 tail-less versions). ATPase activities (values, characterised regions in other ATPases that promote sliding23,28.

We then addressed how ISWI integrates the presence of nucleosomal epitopes. For wild-type ISWI, omission of extranucleosomal DNA (lane 17) greatly impaired sliding, whereas omission of the H4 tail (lane 9, or omitting both, lane 25) prevented sliding. With ISWI(2RA), loss of both the H4 tail and extranucleosomal DNA prevented sliding (lane 29), even though ATPase activity was high; comparable to wild-type ISWI on extended nucleosomes (lane 1). However, ISWI(2RA) could slide extended nucleosomes lacking the H4 tail (lane 13), or core nucleosomes bearing H4 tails (lane 21). Thus, further removal of NegC (ΔC617) restored sliding activity, without an accompanying increase in ATPase activity. Restoration was moderate with ISWI(2RA), loss of both the H4 tail and extranucleosomal DNA prevented sliding (lane 17), and efficient with ISWI(2RA/ΔC617) (lane 6) on extended nucleosomes. Similarly, with core nucleosomes, sliding was low but detectable with ISWI(ΔC617) (lane 18), and efficient with ISWI(2RA/ΔC617) (lane 22). Thus, NegC is a new region that negatively regulates sliding through ATPase/translocation uncoupling. NegC is entirely separate from, and opposed in function to, characterized regions in other ATPases that promote sliding23,28.
both the H4 tail and extranucleosomal DNA contribute to coupling. Importantly, whereas ISWI(2RA/ΔC697) and ISWI(2RA/ΔC648) are unable to slide nucleosomes lacking the H4 tail (g4; lanes 15, 16, 31, 32), removal of NegC (ISWI(2RA/ΔC617)) restored relatively efficient sliding of extended g4 nucleosomes (lane 14), and moderate sliding of core g4 nucleosomes (lane 30). Furthermore, removal of NegC from ISWI(2RA) restores sliding ability with nucleosomes bearing amino-acid substitutions in both H4K12 and the basic patch (Supplementary Fig. 4). Thus, with ISWI(2RA), NegC omission enables sliding of nucleosomes without a basic patch or extranucleosomal DNA (Fig. 3e, lanes 6, 14, 22, 30); properties that normally define SWI/SNF-family remodellers.

To examine the effects of AutoN and NegC in vivo, we expressed (in Saccharomyces cerevisiae) mutations/truncations in Isw1 equivalent to those from our ISWI biochemistry (see Figs 1b and 3a) and tested complementation (Supplementary Fig. 5) or dominant phenotypes (using a sensitized *rsc7* strain; also known as *NPL6* background, synthetically lethal with a wide range of chromatin mutants). Interestingly, expression of an Isw1 derivative lacking both AutoN and NegC regulation prevented growth (Fig. 4a), consistent with rogue chromatin misregulation activity (although loss of proper regulation by Isw1-associated proteins may also contribute).

Taken together, our work provides several conceptual advances that affect both ISWI regulation and mechanism (Fig. 4b). Regarding regulation, the prior model involved ‘positive’ regulation of ISWI by the H4 basic patch and by extranucleosomal DNA. Here, we replace that conception with an inhibition-of-inhibition model, whereby these two nucleosomal epitopes function to relieve an intrinsic autoinhibition conferred by two new negative regulatory domains, AutoN and NegC. Notably, whereas AutoN functions primarily to inhibit ATPase activity, NegC functions to inhibit ATPase coupling to DNA translocation, with structural models for NegC supporting this function. By this model, interaction of the HSS domain with extranucleosomal DNA does not activate the ATPase per se, it relieves NegC and restores ATPase coupling. Considering mechanism, a key current model involves the HSS pushing extranucleosomal DNA into the nucleosome, providing the initial mechanical power stroke to form a translocated DNA loop on the surface of the nucleosome. However, as we observe efficient DNA translocation and nucleosome sliding following deletion of the HSS (when combined with NegC omission), the primary function of the HSS is not mechanical, but rather to regulate DNA translocation by antagonizing NegC, ensuring that coupling occurs only when extranucleosomal DNA is of sufficient length.

**METHODS SUMMARY**

ISWI protein derivatives were produced as N-terminal fusions to proteins coding for trigger factor and the Tet repressor DNA-binding domain (TF-TetR-ISWI), which heterodimerizes with an unused TetR to enable *tetO* binding for DNA-translocation assays. Proteins were expressed in *Escherichia coli* BL21CodonPlus(DE3)RII, and were purified to homogeneity as monodisperse derivatives. Chemically synthesized H4 tail peptides encompass residues Lys8 to Asp24 and AutoN peptide includes Lys17 to Asp93, as indicated.

Mononucleosomes used purified recombinant *Drosophila* histones expressed in *E. coli* BL21-CodonPlus(DE3)RII, assembled with 147-bp or 200-bp DNA fragments (isolated from a plasmid by restriction digests, and containing the 601 strong-positioning sequence) by a salt-dialysis linear gradient. For the 200-bp fragment, the 601 sequence was centrally located.

The DNA-translocation assays measured plasmid supercoils generated by a single ISWI protein anchored through its TetR fusion to a previously relaxed plasmid (by *E. coli* topoisomerase I) plasmid DNA containing a single *tetO* operator sequence (Supplementary Fig. 2). Deproteinized samples were loaded on a 1.3% agarose gel, subsequently stained in ethidium bromide, and scanned on a Typhoon Trio (Amersham, GE).

The nucleosome-sliding assays were performed at 26 °C using a 1:2 enzyme:substrate molar ratio, except in the titration series. The reactions were stopped by adding competitor pbLueScript plasmid, and loaded on a 6.3% agarose gel, subsequently stained in ethidium bromide, and scanned on a Typhoon Trio.

Full Methods and any associated references are available in the online version of the paper.

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METHODS

Reagents. ISWI protein derivatives were expressed in *Escherichia coli* BL21CodonPlus(DE3)RIIL, upon 0.5 mM isopropyl-β-D-thiogalactoside induction at *D*~0.600~ ~0.5 for 24 h at 15 °C, as complexes from two vectors: one—pCDFDuet-1—bears a gene coding for TetR-(His)7, and the other—pCold TF (Takara) lacking the His tag—is a gene fusion containing TF-3C cleavage site-TetR-ISWI-Flag. A sequence of eight amino acids (GGQGGQGGQ) was inserted between the genes coding for TetR and ISWI. The properly assembled complex contains a heterodimer of TetR obtained by two successive affinity purifications. The bacterial-cell extracts were first mixed with nickel-nitrilotriacetic acid agarose resin (*Qiagen*), capturing the complex and the unwanted TetR homodimer. The sample eluted from the resin was then purified using the Flag tag and anti-Flag M2 affinity gel and eluted with 3×Flag peptide (Sigma) to obtain the desired complex. Then, trigger factor was cleaved from the complex using human rhinovirus 3C protease (*Novagen*) using 1 unit of protease per 0.1 mg of complex by incubating 16 h at 4 °C, in the presence of 10 mM β-mercaptoethanol and 0.5 mM EDTA. Finally, the purification of the complex was achieved by a gel-filtration step on two S200GL 10/300 (Amersham, GE) in series. Obtained complexes were homogen and monodisperse.

Chemically synthesized H4 tail peptides encompass residues Lys 8 to Asp 24; AutoN peptide includes ISWI residues Lys 76 to Glu 98.

Mononucleosomes were produced from single recombinant *Drosophila* histones expressed in *E. coli* BL21CodonPlus(DE3)RIIL, purified as inclusion bodies, and assembled in octamers by salt-dialysis, essentially as described in ref. 31. The 200- and 147-bp DNA fragments containing the 601 positioning sequence were respectively produced from plasmids pUC12 × 601 (digested by Aval) and pTS55-16 × NCP601a (digested by EcoRV). For the 200-bp fragment, the 601 sequence was centrally located. The DNA fragments were purified from the backbone using preparative electrophoresis (PrepCell, Bio-Rad) at 400 V constant in 0.5 TBE (Tris-borate 45 mM, pH 8, 3 mM EDTA) with TE (Tris 10 mM, 1 mM EDTA) as elution buffer. Using preparative electrophoresis (PrepCell, Bio-Rad), 4.5% (37.5:1) native polyacrylamide gel running at 400 V constant was used. DNA fragments were purified from the backbone using preparative electrophoresis (PrepCell, Bio-Rad) at 400 V constant in 0.5 TBE (Tris-borate 45 mM, pH 8, 3 mM EDTA) with TE (Tris 10 mM, 1 mM EDTA) as elution buffer.

DNA-translocation assay. The 20-μl reaction contained 10 pmol ISWI, with or without 15 μM peptide, in the presence of 250 ng of previously relaxed plasmid, 1 mM ATP, 2.5 U of topoisomerase I (NEB) in NEB1 1× buffer, 1 mg ml⁻¹ BSA. Experiments were performed at 30 °C for 2 h (or shorter during time course), followed by heat inactivation at 65 °C for 20 min. De-proteinization was performed by adding 2 μl of proteinase K at 10 mg ml⁻¹ and 1 μl SDS 20% and incubated at 50 °C for 1 h. Reactions were subsequently precipitated in ethanol, prior to loading on a 1.3% agarose gel run for 3 h at 130 V. Gels were stained for 20 min in a 1 μg ml⁻¹ ethidium bromide solution, and scanned on a Typhoon Trio (Amersham, GE). Time-course experiments are performed using a starting reaction corresponding to a scale-up of amounts above, proportional to the number of desired aliquots.

Sliding assay. 100 fmol (or variable for titration) ISWI was incubated in 10 mM Tris buffer, pH 7.4, 50 mM KCl, 3 mM MgCl₂, 0.1 mg ml⁻¹ BSA, 1 mM ATP, in the presence of 200 fmol of mononucleosome for 90 min, with shaking at 300 r.p.m. in a Thermomixer (Eppendorf). 10-μl reactions were stopped by adding 200 ng competitor DNA (pBluescript plasmid) and incubated for an additional 30 min as previously. Reactions were loaded using glycerol 10% on a 4.5% (37:5:1) native polyacrylamide gel and run in 0.4 × TBE for 55 min at 110 V constant. Gel was stained for 10 min in a 1 μg ml⁻¹ ethidium bromide solution, and scanned on a Typhoon Trio (Amersham, GE). Time-course experiments were performed using a starting reaction corresponding to a scale-up of amounts above, proportional to the number of desired aliquots.

In vivo yeast experiments. Plasmids expressing Isw1 derivatives were generated by marker conversion to LEU2 (from *S. cerevisiae*) of pRS416-Isw1 (ref. 33), followed by site-directed mutagenesis (via PCR) generating AAA mutations and AC truncations. Final constructs were verified by sequencing.

Complementation assay: the growth of *S. cerevisiae* strain TOH1358 lacking CHD1 and ISW1 genes is temperature sensitive. Complementation experiments were performed by transforming the strain with plasmids expressing various derivatives of Isw1 under control of the endogenous promoter, performing tenfold spot dilutions, and observing the rescue of the growth ability at high temperature. Plate medium is SC-Leu.

Testing the dominant-negative phenotype: here, we used a sensitized genetic background strain YBC2333, lacking ISW1 and RSC7. The strain was transformed with plasmids expressing separate Isw1 derivatives under control of the endogenous ISW1 promoter, selecting for colonies on SC-Leu. Growth ability (in spot dilution format) was tested at 30 °C on SC-Leu plates, which either lacked or contained 5-FOA (which enforces the loss of a URA3-marked plasmid bearing RSC7).

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