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ACF1 improves the effectiveness of nucleosome mobilization by ISWI through PHD–histone contacts

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The nucleosome remodelling ATPase ISWI resides in several distinct protein complexes whose subunit composition reflects their functional specialization. Association of ISWI with ACF1, the largest subunit of CHRAC and ACF complexes, improves the efficiency of ISWI-induced nucleosome mobilization by an order of magnitude and also modulates the reaction qualitatively. In order to understand the principle by which ACF1 improves the efficiency of ISWI, we mapped their mutual interaction requirements and generated a series of ACF complexes lacking conserved ACF1 domains. Deletion of the C-terminal PHD finger modules of ACF1 or their disruption by zinc chelation profoundly affected the nucleosome mobilization capability of associated ISWI *in trans*. Interactions of the PHD fingers with the central domains of core histones contribute significantly to the binding of ACF to the nucleosome substrate, suggesting a novel role for PHD modules as nucleosome interaction determinants. Connecting ACF to histones may be prerequisite for efficient conversion of ATP-dependent conformational changes of ISWI into translocation of DNA relative to the histones during nucleosome mobilization.

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Introduction

Nucleosomal arrays are rendered dynamic through the action of nucleosome remodelling factors, which use the energy freed by ATP hydrolysis to disrupt histone–DNA contacts. Nucleosome remodelling increases the accessibility of nucleosomal DNA and induces histone octamer translocation on DNA (Becker and Hörz, 2002; Narlikar *et al*, 2002; Lusser and Kadonaga, 2003). The enzymes dedicated to nucleosome remodelling belong to the SNF2/SWI2 family of ATPases (Eisen *et al*, 1995). These enzymes typically reside in multiprotein complexes of variable composition. Proposed roles for associated subunits include the modulation of remodelling activity, the targeting of the enzymes to particular sites of action as well as the integration of the remodelling process into more complex regulatory programmes. The ATPase ISWI serves as a paradigm for understanding the mechanism of ATP-dependent nucleosome remodelling and its regulation (Längst and Becker, 2001b). Currently, a number of ISWI-containing complexes have been identified in various species from yeast to man (Längst and Becker, 2001b; Corona and Tamkun, 2004; Mellor and Morillon, 2004). Although their overall subunit composition may vary, ISWI is frequently associated with a larger subunit belonging to a family of related proteins. Members of this family, such as ACF1, WSTF, WCRF, TIP5 and NURF301, share C-terminal plant homeo domain (PHD) finger and bromodomain (Brd) modules besides several conserved sequence motifs of unknown structure and function. Brds and PHD fingers are found in other chromatin regulators as well. Brds can serve to interact with acetylated histone H4 and H3 N-termini and may possibly sensitize a nucleosome remodelling machine for histone acetylation marks (Jenuwein and Allis, 2001). PHD fingers (Aasland *et al*, 1995) are found in a large number of nuclear proteins involved in chromatin structure modulation. Their mutation or deletion in the context of various regulators, such as the ATRX, ING1 and AF10 proteins, leads to various disease syndromes (references cited in Capili *et al*, 2001). PHD fingers are supposed to mediate contacts with other proteins and sometimes this function requires coordination with a neighbouring Brd (Aasland *et al*, 1995; Hsu *et al*, 2001; Schultz *et al*, 2001).

The first ISWI interaction partner from this family of PHD–Brd proteins to be identified was *Drosophila* ACF1, which associates with ISWI to form ACF (Ito *et al*, 1999) and CHRAC complexes (Poot *et al*, 2000; Eberharter *et al*, 2001). These complexes potentially have dual functions as *in vitro* they may contribute to the assembly of nucleosomal arrays (Ito *et al*, 1999; Clapier *et al*, 2002; Vary *et al*, 2004) and also render these arrays dynamic by mobilizing histone octamers to slide on DNA (Corona *et al*, 1999; Längst *et al*, 1999; Eberharter *et al*, 2001). In certain chromatin assembly protocols, ISWI will not function without ACF1 (Ito *et al*, 1999; Fyodorov and Kadonaga, 2002b). When it comes to nucleosome sliding, ISWI is able to carry out a basal reaction, but its performance can be boosted by an order of magnitude upon association of ACF1 (Eberharter *et al*, 2001). Interestingly, ACF1 improves the effectiveness ('energy-efficiency') of ISWI *in trans*, that is, although the ATP hydrolysis rate of ISWI does not change upon contact with ACF1, nucleosomes are mobilized much more efficiently. In addition, association of ACF1 modulates the remodelling activity of ISWI qualitatively by altering the directionality of nucleosome movement on small DNA fragments. While ISWI alone catalyses the movement of histone octamers from central positions to the

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ends of small DNA fragments, ACF triggers movement of end-positioned nucleosomes to more internal positions (Eberharter *et al*, 2001). The relevance of 'directionality' of histone octamer translocation on nonphysiological mononucleosome substrates should not be overemphasized, but the phenomenon reveals a regulatory effect of ACF1 on ISWI function *in trans,* whose explanation in molecular terms will undoubtedly contribute to describing the mechanics of nucleosome remodelling.

The effect of ACF1 on ISWI may be due to an allosteric effect upon interaction of the two proteins, or alternatively be mediated by contacts of ACF1 with the nucleosome substrate. So far, interactions of ISWI and ACF1 with nucleosomal DNA have been characterized (Grüne *et al*, 2003), but interactions with the histone moiety, although suspected from the substrate requirements of nucleosome remodelling (Clapier *et al*, 2001), have not been described. In order to distinguish between the two possibilities, we reconstituted ACF complexes by coexpression of both subunits in insect cells from baculovirus vectors and mapped the interaction domains on either factor. Through construction of variant ACF complexes, in which conserved ACF1 domains were deleted, we were able to separate the effect of ACF1 on the directionality of nucleosome mobilization from its contribution to the energy efficiency of the remodelling process. Intriguingly, deletion of the PHD modules largely abolishes the positive effect of ACF1 on nucleosome mobilization. The PHD fingers of ACF1 make crucial contacts with histones and nucleosome particles, a property that has not been previously noted. Our results are consistent with a scenario, according to which contacts of ACF1 with the histone component of the nucleosome substrate improve the effectiveness, with which ISWI can translocate DNA relative to the histone octamer.

Results

Mapping the ACF1 interaction determinant on ISWI

ISWI can be roughly subdivided into an N-terminal ATPase domain and a C-terminal substrate recognition function consisting of HAND, SANT and SLIDE domains (Grüne *et al*, 2003; Figure 1A). In order determine, which part of ISWI would interact with ACF1, we expressed a series of ISWI fragments (Figure 1A) as glutathione-*S*-transferase (GST) fusion proteins in *Escherichia coli* and purified them by chromatography on glutathione-Sepharose. As a binding partner, we expressed FLAG-tagged ACF1 in Sf9 cells (Eberharter *et al*, 2001) using baculovirus vectors and immobilized it on anti-FLAG (M2) beads. The ISWI fragments were allowed to interact with immobilized ACF1 under stringent conditions and bound protein was resolved by PAGE and detected by Western blotting (Figure 1B). The ACF1 binding determinant localized to the C-terminal part of ISWI (Figure 1B) and could be narrowed down to the very C-terminus of ISWI containing amino acids (aa) 962–991 (Figure 1C). A summary of the interactions is shown in Figure 1A. We will refer to the ISWI domain required for ACF1 binding as AID (for ACF1 Interaction Determinant).

Determinants on ACF1 for ISWI interaction

To establish the requirements for ISWI interaction in ACF1, we coexpressed a series of myc-tagged ACF1 derivatives with FLAG-tagged full-length ISWI in Sf9 cells (see Figure 2A).

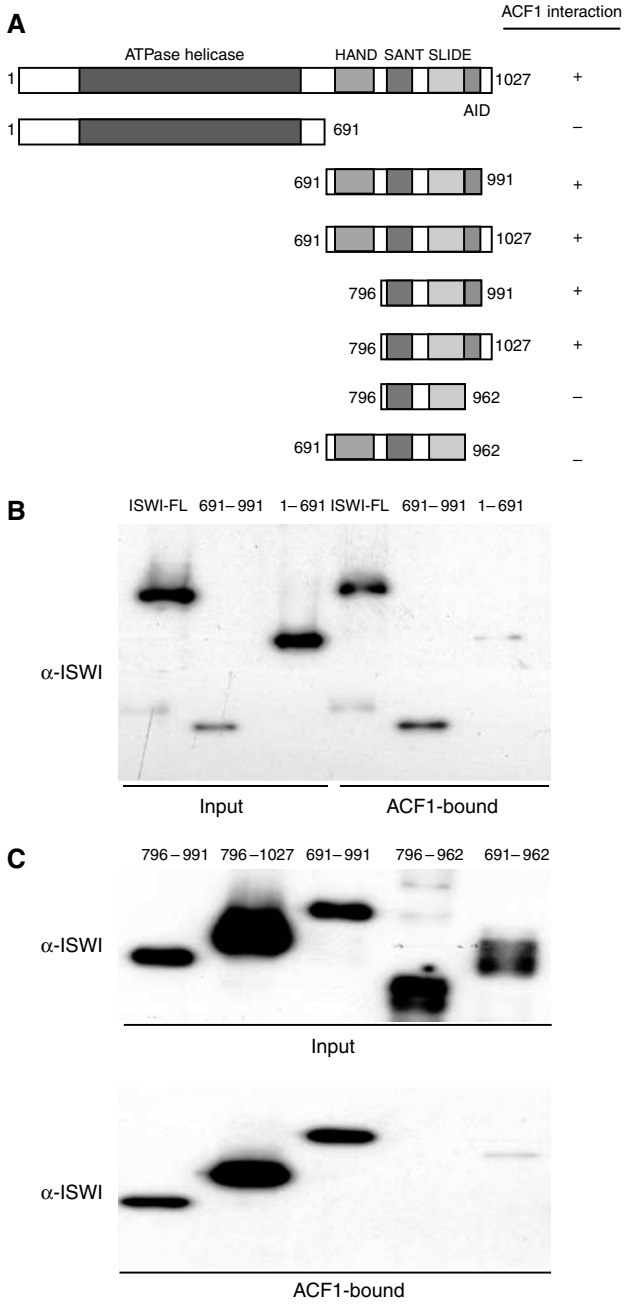

Figure 1 ACF1 interacts with the C-terminus of ISWI. (**A**) Summary of the domain organization of ISWI known to date. HAND (aa 697–795), SANT (aa 796–850) and SLIDE (aa 886–977) domains were recently defined by Grüne *et al* (2003). '+' and '–' behind each derivative indicate the ability to bind as determined in (B) and (C). The AID (between aa 962 and 991) was defined here. (**B**) FLAG-tagged ACF1 was immobilized on M2 anti-FLAG agarose. The resulting affinity resin was extensively washed and then used in pull-down experiments to monitor the interaction of ISWI. Bacterially expressed ISWI derivatives as indicated were incubated with the ACF1 beads. After extensive washes 30% of bound material was separated by SDS–8% PAGE and detected by Western blotting with an ISWI antibody ('ACF1-bound'). As a control for interaction with full-length ISWI, we used a whole-cell extract of baculoviral-expressed, untagged ISWI. As reference, 10% of the input was loaded. (**C**) Smaller parts of ISWI (numbers above lanes correspond to first and last amino acids) were expressed in *E. coli* and tested for interaction with ACF1, as in (B). The upper panel displays the input of ISWI derivatives, and the lower panel reveals the bound protein.

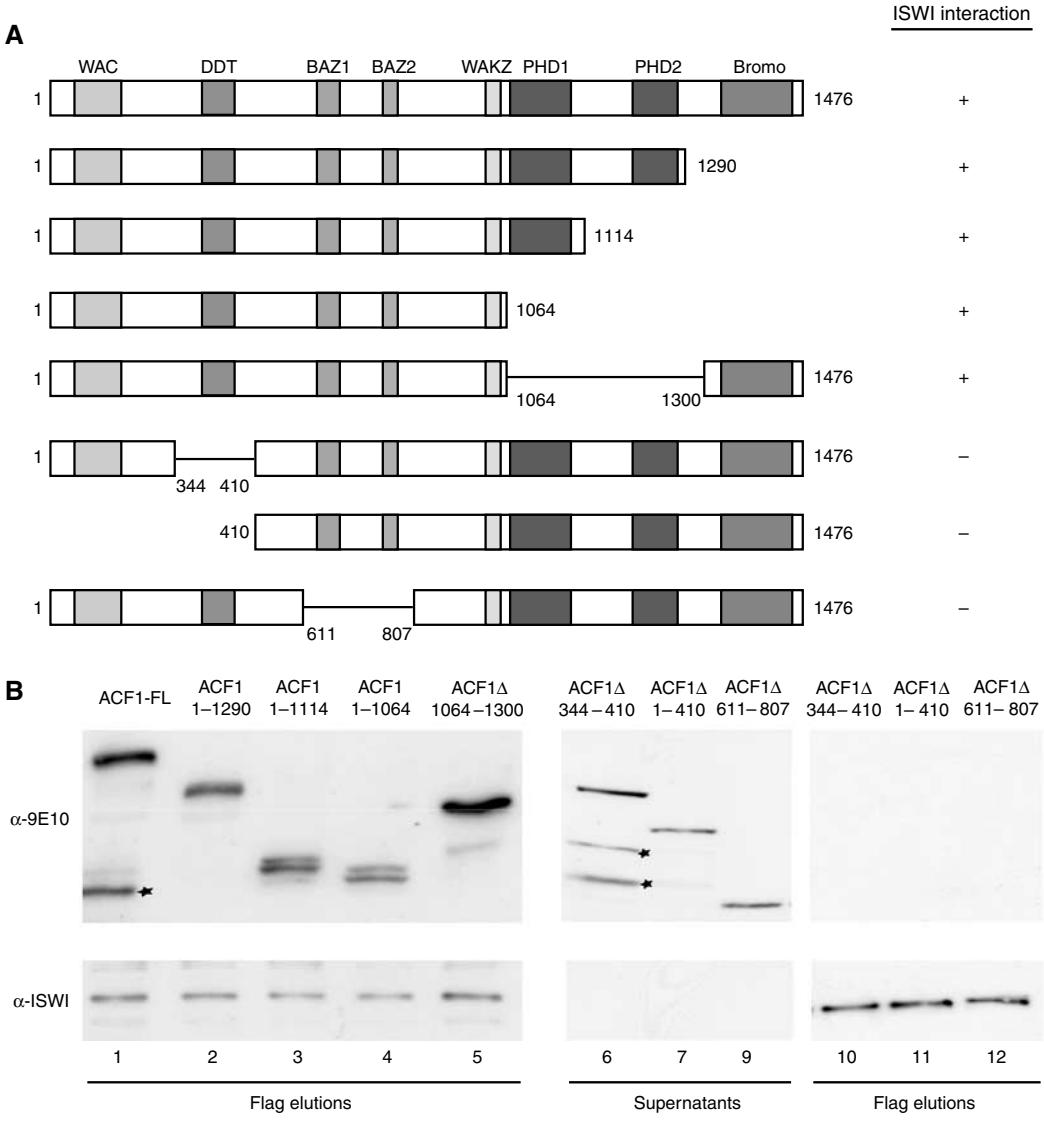

Figure 2 Mapping the ISWI interaction determinants on ACF1. (**A**) Summary of the known domain organization of ACF1 (Ito *et al*, 1999). The C-terminal domains extend between the following coordinates: PHD1: aa 1064–1114; PHD2: aa 1240–1300; Brd: aa 1361–1463. '+' and '–' indicate the ability to bind as determined in (B). (**B**) Full-length (FL) myc-tagged ACF1 and derivatives bearing various deletions as indicated were coexpressed with FLAG-tagged ISWI in Sf9 cells and resulting complexes were affinity-purified via the FLAG tag. Equal amounts of FLAG-eluted ACF complexes (i.e. ACF1 bound to ISWI) and the corresponding supernatants (i.e. proteins not interacting with ISWI) were separated by SDS–PAGE. ACF1 and ISWI were detected by Western blotting and probing with either monoclonal anti-myc antibody 9E10 for ACF1 detection or with antibody directed against ISWI (provided by J Tamkun).

Complexes were purified from the cell lysate by affinity chromatography over an anti-FLAG resin and elution by a competing FLAG peptide. The interacting ACF1 derivatives or unbound ACF1 in the supernatant were detected by Western blotting. Deleting the C-terminal Brd and PHD fingers of ACF1 did not affect complex formation (Figure 2B). Deletions within a rather broad region within the N-terminus effectively removing the DDT and BAZ motives (Jones *et al*, 2000) abolished coelution with ISWI and led to the accumulation of the corresponding ACF1 derivatives in the supernatant (Figure 2B). Similar results were obtained in experiments where *in vitro*-translated ACF1 fragments were tested in 'pull-down assays' with immobilized ISWI beads (data not shown). Our mapping of the ISWI interaction domain to a broad, central part of ACF1 is in agreement with previous results from the Kadonaga laboratory (Fyodorov and Kadonaga, 2002a).

Functional impact of C-terminal ACF1 deletions

Since the PHD fingers and the Brd motif were dispensable for ACF complex formation (see Figure 2), we were able to investigate whether these domains contributed to its nucleosome remodelling activity. Wild-type (wt) ACF and complexes bearing deletions within the C-terminus of ACF1 exhibited comparable nucleosome-stimulated ATPase activity, when the enzyme input was standardized by the amount of ISWI (data not shown). As a measure of remodelling activity, we monitored the ability of wt and mutated ACF complexes to catalyse the movement of a mononucleosome positioned at the end of a short DNA fragment to more central

positions on the DNA (Längst *et al*, 1999; Eberharter *et al*, 2001) by a gel retardation assay (Figure 3A). Titrating substoichiometric amounts of wt ACF into a sliding reaction led to progressively more nucleosome movement (Figure 3A, lanes 2–6). Deleting the Brd of ACF1 (ΔBrd) did not impair the nucleosome sliding activity, but even improved the reaction efficiency somewhat (Figure 3A, lanes 7–11). However, further deletion removing the C-terminal PHD finger (ΔPHD2-

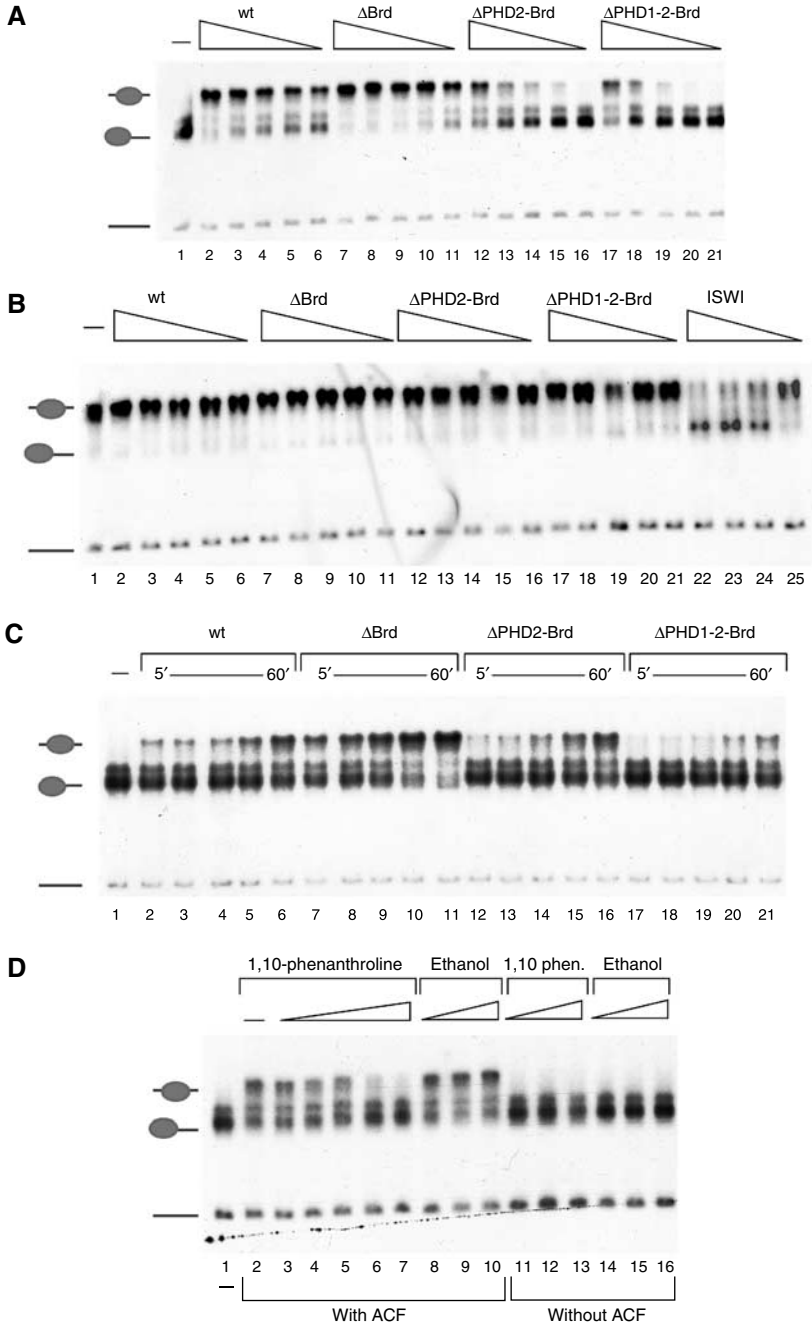

Figure 3 The PHD fingers of ACF1 are important for nucleosome mobilization. (**A**) 60 fmol of mononucleosomes positioned at the end of a 248 bp DNA fragment were incubated for 1 h with affinity-purified ACF or variant complexes bearing the indicated deletions of ACF1. In order to visualize nucleosome movement, samples were separated on a native 4.5% polyacrylamide gel. Dried gels were exposed to film overnight at −80°C. The reactions contained the following amounts of ACF complexes: 3 fmol (lanes 2, 7, 12 and 17); 1.5 fmol (lanes 3, 8, 13 and 18); 1 fmol (lanes 4, 9, 14 and 19); 0.75 fmol (lanes 5, 10, 15 and 20) and 0.375 fmol (6, 11, 16 and 21). Lane 1 shows the band corresponding to the mononucleosome in the absence of enzyme. The positions of free DNA, end- and centre-positioned nucleosomes are indicated to the left. (**B**) Nucleosome sliding reaction as in (A), but starting with 60 fmol of a centrally positioned mononucleosome. Lanes 22–25 show reactions driven by 6, 3, 1.5 and 0.75 fmol of ISWI, respectively. The untreated centre-positioned nucleosome is shown in lane 1. (**C**) Time course of nucleosome sliding. Reactions as in (A) contained 0.5 fmol of each of the indicated ACF complexes and 60 fmol of nucleosomes. Reactions were stopped at different times (5, 10, 15, 30 and 60 min as indicated) by the addition of 200 ng of unlabelled competitor DNA. (**D**) Nucleosomal sliding reactions as in (A) in the absence (lane 2) or presence of either 1,10 phenanthroline (0.1, 0.5, 1, 2.5 and 5 mM in lanes 3–7, respectively) or corresponding concentrations of the solvent ethanol (0.5, 1.25 or 2.5% in lanes 8–10, respectively). For controls, mononucleosomes were incubated with either 1, 2.5 or 5 mM of 1,10 phenanthroline or 0.5, 1.25 or 25% ethanol and resolved on the gel in lanes 11–13 and 14–16.

Brd) clearly reduced the sliding efficiency about four-fold when compared to wt activity and about 10-fold when compared to the ΔBrd mutant (Figure 3A, lanes 12–16). Further deletion of PHD1 also resulted in an impaired enzyme (Figure 3A, lanes 17–21).

Previously, we had seen that the stimulatory effect of ACF1 on ISWI activity correlated with a changed 'directionality' of nucleosome movement by ACF (Eberharter *et al*, 2001). It was therefore possible that the reduced sliding of end-positioned nucleosomes upon deletion of the PHD fingers would be accompanied by a corresponding increase of nucleosome movement from a central position. We therefore analysed the ACF complexes deleted in the C-terminus of ACF1 in reactions geared at sliding nucleosomes off central positions (Figure 3B). Recombinant ISWI, devoid of ACF1, was able to catalyse this reaction efficiently (Figure 3B, lanes 22–25). None of the ACF complexes was able to mobilize nucleosomes off the centre of the fragment. Evidently, we have separated the improvement of energy efficiency of ISWI from the qualitative modulation of directionality: whereas the PHD fingers appear crucial for improved energy efficiency, the determinant of ACF-type directionality (end to centre) of nucleosome sliding may reside in the N-terminus of ACF1 or correlate with the mere binding of ACF1 to ISWI.

We further explored the requirement of the ACF1 PHD fingers for efficient nucleosome mobilization by time courses under stringent, low enzyme conditions (60 fmol of nucleosomes versus 0.5 fmol of ACF complex; Figure 3C). The reaction was tuned such that about 40% of nucleosomes were moved by wt ACF during the course of the 60 min incubation (Figure 3C, lanes 2–6). Deletion of Brd improved the sliding efficiency significantly, as before (Figure 3C, lanes 7–11). Further deletion of PHD2 abolished this stimulatory effect and the ΔPHD1-2-Brd derivative was considerably less active than wt ACF (compare 15 min time points, lanes 4, 9, 14 and 19).

PHD domains require zinc for proper folding (Pascual *et al*, 2000; Capili *et al*, 2001). Removal of zinc is therefore expected to lead to local unfolding of the domain. To determine whether the activity of ACF1 indeed depended on proper folding, we thought of removing zinc from the protein by inclusion of 1,10-phenanthroline in nucleosome sliding reactions. 1,10-phenanthroline had been used in analogous experiments to evaluate the contribution of zinc-finger structures to the functions of the acetyltransferase MOF (Akhtar and Becker, 2001), the GAGA factor (Pedone *et al*, 1996), CBP (Kalkhoven *et al*, 2002) and p300 (Bordoli *et al*, 2001). Increasing amounts of 1,10-phenanthroline dramatically reduced the sliding ability of ACF (Figure 3D, lanes 3–7), whereas corresponding concentrations of the solvent, ethanol, did not affect the reaction (Figure 3D, lanes 8–10). Neither the drug nor the solvent affected sliding by ISWI (data not shown) or the mobility of nucleosomes alone (Figure 3D, lanes 11–16).

To explore the role of the ACF1 PHD fingers in nucleosome mobilization by ACF, we further expressed an ACF mutant complex lacking both PHD fingers (ACFΔPHD1-2) and analysed it in parallel with the wt ACF complex (Figure 4). wt ACF and ACFΔPHD1-2 were expressed and purified in parallel and carefully standardized with respect to subunit concentrations (Figure 4A). As we have observed for the different ACF complexes (Figure 3), equal amounts of wt and ACFΔPHD1-2

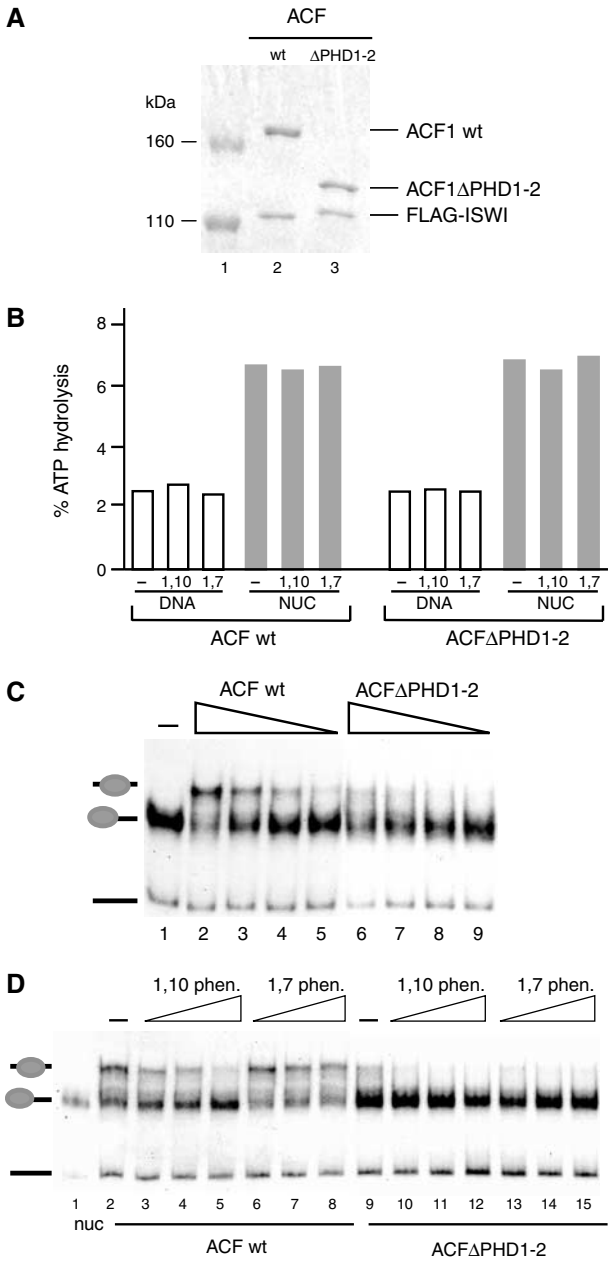

Figure 4 Requirement of the ACF1 PHD fingers for nucleosomal mobilization. (**A**) Normalization of proteins in ACF wt and ACFΔPHD1-2 complexes to be compared in the following experiments by 6% denaturing PAGE and Coomassie staining. Lane 1: size marker. (**B**) ATPase assays with 120 ng of naked DNA or 120 ng of nucleosomal DNA using 2 fmol of either wt ACF or the indicated mutant ACF complex. The reactions were performed in the absence (−) or presence of 3 mM of either 1,10-phenanthroline or 1,7-phenanthroline. (**C**) Nucleosome sliding reactions as in Figure 3A comparing ACF (lanes 2–5) and ACFΔPHD1-2 (lanes 6–9). Protein concentrations were 1 fmol (lanes 2 and 6), 0.5 fmol (lanes 3 and 7), 0.25 fmol (lanes 4 and 8) or 0.125 fmol (lanes 5 and 9). The untreated nucleosome is indicated in lane 1. (**D**) Nucleosome sliding reactions as in (C) with 1 fmol of either ACF wt (lanes 2–8) or ACFΔPHD1-2 (lanes 9–15). Reactions were carried out in the absence (lanes 2 and 9) or presence of either 1,10-phenanthroline at 2 mM (lanes 3 and 10), 3 mM (lanes 4 and 11) and 4 mM (lanes 5 and 12) or 1,7-phenanthroline at 2 mM (lanes 6 and 13), 3 mM (lanes 7 and 14) and 4 mM (lanes 8 and 15). The untreated nucleosome is indicated in lane 1 (nuc).

exhibited similar ATPase activity, both in the presence of DNA and nucleosomal substrates (Figure 4B). Moreover, the ATPase activity of these complexes was not affected by 1,10-phenanthroline, under conditions where the zinc chelator inhibits sliding (Figure 4B). 1,7-phenanthroline, which does not chelate zinc (Bird *et al*, 2003), also had no effect on the ATPase activities. Despite its ability to hydrolyse ATP in response to the nucleosome substrate, ACFΔPHD1-2 was essentially unable to mobilize nucleosomes under conditions where intact ACF functioned efficiently (Figure 4C, compare lanes 2 and 3 with 6 and 7). The addition of 1,10-phenanthroline to the reaction inhibited nucleosome sliding, whereas the nonchelating 1,7-phenanthroline isomer had only a minor effect (Figure 4D, lanes 2–8). The weak sliding activity of ACFΔPHD1-2 (Figure 4D, lane 9) was slightly affected by both 1,10- and 1,7-phenanthroline, pointing to a nonspecific effect (see lanes 10–15).

This suggests that disruption of the ACF1 PHD fingers neither affects substrate recognition nor the ATPase activity of ISWI, but rather the coupling of ATPase to DNA relocation. This is an example where the activity of a remodelling ATPase is affected *in trans* by a domain of an associated subunit.

ACF1 binds nucleosomes

Since the PHD fingers of ACF1 are not involved in binding ISWI, we considered interactions with the nucleosome substrate. ACF forms a single, well-defined complex with the mononucleosome sliding substrate in electrophoretic mobility shift assays (EMSAs) (Figure 5A). Deletion of the ACF1 C-terminus including Brd and both PHD fingers did not change this interaction significantly (Figure 5A, lanes 2–13). This overall binding activity is the result of multiple contacts between both subunits of the remodelling factor and the substrate. ISWI alone has a strong preference to interact with nucleosomal DNA (Figure 5A, lanes 14–16; Längst and Becker, 2001a; Grüne *et al*, 2003), which is likely to dominate the EMSA. A further DNA binding function has been suggested to reside in the N-terminus of ACF1 (Fyodorov and Kadonaga, 2002a). In agreement with dominant DNA binding activities of ACF that are not mediated by the PHD fingers, deletion of the PHD fingers did not abolish the band-shift and 1,10-phenanthroline did not affect this nucleosomal interaction (Figure 5B). The nucleosome binding properties of ACF1 alone have never been analysed since the protein could not be expressed in a soluble form previously (Fyodorov and Kadonaga, 2002a). We optimized baculovirus expression to obtain soluble ACF1 without ISWI, and demonstrate that ACF1 alone binds nucleosomes well (Figure 5A, lanes 17–19; Figure 5C, lanes 2–11). Deletion of the PHD finger did not diminish the affinity to the nucleosome; however, the complexes that were formed were less distinct (the band-shift smeary), possibly because the complex formed under these conditions was less rigid.

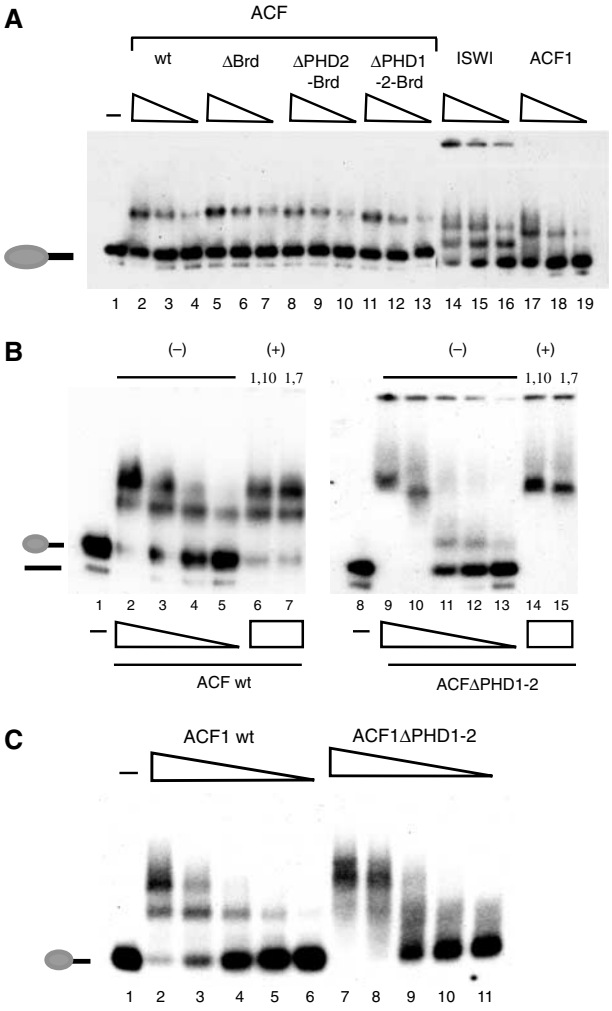

Figure 5 The PHD fingers of ACF1 are important for nucleosome recognition. (**A**) EMSA monitoring the interaction of various remodelling factors with mononucleosome substrates. Affinity-purified ACF complexes bearing the indicated ACF1 deletions, ISWI and ACF1 were incubated with 60 fmol of end-positioned nucleosome for 15 min. The reactions were then separated on a 1.4% agarose gel, which was dried and exposed to film overnight at −80°C. Protein concentrations were 60 fmol (lanes 2, 5, 8, 11, 14 and 17), 30 fmol (lanes 3, 6, 9, 12, 15 and 18) or 15 fmol (4, 7, 10, 13, 16 and 19). Lane 1 shows the migration of the nucleosome alone. (**B**) EMSA as in (A) using wt ACF complex (lanes 2–7) and ACFΔPHD1-2 (lanes 9–15). Protein concentrations were 120 fmol (lanes 2, 6, 7, 9, 14 and 15), 90 fmol (lanes 3 and 10), 60 fmol (4 and 11), 30 fmol (lanes 5 and 12) and 15 fmol (lane 13). Where indicated, either 3 mM 1,10-phenanthroline (lanes 6 and 14) or 3 mM 1,7-phenanthroline was added to the reaction. Lanes 1 and 8 show the migration of the nucleosome alone. (**C**) EMSA as in (A) analysing ACF1 full length (lanes 2–6) and ACFΔPHD1-2 (lanes 7–11). Protein concentrations were 90 fmol (lanes 2 and 7), 60 fmol (lanes 3 and 8), 30 fmol (lanes 4 and 9), 15 fmol (lanes 5 and 10) or 7.5 fmol (lanes 6 and 11). The migration of mononucleosomes in the absence of protein is shown in lane 1.

PHD fingers of ACF1 bind histones

PHD finger structures are known to interact with proteins in other contexts (Aasland *et al*, 1995; Zhang *et al*, 1998). In order to address their function directly without interference of the rest of ACF1, we expressed a region of ACF1 containing both fingers (aa 1039–1290) as a C-terminal fusion to a GST (GST-PHD1-2) in bacteria and bound them to glutathione-Sepharose beads, thereby generating an affinity resin. Since we were able to detect significant binding of nucleosomes but not of free DNA to this resin (data not shown), we explored whether the PHD region was able to interact with histones. For reference, we also expressed a GST-Brd protein and coupled it to Sepharose for pull-down studies (GST-Brd). Equivalent volumes of affinity beads were incubated with 1 μg of either purified embryonic *Drosophila* histones (Figure 6A, upper panel) or recombinant *Drosophila* histones

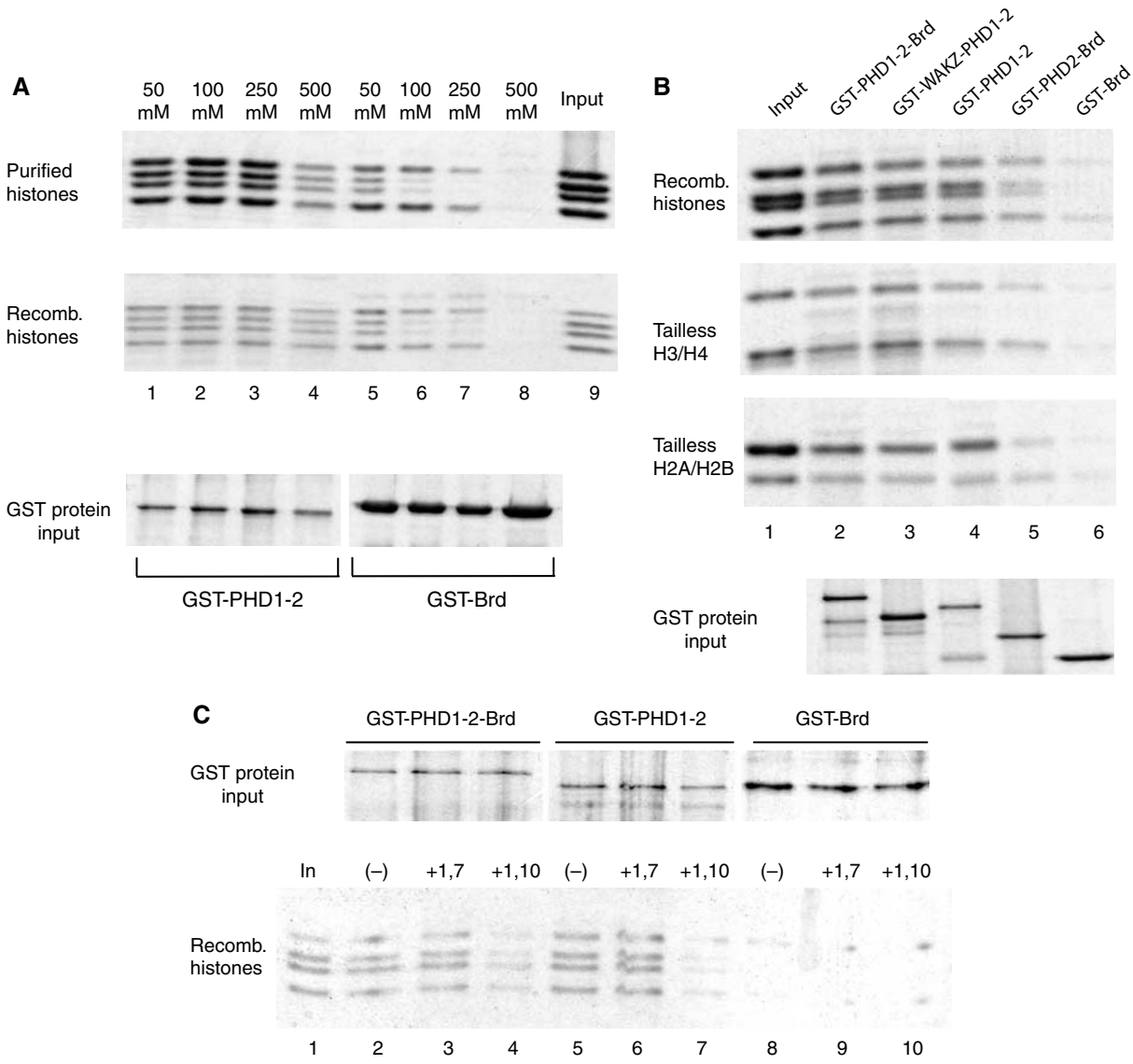

Figure 6 The ACF1 PHD fingers are required for interaction with histones. (**A**) GST-PHD1-2 and GST-Brd fusion proteins were expressed in bacteria, bound to glutathione-Sepharose 4B and used in 'pull-down' experiments. About 500 ng of immobilized protein was incubated with 1 μg of *Drosophila* histone mixtures either purified from embryos (purified histones) or expressed in bacteria (recombinant histones) at the indicated salt concentrations. Histones that remained bound through excessive washes were separated by 15% SDS–PAGE and visualized by Coomassie blue staining. Lane 9 shows the histone input. (**B**) Indicated domains of ACF1 were expressed as GST fusion proteins and used in 'pull-down' experiments as in (A). A 1 μg portion of all four recombinant histones (recomb histones) or of recombinant H3/H4 tetramers (tailless H3/H4) or recombinant H2A/H2B dimers (tailless H2A/2B), lacking their N-terminal tail domains, was tested in 'pull-down' experiments. Bound material (50%) was separated by 15% denaturing PAGE and stained with Coomassie blue. The relative amount of each GST construct is shown in the bottom panel. (**C**) The indicated domains of ACF1 (upper panel) were expressed and used in pull-downs as described in (A). Before incubation with histones, the indicated GST beads were pretreated for 4 h at RT with buffer alone (−), 3 mM of 1,7-phenanthroline (+1,7) or 3 mM of 1,10-phenanthroline (+1,10). Lane 1 shows the histone input.

(middle panel), bound protein was washed stringently, resolved by PAGE and detected by Coomassie blue staining. Both fusion proteins were able to bind a major fraction of the input histones at low and physiological salt, but considering the higher input of GST-Brd over GST-PHD1-2 (Figure 6A, 'input' lower panel), the latter was more effective. GST beads alone did not bind any histones (not shown). When the stringency of the binding reaction was raised by increasing the ionic strength during the binding reaction, two phenomena were observable. First, the interaction of PHD1-2 appeared considerably stronger (binding at 500 mM salt) than the Brd interaction, since the latter faded as the ionic strength was increased and did not resist to 500 mM salt washes. Second, Brd showed a binding preference for histones H3 and H4 over H2A/H2B, whereas the PHD finger domains contacted all histones equally well. In order to determine whether the PHD finger functioned in the context of a larger structure, we generated additional GST fusion proteins (Figure 6B). As before, our reference was a weak interaction of the GST-Brd protein with H3 and H4. Addition of PHD2 improved the interaction with all four histones somewhat (Figure 6B, lane 5), but inclusion of PHD1 led to profound histone binding (Figure 6B, lane 2). The two PHD fingers alone bound the histones well and this binding was not

improved by the presence of the WAKZ domain (lanes 4 and 3). We conclude that the PHD fingers are major determinants of histone interaction. The finding that all four core histones were bound equally well by the PHD finger domain led us to consider that the interaction may not be due to the flexible histone tails but to the central parts of the histones, which all share a common histone fold. Indeed, deletion of the N-termini in the context of histone pairs (labelled 'tailless' in Figure 6B) did not abolish the interaction (Figure 6B, middle panels). No interaction, however, was observed when we used a dimer of the two histone-fold CHRAC components, CHRAC14/16, in the pull-down assays (data not shown).

In a final experiment, we wished to determine whether the PHD–histone interactions were sensitive to 1,10-phenanthroline. We chelated zinc from the GST fusion proteins by treatment with 3 mM 1,10-phenanthroline before performing pull-down assays. The nonchelating 1,7-phenanthroline served as a control as before (Figure 6C). Interaction of the PHD fingers with histones was sensitive to zinc chelation (lanes 7 and 4), while the 1,7-phenanthroline did not have an effect (lanes 6 and 3). The weak interaction of Brd with H3 and H4 was insensitive against phenanthroline treatment (Figure 6C, lanes 8–10). Taken together, our data suggest that the PHD finger domains of ACF1 contribute to nucleosome mobilization by making crucial contacts with the central histone moiety of the nucleosome substrate.

Discussion

PHD–histone contacts are required for efficient nucleosome remodelling by ISWI

Binding of ACF1 to ISWI leads to a remarkable increase in energy efficiency of the nucleosome remodelling reaction catalysed by ISWI: while the amount of ATP hydrolysed in response to the nucleosome substrate remains unchanged upon ACF1 interaction, the complex moves nucleosomes an order of magnitude more efficiently than ISWI alone (Eberharter *et al*, 2001). Now, we have found that the PHD modules in the C-terminus of ACF1 are crucially involved in this activation. Previously, we observed this boost of activity only in conjunction with a change of nucleosome sliding directionality (Eberharter *et al*, 2001). Deletion of the PHD domain selectively affects the efficiency of the sliding reaction, but does not alter the type of nucleosome movement (this study). It appears, therefore, that structures in the N-terminus of ACF1, or simply the fact that ACF1 interacts with ISWI, determine the qualitative outcome of nucleosome mobilization.

PHD fingers have been proposed to serve as protein interaction surfaces (Aasland *et al*, 1995; Zhang *et al*, 1998), but our demonstration of contacts with the central parts of core histones in the context of a nucleosome remodelling factor is novel. Disruption of the PHD structure through zinc chelation destroys the interaction of ACF1 with histones and at the same time abolishes the stimulatory effect of ACF1 on the efficiency of nucleosome sliding. Evidently, ACF1–histone contacts are crucial for efficient nucleosome remodelling. Our results are in conflict with those obtained earlier by Fyodorov and Kadonaga (2002a), who did not observe a detrimental effect of deleting the PHD or Brd modules in an ACF-dependent chromatin assembly system. We think that this discrepancy may be due to the different functional assays employed (chromatin assembly versus nucleosome sliding) and perhaps also to differences in the protein expression protocol: whereas Fyodorov and Kadonaga (2002a) were unable to express soluble ACF1, our procedure yielded soluble protein that could be functionally characterized.

According to our favourite model, ISWI-containing remodelling factors lift DNA off the histone surface at the edge of the nucleosome and distort it into a bulge or loop. Propagation of this distortion over the surface of the histone octamer leads to nucleosome relocation (Längst and Becker, 2001a; Becker and Hörz, 2002; Figure 7). We consider that efficient displacement of nucleosomal DNA relative to the histone octamer requires contacts of the remodelling enzyme with both the DNA and histone moieties. ISWI alone binds nucleosomes mainly through interactions of its C-terminal SLIDE domain with nucleosomal DNA (Grüne *et al*, 2003). Although a segment of the H4 N-terminus is absolutely required for ISWI function (Clapier *et al*, 2001), we are so far not aware of any stable interactions of ISWI with histones. ISWI may be a relatively inefficient remodelling enzyme because it lacks a stable anchoring point on the histone body (Figure 7A). The PHD–histone contacts documented in our study may provide such an anchoring point for the enzyme, assuring that the presumed conformational changes triggered by ATP binding and hydrolysis are efficiently converted into positional shifts of DNA relative to histones (Figure 7B). According to a variation of this model, ACF plays an active role in the propagation of the DNA distortion ('the loop') around the histone octamer (Lusser and Kadonaga, 2003; Figure 7C), which would necessitate changing contacts of the remodelling machinery with the histone octamer surface as it traverses around the particle. The observation that the PHD fingers of ACF1 interact with all four histones suggests that they recognize a common struc-

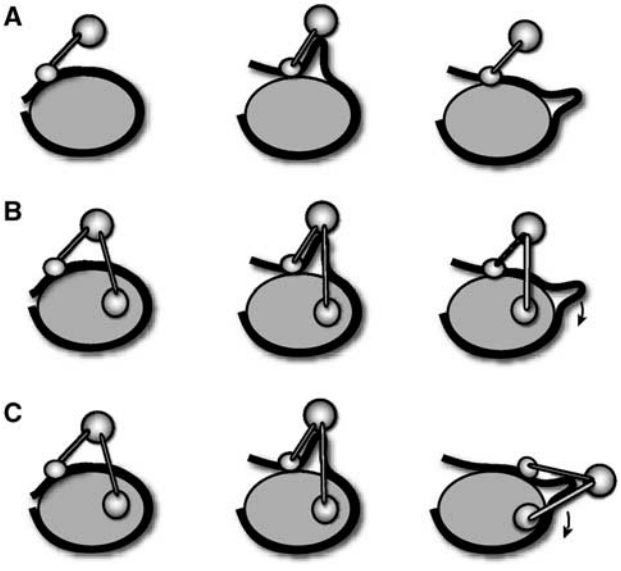

Figure 7 Models explaining the increased effectiveness of nucleosome sliding upon interaction of ACF1 with ISWI. (**A**) ISWI mainly interacts with linker DNA. (**B, C**) Additional contact of ACF1 with histones provides an anchor on the histone moiety of the nucleosome that allows efficient conversion of ATP-dependent conformational changes of ISWI into translocation of DNA relative to the histones during nucleosome mobilization.

tural feature on the histone pairs, and is compatible with models involving multiple, different contacts of the remodeller on the histone octamer.

Widespread occurrence of PHD modules in chromatin modifiers

ACF1 is a prominent member of a family of related proteins in various species, which interact with ISWI to form several distinct nucleosome remodelling complexes such as ACF (Ito *et al*, 1999), CHRAC (Eberharter *et al*, 2001), NURF (Xiao *et al*, 2001), WICH (Bozhenok *et al*, 2002), WCRF (Bochar *et al*, 2000) and NoRC (Strohner *et al*, 2001). These proteins share with ACF1 a similar domain organization including C-terminal PHD and Brds. A region of NURF301, the largest subunit of NURF, containing two PHD fingers and the adjacent Brd binds to all four core histones, but the functional consequences of these interactions have not been determined (Xiao *et al*, 2001). Interestingly, human ACF1 (alias WCRF180) and the related human WSTF, which associate with the human homologue of ISWI, SNF2H (Bochar *et al*, 2000; Poot *et al*, 2000; Bozhenok *et al*, 2002), function with only one PHD module and the hSNF2H-interacting protein RSF1 does not share any sequence similarity with ACF1, except for one PHD module (Loyola *et al*, 2003). It is therefore likely that one PHD module may be sufficient for function.

PHD fingers are diverse in sequence and may connect different proteins in various contexts. Our observation that PHD modules of ACF1 serve to tether a nucleosome remodelling enzyme to its substrate adds a new function to the list that should be tested for the known or suspected modifiers of chromatin structure, such as the remodelling ATPase Mi-2 (Zhang *et al*, 1998), the epigenetic regulators Trithorax, Polycomb-like (Aasland *et al*, 1995), Ash1 (Tripoulas *et al*, 1996), Ash2 (Adamson and Shearn, 1996) and Lid (Gildea *et al*, 2000).

PHD–Brd—an integrated nucleosome recognition module?

PHD modules are frequently found in the direct neighbourhood of a Brd. Examples include the ACF1-related proteins discussed here, and also the histone acetyltransferases CBP and p300 (Bordoli *et al*, 2001; Kalkhoven *et al*, 2002) and the KAP1 repressor (Schultz *et al*, 2001). From their functional analysis of domains involved in KAP-1 repression, Schultz *et al* concluded that both domains form an integrated, cooperative unit involved in binding the Mi2α subunit of the NuRD complex. In the context of histone binding, the idea of cooperativity between PHD fingers and Brds is attractive. While it is well established that Brds interact preferentially with acetylated N-termini of histones H3 and H4 (Jacobson *et al*, 2000; Owen *et al*, 2000), we showed that the PHD modules of ACF1 interact with the central domains of the core histones. This interaction may thus be further modulated by additional contacts of Brd with appropriately modified N-termini, as a means of fine-tuning nucleosome remodelling activity in response to the histone modification status. Whether this principle applies to ACF remains to be seen. However, Aasland and co-workers (Ragvin *et al*, 2004) have recently functionally characterized the Bromo–PHD modules of the histone acetyltransferase p300 and found that both domains cooperated for preferential binding to highly acetylated nucleosomes in a stringent assay.

Interestingly, deletion of the ACF1 Brd alone did not diminish nucleosome sliding, but consistently improved it. Given our limited knowledge on the structure of the ACF1 C-terminus as an entity, it is difficult to interpret this observation. Conceivably, the interaction of Brd with another domain of the remodelling machinery dampens its activity until a conformational change triggered by its interaction with an acetylated histone N-terminus unleashes full remodelling potential. This scenario suggests a strategy by which histone acetylation could regulate remodelling activity other than by simply increasing the affinity of the remodelling machinery for the substrate.

Apposition of functional domains through ACF1–ISWI interaction

We mapped AID to the very C-terminus of ISWI, directly adjacent to the SANT/SLIDE module, the nucleosome interaction determinant of ISWI (Grüne *et al*, 2003). Through this interaction, ACF1 will be brought close to the nucleosome surface, but this interaction will also lead to the apposition of several domains on both ACF subunits: the SANT domain of ISWI, which may be involved in contacting histone tails (Peterson and Logie, 2000; Boyer *et al*, 2002; Grüne *et al*, 2003); the SLIDE domain of ISWI, which contacts nucleosomal DNA (Grüne *et al*, 2003); the PHD fingers of ACF1, which binds the histone octamer surface (this study); and finally Brd with its potential for interactions with appropriately modified histone N-termini. Unravelling the sequence and dynamics of enzyme–substrate contacts during the remodelling process remains a major challenge for future research.

Materials and methods

Expression constructs

Baculovirus expression clones. All ACF1 deletions were generated by PCR from the full-length ACF1 cDNA in the pSport1 vector (Gibco-BRL). Each construct was created with a C-terminal myc tag and cloned with appropriate restriction sites into the vector pFastBac1 (Life Technologies Inc.). The clones were verified by sequencing. Details are available upon request. After recombination according to the manufacturer's protocol (Gibco-BRL) each bacmid was transfected into freshly diluted Sf9 cells together with Cellfectin (Life Technologies Inc.). Baculoviruses were amplified three times to obtain high-titer stocks before they were used in protein expression.

ACF1 constructs for expression in E. coli. ACF1 fragments were generated by PCR from full-length ACF1 in pSport1 vector. The GST fusion constructs PHD1-2-Brd (aa 1059–1476) and PHD2-Brd (aa 1237–1476) were subcloned into the *Eco*RI/*Xho*I sites of pGEX-4T-3 vector (Amersham Pharmacia Biotech), whereas the ACF1 fragments PHD1-2 (aa 1039–1290) and Brd (1300–1476) were subcloned into pET41c vector (Novagen) using the restriction sites *Hin*DIII/*Xho*I and *Spe*/*Xho*I, respectively. Clones were verified by DNA sequencing.

Purification of ISWI, ACF1 and ACF complexes from Sf9 cells

ACF1-FLAG and FLAG-ISWI were synthesized in Sf9 cells as described previously (Eberharter *et al*, 2001). The different ACF complexes were generated by coexpression of FLAG-ISWI and variants of ACF1. The proteins were bound to M2 anti-FLAG agarose beads (SIGMA) or, in the case of myc-tagged ACF1 Δ-PHD1-2, to anti-c-myc agarose (SIGMA), extensively washed and purified as a complex or individual proteins as described (Eberharter *et al*, 2001). In short, Sf9 cells were suspended in HEMG-500 (25 mM HEPES (pH 7.6), 500 mM NaCl, 0.5 mM EDTA, 1.5 mM $MgCl_2$,

10% glycerol, 0.05% NP-40 and protease inhibitors), frozen in liquid nitrogen and sonicated. Elution of the ACF1 Δ-PHD1-2-myc protein was achieved by adding 500 ng/μl MYC peptide (SIGMA) to HEMG-200 containing 1% NP-40 and constant rotation for 4 h at 4°C. To generate ACF1-FLAG beads for ISWI interaction studies, the FLAG peptide elution was omitted and the ACF1 beads were kept in buffer HEMG-250 as a 50% slurry (beads:buffer) at 4°C. A 10 μl volume of slurry of the ACF1-FLAG beads was routinely analysed by SDS–PAGE and Coomassie blue staining.

Synthesis and purification of bacterially expressed proteins

All subfragments of ACF1 were expressed as GST fusion proteins in *E. coli*. For a typical protein expression, 1 l culture was incubated at 37°C until an OD_{600} of 0.6, 0.3 mM IPTG was added and incubation proceeded for an additional 3 h. After collecting the cells by centrifugation, 40 ml of resuspension buffer (25 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1% Triton X-100 and protease inhibitors) was added and the suspension was sonicated (Branson 250-D; amplitude 50%) on ice for 30 s with three repetitions. The material was clarified by centrifugation at 4°C for 20 min at 10 000 *g*. Soluble material was then incubated with pre-equilibrated glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for at least 1 h at 4°C on a rotating wheel and washed five times with an excess of HEMG-200 buffer. The GST subfragments of ACF1 were finally resuspended in HEMG-200 and stored as a 50% (beads:buffer) slurry at 4°C.

ISWI subfragments were cloned into the *Bam*HI/*Eco*RI sites of vector pGEX-4T-3 and expressed in 450 ml of *E. coli* as GST fusion proteins by induction with 0.15 mM IPTG for 3 h at 37°C. For ISWI 691–991, 796–991, 691–1027 and 796–1027, cells were resuspended in 25 ml of TBS (50 mM Tris–HCl (pH 8.0) and 150 mM NaCl) + 10% Triton X-100 and protease inhibitors, sonicated (2 × 20 s, Branson 250-D; amplitude 60%) and cleared by centrifugation (4°C, 20 min, 10 000 *g*). The supernatant was incubated with 400 μl glutathione-Sepharose 4B beads for 1 h at 4°C. After extensive washes with TBS, proteins were eluted with 5 mM reduced glutathione for 15 min at 4°C under constant mixing. Glycerol was added to a final concentration of 25% and GST subfragments were kept at −20°C. In the case of ISWI 691–962 and 796–962, proteins were isolated from inclusion bodies by resuspending the cells in SAU200 (8 M urea, 20 mM NaAc (pH 5.2), 200 mM NaCl, 1 mM EDTA and 5 mM β-mercaptoethanol) overnight at RT under constant mixing. The material was cleared by centrifugation at 10 000 *g* for 20 min at 4°C and the supernatant was dialysed three times for 2 h and then overnight against refolding buffer (25 mM HEPES (pH 7.6), 500 mM KCl, 1.25 mM $MgCl_2$, 0.1 mM EDTA, 25% glycerol, 0.05% NP-40 and 1 mM DTT). After dialysis, the material was cleared again by centrifugation and the supernatant was incubated with 300 μl glutathione-Sepharose 4B beads for 1 h at 4°C. Washes, elution and storage of the ISWI subfragments were as above.

ATPase assays

The ATPase assays were performed as described previously (Eberharter *et al*, 2001).

Nucleosome mobility and binding assays

The generation of mononucleosome particles and the nucleosome sliding reactions were as described (Eberharter *et al*, 2004). Reactions were stopped by the addition of 200 ng of competitor DNA. The samples were kept on ice before electrophoresis on native 4.5% PAG in 0.5 × TBE. Incubations for the EMSAs were performed at 26°C for 15 min using 60 fmol of nucleosomal substrate and different amounts of the affinity-purified proteins. Samples were electrophoresed on 1.4% agarose gels in 0.3 × TBE at 4°C for 75 min at 150 V. Gels were dried and exposed to film overnight at −80°C. 1,10 phenanthroline or 1,7 phenanthroline (SIGMA-Aldrich) were dissolved as 200 mM stock solutions in ethanol and stored at −20°C. Prior to the experiment, the phenanthroline solutions were diluted into the nucleosome mobility/band-shift buffer.

GST pull-down experiments

Histone octamers were either isolated from *Drosophila* embryo extract (Simon and Felsenfeld, 1979; Eberharter *et al*, 2004) or individual recombinant histones were expressed in *E. coli* (Luger *et al*, 1999; Clapier *et al*, 2001). In a typical GST pull-down assay, equal amounts of ACF1-fragment-GST beads were incubated with 1 μg of histones in a final volume of 150 μl of HEMG-X (X for variable salt concentrations) for 4 h at 4°C under constant mixing. Beads were then washed five times with an excess of HEMG-200 or indicated buffers and finally resuspended in 20 μl of loading buffer for SDS–PAGE. Bound material was separated on 15% polyacrylamide gels and stained with Coomassie blue.

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