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# The histone chaperone Asf1 is dispensable for direct de novo histone deposition in *Xenopus* egg extracts

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**Abstract** Histone chaperones that escort histones during their overall lifetime from synthesis to sites of usage can participate in various tasks. Their requirement culminates in the dynamic processes of nucleosome assembly and disassembly. In this context, it is important to define the exact role of the histone chaperone Asf1. In mammals, Asf1 interacts with two other chaperones, CAF-1 and HIRA, which are critical in DNA synthesis-coupled and synthesis-uncoupled nucleosome assembly pathways, respectively. A key issue is whether Asf1 is able or not to deposit histones onto DNA by itself in both pathways. Here, to delineate the precise role of Asf1 in chromatin assembly, we used *Xenopus* egg extracts as a powerful system to assay de novo chromatin assembly pathways in vitro. Following characterization of both *Xenopus* Asf1 and p60 (CAF-1), we used immunodepletion strategies targeting Asf1, HIRA, or CAF-1. Strikingly, the depletion of Asf1 led to the simultaneous depletion of HIRA and consequently impaired the DNA synthesis-independent nucleosome assembly pathway. The

rescue of nucleosome assembly capacity in such extracts was effective when adding HIRA along with H3/H4 histones, yet addition of Asf1 along with H3/H4 histones did not work. Moreover, nucleosome assembly coupled to DNA repair was not affected in these Asf1/HIRA-depleted extracts, a pathway impaired by CAF-1 depletion. Thus, these data show that Asf1 is not directly involved in de novo histone deposition during DNA synthesis-independent and synthesis-dependent pathways in egg extracts. Based on our results, it becomes important to consider the implications for Asf1 function during early development in *Xenopus*.

## Introduction

Histone chaperones can play a variety of roles involved in histone metabolism (Loyola and Almouzni 2004). By definition, a histone chaperone is a factor that associates with histones to facilitate and control their interaction with other molecules without being a component of the final reaction product (Polo and Almouzni 2006). Their requirement culminates in the dynamic processes of nucleosome assembly and disassembly. Our current understanding of the assembly mechanisms enables to define two distinct pathways: (1) associated with DNA synthesis during DNA replication and specific repair events or (2) independently of DNA synthesis. The latter situation can be encountered as part of a histone exchange mechanism to introduce histone variants (Henikoff and Ahmad 2005; Kamakaka and Biggins 2005; Sarma and Reinberg 2005) or following eviction of histones from chromatin as proposed for activation of the PHO5 promoter in yeast (Boeger et al. 2003; Reinke and Horz 2003). To date, two H3/H4 histone chaperones, CAF-1 (chromatin assembly factor 1) and HIRA (histone regulating protein A), have been characterized as directly involved

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in histone deposition per se. CAF-1, which consists of three subunits, p150, p60, and p48 in human cells (Ridgway and Almouzni 2000) is considered as the main histone chaperone that assists nucleosome assembly coupled to DNA synthesis (Groth et al. 2007) both during DNA replication (Smith and Stillman 1989) and nucleotide excision repair (NER) (Gaillard et al. 1996; Polo et al. 2006). In contrast, HIRA has been involved in a nucleosome assembly pathway independent of DNA synthesis in *Xenopus* egg extracts (Ray-Gallet et al. 2002) and during sperm nucleus decondensation in *Drosophila* (Loppin et al. 2005) and in mouse (van der Heijden et al. 2005). The isolation of a specific predeposition complex containing the replicative histone variant H3.1 together with CAF-1, distinct of another predeposition complex containing the replacement histone variant H3.3 together with HIRA supports this view (Tagami et al. 2004). The H3.3 variant, incorporated into chromatin independently of DNA synthesis (Ahmad and Henikoff 2002; Tagami et al. 2004) and proposed to mark transcriptionally active genes (Schwartz and Ahmad 2005), could thus use this latter chaperone (Loppin et al. 2005; Nakayama et al. 2007; van der Heijden et al. 2007).

Asf1 (Anti-silencing function 1), initially identified in *S. cerevisiae* (Le et al. 1997), is another H3/H4 chaperone whose orthologs in human consist of two genes, Asf1a and Asf1b, whereas only one Asf1 was characterized in *Drosophila*. The copurification of Asf1a and b with both CAF-1 in the H3.1 complex and HIRA in the H3.3 complex suggests that Asf1a and b could potentially participate in both nucleosome assembly pathways coupled and uncoupled to DNA synthesis (Tagami et al. 2004). Furthermore, Asf1 interacts directly with CAF-1p60 subunit and synergizes with CAF-1 to facilitate nucleosome assembly in *Drosophila* embryo extracts onto newly replicated DNA (Tyler et al. 1999) and in human cell extracts onto repaired DNA (Mello et al. 2002) or newly replicated DNA (Groth et al. 2005). Asf1a, but not Asf1b, interacts specifically with HIRA in human cells (Daganzo et al. 2003; Tagami et al. 2004), and both Asf1a and HIRA have been involved in the formation of macroH2A-containing senescence-associated heterochromatic foci (SAHF; Zhang et al. 2005). The fact that both HIRA and p60 CAF-1 use similar motifs for binding to Asf1a suggests a competing interaction with this chaperone (Tang et al. 2006). How this specificity applies to chromatin assembly is still an enigma. In any case, Asf1 function seems to generally relate to disassembling and reassembling histones, as proposed based on work in *S. cerevisiae* in which Asf1 importance was evaluated in the context of transcriptional activation (Adkins et al. 2004; Adkins and Tyler 2004; Schwabish and Struhl 2006).

Although the general view involving the histone chaperone Asf1 in chromatin assembly is consistent with series of

experiments in various organisms (Mousson et al. 2007), its exact position in the assembly line leading to nucleosome formation remains unclear. A major question is whether Asf1 is able or not to deposit histones onto DNA by itself or if it does act through another partner. In which case, its role would rather consist in being a “histone provider” as already proposed (Groth et al. 2005; Loyola and Almouzni 2004; Mello et al. 2002). Here, we aim to delineate the precise role of Asf1 in chromatin assembly. For this, we use *Xenopus* egg extracts as a powerful source of histones and chaperones to test de novo chromatin assembly pathways in vitro (Almouzni and Mechali 1988; Laskey et al. 1977; Ray-Gallet and Almouzni 2004). First, it was necessary to develop the adequate tools in *Xenopus*. We thus characterized both *Xenopus* Asf1 and p60 (CAF-1). Then, we could exploit immunodepletion strategies targeting Asf1, HIRA, p150, or p60 in *Xenopus* high-speed egg extracts (HSE) to examine the role of Asf1 in histone deposition in comparison with the other chaperones. We carried out this analysis for both DNA synthesis-independent and synthesis-dependent nucleosome assembly pathways. Taken together our results argue that Asf1 is not critically required for de novo histone deposition per se neither coupled nor uncoupled to DNA synthesis in *Xenopus* egg extracts.

## Materials and methods

### Cloning and sequence analysis of Asf1 and p60 *Xenopus* cDNAs

*Xenopus* Asf1 cDNA isolated from a lambda gt11 *Xenopus* oocyte cDNA library (Clontech) was cloned in pBluescript SK (-) plasmid (accession number AY351903).

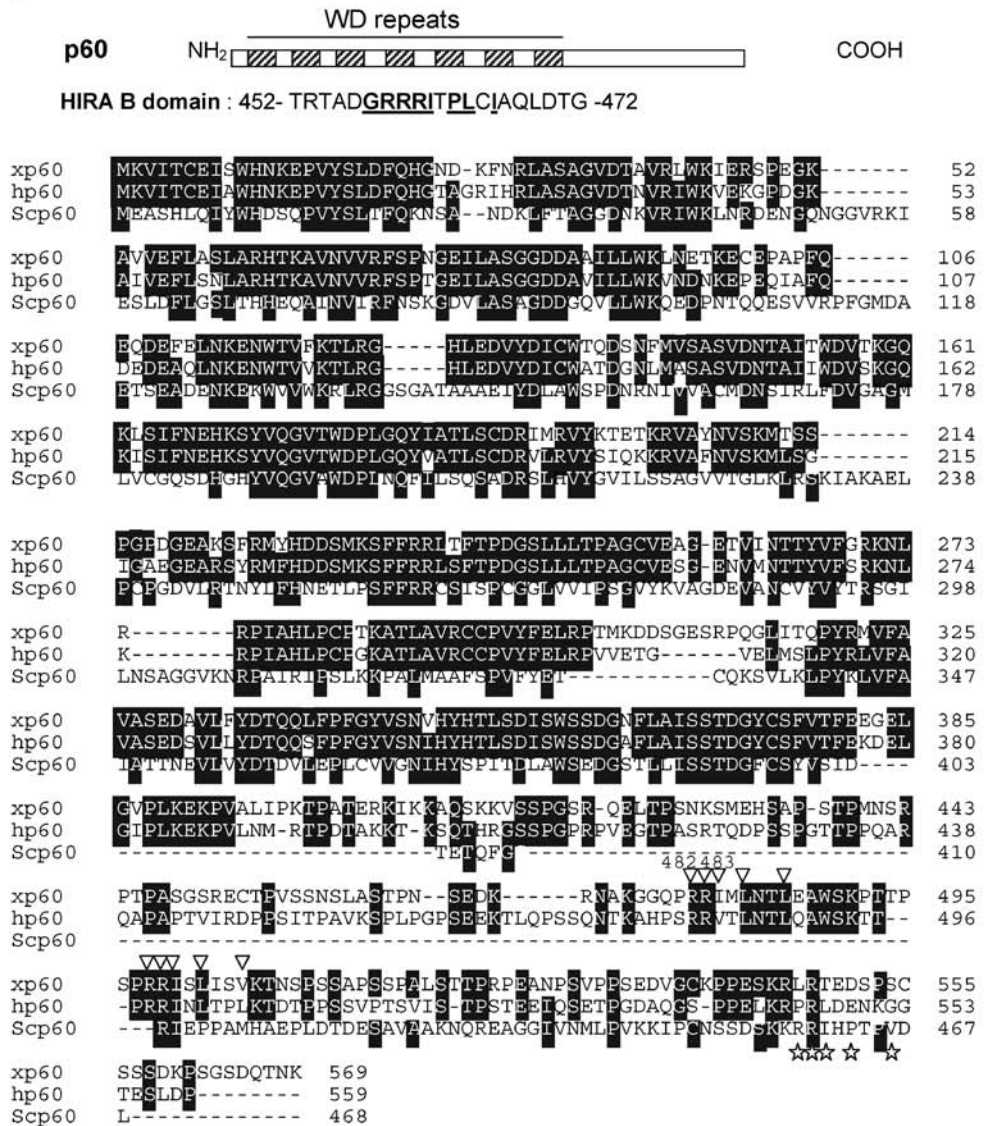
*Xenopus* p60 cDNA (clone XL061p11) was obtained from the NIBB Mochii normalized *Xenopus* tailbud library in the pBlueScript SK(-) plasmid (accession number AJ579309).

**Fig. 1** Asf1 and p60 proteins in *Xenopus Laevis*. **a** Amino acid sequences of *Xenopus* Asf1 (*xAsf1*), human Asf1a (*hAsf1a*), human Asf1b (*hAsf1b*) and *S. cerevisiae* Asf1 (*ScAsf1*). The acidic amino acid stretch in *ScAsf1* is underlined. The two conserved aspartic residues D37 and D58, which are crucial for Asf1 complex formation with HIRA or CAF-1 p60, are indicated (Sanematsu et al. 2006; Tang et al. 2006). **b** *Xenopus* p60 (*xp60*), human p60 (*hp60*) and *S. cerevisiae* p60 (*Scp60*). The sequence of the B domain of human/*Xenopus* HIRA, which is involved in Asf1a binding is shown (HIRA residues that contact Asf1a are in **bold** and **underlined**). HIRA B domain-like motifs of CAF-1 p60 are indicated by marking the residues that should contact Asf1a (**arrowhead** for the two motifs in both *Xenopus* and human p60; **star** for the unique motif in yeast p60). The two arginine residues of the first HIRA B domain like-motif, R482 and R483, which are critical for HIRA and p60 CAF-1 binding, are indicated (Tang et al. 2006). We used ClustalW for alignments and shaded identical residues found at least in two of the sequences

**a**

		37	58	
xAsf1	MAKVQVNNVVLDNPSFFYNPFQFEITFECIEDLSEDLWKIIYVGSASEEYDQVLD			60
hAsf1a	MAKVQVNNVVLDNPSFFYNPFQFEITFECIEDLSEDLWKIIYVGSASEEYDQVLD			60
hAsf1b	MAKVSVLNVVLDNPSFFYNPFQFEITFECIEDLSEDLWKIIYVGSASEEYDQVLD			60
ScAsf1	MSTVSLLGIKVLNNAKFTDYEFEITFECLESLKHDLEWKLTVGSSRSLDHQDEL			60
xAsf1	LVGPVPAGRHMVFVQADAPNPLIPDADAVGTVVLLITCTYRGOEFIRVGYVNNYET			120
hAsf1a	LVGPVPAGRHMVFVQADAPNPLIPDADAVGTVVLLITCTYRGOEFIRVGYVNNYET			120
hAsf1b	LVGPVPAGRHMVFVQADAPNPLIPDADAVGTVVLLITCTYRGOEFIRVGYVNNYET			120
ScAsf1	LVGPVPGVGNKVFVSADPPSABLIPASELVSVTVVLLITCTYRGOEFIRVGYVNNY			120
xAsf1	ELRENPPVKPDFSKLQRNILASNPRVTRFHINWEE			156
hAsf1a	ELRENPPVKPDFSKLQRNILASNPRVTRFHINWED			156
hAsf1b	ELRENPPVKPDFSKLQRNILASNPRVTRFHINWDN			156
ScAsf1	ELRENPPKQVNDHLVRNILASNPRVTRFNIWDNENEGDLYPPEQPGVDDDEEEDDEE			180
xAsf1	--TEKLEDAESSNPILQSLSTLALPSASKGWSTSENSLNVMLSEHMDCM			201
hAsf1a	--TEKLEDAESSNPILQSLSTLALPSASKGWSTSENSLNVMLSEHMDCM			204
hAsf1b	--MDRLELLETQDPSLGCCLPLNCLP--IKGGLPGCIPGLLLENSMDCM			202
ScAsf1	DDDEDLEDEDDEDDLELCEGAAEEAEEEEEEKEEDNETNLEEEEDLENSDGDDEE			240
xAsf1	-----			
hAsf1a	-----			
hAsf1b	-----			
ScAsf1	EEVGSVDKNEGDNDKRRKIEGGSTDIESTPKDAARSTN	279		

**b**



## Asf1 and p60 recombinant *Xenopus* proteins and antibody production

PCR fragments containing the coding sequences of xAsf1 and xp60 cDNAs were subcloned in pGEX4T1 and PET 30 (for xAsf1) plasmids to produce GST and His fusion proteins, respectively. GST fusion and untagged HIRA *Xenopus* proteins were produced (Ray-Gallet et al. 2002). A rabbit polyclonal antiserum against *Xenopus* p60 was obtained by immunization with GST-p60 and against human Asf1 proteins by immunization of rabbits with GST-hAsf1b or GST-hAsf1a (Groth et al. 2005).

## Protein interactions and immunoprecipitations

A TNT-coupled rabbit reticulocyte lysate kit (Promega) was used for in vitro translation of the full-length HIRA and Asf1 *Xenopus* proteins from pBluescript clones and GST protein interactions performed (Ray-Gallet et al. 2002).

For HSE immunoprecipitation, 20  $\mu$ l of HSE were incubated with either anti-HIRA antiserum or preimmune serum for 2 h at 4°C in 10 mM Hepes (pH 7.6), 150 mM KCl, 0.5% NP40 and protease inhibitors.

## Depletions and nucleosome assembly reactions

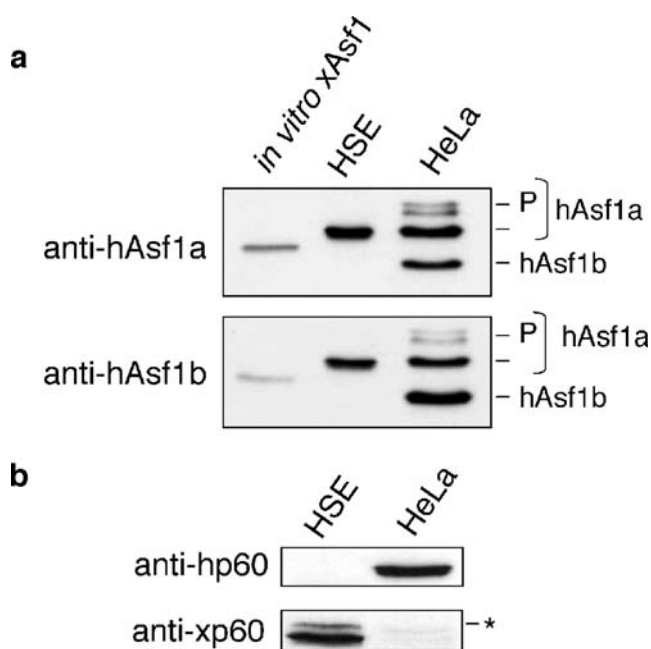
The HSE, prepared as described (Almouzni 1998), were immunodepleted (Ray-Gallet et al. 2002) using antibodies against *Xenopus* p60, *Xenopus* HIRA (Ray-Gallet et al. 2002), *Xenopus* p150 (Quivy et al. 2001), and human Asf1b (Groth et al. 2005) or mock-treated by incubation with noncoupled protein A sepharose.

In vitro nucleosome assembly reactions for 3 h at 23°C with HSE (8  $\mu$ l) used (1) nonirradiated pBS plasmid (-UV) for the pathway independent of DNA synthesis (2) or pBS plasmid irradiated with UV-C (500 J/m<sup>2</sup>; +UV) in the presence of ( $\alpha$ -<sup>32</sup>P)dCTP for the pathway coupled to nucleotide excision repair (NER) (Gaillard et al. 1999; Ray-Gallet and Almouzni 2004). Supercoiling and DNA synthesis analyzed by agarose gel electrophoresis were visualized by ethidium bromide staining (to detect Total DNA) and autoradiography (to detect labeled repaired DNA). For rescue of p150-depleted HSE, human CAF-1 complex (p150, p60, p48) (15 ng) (Verreault et al. 1996) was added to the nucleosome assembly reaction. For complementation of Asf1/HIRA-depleted HSE, *Xenopus* His-Asf1 (10 ng) and/or untagged *Xenopus* HIRA protein (10 ng) and/or (H3-H4)<sub>2</sub> tetramers (150 ng) purified from chicken erythrocytes (Simon and Felsenfeld 1979) were preincubated 10 min on ice before addition to the nucleosome assembly reaction after 10 min incubation.

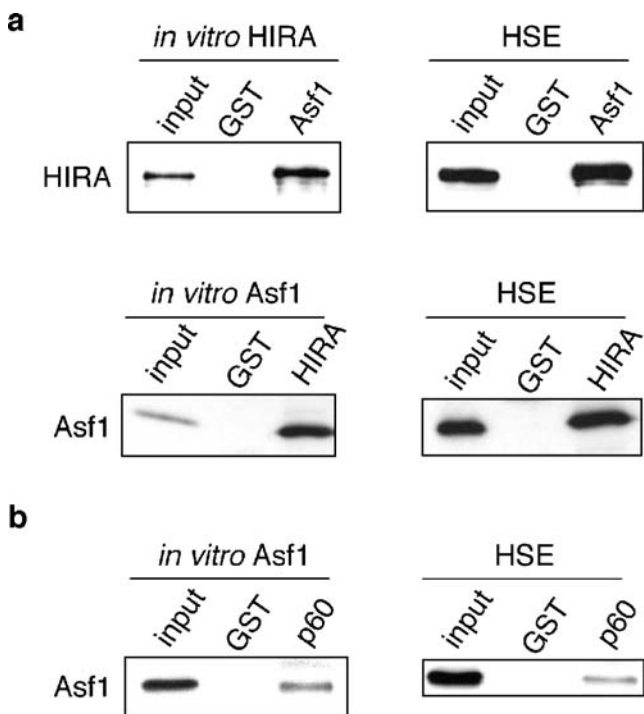
## Results

### *Xenopus* Asf1 and p60 CAF-1

To dissect in the *Xenopus* system the position of Asf1 in a chromatin assembly line and its relationship with CAF-1 and HIRA, we first cloned Asf1 and the p60 subunit of CAF-1 in *Xenopus laevis*. We found that *Xenopus* Asf1 cDNA encodes a protein of 201 amino acids which shares 94 and 70% amino acid identity with human Asf1a and Asf1b, respectively (Sillje and Nigg 2001), and 45% identity with the yeast *S. cerevisiae* Asf1 (Le et al. 1997) (Fig. 1a). The C-terminal portion of these proteins is the least conserved and this divergence is highly pronounced when comparison is made with the yeast sequence which possesses an acidic stretch not observed in human and *Xenopus* proteins. The two aspartic residues in Asf1, Asp37, and Asp58, that were described as crucial for human Asf1a complex formation with HIRA or CAF-1 p60 are conserved in the *Xenopus* protein (Sanematsu et al. 2006; Tang et al. 2006). Moreover, residues in Asf1 which are critical for H3/H4 binding are also conserved in the *Xenopus* counterpart, as Val45, Asp54, Val94, and Tyr112 (English et al. 2006; Mousson et al. 2005; Natsume et al. 2007). The conservation of these critical amino acids emphasizes that Asf1 may share common function(s) in various species.



**Fig. 2** Western blot analysis of Asf1 and p60 in *Xenopus*. **a** In vitro translated *Xenopus* Asf1, HSE (1  $\mu$ l) and human HeLa nuclear cell extracts (20  $\mu$ g) were analyzed by Western blotting with anti-human Asf1a or anti-human Asf1b antibodies. **b** HSE (1  $\mu$ l) and HeLa cell nuclear extracts (20  $\mu$ g) were analyzed by Western blotting using antibodies against human p60 (*anti-hp60*) or *Xenopus* p60 (*anti-xp60*). The asterisk indicates a minor band detected with the anti-xp60 antibody, potentially representing a p60 phosphorylated form



**Fig. 3** *Xenopus* Asf1 interacts with HIRA and p60 *Xenopus* proteins in vitro. **a** In vitro translated Asf1 or HSE were tested for binding to GST and GST-HIRA (*HIRA*) and conversely, in vitro translated HIRA or HSE for binding to GST or GST-Asf1 (*Asf1*). Input (10%) and bound proteins analyzed by Western blotting using anti-Asf1 or anti-HIRA antibodies. **b** In vitro translated Asf1a or HSE were used for binding to GST or GST-p60 (*p60*) and bound proteins analyzed by Western blotting using anti-Asf1 antibody

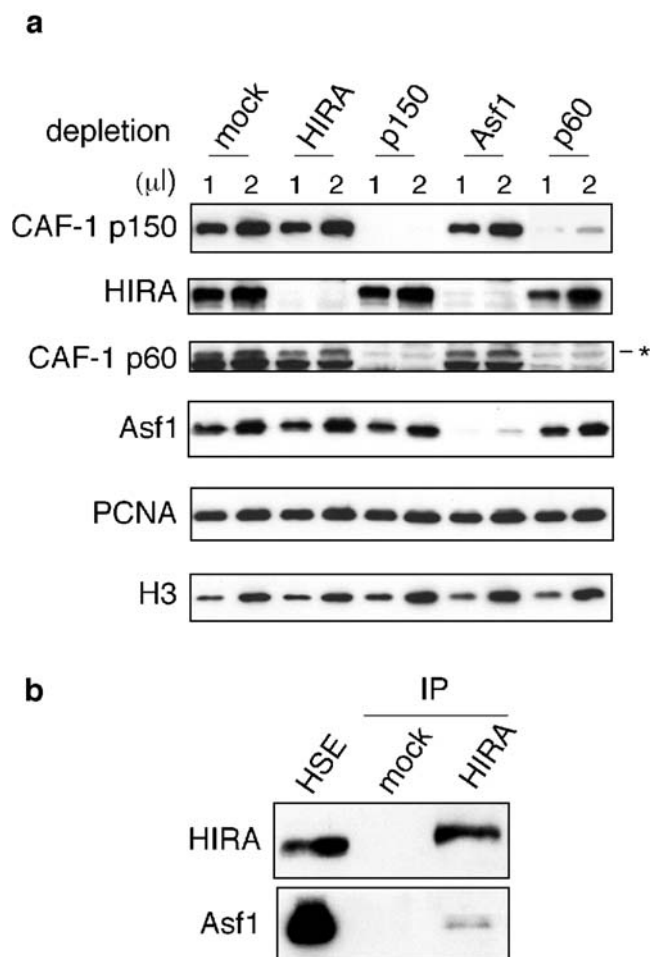
Western blot analysis using antibodies raised against either human Asf1a or Asf1b, which recognize both proteins in human cell extracts, detected the in vitro translated *Xenopus* Asf1 protein and one band in HSE (Fig. 2a). The Asf1 protein in egg extracts that exhibits a distinct migration profile when compared to in vitro translated protein can possibly be posttranslationally modified in *Xenopus* eggs. Indeed, the human counterparts can be phosphorylated by Tousled-like kinases (Tlk) (Sillje and Nigg 2001).

The *Xenopus* p60 cDNA encodes a protein of 569 amino acids sharing 63% amino acid identity with human p60 and 32% identity with yeast *S. cerevisiae* p60 (CAC2) (Fig. 1b). The most conserved region lies in the WD repeat domain in the N-terminal part of the protein. Interestingly, the two HIRA B domain-like motifs found in the C-terminal part of human CAF-1 p60 and whose the first one appears primarily responsible for Asf1a binding (with the two critical arginine residues Arg482 and Arg483) are conserved in *Xenopus* (Tang et al. 2006). To search for the presence of p60 CAF-1 in *Xenopus* eggs, a specific rabbit polyclonal antibody was raised using a recombinant *Xenopus* p60 protein. Indeed, despite the conservation between human and *Xenopus*, an antibody raised against the human p60 was unable to reveal its *Xenopus* counter-

part in egg extracts by Western blot analysis (Fig. 2b). We found significant amount of p60 in *Xenopus* eggs, which thus represent a valuable source of material to explore its function (Fig. 2b).

*Xenopus* Asf1 interacts with both HIRA and p60 in vitro

*Xenopus* GST-Asf1, GST-HIRA and GST-p60, in vitro translated HIRA and Asf1 proteins, and HSE were produced for pull-down assays. A specific interaction was detected between Asf1 and HIRA (Fig. 3a) and GST-p60 was able to pull down Asf1 from an in vitro translation reaction or from HSE (Fig. 3b). These results demonstrate that the identified interactions of human Asf1a with human HIRA (unpublished results; Daganzo et al. 2003; Tagami et al. 2004; Zhang et al. 2005) and human p60 (Mello et al. 2002) are conserved in *Xenopus* egg extracts. Thus, our



**Fig. 4** Depletion of Asf1 leads to co-depletion of HIRA in HSE. **a** 1 and 2  $\mu$ l of mock-, HIRA-, Asf1-, p60-, and p150-depleted HSE analyzed by Western blotting using the indicated antibodies. The asterisk is as in Fig. 2b a potential modified p60 form. **b** Input HSE (10%) and immunoprecipitated proteins with either preimmune serum (*mock*) or HIRA antibody (*HIRA*) analyzed by Western blotting with anti-HIRA and anti-Asf1 antibodies

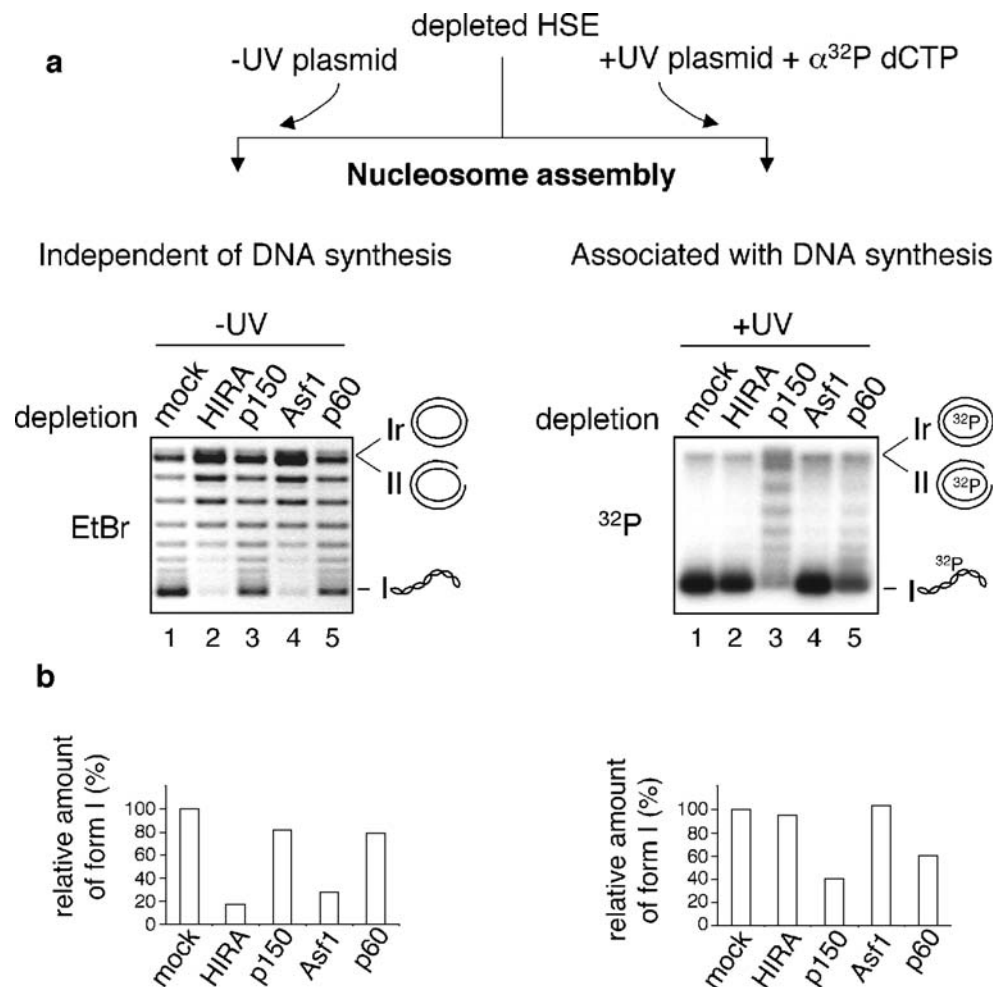
system, which recapitulates known properties of Asf1 in somatic human cells, appears relevant to gain information concerning the role of Asf1 and its relationship with CAF-1 and HIRA in chromatin assembly.

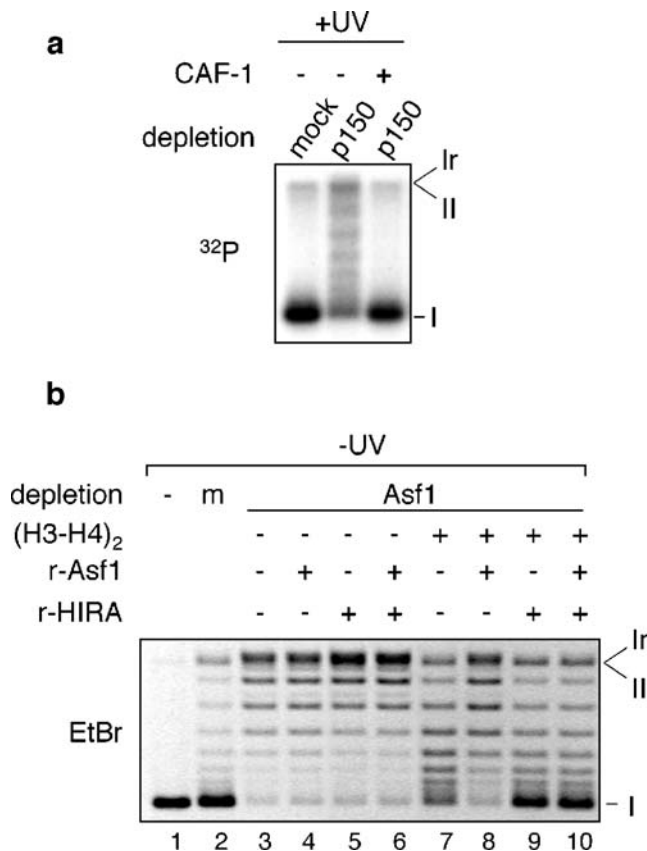
#### Depletion of Asf1 from HSE leads to codepletion of HIRA

To study the role of Asf1 in chromatin assembly, HSE were immunodepleted for Asf1. We then tested the activity of the Asf1-depleted extracts in *in vitro* nucleosome assembly assays. For comparison, immunodepletions of HIRA, p60, and p150 CAF-1 from HSE were carried out in parallel using appropriate antibodies. These depletions were assessed by Western blot analysis as compared to a control HSE (mock-depleted; Fig. 4a). They were specific for the protein of interest and did not affect the amount of proliferating cell nuclear antigen (PCNA) nor histone H3 present in the extract. Although these chaperones are all known to interact with H3, the absence of codepletion of

H3 in the egg can be explained because the vast majority of the large maternal stores of histones H3/H4 are sequestered by the chaperone N1/N2 (Kleinschmidt and Franke 1982; Laskey et al. 1978). Depletion of p60 resulted in a codepletion of p150 and conversely, showing that CAF-1 exists as a stable complex in these extracts. Strikingly, the Asf1 depletion led to an extensive codepletion of the HIRA protein, which compares to the efficiency achieved using antibodies directed against HIRA (Fig. 4a). In the latter case, the use of the HIRA polyclonal antiserum could not deplete Asf1 (Fig. 4a and b), possibly due to the fact that Asf1 is present in excess over HIRA or that binding of the anti-HIRA antibody disrupts the association between HIRA and Asf1. In other species, Asf1 proteins have been found interacting with several partners (Chimura et al. 2002; Emili et al. 2001; Moshkin et al. 2002), arguing that Asf1 likely also exists independently of the HIRA complex. In any case, we can conclude that most of the HIRA proteins are associated with Asf1 proteins as a stable complex in HSE.

**Fig. 5** Depletion of Asf1/HIRA or CAF-1 and nucleosome assembly activity in HSE. **a** Nucleosome assembly reactions, with mock-, HIRA-, Asf1-, p60-, or p150-depleted HSE, were performed using either non-treated plasmid (-UV) to follow the pathway independent of DNA synthesis (*left panel*) or plasmid irradiated with 500J/m<sup>2</sup> UV-C (+UV) in presence of ( $\alpha$ -<sup>32</sup>P)dCTP to examine the pathway coupled to DNA synthesis (*right panel*). After 3 h incubation, DNA was analyzed on agarose gel and visualized by ethidium bromide (EtBr) or by autoradiography (<sup>32</sup>P). Migration positions of DNA plasmid form I (supercoiled), form II (nicked circular) and form Ir (closed circular) are indicated. **b** Graphs provide a quantification of the data (with mock depletion as 100% reference)





**Fig. 6** Rescue of the nucleosome assembly capacities of p150- and Asf1/HIRA-depleted HSE. **a** Nucleosome assembly reactions using UV-treated plasmid (+UV) and either mock- or p150-depleted HSE supplemented or not with human CAF-1 complex were analyzed as in Fig. 5a. **b** Various combinations of recombinant proteins (*r-HIRA* and *r-Asf1*) and (H3-H4)<sub>2</sub> tetramers were added to the nucleosome assembly reaction mix using nontreated plasmid (-UV) and either mock (*m*) or Asf1-depleted HSE and analyzed as in Fig. 5a. Input DNA run in parallel (*lane 1*). Migration positions are as in Fig. 5a

Asf1 depletion, with HIRA codepletion, affects nucleosome assembly independent of DNA synthesis but not coupled to DNA repair

We compared the de novo nucleosome assembly capacities of HSE depleted for Asf1, HIRA, p60, and p150 in both DNA synthesis-coupled and synthesis-uncoupled pathways. To examine nucleosome assembly independent of DNA synthesis we used undamaged plasmid (-UV). In the HSE control (mock-depleted) or depleted for p60 or p150, nucleosome assembly occurred efficiently as revealed by the appearance of supercoiled plasmid (form I) after 3 h incubation (Fig. 5a, -UV: lanes 1, 3, and 5). In contrast, in HIRA- or Asf1-depleted extracts, this DNA synthesis-independent assembly was severely impaired (Fig. 5a, -UV: lanes 2 and 4). Next, to examine nucleosome assembly coupled to DNA synthesis, we performed a similar analysis using UV-irradiated plasmid (+UV). In this system, the repaired DNA is detected by the incorporation of radio-labeled nucleotides ( $\alpha^{32}\text{P}$  dCTP). While the assembly

pathway linked to DNA synthesis was intact in the control, HIRA- or Asf1-depleted HSE (Fig. 5a, +UV: lanes 1, 2, and 4), this assembly was affected in the p150- or p60-depleted HSE (Fig. 5a, +UV: lanes 3 and 5). Quantification of these data is shown on the graphs in Fig. 5b. Thus, in this system, Asf1 appears dispensable for direct de novo histone deposition in the nucleosome assembly pathway coupled to DNA synthesis associated with NER whereas both p60 and p150 are essential. Remarkably, Asf1-depleted extracts codepleted for HIRA were impaired in their ability to assemble nucleosomes independently of DNA synthesis in a manner comparable to extracts depleted of HIRA alone.

Addition of Asf1 cannot rescue the nucleosome assembly capacity of Asf1/HIRA depleted extracts

Rescue experiments of the Asf1/HIRA-depleted extracts showed that neither the addition of Asf1 or HIRA recombinant proteins nor H3/H4 histones added separately is sufficient for recovery of DNA synthesis-independent nucleosome assembly (Fig. 6b, lanes 4, 6, and 8). In contrast, the simple addition of CAF-1 subunits is able to restore nucleosome assembly coupled to DNA repair in p150-depleted HSE (Fig. 6a). However, the combined addition of HIRA along with H3/H4 markedly improved assembly efficiency of Asf1/HIRA-depleted extracts (Fig. 6b, lane 9) as seen for HIRA depleted extracts (Ray-Gallet et al. 2002). Strikingly, a similar combined addition of Asf1 along with H3/H4 has no effect (Fig. 6b, lane 8), and addition of Asf1 with both HIRA and H3/H4 did not improve further the rescue (Fig. 6b compare lanes 9 and 10). Although we cannot totally exclude that the small amount of Asf1 remaining in the Asf1-depleted extracts (Fig. 4a) may contribute in combination with HIRA to the rescue of the nucleosome assembly, our results strongly suggest that Asf1 is not critical for de novo deposition of histones per se in the assembly process independent of DNA synthesis using egg extracts.

## Discussion

To assign to the histone chaperone Asf1 a position in a chromatin assembly line and to relate it to CAF-1 and HIRA functions, we took advantage of the properties of *Xenopus* egg extracts. These extracts can be depleted of a chosen chaperone to evaluate its respective contribution to DNA synthesis-dependent or synthesis-independent de novo nucleosome assembly. To exploit the *Xenopus* system, it was necessary to first clone and identify Asf1 and p60 subunit of CAF-1 in *Xenopus*. Then, using recombinant proteins, we showed that *Xenopus* Asf1 interacts with both p60 and HIRA (Fig. 3). Furthermore, the codepletion of HIRA from



HSE using Asf1 antibodies indicated that the HIRA protein is mostly complexed with Asf1 in egg extracts (Fig. 4a). Although Asf1 and p60 interact in vitro, the depletion of either Asf1 or CAF-1 (p60 or p150) from HSE did not lead to their codepletion suggesting that only a small proportion of the proteins may exist in a stable complex in egg extracts (Fig. 4a). The Asf1/HIRA-depleted HSE remained efficient to assemble nucleosome coupled to DNA synthesis, a pathway impaired in CAF-1 depleted extracts (Fig. 5). In egg extracts, large amount of Asf1 appears therefore dispensable to assemble nucleosomes coupled to DNA repair. This is different from the situation in extracts derived from human somatic cells for which a similar depletion of Asf1 can lead to a severe defect in this nucleosome assembly pathway (Groth et al. 2005). Thus, the replicative pool of histones available for CAF-1 is not as limiting in egg extracts, possibly due to the existence of alternative storage forms, not necessarily mediated by Asf1. Indeed, in *Xenopus* eggs the maternal pool of H3/H4 is associated with the chaperone N1/N2 (Kleinschmidt and Franke 1982; Laskey et al. 1978). In this context, we can therefore hypothesize that N1/N2 is the direct “histone provider” for CAF-1 and that Asf1 is dispensable for this function. In the Asf1/HIRA-depleted HSE, the nucleosome assembly independent of DNA synthesis was severely impaired to an extent comparable to the HIRA depletion (Fig. 5). This observation further supports the importance of HIRA in this pathway, as shown by the rescue obtained by a simple addition of HIRA and histones (Fig. 6b). Importantly, the readdition of Asf1 along with histones H3/H4 failed to restore the assembly activity. Recent studies showed that Asf1 interacts with H3-H4 as dimers (Antczak et al. 2006; English et al. 2006; Natsume et al. 2007). Thus, we cannot exclude that the source of H3-H4 as tetramers that we used in our set up could account for a lack of efficiency in the *Xenopus* system. However, this seems unlikely because a similar set up was used successfully with human cell extracts (Groth et al. 2005; Mello et al. 2002), and recent data showed that Asf1 can disrupt (H3-H4)<sub>2</sub> tetramers to form Asf1-H3-H4 complex (Natsume et al. 2007). From our data, we emphasize that, although Asf1 can promote reconstitution of nucleosomes when using pure components, a general property expected from any “histone chaperone” under such conditions (Green et al. 2005), we show here that this property does not suffice to make it a deposition factor.

Taken together, in the complex system of *Xenopus* egg extracts, the emerging picture is thus that Asf1 is either dispensable or likely functions as a “histone provider” for de novo histone deposition while CAF-1 and HIRA act as deposition factors in an assembly pathway. The specific properties of *Xenopus* egg extracts, which contain large amounts of the proteins needed for the rapid nuclear divisions occurring after fertilization in the absence of

transcription, may explain why Asf1 appears not critically required at this stage. In egg extracts, CAF-1 and HIRA could already be present in complexes ready for histone deposition and in amounts not limiting for the in vitro nucleosome assembly assays (and therefore not depending on the Asf1 “histone provider” function). Alternatively, at this stage, they would function independently of Asf1 with other “histone providers” such as N1/N2. Finally, we should bear in mind that the role of Asf1 may also relate to nucleosome disassembly by disrupting (H3-H4)<sub>2</sub> tetramers. Yet, we could not evaluate this property, using naked DNA as a template for our in vitro de novo assembly assays. Indeed, the recent determination by several groups of the crystal structure of Asf1 bound to histones revealed that Asf1 binds to a heterodimer of histones H3/H4 (Antczak et al. 2006; English et al. 2006; Natsume et al. 2007). This is consistent with previous arguments, which put forward a similar CAF-1 and HIRA interaction with H3/H4 heterodimers (Tagami et al. 2004). The possible disruptive activity of Asf1 is consistent with its reported role in nucleosome disassembly in yeast (Adkins et al. 2004; Adkins and Tyler 2004). It will be important to further evaluate the role of Asf1 in the context of chromatin templates and thus be able to test a combined function to coordinate both disassembly and reassembly events.

Finally, to properly determine the relative importance of Asf1 in comparison with CAF-1 and HIRA, it will be interesting to examine its requirement during development at specific stages. Indeed, a critical role for CAF-1 was demonstrated during the early cleavage stages of *Xenopus* embryogenesis (Quivy et al. 2001) and knock down of HIRA in mouse resulted in embryonic lethality at the gastrulation stage (Roberts et al. 2002). HIRA is involved in the incorporation of the histone variant H3.3 in the male pronucleus before the first round of DNA replication in *Drosophila* (Loppin et al. 2005) and mouse (van der Heijden et al. 2005), an event which appears critical for the embryo development. Determining how Asf1 is required or not during these development steps should help to understand its specific function at the level of an organism at key developmental stages.

In conclusion, our results emphasize that Asf1 is not directly involved in de novo histone deposition in *Xenopus* egg extracts, a role played by CAF-1 and HIRA in DNA synthesis-coupled and synthesis-uncoupled pathways, respectively.

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