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# The Program for Processing Newly-synthesized Histones H3.1 and H4

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## Abstract

The mechanism by which newly synthesized histones are imported into the nucleus and deposited onto replicating chromatin alongside segregating nucleosomal counterparts is poorly understood, yet this program is expected to bear on the putative epigenetic nature of histone posttranslational modifications. In order to define the events by which naïve pre-deposition histones are imported into the nucleus, we biochemically purified and characterized the gamut of histone H3.1-containing complexes from human cytoplasmic fractions and identified their associated histone PTMs. Through reconstitution assays, biophysical analyses, and live cell manipulations, we describe in detail this series of events, namely the assembly of H3-H4 dimers, the acetylation of histones by the HAT1 holoenzyme, and the transfer of histones between chaperones that culminates with their karyopherin-mediated nuclear import. We further demonstrate the high degree of conservation for this pathway between higher and lower eukaryotes.



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**Author Contributions**

E.I.C. performed the tissue culture-related work and biochemical purification of eH3.1, sNASP and ASF1B; protein interaction and enzymatic assays, with assistance from Z.G; sub-cloning and mutagenesis; crosslinking experiments, with the assistance of P.V.; AUC runs, with the assistance of L.D.; and RNAi experiments. J.F. performed the work in S. cerevisiae with assistance from W.H.W.K. and H.S. G.L. cloned sNASP and generated stable sNASP clones. H.Z. performed the mass spectrometry analyses. The manuscript was written by E.I.C. and D.R. with assistance from the other authors.

## Introduction

Canonical nucleosomal histone octamers are formed of a stable $(H3\text{-}H4)_2$ tetrameric core flanked by two relatively labile H2A-H2B dimers[1]. Each histone octamer is enfolded by 147 bp of DNA[2] to compact, organize, and regulate access to the underlying genetic material[3]. There are three major canonical H3 variants in humans, histones H3.1, H3.2, and H3.3[4]. H3.1 and H3.2 differ by a single amino acid substitution (C96S in H3.2), are expressed in S-phase[4] and thus termed replication-dependent. While H3.2 is expressed from a single gene, H3.1 levels predominate as it is expressed from ten genes[5]. H3.3 is replication-independent and expressed at low levels throughout the cell cycle[4].

During DNA replication, pre-existing parental histones segregate onto both leading and lagging strands behind the replication fork[6], co-depositing alongside newly synthesized counterparts. Early biochemical studies determined that *in vitro*, nucleosomes predominantly dissociate into a stable $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers as a function of salt concentration, temperature, and pH[1]. It was also previously reported that during DNA replication, segregating nucleosomal H3-H4 histones are predominately tetrameric, whereas H2A-H2B histones remain as dimers[7, 8]. Furthermore, chromatin is thought to be assembled in a sequential manner, through transitory DNA contacts with a central H3-H4 tetramer followed by the addition of two H2A-H2B dimers to complete the nucleosome particle[9].

Altogether these findings implied a ‘two-step deposition’ model by which pre-existing nucleosomal histones dissociate ahead of the replication fork to re-distribute on both leading and lagging strands as $(H3\text{-}H4)_2$ tetrameric blocks and H2A-H2B dimers. Yet, recent analyses of H3.1 and H3.3 complexes indicated that pre-deposition H3-H4 histone units are handled as dimers *in vivo* since immunoprecipitation of exogenously expressed epitope-tagged histones would not co-precipitate endogenous counterparts[10, 11]. Furthermore, biochemical and crystallographic analyses on the anti-silencing factor 1 (ASF1), a major H3-H4 chaperone, indicated that ASF1 binds exclusively an H3-H4 dimer rather than a tetramer[12, 13, 14]. Since ASF1 co-purifies with subunits of the MCM helicase[15], it was proposed that segregating nucleosomal H3-H4 histones dissociate as dimers[15, 16]. The discrepancy between these recent reports and the earlier ones will only be resolved once the molecular mechanism by which histones are chaperoned and assembled *in vivo* is thoroughly established. The outcome is important since it may dictate the way cells handle histones as potential carriers of epigenetic information.

Little is known regarding the processing of newly synthesized histones. In humans, newly synthesized histone H4 is acetylated on lysines 5 and 12 by the HAT1-RbAp46 holoenzyme[17]. Additionally, mass spectrometric analysis of pre-deposition H3.1 histones showed that over a third of this pool contains lysine 9 monomethylation as the sole H3 posttranslational modification[18]. More recently, the HAT1-RbAp46 holoenzyme and the nuclear autoantigenic sperm protein (NASP) were detected in ASF1 immunoprecipitates from cytosolic fractions[16], although the significance of this finding was not clarified. Once in the nucleus, the PCNA-tethered chromatin assembly factor-1 (CAF-1) is essential for the deposition of H3.1-H4 histones onto chromatin during DNA replication[19, 10]. By interacting

with the p60 subunit of CAF-1 (p105 in *Drosophila*), ASF1 is believed to supply histones to newly replicated or repaired DNA[20, 21].

Here, we report the first comprehensive biochemical purification and characterization of cytosolic H3.1 complexes. With this gamut of species, we were able to detect the ongoing transactions whereby histones are transferred from one chaperone to another. These findings bridge the gaps between previous studies and identify the key steps required for the import of newly synthesized histones.

## Results

### Distinct H3.1 Chaperones in Different Cell Compartments

To ascertain the program whereby naïve pre-deposition histones are handled before incorporation onto chromatin, we purified replication-dependent H3.1 complexes from cytosolic extracts. We employed a HeLa S3 lineage (eH3.1-HeLa)[10] that expresses low levels of the replication-dependent histone H3.1 tagged with FLAG and HA epitopes at its C-terminus (eH3.1), from a constitutive viral promoter. Cells were lyzed and fractionated into cytosolic, nuclear, and chromatin-bound fractions. Silver staining of tandem affinity purified eH3.1 samples followed by mass spectrometry allowed the detection of different eH3.1-associated polypeptides, as a function of their subcellular compartments of origin (Fig. 1a). In the cytosolic fraction (S100), six chaperones associated with eH3.1 (HSC70, HSP90, tNASP, sNASP, RbAp46, and ASF1B), in addition to importin-4, histone H4, and the histone acetyltransferase HAT1, among other polypeptides (Fig. 1a). Although the nuclear fraction (NE) also included most of these proteins, western blot analysis revealed stark differences, such as the expected enrichment in CAF1 (Fig. 1b). Similarly, ASF1 was enriched in the cytosolic fraction, and the MCM7 polypeptide was enriched in the chromatin-bound soluble nuclear pellet (SNP) that was devoid of (or drastically reduced in) ASF1 and HAT1 (Fig. 1b). Therefore, histone H3.1 is differentially chaperoned through these different subcellular compartments.

As per previous reports[10, 11], immunoprecipitates containing the epitope tagged histone H3.1 showed a lack of its endogenous counterpart (Fig. 1c). However, while the exogenous eH3.1 gene is constitutively expressed throughout the cell cycle, most cells are normally found outside S-phase. To assess possible association between exogenously and endogenously expressed H3.1 during S-phase, we synchronized the eH3.1-HeLa cells at the G1/S border and verified the level of histones every hour following their release into S-phase (Fig. 1c and Supplementary Fig. 1a). Little change in histone levels was observed in the cytoplasm, highlighting the importance of soluble histone pools outside S-phase. Importantly, barely detectable levels of endogenous histone H3 co-precipitated with exogenous eH3.1 in S-phase, even though endogenous H3 levels surpassed those of the exogenous protein (Fig. 1c). This result corroborates that histones H3-H4 exist as dimeric rather than tetrameric species in the cytoplasm *in vivo*.

## Four Distinct H3.1 Complexes in the Cytoplasm

To evaluate the significance of the proteins associated with cytoplasmic histone H3.1, affinity purified cytoplasmic eH3.1 was first applied to a Superdex 200 gel filtration column. Although all proteins closely eluted, the HAT1 and RbAp46 protein peak was separated by two fractions from the importin-4 protein peak (data not shown). The mass of all proteins combined surpassed that at which they eluted (~200 kDa) suggesting that the eH3.1 immunoprecipitate consists of more than one core complex. We therefore proceeded to further partition the affinity purified eH3.1 material as depicted in Figure 2a.

None of the affinity-purified histones bound to a Mono S column (not shown), although pure histones are expected to avidly bind cation exchange resins[22]. Indeed, eH3.1-containing histone octamers purified to homogeneity from this HeLa lineage tightly bound the cation exchange resin Mono S (not shown), whereas eH3.1 complexes solely bound the anion exchange resin Mono Q (Fig. 2b). This suggested that cellular histones are not present in isolation, but instead are complexed with other proteins that neutralize their positive charge *in vivo*.

Before proceeding to a large-scale purification, we assessed the effect of cell cycle stage on the assembly and import of cytoplasmic H3.1 histones. To this end, we compared the profile of eH3.1 species obtained after fractionating cells in early or late S-phase (Supplementary Fig. 1b), to that observed in an asynchronous state. No differences were observed among the three cell populations, suggesting a main pathway for the processing and import of newly synthesized histones (compare Fig. 2b with Supplementary Fig. 1b).

Our large-scale purification of cytoplasmic H3.1 complexes revealed four main eH3.1 complexes eluting from the Mono Q column (Fig. 2b). These were numbered (I–IV) to reflect the likely order in the sequence of events during H3.1 transfer, as detailed throughout the text. Fractions were analyzed for suspected enzymatic activity (Fig. 2c) and further purified by gel filtration (Fig. 2e–g).

Fractions 10–16 that eluted at low salt concentrations (170–205 mM KCl) contained the least abundant complex (I) (Fig. 2b), comprising the heat-shock cognate 70 protein (HSC70)

pathway given that the sole detectable PTM was lysine 9 monomethylation, while other normally abundant PTMs (such as trimethylation of lysines 4, 9, or 27) were undetectable by western (not shown). The purified histones migrated as a single sharp band by SDS-PAGE and ubiquitinated species were not detected (not shown).

The Mono Q complex that was next in abundance (II) contained HSP90, tNASP, histone H4, and histone eH3.1 (Fig. 2b). The human *NASP* gene encodes a full-length transcript that is highly expressed in testes and thus termed ‘testicular’ NASP (tNASP), as well as a splicing variant termed ‘somatic’ NASP (sNASP)[24]. The tNASP-HSP90 complex (fractions 46–48) shoulders a much more abundant complex (Complex III -fractions 36–42) containing the sNASP variant. To ascertain the interactions between these proteins, fractions 46-48 were

of a core complex composed of the HAT1 holoenzyme and histones H3-H4 that closely interact with ASF1B.

As expected, the Mono Q fractions containing the cytoplasmic sNASP-HAT1 complex exhibited robust acetyltransferase activity towards histone H4, predominately at lysines 5 and 12 (Fig. 2c). Of note, western analysis revealed a sharp reduction in the levels of H3 lysine 9 monomethylation compared to those within Complexes I and II (Fig. 2b). Mass spectrometric analysis of the histones that co-eluted with Complex III confirmed the overwhelming acetylation of histone H4 and trace amounts of other PTMs (Supplementary Tables 1 and 2). Based on this, we considered Complex III to be the third step in histone H3 processing.

## ASF1 Associates with Karyopherins

The last cytoplasmic H3.1 complex (IV) is the one responsible for the nuclear import of the H3.1-H4 histones as it contained the ASF1B chaperone and the importin-4 protein (Fig. 2b, fractions 20–26). Three complexes of identical composition eluted from the Mono Q column, each composed of high levels of ASF1B and importin-4 proteins along with the two histones, and each exhibiting perfect co-elution of these components upon subsequent gel filtration (Fig. 2g; Supplementary Fig. 2b); slight shifts in molecular weights again suggests changes in stoichiometry or posttranslational modifications. Residual peptides from Complex III were sometimes detected, again suggesting a close interaction between the two core complexes. Interestingly, ASF1A was not detected in these fractions by either western blotting or mass spectrometry (not shown).

As in the case of sNASP, stable HEK293F clones expressing FLAG-tagged ASF1B were generated. In agreement with the results above, most of the ASF1B protein eluted with importin-4 and histones H3 and H4 (Supplementary Fig. 3b, fractions 20–22), whereas a less abundant fraction of ASF1B eluted with sNASP, RbAp46, and HAT1 (fractions 26–28). Interestingly, a slower migrating ASF1B species was observed. Mass spectrometric analysis confirmed that the upper band was ASF1B, not ASF1A (not shown). ASF1B

interaction between sNASP and the HAT1 holoenzyme (Fig. 3a). Recombinant RbAp46 (without HAT1) also co-precipitated with sNASP only in the presence of histones H3-H4

The 2:2:2 species might arise from two sNASP molecules each interacting with two H3-H4 dimers or, alternatively, two sNASP molecules binding an $(H3\text{-}H4)_2$ tetramer. To differentiate between these two possibilities, we repeated the experiments under conditions non-conducive to tetrameric $(H3\text{-}H4)_2$ formation. Five residues are predominantly involved in the H3-H3' interface responsible for the formation of $(H3\text{-}H4)_2$ tetramers[2]. Interaction through the H113 residue involves hydrogen bonds and its substitution with alanine reduces the tetrameric pool[29]. We further mutated combinations of C110, L126, and I130 to aspartate to abolish hydrophobic interactions. The H3H113A/C110D/I130D combination abolished the formation of H3-H4 tetramers (Fig. 4a), with weak tetramer formation being visible only under conditions of extensive crosslinking at salt concentrations of 400 mM (Supplementary Fig. 5c–d). In spite of this, these obligate H3-H4 dimers (200 mM NaCl, 100 molar excess BS[3]) formed the same 1:1:1 and 2:2:2 species as wt histones when increasing amounts of sNASP were added to a fixed amount of histones (Fig. 4b). The addition of increasing amounts of obligate histone dimers onto a fixed concentration of sNASP yielded the same result (Supplementary Fig. 5e). These findings strongly suggest that sNASP dimerizes and forms a complex with two histone H3-H4 dimers.

To corroborate this interpretation, sedimentation equilibrium (SE) analyses were performed by analytical ultracentrifugation (Fig. 4c). For this purpose, we isolated the peak fractions after gel filtration chromatography of HIS-tagged sNASP and $(H3\text{-}H4)_2$, as well as pre-mixed HIS-sNASP + wt H3-H4 at 1:1, 1:2 or 2:1 molar ratios. We considered several

### Assembling Naïve H3-H4 Dimers

To prevent misfolding and aggregation, various protein chaperones congregate near the ribosomal polypeptide exit site[36]. Although they do not directly bind ribosomes, the Heat-shock Protein 70 family of proteins (of which HSC70 is the major human cytosolic member) are recruited to these sites to adjust hydrophobic segments within a large number of client proteins through binding and release cycles accelerated by ATP hydrolysis[36]. Upon release

### Enzymatic Activity Assays

An aliquot of each purified fraction was diluted ten fold and one microliter mixed in 25 µl HAT buffer [10 mM Tris, pH 7.6, 250 mM NaCl, 10% Glycerol (v/v), 0.5 mM EDTA, and 1

and 18100 × *g*. All analyses were performed at 4°C. Data was analyzed using the Sedfit and Sedphat softwares[30].

### RNAi

Cells were transfected using the RNAiMAX reagent (Invitrogen) using 30 μM pooled siRNA oligos (Dharmacon) for 72 hrs. Importin-4 target sequences: GCAUUUCGCUGUACAAGUU, AGUCAGAGGUGCCGGUCAU, CCUCGCAAGUUGUACGCAA, AUGGAGCACCUGCGGGAAU. ASF1B target sequences: GCAGGGAGACACAUGUUUG, AGUGGAAGAUCAUUUAUGU, GACCUGGAGUGGAAGAUCA, CGGACGACCUGGAGUGGAA. NASP target sequences: GGAAAUCACUUCUGGAGUU, GGAAGCAGCUAGUCUUUUA, CCGAAGAAAUGCCAAAUGA, GGAACUGCUACCCGAAAUU. HAT1 target sequences: GCACAAACACGAAUGAUUU, GAAGAUUACCGGCGUGUUA, GCUACAGACUGGAUAUUAA, CUAAUUAGCCCAUAUAAGA.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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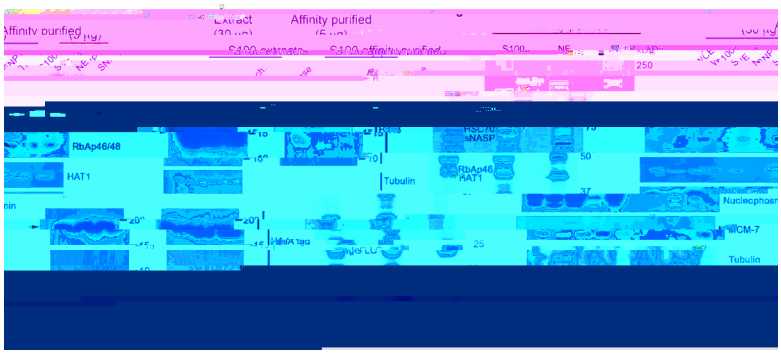


**Figure 1.**

Subcellular distribution of H3.1-containing complexes. HeLa S3 cells expressing a FLAG-HA tagged histone H3.1 (eH3.1) were fractionated into cytoplasmic (S100), nuclear (NE), and chromatin-bound soluble nuclear pellet (SNP) fractions. (a) Extracts were tandem-affinity purified and resolved by SDS-PAGE for silver staining. (b) Western analysis comparing crude whole cell extracts (WCE), S100, NE, SNP, and total nuclear pellets (TNP) with matching affinity-purified material. (c) Western analysis of endogenous H3.1 co-purifying with exogenous eH3.1 at different cell cycle stages. Arrows mark eH3.1 histones whereas the arrowhead indicates native histone H3.

**Figure 2.**

Purification of eH3.1 from cytosolic extracts. (a) Purification scheme. (b) Anion exchange chromatography (Mono Q) of affinity-purified cytoplasmic eH3.1 protein complexes. Numbers at the bottom indicate the concentration of potassium chloride (mM) at which peak fractions eluted. Cytoplasmic fractions contain four predominant H3.1 complexes (I–IV). (c) Histone acetyltransferase assay using purified histones, the Mono Q fractions, and tritiated acetyl-CoA as substrate. Top panel, autoradiograph. Bottom panel, Coomassie Blue-stained histone substrates. All HAT activity was specific for histone H4. Note that these fraction numbers are displaced by one (odd numbers), relative to those used for the silver stain and western analyses (even numbers). (d) Endogenous HSC70 co-precipitates with endogenous histone H3. Size exclusion chromatography (S200) of the eH3.1 peaks eluting at (e) 380 mM, (f) 330 mM, and (g) 265 mM KCl from the Mono Q column.

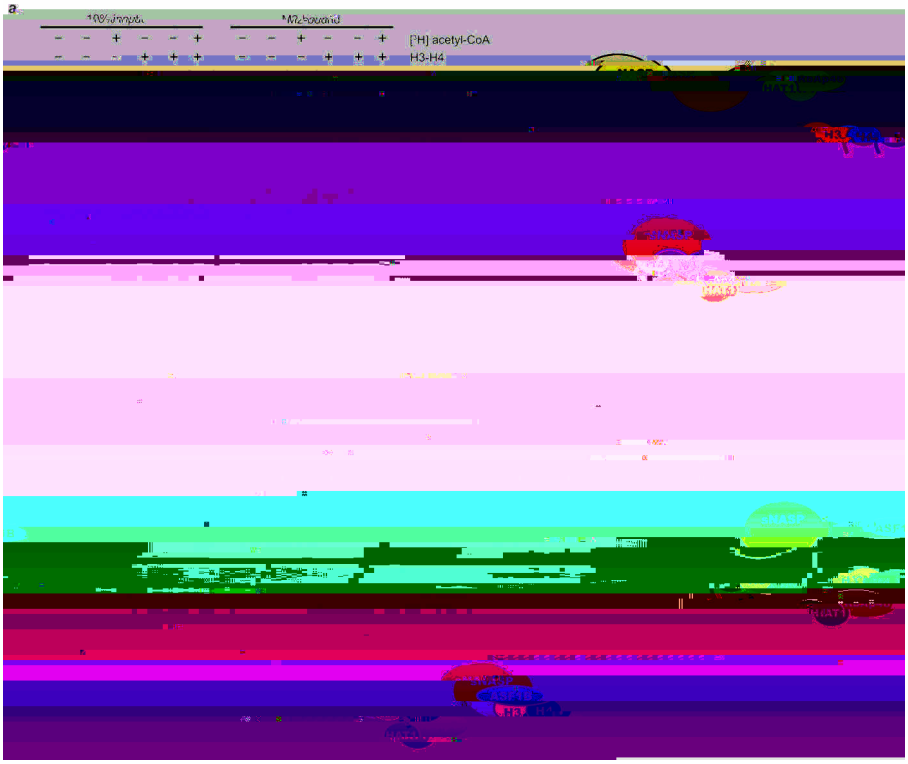


**Figure 3.**

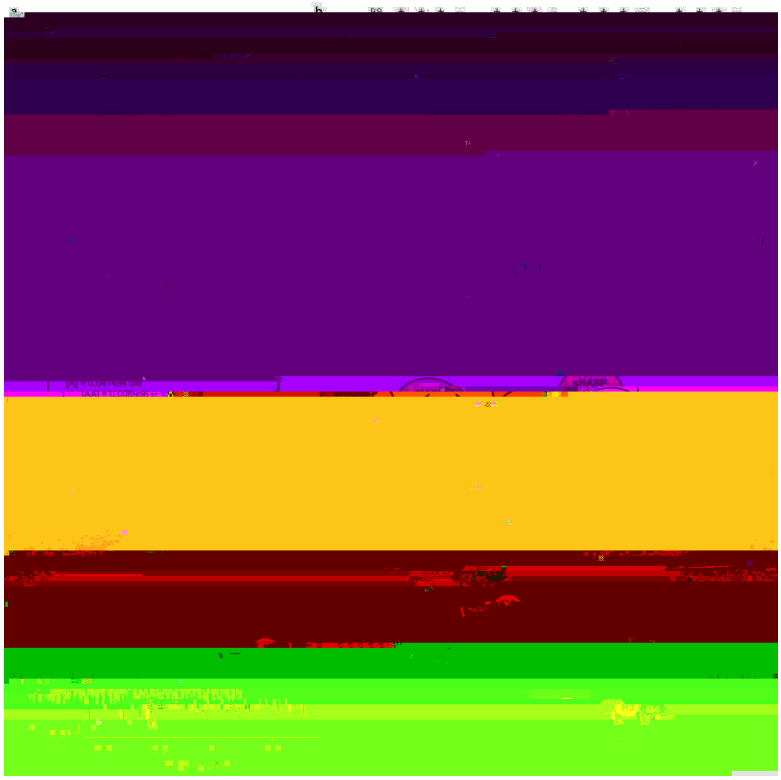

**Figure 4.**

sNASP homodimerizes but binds H3-H4 heterodimers. (a) Chemical crosslinking of recombinant histones and sNASP or (b) sNASP pre-incubated with H3-H4 at different molar ratios. Crosslinking was performed using wt histones or H3 bearing the indicated point mutations. (c) Sedimentation equilibrium analysis of the complexes between H3-H4 and sNASP. Shown are individual absorbance values at 276 nm recorded at multiple radial positions after equilibrium was reached at 8 krpm (blue), 12 krpm (yellow), and 15 krpm (red). The fitted curves (top graph) are from a single simultaneous fitting of three independent samples at the three speeds. Based on the fixed molecular weights for the postulated species, the data were fitted to the following interaction scheme: (A+A)+B +B ⇔ A+AB+B ⇔ (AA)B+B ⇔ AABB, where A = H3-H4, (AA) is a dimer of A and (A+A) represents self-association, B is the sNASP monomer, AB is the 1:1:1 complex of sNASP:H3:H4, and AABB is its 2:2:2 complex. Bottom graph shows the mean root square deviations.

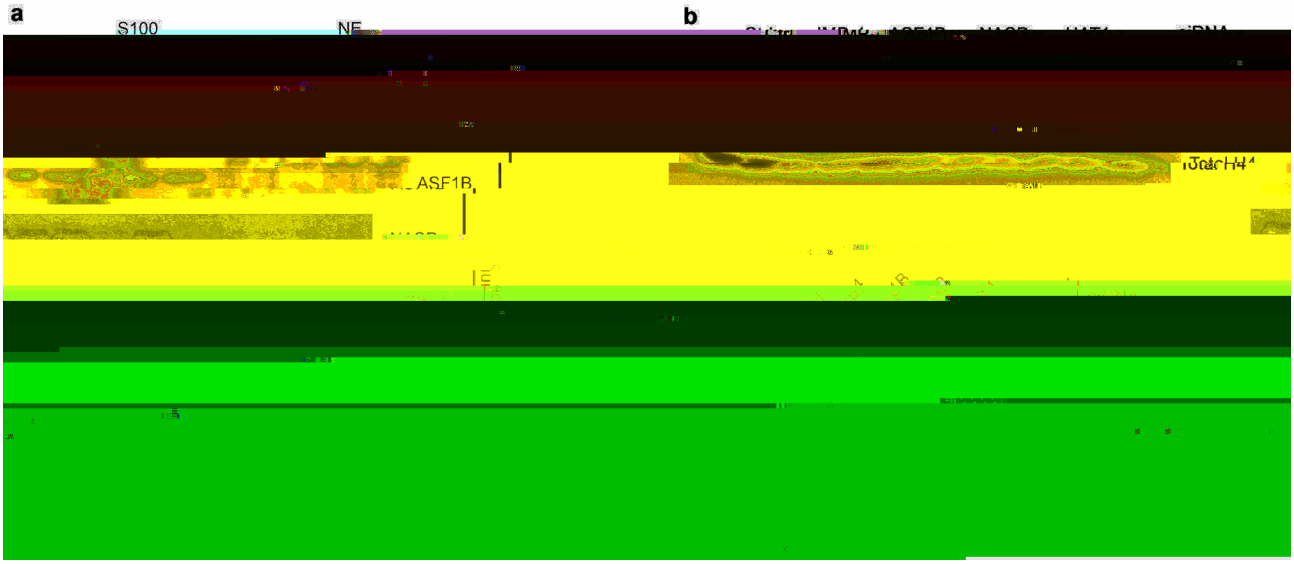


**Figure 5.**

Importin-4 and ASF1B function downstream of s/tNASP and HAT1 *in vivo*. (a) Western analysis of H4K12ac levels in cells transfected with siRNA oligos targeting importin-4, ASF1B, NASP (both isoforms), and HAT1. (b) *In vitro* acetyltransferase activity towards recombinant histones using extracts from cells treated with RNAi against the proteins indicated on top. (c) Immunoprecipitation of histone eH3.1 from cytosolic extracts of RNAi-treated cells.


**Figure 6.**

Conservation of the H3-H4 pre-deposition pathway in *S. cerevisiae*. (a) TAP-tagged Hif1 was purified from strains wherein different pre-deposition components were deleted as indicated on top, resolved by SDS-PAGE and silver stained (left panel) or analyzed by western (right panel). (b) TAP-tagged Asf1 was purified from strains lacking Hif1, Hat1, or Hat2 and analyzed by western. (c) Histone dependency for Asf1-Hif1 interactions using TAP-tagged versions of wt Asf1 versus the Asf1 mutant V94R.

**Figure 7.**

Model for nuclear import of pre-deposition replication-dependent histones. In humans, H3.1 folding is first assisted by the HSC70 chaperone at the ribosomal exit. H3.1 is transferred to HSP90 that, along with the tNASP co-chaperone, assembles it into H3-H4 units. The sNASP chaperone binds H3.1-H4 heterodimers and presents the H4 carboxyl domain to RbAp46 that recruits HAT1 activity. After acetylation of histone H4, the complex is stabilized and the histones transferred to ASF1B. ASF1B associates with importin-4 and the histones are then transported into the nucleus. In budding yeast, Hif1 associates with the H3-H4 histones and the Hat1-Hat2 holoenzyme. The transfer of acetylated histones to Asf1 is mediated through histones.