Unveiling novel interactions of histone chaperone Asf1 linked to TREX-2 factors Sus1 and Thp1

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Keywords: (5-10) yAsf1, TREX-2, Sus1, histones, TAP-MS strategy, SAGA, yeast

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; NPC, nuclear pore complex; PTM, post-translational modification; TAP, tandem affinity purification; WCE, whole cell extract; WT, wild-type

Anti-silencing function 1 (Asf1) is a conserved key eukaryotic histone H3/H4 chaperone that participates in a variety of DNA and chromatin-related processes. These include the assembly and disassembly of histones H3 and H4 from chromatin during replication, transcription, and DNA repair. In addition, Asf1 is required for H3K56 acetylation activity dependent on histone acetyltransferase Rtt109. Thus, Asf1 impacts on many aspects of DNA metabolism. To gain insights into the functional links of Asf1 with other cellular machineries, we employed mass spectrometry coupled to tandem affinity purification (TAP) to investigate novel physical interactions of Asf1. Under different TAP-MS analysis conditions, we describe a new repertoire of Asf1 physical interactions and novel Asf1 post-translational modifications as ubiquitination, methylation and acetylation that open up new ways to regulate Asf1 functions. Asf1 co-purifies with several subunits of the TREX-2, SAGA complexes, and with nucleoporins Nup2, Nup60, and Nup57, which are all involved in transcription coupled to mRNA export in eukaryotes. Reciprocally, Thp1 and Sus1 interact with Asf1. Albeit mRNA export and GAL1 transcription are not affected in asf1Δ a strong genetic interaction exists between ASF1 and SUS1. Notably, supporting a functional link between Asf1 and TREX-2, both Sus1 and Thp1 affect the levels of Asf1-dependent histone H3K56 acetylation and histone H3 and H4 incorporation onto chromatin. Additionally, we provide evidence for a role of Asf1 in histone H2B ubiquitination. This work proposes a functional link between Asf1 and TREX-2 components in histone metabolism at the vicinity of the nuclear pore complex.

Introduction

Chromatin comprises a repeated array of nucleosome core particles of the 147 bp DNA wound 1.7 times around the outside of a core histone octamer, which includes two molecules of histones H2A, H2B, H3, and H4. Hundreds of proteins regulate the dynamic transitions in the chromatin structure, including histone chaperones, non-histone DNA-binding proteins, ATP-dependent chromatin remodelling complexes, and post-translational modification enzymes. Anti-silencing function 1 (Asf1), identified as a single protein in yeast, is a highly conserved chaperone of histones H3/H4 that assembles or disassembles chromatin during replication, transcription and repair. Asf1 is the only histone chaperone to be implicated in both replication-dependent and replication-independent chromatin assembly by assisting the CAF-1 or the HIR complexes, respectively. The main part of non-chromatin associated histones is bound to Asf1, which underscores its fundamental role as a central histone chaperone in eukaryotes. The Asf1-H3/H4 structure suggests a “strandcapture” mechanism whereby the H4 tail acts as a lever to facilitate chromatin disassembly and/or assembly, which may be used ubiquitously by histone chaperones. In addition to binding histones, Asf1 also binds to Rad53, but not to both simultaneously. Asf1 plays a role in monitoring replication and chromatin assembly precisely through an interaction with this checkpoint kinase Rad53. It has been shown that Asf1 is required for H3 lysine 56 acetylation catalyzed by Rtt109, a modification associated with S-phase in mitosis and meiosis, and that it also contributes to DNA replication by presenting dimer H3/H4 to the Rtt109-Vps75 complex and by forming a ternary complex although both proteins, Asf1, and Rtt109, seems to interact only weakly. This H3K56 acetylation also promotes chromatin disassembly during transcriptional activation and is required for RNA polymerase

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Submitted: 01/24/2014; Revised: 05/07/2014; Accepted: 05/07/2014; Published Online: 05/13/2014
http://dx.doi.org/10.4161/nucl.29155

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II transcription elongation through heterochromatin. The absence of this histone modification has been associated with extreme sensitivity to genotoxic agents. Trimethylation of H3K36 catalyzed by Set2, also depends on Asf1 binding to H3/H4. In addition to its interaction with Rad53, Asf1, and the protein components of the HIR complex (Hir1, Hir2, Hir3, and Hpc2) exist as a complex in vivo. As cited above, Asf1 also interacts with other factors to carry out several cellular functions.

In this study, mass spectrometry-based proteomics has been used as a powerful technique to investigate more deeply the repertoire of Asf1’s protein interactions. We find novel Asf1 co-purifying proteins that functionally connect Asf1 to components of the molecular machineries working at the interface of transcription and mRNA export. These include components of the transcription regulatory complex SAGA (Spt-Ada-Gcn5 Acetyltransferase), subunits of the TREX-2 complex (TRanscription and EXport-2), and several nucleoporins.

**Results**

Asf1 co-purifies with factors involved in distinct gene expression steps

Asf1 is a histone chaperone that interacts with histones H3 and H4 and with several other chromatin-associated proteins, including the components of the HIR complex, Bdf1 and checkpoint kinase Rad53. A complete list of Asf1 interactors is found in the BIOGRID database (BioGRID: http://thebiogrid.org). In all, 170 physical interactions have been proposed through different technical approaches. To further explore the repertoire of Asf1 physical interactions, Asf1 was TAP-tagged and purified. Figure 1A shows the proteins that co-purify with Asf1 resolved by a Tricine-SDS-polyacrylamide gel; Figure S1 shows a control of a TAP purification of a strain lacking the TAP-tag ensuring the specificity of the co-purifying bands. From Figure 1A, the most prominent band and a second band running just below were independently identified as Asf1 by an

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**Figure 1.** Novel Asf1 interactors revealed by TAP-Mass spectrometric analysis. (A) Proteins co-purifying with Asf1-TAP in yeast cells extract obtained with Buffer A, resolved by 12% Tricine-SDS. Indicated protein bands and gel fragments were excised and analyzed. Each band labeled as Asf1-CBP(*) or Asf1-CBP were cut and independently analyzed. (B) List of proteins identified in gel slices 1 and 2. Novel interactors of Asf1 are indicated in bold. The total number of peptides found for each protein is listed. (C) List of PTMs identified from Asf1-CBP(*) band. The specific modification and residues are indicated.
LC-MS/MS analysis after extraction from the gel. In order to find out Asf1 co-purifying proteins, the gel seen in Figure 1A was cut into two slices and was MS-analyzed. Figure 1B provides the list of the most representative Asf1 co-purifying proteins and the number of peptides found in each case. Apart from the known Asf1 interactors (Rad53, HiR subunits, and histones), several peptides corresponding to distinct factors involved in different gene expression steps were found. Among them, the analyses identified some previously unknown Asf1 interactors (labeled in bold), such as: Taf12 (a subunit of the transcription regulatory complex SAGA (Spt-Ada-Gcn5 Acetyltransferase), Tfg2 (transcription factor G involved in transcription initiation and elongation), Iws1 (a protein involved in RNA polymerase II transcription), Ubp3 (the ubiquitin-specific protease involved in transcription elongation), and nucleoporins Nup60 and Nup2, which are components of the nuclear pore complex (NPC). In conclusion, Asf1 co-purifies with factors related to transcription and some nucleoporins.

Novel post-translational modifications of Asf1 revealed by mass spectrometry

As shown in Figure 1A, Asf1-TAP is resolved in two bands both with an apparent size different to the expected accordingly to the estimated molecular mass of the fusion protein. It has been reported that Asf1 is modified by multiple phosphorylations in several residues as S264, T265, S269, and T270 (http://thebiogrid.org). To explore whether Asf1 could be subjected to other post-translational modifications (PTMs) which could explain this migration pattern, each band corresponding to Asf1 from the gel shown in Figure 1A was analyzed independently using the Paragon Algorithm of the Protein Pilot Software (Protein Pilot software, AB Sciex). Our analyses showed that the N-terminal methionine was eliminated and the resulting serine residue at position 1 was acetylated. However, we maintained the standard Asf1 amino acids positions nomenclature to avoid confusion with previous literature. Several novel PTMs were identified by the analysis of the corresponding spectra. These analyses revealed that the upper band from the gel shown in Figure 1A (Asf1-CBP*), contains Asf1 modified by ubiquitination on lysine 143 and lysines 71 and 259 (Fig. 1C; Fig. S2). Moreover, we also identified novel methylations at positions R123, K143, and K259 (Fig. 1C; Fig. S2). These spectra also showed that K259 was acetylated. In relation to the phosphorylation sites, we found that, in addition to the already described sites S264 and S269, serine at position 98 was phosphorylated in Asf1 upper band. The Asf1 band migrating lower (Asf1-CBP from Fig. 1A) was also analyzed and trimethylation in lysine K272 was identified (Fig. S2). In summary, we found novel PTMs in Asf1 that might be relevant for its regulation.

Asf1 interacts with SAGA and TREX-2 factors

The above-presented data indicate that Asf1 co-purifies with a SAGA subunit (Taf12) and several nucleoporins. The transcriptional coactivator SAGA interacts with the NPC through its association with TREX-2, a five subunit complex that is involved in transcription coupled to mRNA export. Moreover, nucleoporin Nup60, is a component of the NPC that is functionally connected to SAGA and TREX-2 since they are all required for the gene gating of GAL1 locus. Interestingly, experimental evidences support a functional link between Asf1 and the SAGA complex. However, no physical interaction between Asf1 and the SAGA or TREX-2 components has been demonstrated to date. To further assess whether Asf1 interacts with other SAGA and NPC components, we prepared new extracts from cells expressing Asf1-TAP using an alternative extraction buffer: Buffer W (see M&M for details). This buffer was previously used to increase the chances of finding interactors that are chromatin dependent. LC-MS/MS analysis was performed by creating an exclusion list with all the peptides identified with a confidence higher than 95%. The application of exclusion lists in LC-MS/MS analysis significantly enhances the sensitivity and resolution of label-free protein identification. In this analysis, the protein sequence coverage, its score, and the emPAI score were obtained. A TAP purification of a non-TAP-tagged strain was used as a negative control to show specificity of the interactions (Table S1). Although the Coomassie

| Complex | Protein | Protein Sequence Coverage (%) | MASCOT Protein score | emPAI score |
|---------|---------|-------------------------------|---------------------|-------------|
| SAGA    | Tra1    | 52.62                         | 1621                | 0.29        |
|         | Spt7    | 46.40                         | 602                 | 0.29        |
|         | Taf5    | 54.90                         | 594                 | 0.49        |
|         | Sgf73   | 51.30                         | 556                 | 0.49        |
|         | Ada3/Ngg1| 48.29                        | 395                 | 0.44        |
|         | Gcn5    | 46.01                         | 351                 | 0.55        |
|         | Ada2    | 32.95                         | 342                 | 0.46        |
|         | Ubp8    | 53.93                         | 326                 | 0.43        |
|         | Ada1/Hlf1| 37.09                        | 313                 | 0.51        |
|         | Sus1    | 93.75                         | 307                 | 7.71        |
|         | Taf12   | 45.64                         | 279                 | 0.23        |
|         | Spt3    | 62.02                         | 278                 | 0.51        |
|         | Taf6    | 35.08                         | 263                 | 0.32        |
|         | Spt20   | 58.11                         | 254                 | 0.33        |
|         | Sgf29   | 43.63                         | 221                 | 0.54        |
|         | Taf9    | 59.87                         | 191                 | 1.04        |
|         | Taf10   | 48.06                         | 164                 | 0.31        |
|         | Sgf11   | 77.78                         | 107                 | 0.70        |
| TREX-2  | Sac3    | 66.49                         | 1033                | 0.61        |
|         | Thp1    | 72.09                         | 611                 | 1.34        |
|         | Cdc31   | 48.45                         | 362                 | 1.70        |
|         | Sus1    | 93.75                         | 307                 | 7.71        |

List of proteins co-purifying with Asf1-TAP using Buffer W in TAP purifications. The proteins have been ordered by the MASCOT protein score. The exponentially modified protein abundance index (emPAI) obtained by MASCOT is also shown. It offers an approximate, label-free, relative quantification of the proteins present in a mixture.
Table 2. Proteins co-purifying with Asf1-TAP identified by Triple-TOF Mass spectrometric analysis

| Protein      | Protein Sequence Coverage (%) | Number of Matched Peptides | PTM                        |
|--------------|-------------------------------|----------------------------|----------------------------|
| Asf1         | 68.5                          | 113                        | S269ph K272me             |
| H4/HHF1      | 86.4                          | 26                         | K12ac K16ac               |
| Rad53        | 35.4                          | 22                         | M1ac                      |
| H3/HHT1      | 66.9                          | 29                         | K23ac K36me K56ac K79me K79me |
| Ubp3         | 25.4                          | 17                         |                           |
| Iws1         | 54.4                          | 15                         | S1ac                      |
| Yra1         | 49.1                          | 12                         | S1ac                      |
| Nup2         | 14.6                          | 11                         |                           |
| H2B1/HB1     | 38.2                          | 9                          |                           |
| Rtt103       | 25.4                          | 8                          |                           |
| Spt5         | 6.6                           | 6                          |                           |
| Taf12        | 10.8                          | 5                          |                           |
| Nup57        | 12.9                          | 5                          |                           |
| Nup60        | 12.4                          | 4                          |                           |
| H2A2/HTA2    | 34.9                          | 3                          |                           |
| Hir2         | 4.1                           | 3                          |                           |
| Smt3         | 24.8                          | 3                          |                           |
| Rtt109       | 7.8                           | 2                          |                           |
| Hir1         | 3.6                           | 2                          |                           |
| Bre5         | 6.8                           | 2                          |                           |
| Hpr1         | 3.6                           | 2                          |                           |
| Spt2         | 6.0                           | 2                          |                           |
| Sir4         | 0.6                           | 1                          |                           |
| Spt4         | 18.6                          | 1                          |                           |
| H2A2/HTZ1    | 6.7                           | 1                          |                           |

List of proteins co-purifying with Asf1-TAP using Buffer W. The list shows the most representative proteins of a total of 343 and they are ordered according to the number of matched peptides. The novel interactors are indicated in bold. Post-translational modifications (PTMs) at ≥95% (ph, phosphorylation; ac, acetylation; me, monomethylation; me, trimethylation) identified in Asf1, H4, Rad53, H3, Iws1, and Yra1 are shown.

stained-protein pattern of the Asf1-TAP eluate obtained using Buffer W resembles the one observed in Figure 1A (data not shown). LC-MS/MS analysis identified not only Rad53, HIR and histones, but also peptides that match all proteins corresponding to the SAGA complex and, TREX-2 components Sus1, Sac3, Thp1, and Cdc31 (Table 1). Notably Sus1, a protein shared by the SAGA and TREX-2 complexes, is one of the best represented (93.75% protein sequence coverage and an emPAI score of 7.71). A second LC-MS/MS analysis of Asf1-TAP co-purifying proteins was performed with 5600 Triple TOF without using an exclusion list. The total number of identified proteins rose to 343 proteins (Table S2). Together with Asf1, the most abundant proteins were Rad53 and histones H3 and H4 (Table 2). Other well-represented proteins were components of the HIR complex (Hir1, Hir2), subunits of the THO-TREX complex (Yra1, Hpr1) and Ubp3 (an ubiquitin-specific protease that interacts with Br5, also identified herein). Taf12, a SAGA component, was also identified by the MS-analysis. As expected from our data in Figure 1B, Asf1 co-enriched nucleoporins Nup2, Nup57, and also Nup60, which are components of the NPC and are functionally linked to TREX-2. Two peptides matching Rtt109, histone acetyltransferase (HAT) which acetylates H3 at position 56 depending on Asf1, were also identified. Interestingly, unlike other MS data, in this analysis we did not found the remaining components of the HIR complex (Hir3 and Hpc2). Our results extend the landscape of knowledge on Asf1 protein interactions and suggest that Asf1 is transiently associated with the functionally related protein complexes, such as SAGA, TREX-2, and also with some components of the NPC, as illustrated in Figure 2 by the STRING representation.

TREX-2 subunits interact physically and genetically with Asf1

While some previous reports suggest functional links between Asf1 and SAGA, the physical interaction Asf1-TREX-2 was unexpected. Thus, we decided to investigate this further. To this end, Asf1 was TAP-purified and the presence of Sus1 was analyzed by western blot using anti-Sus1 specific antibodies. As shown in Figure 3A, Sus1 protein was immunodetected from Asf1-TAP and Tafl9-TAP purifications in contrast to the negative controls (Asf1-TAPsus1Δ and a non-tagged strain). These experiments confirm the mass spectrometry results showing a specific physical interaction between Asf1 and Sus1. Conversely, Asf1 was also specifically detectable by western blot in a Sus1-TAP purification, but not in a Sus1-TAPasf1Δ when an anti-Asf1 antibody was employed (Fig. 3B). To follow up the interaction between Asf1 and TREX-2, we decided to test the physical association with another TREX-2 complex subunit. Therefore, Thp1-TAP was affinity-purified and its interacting partners were investigated by mass spectrometry. As expected, the analysis of Thp1-TAP co-purifying proteins by LC-MS/MS identified all the TREX-2 subunits (Fig. 3C). Notably, this analysis also found peptides matching Asf1, Rad53, histones H4 and H2A, and the Sas5 protein. In conclusion, TREX-2 components Sus1 and Thp1 co-purify with Asf1 and some Asf1-interacting proteins such as Rad53 and Sas5. Thus, we infer that Asf1 interacts physically with TREX-2 components in vivo, probably in a transient way.

The factors functioning in related pathways are usually genetically connected. To check whether Asf1 interacts genetically with SUS1, a double mutant bearing deletions of both SUS1 and ASF1 was created. As seen in Figure 3D, the double deletion of both genes conferred significant growth impairment when compared with each single mutant. Since these interactions can be indicative of a functional link, we wondered whether Asf1 is required for mRNA export, similarly to mutants on TREX-2 factors. Therefore, an analysis of the export of bulk poly(A)⁺
mRNA in asf1Δ was performed and compared with a sus1Δ used as a positive control. The results show that Asf1 is dispensable for mRNA export under the conditions tested in this study (Fig. 3E).

Asf1 is dispensable for Sus1 recruitment to GAL1 gene and its induction

Asf1 interacts with some SAGA-regulated genes, such as GAL1, and it travels with the elongating RNA Pol II. Therefore, we decided to investigate the impact of Asf1 on the association of Sus1 with GAL1. Chromatin immunoprecipitations (ChIP) were performed using a strain expressing TAP-tagged Sus1 in wild-type and asf1Δ cells to monitor the recruitment of Sus1 to the GAL1 gene. When comparing recruitment of Sus1 to the different GAL1 regions studied (promoter and the gene body), no differences were observed between wild-type and asf1Δ cells (Fig. 4A). Consequently, this correlates with a normal GAL1 induction in asf1Δ cells (Fig. 4B). However, we noticed that a significant difference between the levels of Sus1 at GAL1 promoter vs. the levels of Sus1 at GAL1 gene body exists in the absence of Asf1 (Fig. 4C). From these experiments we conclude that Asf1 is dispensable for Sus1 recruitment to at least GAL1 gene and its induction after galactose addition. Nevertheless, it affects the ratio of Sus1 binding to GAL1 promoter vs. ORF.

Histones H3 and H4 incorporation into chromatin and H3K56 acetylation are affected in sus1Δ and thp1Δ mutants

In this study we have shown that the physical interaction between Asf1 and TREX-2 is accompanied neither by a functional requirement of Asf1 in mRNA export nor by defects in Sus1-dependent transcription. Asf1 participates in chromatin remodeling during replication, transcription, and repair. Replication and transcription are accurately coordinated to prevent genome instability. Notably, TREX-2 is involved in the transitions at the forks encountering nuclear pore gated genes during replication. As cited above, Asf1 is also required for the appropriate acetylation of H3K56, which is important for genome stability. Thus it is plausible that TREX-2 factors can contribute to Asf1-dependent chromatin remodeling. To sustain this idea, we investigated the levels of H3 and H4 incorporated into chromatin and the amount of incorporated histone H3K56ac in sus1Δ, asf1Δ and double sus1Δasf1Δ mutants. Figure 5A summarizes the quantification of three biological replicates and shows one representative western blot of the incorporation into chromatin of H3 and H4 for each strain. Chromatin incorporated H3 and H4 (referred to total H3 and H4 respectively) have been set to 1 for wild-type and the other strains have been compared with this value. Interestingly, and unlike asf1Δ, the deletion of
SUS1 significantly enriched association of histones H3 and H4 to chromatin. Notably, deletion of SUS1 in asf1Δ mutant cells increases H3 incorporation. In addition, levels of H3K56 acetylation are increased in sus1Δ cells (Fig. 5B). H3K56 acetylation was almost absent in both asf1Δ and the double mutant sus1Δasf1Δ (Fig. 5B). These data suggest that the absence of SUS1 impacts on H3 and H4 levels incorporated into chromatin and in H3K56 acetylation.

Likewise, we investigated the levels of chromatin-associated histones and acetylation of H3K56 in cells lacking THP1, which also interacts physically with Asf1. As shown in Figure 5C, similarly to asf1Δ, loss of THP1 reduces histones H3 and H4 levels incorporated into chromatin compared with the wild-type cells and also lowers the K56ac levels of H3-chromatin bounded. In conclusion, two TREX-2 components, Sus1 and Thp1, are required for adequate incorporation of histones H3 and H4 onto chromatin and their absence affect H3K56 acetylation, which could account for the functional connection of Sus1, Thp1, and Asf1 in DNA metabolism.

Absence of ASFI reduces global levels of H2B ubiquitination and counteracts the effects of SUS1 deletion

Monoubiquitination of H2B (H2B-ub) is an important chromatin modification with roles in telomeric silencing, transcription, DNA repair and mRNA processing. H2B-ub permits FACT (FAcilitator of Chromatin Transactions) to efficiently displace H2A/H2B histone dimers and both FACT and Asf1, evict histones from the DNA in a coordinated manner in some promoters. Notably, Sus1 plays a prominent role on deubiquitination of histone H2B as part of the SAGA deubiquitination module. Combinations of histone modifications alter chromatin to regulate gene expression. Therefore, we ought to analyze whether Asf1 contributes to H2B ubiquitination levels. As shown in Figure 6, in contrast to sus1Δ mutant, deletion of ASFI leads to a significant H2B ubiquitination (H2Bub) reduction both in chromatin (Fig. 6A) and in total protein extracts (Fig. 6B). This decrease is partially suppressed by the deletion of SUS1 (see sus1Δasf1Δ lanes). In

Figure 3. Asf1 interacts physically and genetically with TREX2. (A) Sus1 co-purifies with Asf1-TAP. Calmodulin eluates from TAP purifications of ASFI-TAPsus1Δ, ASFI-TAP, TAF9-TAP, and a non-tagged strain cells were assayed by western blot using the anti-Sus1 antibody (*). Size of proteins of the molecular marker (MM) is indicated and unspecific reacting bands are labeled with diamonds. (B) Asf1 co-purifies with Sus1-TAP. Calmodulin eluates from TAP purifications of SUS1-TAPasf1Δ and SUS1-TAP were assayed by western blot using anti-Asf1 (upper part) or anti-TAP showing Sus1 (lower). Unspecific reacting bands are labeled with diamonds. (C) List of proteins indicating the number of peptides identified from Thp1-TAP eluate by multidimensional protein identification technology. TREX-2 factors are indicated in red. (D) Cultures of wild-type, sus1Δ, asf1Δ, and sus1Δasf1Δ cells were diluted in 10⁻¹ steps, and equivalent amount of cells were spotted onto YP + Glucose and incubated at 30 °C for 24 h. (E) Representative images of poly(A) RNA localization in wild-type, sus1Δ, and asf1Δ cells assayed by in situ hybridization using Cy3-labeled oligo(dT) probes. Cells were grown at 39 °C in YP + Glucose for 3 h.
conclusion, Asf1 is required for maintaining the correct levels of histone H2B ubiquitination.

**Discussion**

This study reveals novel physical interactions between the conserved histone chaperone Asf1 and several proteins involved in different gene expression stages. From these novel interactors, we have focused on understanding the functional relevance of the Asf1 association with TREX-2 and SAGA. These complexes play an important role in the coupling of gene expression and mRNA export at the NPC. Notably, three NPC nucleoporins—Nup60, Nup2, and Nup57—were found as co-purifying Asf1’ factors. This study also describes novel post-translational modifications at different residues of Asf1, including ubiquitination, methylation, and acetylation. In order to answer how Asf1 PTMs are regulated and whether they impact on its cellular roles, further work is required.

As suggested by the Asf1/TREX-2 interaction observed by proteomic approaches using Asf1 as the bait, here we provide evidence for a reciprocal physical interaction between Sus1 and Thp1 with Asf1. Although Asf1 is not a bona fide component of TREX-2, it may participate in TREX-2-related functions. TREX-2 is constituted by Sac3, Thp1, Sus1, Cdc31, and Sem1 proteins. Mutants in TREX-2 genes lead to defects in mRNA export, transcription elongation, hyperrecombination and genome stability. Moreover, TREX-2 is required for the association of transcribed genes to the NPC. Based on our results, it is tempting to speculate that Asf1 can associate to SAGA, TREX-2, and NPC components when these complexes interact. When does this situation occur? One scenario in which these complexes interact is to couple transcription with the mRNA export, mainly during the NPC-tethering of regulated genes. Accordingly, Asf1 may contribute to organize the chromatin context to facilitate the coupling mechanism. In line with this, Asf1 is the first example of a factor, apart from Sus1, that is able to purify both SAGA and TREX-2. Notably, Asf1 also co-purifies some nucleoporins. The role of nucleoporins and the NPC in transcription and in other chromatin-mediated processes is well established. The fact that Nup2 and Nup60 are nucleoporins located on the nucleoplasmic side is consistent with a transient association of the histone chaperone Asf1 with the NPC. This interaction might be of functional relevance to
modify chromatin as a way to regulate the NPC-chromatin interaction. Notably, it has been shown that the transcriptional memory of those genes gated at the nuclear periphery requires H2A.Z incorporation after repression.\(^{37}\) This indicates that chromatin actively participates in this association. Asf1 could help to establish the correct chromatin environment in order to facilitate these physical interactions. Our results do not allow us to propose a model in which Asf1 contributes to the role on transcription coupled to mRNA export of SAGA/TREX-2. Our data indicate that the presence of Asf1 is not required for poly(A)+ RNA export, neither for \textit{GAL1} expression. However, it is faceable that some minor defects in transcription and export occur indeed in \textit{asf1}\(^{\Delta}\) mutant, but the cell could compensate them. We cannot rule out the possibility that Asf1 participates in the export of a subset of mRNAs, for instance SAGA-regulated transcripts, or that the experimental conditions required to observe this phenomenon are more specific. For instance, as Asf1p is involved in chromatin assembly throughout the cell cycle,\(^{38}\) it is possible that the cell cycle phase is important to observe a phenotype. Along these lines, we consistently observed a small number of cells in the \textit{asf1}\(^{\Delta}\) mutant that presented a partial mRNA export block compared with the wild type although the experiments did not allow us assume that this difference was significant.

According to Figure 4, it is shown thatSus1 recruitment to chromatin is not affected by the absence of \textit{ASF1}. However, a significant difference between Sus1 levels at \textit{GAL1} promoter vs. \textit{GAL1} gene body exists in the absence of Asf1. It seems that the output of this unbalance is not affecting \textit{GAL1} expression, but it could reflect how the chromatin environment created by \textit{ASF1} deletion affects Sus1 recruitment. The literature contains some controversial results that are not easy to accommodate in a simple model. Struhl’s lab reported that Asf1 is recruited to the \textit{GAL1} promoter and ORF,\(^{19}\) and that it affects the recruitment of RNA Polymerase II (RNAP II) to other genes such as \textit{GAL10} and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Deletion of \textit{SUS1} and \textit{THP1} affect H3 and H4 chromatin incorporation and H3K56 acetylation. (A) Absence of \textit{SUS1} increases H3 and H4 incorporation into chromatin. Two dilutions of chromatin extracts from each strain wild-type (wt), \textit{sus1}\(^{\Delta}\), \textit{asf1}\(^{\Delta}\), and \textit{sus1}\(^{\Delta}\)\textit{asf1}\(^{\Delta}\) were obtained and subjected to SDS-PAGE and probed with anti-H3 and H4. A representative western blot is shown. Bar graph shows total histones normalized levels of chromatin incorporated H3 and H4 for each strain after quantification of western blot signals. Data are representative of three independent experiments. (B) As in (A) using anti-H3K56ac antibody. (C) Bar graph shows total histones normalized levels of chromatin incorporated H3, H4 and H3K56 for wild-type (wt) and \textit{thp1}\(^{\Delta}\) after quantification of western blot signals. Data are representative of three independent experiments.}
\end{figure}
Asf1 in the context of DNA replication at NPC-gated genes in of histone H3.

Strikingly, in human cells, the histone acetyltransferase Gcn5, which is part of SAGA, participates in the acetylation of K56. Interestingly a double mutant bearing both deletions is able to suppress partially the phenotypes of the single mutant defects. Moreover, the deposition of H3 acetylated in lysine 56 is linked to transcription, replication and DNA repair and Asf1 promotes this modification. Our work shows, for the first time, that the absence of Sus1 or Thp1 affects the acetylation status of H3K56 incorporated onto chromatin (Fig. 5). However, since H3K56ac dynamics varies along the cell cycle it is formally possible that alterations in cell cycle progression in TREX-2 mutants indirectly affect H3K56ac levels. A possible model to explain Asf1 interaction with SAGA/TREX-2 would be based on an interaction among the nuclear pore complex (Fig. 7). Asf1 is a factor involved in the assembly/reassembly of chromatin after DNA replication and H3K56ac is a signal of the success of the process. SAGA and TREX-2 regulate the tethering of some genes to the NPC and Rad53-dependent replication checkpoint promotes replication fork stability by controlling gene gating. Thus, gene gating and replication must be very well orchestrated, not only by the checkpoint, but also by the chromatin environment imprinted via histone modifications. In these lines, ubiquitination of histone H2BK123 is involved not only in transcription, but also in DNA Damage Response (DDR) as well as acetylation of histone H3K56. H2B monoubiquitination is the major target of the DUB activity mediated by SAGA in which Sus1 plays a crucial role. However, it hasn’t been investigated whether ASFI is required for H2B ubiquitination. Here we demonstrate that monoubiquitination of H2B depends on Asf1. It happens for H3K56 acetylation, deletion of SUSI or ASFI leads to opposite defects. Interestingly a double mutant bearing both deletions is able to suppress partially the phenotypes of the single mutant suggesting that they contribute coordinately to the same process.

Materials and Methods

Yeast strains DNA recombinant work and microbiological techniques

The *Saccharomyces cerevisiae* yeast strains used in this study are listed in Table S3. Yeasts were grown at 30 °C in yeast peptone dextrose (YPD) medium containing, or not, supplementary adenine. Epitope tags and gene replacements were introduced into the genome by PCR-mediated one-step gene replacement.
Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as formerly described using 50 mL of early-log-phase cultures grown in YP + Glucose (repressing), YP + Raffinose (derepressing) or YP + Galactose (inducing). Cultures were cross-linked with 1% formaldehyde solution (Sigma) for 20 min at room temperature and were then quenched with 125 mM glycine. Cells were collected by centrifugation and washed 4 times with 25 mL cold Tris-saline buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5). Cell breakage was performed in 300 μL of lysis buffer (50 mM HEPES-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 1 mM PMSF, and proteases inhibitors) with glass beads, and cell extracts were sonicated in a Bioruptor sonicator (Diagenode) for 30 min in 30 s on and 30 s off cycles. Ten microliters of extract were reserved as input. The rest volume was used for immunoprecipitation with magnetic beads (Dynabeads®) coated with monoclonal human anti-mouse IgG antibodies for 2 h at 4 °C. Beads were washed twice with lysis buffer, twice with lysis buffer supplemented with 360 mM NaCl, twice with wash buffer (10 mM TRIS-HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 125 mM Nadeoxochol, 1 mM EDTA) and once with TE. Samples were eluted twice at 65 °C for 10 min with 100 μL elution buffer (50 mM TRIS-HCl, pH 8, 10 mM EDTA, 1% SDS). Input and immunoprecipitations (IP) samples were incubated overnight at 65 °C to reverse the crosslink. Samples were then treated with proteinase K (Ambion) at 100 μg/250 μL of chromatin at 45 °C for 1.5 h, and DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1), and was ethanol-precipitated and resuspended in 40 μL TE. DNA was used for the qPCR reaction by employing the following primers: GALI Promoter: FW: aaaatggga gtaacctggc c and RV: ccccaaat aaggttaaa aactaat; GALI ORF: FW: ttgctagtac gccggtaga gtc and RV: ggcggacaa atatacaat c; INTergenic: FW: tgtgctttta agaggtgtag gta and RV: ggctggacga ctggggaaac c). In all cases, at least three biological replicates were performed to obtain the standard deviation.

RNA isolation and RT-PCR quantitative PCR

Total RNA was harvested from the yeast early-log-phase cultures grown in YP + Glucose (repressing), YP + Raffinose (derepressing), or YP + Galactose (inducing) by Phenol/Chloroform extraction. RNA was quantified using NanoDrop®. The RT-PCR analysis was performed using 1 μg of total RNA. After DNase-I treatment, RNAs were purified by Phenol/Chloroform extraction. Reverse transcription was performed by following standard procedures with random hexamers and M-MLV reverse transcriptase (Invitrogen®).

Specific pairs of primers were used to amplify the qRT-PCR products of transcripts SCR1 or GAL1 using 3 μL of cDNA as a template (previously diluted at 1/10). For qPCR, SYBR® Premix Ex Taq (Tli RNaseH Plus) (TAKARA) was used in 10 μL of the final volume. Each sample was analyzed in triplicate and qPCR was performed in a LightCycler® Roche 480. Real-time PCR amplification efficiency was calculated from the given slopes in the LightCycler® Roche 480. All the calculations were made by taking into account real primer efficiencies. The GAL1 mRNA
fold-change levels were normalized to the reference gene SCR1 and were expressed in relation to the transcript level for each strain at the zero time point, defined as 1.0. In all cases, at least three biological replicates were performed to obtain the standard deviation. The set of primers used was: ggtatgggactggctctc (FW) and aatctctggaaatattcaggg (RV) for GAL7 and aacgcttttctcctcgtgta (FW) and aagaactcttgccgacca (RV) for SCR1. 

Fluorescence in situ hybridization

Fluorescence in situ hybridization against poly(A)+ RNA was done by growing yeast cells in 50 mL of YP + Glucose medium overnight at 30 °C and shifted to 39 °C for 3 h at 0.5 O.D. 600 . Cells were fixed by adding formaldehyde to 10% and incubation for 60 min at room temperature. The fixative was removed by two rounds of centrifugation and wash with 0.1 M potassium phosphate (pH 6.4). Cells were resuspended in ice-cold washing buffer (1.2 M sorbitol and 0.1 M potassium phosphate, pH 6.4). Subsequently, the cell wall was digested with 0.5 mg/mL of Zymolyase 100T, while samples were applied on poly-L-lysine-coated slide wells. Non-adhering cells were removed by aspiration, cells were rehydrated with 2× SSC (0.15 M NaCl and 0.015 M sodium citrate) and were hybridized overnight at 37 °C in 20 μL of prehybridization buffer with 0.8 pmol of Cy3-end-labeled oligo(dT) in a humid chamber. After hybridization, slides were washed with 1×SSC at room temperature, air-dried and mounted using VECTASHIELD® Mounting Medium with DAPI. Cy3-oligo(dT) was detected under Leica TCS SP2 AOLS confocal microscope.

Preparation of yeast cell extracts and cellular fractionation

For the preparation of the protein cell content in a soluble and in chromatin-associated fractions, yeast cultures were grown until O.D. 600 ≈ 2.0–3.0, except mutant tbp1 (O.D. 600 1.0), and harvested cells were subjected to the previously described procedure. For the western blot assays, the proteins of the chromatin fraction pellet (1 μL/0.1 O.D. 600 units of cells), from 0.35 O.D. 600 of cells, were separated by 2× SDS PAGE and electrotransferred to 0.2 μm pore nitrocellulose membranes, as previously described. Membranes were routinely stained with Ponceau S, as a transferring control, and were probed with specific antibodies: α-H3Ct (ab1791 Abcam), α-H4 Pan (05-858 Upstate), α-H3 acetylated at position 56 (07-677 Upstate), and α-H2B ubiquitinated at position 123 (Cell Signaling). Proteins were detected with horseradish peroxidase-conjugated anti-rabbit secondary antibodies and ECL Advanced reagents (GE Healthcare). Specific signals were quantified by the ImageJ program (http://rsbweb.nih.gov/ij/).

Mass spectrometric analyses

TAP-tagged co-purified proteins were identified as previously described by LC-MS/MS at the 95% confidence level. Proteins were precipitated with TCA (final concentration of 10%) overnight at 5 °C. The pellet was washed with cold acetone by centrifugation, and was then redissolved with 50 μL of 100 mM ABC (ammonium bicarbonate). The protein concentration was estimated by absorbance at 280 nm in a NanoDrop instrument. Cysteine residues were reduced by 2 mM DTT (DL-Dithiothreitol) in 50 mM ABC at 60 °C for 20 min. The sulfhydryl groups were alkylated with 5 mM IAM (iodoacetamide) in 50 mM ABC in the dark at room temperature for 30 min. IAM excess was neutralised with 10 mM DTT in 50 mM ABC, 30 min at room temperature. The sample was subjected to trypsin digestion with 500 ng (100 ng/μL) of sequencing-grade modified trypsin (Promega) in 50 mM ABC at 37 °C overnight. The reaction was stopped with TFA (trifluoroacetic acid) at a final concentration of 0.1%. The final protein concentration was 0.2 μg/μL.

QSTAR-XL analysis

LC-MS/MS was performed as described before. Briefly, the resulting peptides from the above-mentioned digestions were resuspended in 6 μL of 5% acetonitrile, 0.1% TFA, and 5 μL of the sample was loaded onto a trap column (PepMap C18, 300 μm × 5 mm, LC Packings) and desalted with 0.1% TFA at a flow rate of 30 μL/min for 3 min. Peptides were then loaded into an analytical column (PepMap C18 3 μm 100 Å, 75 μm × 15 cm, LC Packings) equilibrated in 5% acetonitrile and 0.1% formic acid. Elution was performed with a linear 5–40% gradient of solvent B (95% acetonitrile, 0.1% formic acid) for 120 min at a flow rate of 300 μL/min. The eluted peptides were analyzed in a nanoESI-Q-TOF mass spectrometer (QSTAR-XL, AB Sciex) in an information-dependent acquisition mode (IDA), in which a 1-sTOF MS scan from 400–1800 m/z was performed, followed by 3 s product ion scans from 65–1800 m/z on the three most intense doubly- or triply-charged ions.

Exclusion list analysis

After protein identification with Protein Pilot, an exclusion list was generated with all the peptides identified with a confidence higher than 95% was created. This list was imported to the Analyst method. Then the same amount of sample was analyzed under the same conditions, excluding all the precursors on the exclusion list. The new data file (wiff file) was combined with the previous one for the database search. At the same time, two aliquots of the same amount of sample were analyzed under the conditions described previously. Yet in one analysis, the TOF MS scan was set from 300 to 900 m/z; and the second aliquot was analyzed with the TOF MS scan set from 900 to 1800 m/z for gas phase fractionation.

5600 triple TOF analysis

For liquid chromatography and tandem mass spectrometry (LC-MS/MS): 5 μL of each sample were loaded into a trap column (NanofL Column, 3 μm C18-CL, 75 μm × 15 cm; Eksigen) and desalted with 0.1% TFA at 2 μL/min for 10 min. Peptides were then loaded into an analytical column (LC Column, 3 μm C18-CL, 75 μm × 25 cm, Eksigen) equilibrated in 5% acetonitrile 0.1% Formic Acid (FA). Elution was performed with a linear gradient of 5% to 35% B in A for 300 min (A, 0.1% FA; B, ACN, 0.1% FA) at a flow rate of 300 nL/min. Peptides were analyzed in a mass spectrometer nanoESI qQTOF (5600 Triple TOF, AB Sciex). The Triple TOF was operated in information-dependent acquisition mode, in which a 0.25 s TOF MS scan from 350–1250 m/z, was performed, followed by 0.05 s product ion scans from 100–1500 m/z on the 50 most intense doubly charged ions.

Database searching and protein identification

Database searching and protein identification were performed as described before. Protein Pilot v4.5 (AB Sciex)
and Mascot v.2.2 (Matrix Science) were used to mine the protein databases. Protein Pilot default parameters were used to generate peak lists directly from the wiff files from the QSTAR or 5600 Triple TOF instruments for both algorithms. The Paragon algorithm of Protein Pilot was used to search the Expaasy protein database with the following parameters: trypsin specificity, cysteinylation and restricted taxonomy to yeast and any biological modifications. Only the primary proteins are shown in the results. A Protein Pilot unused score above 1.3, which is the equivalent to a protein confidence threshold over 95%, was considered significant. The carbamidomethylation of Cys was set as a fixed modification, and Met oxidation and Asn/Gln deamidation were set as variable modifications. To determine the protein modification, another error-tolerant search was performed. The relative abundance of a protein in the sample was quantified using the protein abundance index (PAI), defined as the number of peptides observed in the experiment divided by the number of observable tryptic peptides per protein within a given mass range of the mass spectrometer employed. The PAI was modified exponentially to give emPAI, the exponential form of PAI minus one, which is directly proportional to the protein content in a sample.54,55

Additional proteomics methods

The PTMs identified by ProteinPilot were analyzed in further detail using Pep TideShaker.56,57 This open-source software employed the mascot generic files generated by ProteinPilot to search a UniProt yeast database using both the OMSSA and X-Tandem search engines. The PTM types of interest were selected as variable modifications. The identified modifications were validated by visual inspection of the spectra.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work has been supported by MINECO, Spain (BFU2011-23418) and by the GV (PROMETEO/2013/061 Valencian Regional Government) grants to S.R.-N. M.P. is funded by MICINN, Spain (BFU2008-01976), and the GV (ACOMP2011/057 Valencian Regional Government). P.O.-C. and E.G.-O. are holders of a MINECO FPI grant and CIFP PhD grant respectively.

We are grateful to Dr C Martínez-Jiménez recipient of a FEBS fellowship for making important contributions to this work. Special thanks to Dr Sendra for scientific comments and all the I-25 members.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/nucleus/article/29155/
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