Elsevier Editorial System(tm) for The International Journal of Biochemistry & Cell Biology
Manuscript Draft

Manuscript Number: BC-D-16-00489R2

Title: A Novel Role for Nhp6 Proteins in Histone Gene Regulation in Saccharomyces cerevisiae

Article Type: Research paper

Keywords: chromatin; transcription; nucleosome occupancy; gene expression; histone amount

Corresponding Author: Dr. Giorgio Camilloni, Associate Professor

Corresponding Author's Institution: University of Rome, Sapienza

First Author: Diletta Durano

Order of Authors: Diletta Durano; Andrea Lukacs; Francesca Di Felice; Gioacchino Micheli; Giorgio Camilloni, Associate Professor

Manuscript Region of Origin: ITALY

Response to Reviewers 2nd revision

Dear Editor,

thank you for the consideration of the revised version of our manuscript.

Please find below our comment to reviewer#1, including minor changes we incorporated in the text.

1) The labels on the x-axis of Figure 2b appear to be shifted (no HTA1 label, the last column is not labeled). Further, there is no explanation for the "break" in the HHF2 bar. Is this meant to indicate that the values are on a different scale than the other columns? If so, what is the scale?

The figure has been redrawn.

2) Figure 4 shows an overall reduction of mRNA in the polysomal fraction for the nhp6 mutant, but the legend only indicates that the qPCR was repeated in triplicate. Were biological replicates performed? That is, is the overall reduction of mRNA in the polysomal fraction reproducible? […]

Yes, it is reproducible. The plots shown in panels a and b show a typical outcome of a sedimentation assay and the three replicates referred to in the legend are three independent biological ones (this is now explicitly stated).

3) […]Further, the authors interpret the decreased level of histones as being due to decreased translation efficiency, but a decrease in the overall amount of histone would be due to a decrease in the absolute amount of translation; lower efficiency of translation coupled with the increased mRNA level observed here could result in normal levels of histone protein. <u>It is therefore more important to determine the level of histone mRNA associated with polysomes</u>, <u>which should decrease</u>, not the level of free message. The authors should comment on whether the statistical analysis supports decreased histone mRNA translation, not decreased histone mRNA translational efficiency.

In *nhp6ab* cells the amount of polysome-associated total RNA is reduced (fig. 4b) as compared to wt cells (fig. 4a) and the proportion of untranslated histone mRNA (F) is clearly higher (fig. 4d). On the other hand, the amount of polysome-engaged histone transcripts (H and L) is quite similar in both genetic backgrounds (fig 4d; H and L ratios are much closer to 1 than F ratios). On this, it is reasonable to assume that the histone reduction observed in *nhp6ab* cells (Celona et al. 2011) stems preferentially from reduced translational efficiency. Decreased translation cannot be entirely ruled out. However, considering that the overall histone reduction is comparatively small (about 25%) and involves eight genes, the exact extent of its contribution, if any, cannot be reliably estimated on the basis of only polysome separations. A strict quantitative correspondence of the experimental outcome from the different techniques employed is difficult to obtain. Thus, since there is a clear tendency towards an alteration of free mRNA [with the following statistical details: *HTA1(p=0.03), HTA2(p=0.04), HTB1(p=0.08), HHT1(p=0.06), HHF1(p=0.09), HHF2(p=0.1)*, *HTB2(p=*0.5*), HHT2(p=*0.5)], only efficiency differences in translation can be appreciated. Interestingly this tendency is associated with the overexpressed histone genes (*HTA1, HTA2, HTB1, HHT1, HHF1, HHF2*) and not with the unaltered ones (*HTB2, HHT2*).

Best regards,

Giorgio Camilloni

A Novel Role for Nhp6 Proteins in Histone Gene Regulation in *Saccharomyces cerevisiae*

Diletta Durano [a, ¶], Andrea Lukacs [a, ¶], Francesca Di Felice [a,] Gioacchino Micheli [b,] and Giorgio Camilloni [a, b, c, *]

[a] Dipartimento di Biologia e Biotecnologie "C. Darwin", Sapienza-Università di Roma, Roma 00185, Italy

[b] Istituto di Biologia e Patologia Molecolari, CNR, Roma, 00185, Italy

[c] Istituto Pasteur Italia - Fondazione Cenci Bolognetti e Dipartimento di Biologia e Biotecnologie "C. Darwin"- Sapienza-Università di Roma, Roma, 00185, Italy

[¶] These authors contributed equally to this work

dilettadurano@hotmail.it

ander.lukacs@yahoo.it

francesca.difelice@uniroma1.it

gioacchino.micheli@uniroma1.it

giorgio.camilloni@uniroma1.it

* Corresponding author

E-mail: giorgio.camilloni@uniroma1.it

tel: +390649912808; Fax: +390649912500

A Novel Role for Nhp6 Proteins in Histone Gene Regulation in *Saccharomyces cerevisiae*

Diletta Durano [a, ¶], Andrea Lukacs [a, ¶], Francesca Di Felice [a], Gioacchino Micheli [b], and Giorgio Camilloni [a, b, c, *]

[a] Dipartimento di Biologia e Biotecnologie "C. Darwin", Sapienza-Università di Roma, Roma 00185, Italy

[b] Istituto di Biologia e Patologia Molecolari, CNR, Roma, 00185, Italy

[c] Istituto Pasteur Italia - Fondazione Cenci Bolognetti e Dipartimento di Biologia e Biotecnologie "C. Darwin"- Sapienza-Università di Roma, Roma, 00185, Italy

[¶] These authors contributed equally to this work

dilettadurano@hotmail.it

ander.lukacs@yahoo.it

francesca.difelice@uniroma1.it

gioacchino.micheli@uniroma1.it

giorgio.camilloni@uniroma1.it

* Corresponding author

E-mail: giorgio.camilloni@uniroma1.it

tel: +390649912808; Fax: +390649912500

ABSTRACT

Maintaining a stable and balanced histone pool is of paramount importance for genome stability and fine regulation of DNA replication and transcription. This involves a complex regulatory machinery, exploiting transcription factors as well as histone chaperones, chromatin remodelers and modifiers. The functional details of this machinery are as yet unclear. Previous studies report histone decrease in mammalian and yeast HMGB family mutants. In this study we find that Nhp6 proteins, the *S. cerevisiae* HMGB1 homologues, control histone gene expression by affecting nucleosome stability at regulative regions of the histone clusters. In addition, we observe that histone gene overexpression in the *nhp6ab* mutant is accompanied by downregulated translation, which in turn is responsible for the histone decrease phenotype. Our observations allow us to incorporate Nhp6 proteins into the large group of chromatin factors that tightly regulate histone gene expression.

Keywords:

chromatin; transcription; nucleosome occupancy; gene expression; histone amount, yeast.

Abbreviations:

TSS: transcriptional start site

MNase: Micrococcal nuclease

NTS: non transcribed spacer

1. INTRODUCTION

Chromatin organization banks on a large number of macromolecular complexes interacting with accuracy and stoichiometric proportions. The activity of these components is crucial for the functioning of the genetic apparatus. A hierarchy of chromatin architectures contributes to proper accommodation of DNA inside the nuclear compartment, shaping the microenvironments that maintain genes in their repressed or active state. Even though the individual role of many chromatin components has not been fully determined, a series of basic functions has been attributed to specific elements like histones (Wu and Grunstein, 2000), histone chaperones (Hondele and Ladurner, 2011), chromatin remodelling complexes (Swygert and Peterson, 2014), histone post-translational modifiers (Emre et al., 2005), long non coding RNAs (Fatica and Bozzoni, 2014), and tridimensional architectural proteins (Misteli, 2001).

Histone regulation is crucial for cell life. An increase or decrease in histone content severely affects gene expression (Hu et al., 2014), genome stability (Celona et al., 2011) and aging (Feser et al., 2010). In budding yeast histone amount is tightly determined through different levels of regulation: i) transcriptionally, by positive factors (Spt10 and Spt21), histone chaperones (HIR complex, Asf1, Rtt106), ATP dependent chromatin remodellers (RSC, SWI/SNF, Yta7), and histone modifiers (Rtt109) (Eriksson et al., 2012); ii) post transcriptionally, by the Tramp4, Tramp5 and Rrp6 systems (Beggs et al., 2012, Reis and Campbell, 2007); iii) post-translationally, by Rad53-mediated phosphorylation of the histone proteins followed by ubiquitylation and proteasome-mediated degradation (Singh et al., 2010). Modifications of these pathways lead to histone number variation, consequently altering nucleosome occupancy, and can be regarded as a genome wide level of epigenetic regulation (Celona et al., 2011).

In *S. cerevisiae* there are eight histone genes, encoded by four different clusters. Each one specifies two divergent genes. The *HHT1*/*HHF1* cluster carries one copy of the H3 and H4 genes. Another copy of the same genes resides on the *HHT2/HHF2* cluster. The *HTA1*/*HTB1* and *HTA2*/*HTB2* clusters harbor one copy each of the H2A and H2B genes. Recently, significant histone reduction and transcription alterations have been observed in mammalian and yeast cells lacking the HMGB1 component of the HMGB protein family (Celona et al., 2011). The Nhp6 proteins of *S. cerevisiae* belong to this family and are well known homologs of the human HMGB1 (Agresti and Bianchi, 2003). They are encoded by the *NHP6A* and *NHP6B* genes, contain a single HMG box, share 89% identity in their conserved core region and 96% overall similarity (Kolodrubetz and Burgum, 1990). The Nhp6 proteins are known to preferentially associate with bent DNA without sequence specificity (Yen et al., 1998). In *S. cerevisiae* they are involved in transcriptional regulation either during initiation (by stimulating the formation of the TBP-TFIIA-DNA complex) or during elongation (as part of the FACT complex) (Biswas et al., 2004). Yeast strains lacking either one of the *NHP6* genes show wild type phenotypes for most characters (Costigan et al., 1994), while the double mutant (*nhp6ab)* shows slow growth, increased genome instability and shortened life span (Stillmann, 2010).

Among the phenotypes displayed by *nhp6ab* mutants, we focused our attention on histone decrease, a phenomenon which is likely quite conserved across evolution, as suggested by a very similar behavior of HMGB1 -/- mutant cells in mammals (Celona et al., 2011). The aim of the present work is to investigate on the molecular mechanisms responsible for the histone decrease in a *nhp6ab* genetic background, with particular attention on how this affects transcriptional regulation. We find that Nhp6 proteins act as important regulators of histone gene transcription by stabilizing the nucleosome around the transcriptional start site (TSS).

2. MATERIALS AND METHODS

2.1 Yeast strains and oligonucleotides sequences

Yeast strains used in this work are listed in Table 1. Oligonucleotide sequences are listed in Table 2. Histone cluster sequences were drawn from the SGD Database (www.yeastgenome.org) as specified in Table 3.

2.2 Culture media and conditions

Yeast cultures were grown and manipulated according to standard protocols (Sherman et al., 1983). YPD medium (1% bacto yeast extract, 2% bacto peptone, 2% glucose) was used for all strains.

2.3 RT qPCR

Yeast cells were grown to exponential phase (OD_{600} 0.5 ml^{-1}) and RNA was extracted as previously described (Verdone et al., 1996). After DNase treatment, 1.5 µg RNA was subjected to retro-transcription using 2.5 µM oligo-dT in order to evaluate *HTA1, HTA2, HTB1, HTB2, HHT1, HHT2, HHF1* and *HHF2* expression levels. Samples were incubated with 50U Bioscript Reverse Transcriptase (Bioline) for 1h at 42°C. The reaction was terminated by incubating samples at 85°C for 5 min and successive chilling on ice. Amplification of cDNA was then performed in triplicate for each sample, using Sso Advanced SYBR Green supermix (Bio-Rad) in a Mini Opticon Real-time PCR System (Bio-Rad). The values (obtained in four independent experiments) were normalized to *UBC6* expression (Teste et al., 2009) or to *ACT1*.

2.4 Chromatin immunoprecipitation

Exponentially growing cells were processed for ChIP analysis as previously described (Cesarini et al., 2012). 350 µg chromatin extract were incubated with 2.5 µg antibody against Rpb1 RNA polymerase II subunit (Santa Cruz Biotechnology, sc-25758), HA tag (Santa Cruz Biotechnology, sc-7392) or H4 (Santa Cruz Biotechnology, sc-8658-R). Chromatin-antibody complexes were recovered using Protein A Sepharose beads (Amersham, GE Healthcare) by incubation for 1.5 h at 4°C on a rotating wheel. Input samples were obtained redissolving the material in 200 µl water; immunoprecipitated (IP) and beads-only (bo) samples were redissolved in 50 µl water. DNA amplification was performed using Sso Advanced SYBR Green supermix (Bio-Rad) in a Mini Opticon Real-time PCR System (Bio-Rad). RNA pol II IP samples (three biological replicates, each assayed in duplicate) were normalized to their corresponding input. For both IP and input the relative increase with respect to a constitutively expressed gene (*UBC6*) was calculated according the formula: Relative Increase = $2^{-\Delta Ct}$, with $\Delta Ct = Ct_{X\ GENE} - Ct_{UBC6}$. The final values were obtained as *nhp6ab*/WT ratio. H4 IP samples were normalized to their corresponding input and to the DNA amount present in the sample evaluated by the Qubit (Life Technology) procedure. Nhp6A-HA IP and INPUT samples (three biological replicates, each assayed in duplicate) were normalized to UBC6 or ACT1 promoter regions. Values were reported as Nhp6A-HA-IP/NHP6-HA INPUT ratio.

2.5 Nuc1 stability assay

100 ml of WT or *nhp6ab* cell culture at 0.5 OD_{600}/ml were washed and treated with zimolyase and nystatin, as previously described (Celona et al., 2011). After spheroplasts preparation, EcoRV in vivo digestion was performed with 20U/sample. DNA was purified by phenol-chloroform extraction and isopropanol precipitation (see above). The digestion

products were subjected to qPCR with oligonucleotides amplifying the nuc1 (cleaved by EcoRV) and nuc2 (uncleaved) regions respectively.

2.6 MNase qPCR

Chromatin from wild type and *nhp6ab* yeast cells was prepared and digested with MNase as previously described (Celona et al., 2011). Samples containing mononucleosome-sized DNA (12 units MNase/10^8 cells, 25°C, 15 min) were run on a 1.2% agarose gel. The mononucleosome band was excised from the gel and incubated with 0.3M Na-Acetate overnight at room temperature. DNA was then purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitated. Purified DNA was amplified by Real Time PCR using oligonucleotides specific for the TSS of the eight histone genes. Nuc1, a stable nucleosome near the rDNA NTS (Vogelauer et al., 1998), was used to normalize the Ct values obtained by three independent experiments. The Student's t test was applied for statistical analysis.

2.7 Sucrose Gradient Preparation of Polysomes

10, 20, 30, 40 and 50 % sucrose solutions were prepared in gradient buffer (20 mM Tris-HCl pH 8, 140 mM KCl, 5 mM MgCl2, 0.5 mM DTT, 0.1 mg/ml Cycloheximide). 2.2 ml of each solution were layered on top of each other in a polyallomer tube and stored overnight at 4°C. Cells in exponential phase from 250 ml YPD culture, were treated with 1 ml of 20 mg/ml Cycloheximide and left on ice for 5 minutes with occasional shaking. After centrifugation at 5000 rpm for 5 minutes, the pellet was washed with 4 ml of lysis buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 30 mM MgCl2, 100 µg/ml Cycloheximide) and resuspended in 1 ml of the same buffer. After cell lysis (glass beads), the extract was recovered and 400µl loaded on sucrose gradient. The gradient tube was centrifuged at 36000

rpm in a swing-out rotor (Thermoscientific TH641) for 1.5 hours. Fourteen 0.8ml fractions were collected and the polysome profile was obtained by measuring the absorbance of each fraction at 260nm.

2.8 RNA extraction and qPCR of polysome fractions

Each fraction of a polysome gradient was subjected to phenol:chlorophorm extraction and the RNA was precipitatated with isopropanol. After DNase digestion and retrotrascription (see above) 1µl of cDNA was amplified by qRT-PCR under the conditions reported in section 2.3.

2.9 Budding index

10 ml of cultures of WT and *nhp6ab cells* grown in YPD at OD_{600} 0.5 were chilled in ices and cells were harvested by centrifugation (at 3500rpm), washed once with ice-cold PBS (0,14M NaCl, 0,25mM KCl, 0,8mM Na_2HPO_4, 0,15mM KH_2PO_4, pH 7) and resuspended in 1 ml PBS. 10 µl aliquots, deposited onto micscope slides and covered with coverslips, were observed by phase contrast microscopy. The budding index was determined by counting cells on photographs of randomly chosen fields. Three cell classes were scored (each field was scored by four independent observers and their data averaged): unbudded cells where considered as G1, cells with a bud smaller than 1/3 the size of the mother cell where scored as S, cells with a larger bud where classified as G2/M (Yang Yu et al., 2011). The sum % S cells + % G2/M cells was assumed as budding index.

2.10 DNA curvature

Computer-generated predictions of intrinsic DNA bending were obtained with DICE (DNA Intrinsic Curvature Evaluator), a program developed by one of the authors (GM) for the analysis of sequence-mediated DNA curvature (Prosseda et al., 2010).

3.0 RESULTS

3.1 Transcription of histone genes is upregulated in nhp6ab cells

Histone reduction is known to occur in *S. cerevisiae nhp6ab* mutants (Celona et al., 2011) but the mechanism behind it is as yet ill-understood. We asked whether histone reduction could be ascribed to altered transcription at histone gene loci. Purified RNA from WT and *nhp6ab* exponentially growing cells was subjected to qPCR using previously described primers (Feser et al., 2010) able to distinguish each histone gene (Table 2). Histone gene expression was normalized to that of a constitutive gene (*UBC6*), whose invariance has been demonstrated previously (Teste et al., 2009). Fig. 1a shows the *nhp6ab* histone expression profile as compared to the WT. Six out of eight histone genes are clearly upregulated in the *nhp6ab* background. Only the *HTB2* and *HHT2* genes are not affected by the absence of Nhp6 proteins. We obtained the same outcome when *ACT1* was employed as reference gene (not shown).

Nhp6 mutants display a slow growth phenotype which could lead to a major change in the proportion of S phase cells. This, in turn, could be at least in part responsible for the histone overexpression we have observed. To rule out this possibility we measured the mRNA amount of S-phase specific genes, particularly *MSB1*, *SPC34*, *ADA2* and *MET1* (Spellman et al., 1998). Fig.1 panel b, shows that no significant alteration exits among these genes, suggesting that the proportion of cells engaged in S-phase is unaltered in the *nhp6ab* background as compared to the wt. Budding index analysis confirmed that no alteration of cell cycle in the *nhp6ab* occurs (not shown).

The increased level of histone transcripts observed in the *nhp6ab* mutant could be due to augmented mRNA stability or to enhanced transcriptional rate. In order to discriminate between these hypotheses, we measured the RNA polymerase II (RNA Pol II) enrichment on

histone gene coding regions by ChIP assays using anti-Rpb1 antibodies. Immunoprecipitated (IP) and input fractions were subjected to amplification using primers specific for the coding region of each histone gene and the data were normalized to the *UBC6* coding region. Fig. 1c shows an increased enrichment of RNA Pol II on the genes that are transcriptionally upregulated in the *nhp6ab* mutant as compared to the WT. The non-perfect match between the two patterns (Fig. 1d) (histone gene transcription and RNA Pol II enrichment on histone genes), could be justified considering the high complexity of histone gene regulation. Taken together, these data indicate that Nhp6 proteins are able to act as effective negative regulators of histone gene transcription.

3.2 The presence of Nhp6 proteins affects the occupancy of regulative nucleosome at histone loci

To gain further understanding on the transcriptional upregulation of histone genes in the absence of Nhp6 proteins, we focused on chromatin control mechanisms relevant to histone gene expression. In particular, we investigated i) on the presence of the Nhp6 in proximity of the TSS regions of the histone gene clusters and ii) on the nucleosome occupancy at those sites.

The presence of Nhp6 close to the TSS regions was analyzed by expressing a HA-tagged version of *NHP6A* and performing a ChIP assay with anti-HA antibodies. The results, reported in Fig. 2, show that Nhp6A-HAp is enriched on histone gene promoters as compared to the same regions of *UBC6* and *ACT1* genes.. As for the stronger accumulation we observe around the *HHF2* TSS, computer-generated predictions of intrinsic DNA curvature suggest that it might be related to the specific sequence-mediated bent conformation of this region (not shown).

To investigate on the ability of Nhp6 proteins to influence the occupancy of regulative nucleosomes we performed a micrococcal nuclease (MNase)-PCR assay. After extensive digestion with MNase, the residual DNA fragments (ranging from 150 to 200 bp) were purified and amplified by qPCR using primers specific for the nucleosome region close to the transcription start site of each histone gene. The results are shown in Fig. 3a. The occupancy represents the MNase-resistant material amplified in the specified regions after normalization to a position-invariant nucleosome, nuc1 (Vogelauer et al., 1998), located in the ribosomal DNA repeats. The stability of this nucleosome was further measured in WT and *nhp6ab* cells by comparing the DNA amplification of the nuc1 region obtained after *in vivo* digestion with EcoRV, whose cleavage site is located on the nuc1 of the ribosomal NTS region (Cioci et al., 2002). When the DNA fragment encompassing the digested region was amplified, the amplification efficiency obtained from the two strains was similar, indicating that the nucleosome protection to EcoRV digestion over this DNA region is comparable in the two strains (Fig. 3b). The copy number of this locus is invariant when the WT and the *nhp6ab* mutant are compared (not shown). The comparison between WT and *nhp6ab* profiles highlights a decreased protection towards MNase activity in *nhp6ab* cells. Interestingly, the *HHF2* gene shows a marked hyper-accessibility to MNase digestion close to the TSS, both in the WT and in the *nhp6ab* mutant, suggesting a different chromatin organization of this histone locus as compared to the others.

ChIP assays (Fig. 3c) carried out to evaluate the abundance of H4 in the same regions reveal a general decrease of this protein in the *nhp6ab* mutant, as expected on the reduced occupancy evidenced by the MNase assay. The MNase assay provides functional information whereas the ChIP assay is more structurally oriented. Both approaches strongly support the view that the lack of Nhp6 proteins negatively affects nucleosome occupancy at the histone gene clusters. The decreased stability of the +1 nucleosome in the *nhp6ab* mutant can be

responsible for the enhanced RNA Pol II occupancy thereby eliciting the increased transcription observed for some histone genes (Fig. 1a).

3.3 The nhp6ab mutant shows decreased histone translation as compared to the WT

Decreased histone protein amount and low nucleosome occupancy have been previously observed in a *nhp6ab* background (Celona et al., 2011). On the other hand the present study shows that the transcription of histone genes is highly increased in a *nhp6ab* mutant, thus raising an apparent paradox. It has been reported that an excess of histone mRNA production activates a series of transcriptional (Osley et al., 1986), post-transcriptional (Eriksson et al., 2010) and post-translational (Singh et al., 2009) mechanisms which are essential to maintain the histone levels within physiological limits.

Among control mechanisms acting downstream transcription, the accumulation of histone mRNA and the enrichment of RNA Pol II at the same genes in the *nhp6ab* mutant (Fig. 1) likely rule out transcriptional and post-transcriptional events as major culprits for the histone decrease and the low nucleosome occupancy reported previously (Celona et al., 2011). Thus, we focused our attention on the translation apparatus, in particular studying mRNA-ribosomes association. To this end polysomes from WT and *nhp6ab* cells were prepared by sucrose gradient sedimentation (Fig. 4 a) and pooled fractions of the gradients, corresponding to heavy polysomal particles (H), light ones (L) and free mRNA (F), were assayed by qRT-PCR (Fig. 4 c). The eight histone genes mRNAs from WT and *nhp6ab* cells were amplified and quantified as *nhp6ab*/WT ratio. The comparison of the WT and *nhp6ab* gradient profiles reveals an overall reduction in the abundance of the heavy and light fractions in *nhp6ab* cells, suggesting a decreased translation efficiency in the mutant background. As for individual mRNAs, the amplification profile of histone genes in the *nhp6ab* cells shows a clear increase in the free fraction. Taken together these data indicate

that the increase of histone mRNA synthesis observed in *nhp6ab* cells is accompanied by a reduction in translation efficiency. In essence, this assay allows to reconcile the increased histone transcription we report in this work with the reduction of histone proteins observed previously (Celona et al., 2011): the increase of free histone mRNA accounts well for the histone protein reduction in the *nhp6ab* strain.

4.0 DISCUSSION

In *S. cerevisiae* histone decrease represents a major phenotype of *nhp6ab* mutants. Though this phenotype is associated with a viable condition, it has a marked impact on gene expression, genome stability and aging (Celona et al., 2011; Giavara et al., 2005). Considering the physical and genetic interactions between Nhp6 proteins and histones (Xue and Lehming, 2008; Formosa et al., 2002), in the present work we studied whether the expression of the histone clusters is altered in a *nhp6ab* background.

We analyzed the mRNA production of the eight histone genes, comparing WT and *nhp6ab* cells. Overexpression of the *H3*, *H4*, *H2A* and *H2B* genes is observed in the *nhp6ab* mutant. As far as the expression of *H2B* and *H4* is concerned, only one of the two gene copies encoding these histones is overexpressed (Fig. 1a). While previous studies on histone gene regulation have used only few selected histone genes as representative paradigms (Eriksson et al., 2012), our approach addresses the expression of each histone gene and allows to better appreciate individual differences in their regulation. Fully disentangling the multiplicity of factors affecting histone gene expression obviously calls for further studies, e.g. single gene analysis, which are beyond the scope of this work.

To clarify whether the overexpression of histones is due to altered transcriptional regulation we measured the amount of RNA Pol II associated with histone genes. We observe (Fig. 1) that the increase of RNA Pol II parallels that of histone mRNAs, the most overexpressed genes (*HTA1* and *HHT1*) being associated with the highest amount of RNA Pol II. In addition, the non-overexpressed gene (*HTB2*) displays the lowest RNA Pol II enrichment. This implies the involvement of Nhp6 proteins in the negative regulation of histone genes. So far, Nhp6 proteins have been mainly considered as transcriptional activators (Stillman, 2010), with only one report (Dowell et al., 2010) suggesting that they

might also act as repressors. Our results represent a direct evidence that Nhp6 proteins are able to also exert a repressive function.

Nhp6 proteins are important components of yeast chromatin (Stillman, 2010). In a *nhp6ab* mutant global nucleosome loss and transcriptome alterations have been observed (Celona et al., 2011). The histone gene overexpression we report in this study suggests a possible role for Nhp6 proteins in controlling the chromatin state of histone gene clusters. In order to verify this hypothesis, we measured the DNA protection exerted *in vivo* by nucleosome particles against MNase digestion, focusing our attention on +1 nucleosomes, i.e. the ones in close proximity to the TSS regions (Fig. 3a). The regulative relevance of the nucleosomal organization of this region is well known (Jiang and Pugh, 2009). In the *nhp6ab* mutant we find that at all histone genes +1 nucleosome protection against MNase digestion is decreased, indicating that a significant alteration of chromatin structure occurs at these regions. This agrees with the sharp increase of transcription, reflecting a release of the repression exerted by the regulative nucleosomes, when Nhp6 proteins are lacking. Given the multiplicity of transcriptional regulators of histone genes (Eriksson et al., 2012), the match between a decrease in nucleosome occupancy and an increase of RNA transcription cannot be expected to be perfect. Therefore we suppose that *HTB2* and *HHT2* could be less sensitive to nucleosome occupancy due to additional elements regulating their transcription. We conclude that Nhp6 proteins negatively regulate at least six out of eight histone genes, repressing their transcription by stabilizing +1 nucleosomes.

To verify whether in the *nhp6ab* background nucleosomes have actually reduced their occupancy rather than changing their conformation we measured the amount of H4 in the same regions by ChIP analysis. The strict correspondence between loss of H4 and enhanced nucleosome accessibility (Fig. 3a, b) demonstrates that +1 nucleosomes become more unstable in the absence of Nhp6 proteins. When assayed by ChIP in the WT, Nhp6 proteins

are found in association with all histone clusters (Fig. 2), indicating an important role in maintaining nucleosome stability at histone TSS regions. As opposed to histone genes, the presence of Nhp6 has been reported to make nuclesomes of non-histone genes unstable (reviewed in Stillman, 2010). Our observations that Nhp6p associates effectively with all histone loci (Fig. 2), and that its absence promotes transcription (Fig.1a), Pol II recruitment (Fig. 1b) and MNase hyper-accessibility (Fig. 3a), indicate that Nhp6 proteins control the expression of histone genes by directly modifying their chromatin organization, conferring on these proteins the potential of novel regulators able to stabilize nucleosomes and maintain a controlled level of transcription.

The ability of Nhp6 to bind intrinsically curved DNA has been demonstrated *in vitro* (Yen et al., 1998). Interestingly, we find that *HHF2* gene binds three to four times the amount of Nhp6 as compared to the other histone genes analyzed (Fig. 2). Computer-generated predictions (not shown) do not reveal significant intrinsic DNA curvature along the four histone clusters, except for the *HHF2* TSS. We speculate that bent DNA may be relevant to Nhp6 binding to this region, although further experiments are needed to clarify this point.

The comparison between the data presented in this work with previous findings raises an apparent paradox: histone mRNA overexpression (Fig. 1a) and histone protein reduction (Celona et al., 2011) coexist in the *nhp6ab* background. In agreement with previous data, indicating that an increased amount of histone proteins is toxic for the cell (Singh et al., 2010), we envisage a scenario involving a mechanism able to prevent the excess of histones possibly induced by mRNA overexpression. Our finding (Fig. 4) that histone translation is decreased in the *nhp6ab* mutant supports this hypothesis. The comparison between WT and *nhp6ab* polysomal profiles shows a decreased translation efficiency in the *nhp6ab* strain. This is in agreement with previous observations stressing that NHP6 proteins control key events affecting translation, e.g. tRNA synthesis (Braglia et al., 2007).

Overall, our observations allow to incorporate Nhp6 proteins in the large group of factors that, acting on chromatin, tightly regulate histone gene expression. The prospective impact of our work relates to the responsiveness of histone genes to the cell cycle machinery. Given the evolutionary conservation of HMGB1 proteins from yeast to mammals, an equally conserved mechanism of action may be hypothesized.

ACKNOWLEDGEMENTS

We are grateful to M. E. Bianchi and B. Cairns for yeast strains and S. Dini Modigliani for the help in polysome preparation.

FUNDING

This work was partially supported by: the Istituto Pasteur-Fondazione Cenci Bolognetti, Università di Roma La Sapienza and by the Epigenomics Flagship Project EpiGen, the Italian Ministry of Education and Research, National Research Council.

FIGURE CAPTIONS

Figure 1. Histone gene transcription is upregulated in a *nhp6ab* background.

(a) qPCR analysis of mRNA transcription for each histone gene. The quantification is relative to UBC6 expression. Grey and white bars refer to histone gene expression in the WT and in the *nhp6ab* mutant respectively. (b) Expression analysis of S-phase related genes (ADA2, MSB1, SRP34 and MET1) by qPCR normalized to UBC6 in the WT and in the *nhp6ab* mutant (black and gray bars respectively). (c) ChIP analysis of RNA polymerase II enrichment at histone genes. For both, the WT and the *nhp6ab* mutant, the RNA polymerase II enrichment is relative to the UBC6 gene and normalized to input DNA. (d) Comparison of transcription and ChIP profiles. Grey bars: RNA expression in *nhp6ab* cells relative to WT (data from panel A). Black bars: RNA polymerase II enrichment in *nhp6ab* cells relative to WT (data from panel C). In panels A-C vertical lines refer to standard deviation (four replicates for panel A, three for panels B and C). In panels A and C t-test significance is indicated (*=$p<0.05$; **=$p<0.01$; ***=$p<0.001$) .

Figure 2. Nhp6A-HA protein occurrence at histone gene promoters.

Bars refer to a ChIP qPCR analysis of a NHP6A-HA tagged strain, normalized to UBC6 or ACT1 promoter regions (panel A and panel B, respectively). Values are reported as IP/INPUT ratio. The vertical lines indicate the standard deviation (three replicates).

Figure 3. Decreased occupancy of regulative nucleosomes at histone gene promoters in a *nhp6ab* mutant.

(a) DNA resistant to extensive MNase digestion (nucleosome occupancy) was amplified by qPCR of the regions close to the TSS of each histone gene in WT and in *nhp6ab* mutant cells

(grey and white bars, respectively). The data were normalized to the occupancy of a stable nucleosome (nuc1) lying in the ribosomal gene NTS region. (b) In vivo digestion of DNA with EcoRV. Nuc1 (cleaved region) amplification byqPCR normalized to nuc2 (uncleaved region) in the WT (grey bar) and in the *nhp6ab* mutant (white bar). (c) ChIP analysis of H4 enrichment at histone genes. Input and IP samples were normalized to total DNA amount. The H4 enrichment is relative to the input DNA. In all panels the vertical lines refer to the standard deviation (three replicates). In panels A and B t-test significance is indicated (*=p<0.05; **=p<0.01; ***=p<0.001).

Figure 4. The *nhp6ab* mutant shows reduced translation efficiency

(a, b) Typical sucrose gradient sedimentation profiles of polysomes from WT and, repectively, *nhp6ab* cells. (c) Fractions from sucrose gradients were pooled: fractions 2-3 constitute the heavy pool (H, white), fractions 4-6 the light pool (L, grey) and fractions 7-14 the free mRNA pool (F, black). qRT-PCR of individual histone mRNAs were performed and quantified. Data are reported as *nhp6ab*/WT ratio for each histone gene analyzed. Dotted line represents the WT value (considered as 1). Vertical lines refer to the standard deviation (three independent biological replicates).

REFERENCES

Agresti, A., Bianchi, M.E., 2003. HMGB proteins and gene expression. Current Opinion in Genetics & Development 13, 170–178.

Beggs, S., James, T.C., Bond, U., 2012. The PolyA tail length of yeast histone mRNAs varies during the cell cycle and is influenced by Sen1p and Rrp6p. Nucleic Acids Res. 40, 2700–2711.

Biswas, D., Imbalzano, A.N., Eriksson, P., Yu, Y., Stillman, D.J., 2004. Role for Nhp6, Gcn5, and the Swi/Snf complex in stimulating formation of the TATA-binding protein-TFIIA-DNA complex. Mol. Cell. Biol. 24, 8312–8321.

Braglia, P., Dugas, S.L., Donze, D., Dieci, G., 2007. Requirement of Nhp6 Proteins for Transcription of a Subset of tRNA Genes and Heterochromatin Barrier Function in Saccharomyces cerevisiae. Molecular and Cellular Biology 27, 1545–1557.

Celona, B., Weiner, A., Di Felice, F., Mancuso, F.M., Cesarini, E., Rossi, R.L., Gregory, L., Baban, D., Rossetti, G., Grianti, P., Pagani, M., Bonaldi, T., Ragoussis, J., Friedman, N., Camilloni, G., Bianchi, M.E., Agresti, A., 2011. Substantial histone reduction modulates genomewide nucleosomal occupancy and global transcriptional output. PLoS Biol. 9, e1001086.

Cesarini, E., D'Alfonso, A., Camilloni, G., 2012. H4K16 acetylation affects recombination and ncRNA transcription at rDNA in Saccharomyces cerevisiae. Mol. Biol. Cell 23, 2770–2781.

Cioci, F., Vogelauer, M., Camilloni, G., 2002. Acetylation and Accessibility of rDNA Chromatin in Saccharomyces cerevisiae in Δtop1 and Δsir2 Mutants. Journal of Molecular Biology 322, 41–52.

Costigan, C., Kolodrubetz, D., Snyder, M., 1994. NHP6A and NHP6B, which encode HMG1-like proteins, are candidates for downstream components of the yeast SLT2 mitogen-activated protein kinase pathway. Mol. Cell. Biol. 14, 2391–2403.

Dowell, N.L., Sperling, A.S., Mason, M.J., Johnson, R.C., 2010. Chromatin-dependent binding of the S. cerevisiae HMGB protein Nhp6A affects nucleosome dynamics and transcription. Genes & Development 24, 2031–2042.

Emre, N.C.T., Ingvarsdottir, K., Wyce, A., Wood, A., Krogan, N.J., Henry, K.W., Li, K., Marmorstein, R., Greenblatt, J.F., Shilatifard, A., 2005. Maintenance of Low Histone Ubiquitylation by Ubp10 Correlates with Telomere-Proximal Sir2 Association and Gene Silencing. Molecular Cell 17, 585–594.

Eriksson, P.R., Ganguli, D., Nagarajavel, V., Clark, D.J., 2012. Regulation of Histone Gene Expression in Budding Yeast. Genetics 191, 7–20.

Fatica, A., Bozzoni, I., 2014. Long non-coding RNAs: new players in cell differentiation and development. Nat Rev Genet 15, 7–21.

Feser, J., Truong, D., Das, C., Carson, J.J., Kieft, J., Harkness, T., Tyler, J.K., 2010. Elevated Histone Expression Promotes Life Span Extension. Molecular Cell 39, 724–735.

Formosa, T., Ruone, S., Adams, M.D., Olsen, A.E., Eriksson, P., Yu, Y., Rhoades, A.R., Kaufman, P.D., Stillman, D.J., 2002. Defects in SPT16 or POB3 (yFACT) in Saccharomyces cerevisiae Cause Dependence on the Hir/Hpc Pathway: Polymerase Passage May Degrade Chromatin Structure. Genetics 162, 1557–1571.

Giavara, S., Kosmidou, E., Hande, M.P., Bianchi, M.E., Morgan, A., d' Adda di Fagagna, F., Jackson, S.P., 2005. Yeast Nhp6A/B and Mammalian Hmgb1 Facilitate the Maintenance of Genome Stability. Current Biology 15, 68–72.

Hondele, M., Ladurner, A.G., 2011. The chaperone-histone partnership: for the greater good of histone traffic and chromatin plasticity. Curr. Opin. Struct. Biol. 21, 698–708.

Hu, Z., Chen, K., Xia, Z., Chavez, M., Pal, S., Seol, J.-H., Chen, C.-C., Li, W., Tyler, J.K., 2014. Nucleosome loss leads to global transcriptional up-regulation and genomic instability during yeast aging. Genes & Development 28, 396–408.

Jiang, C., Pugh, B.F., 2009. Nucleosome positioning and gene regulation: advances through genomics. Nature Reviews Genetics 10, 161–172.

Kolodrubetz, D., Burgum, A., 1990. Duplicated NHP6 genes of Saccharomyces cerevisiae encode proteins homologous to bovine high mobility group protein 1. J. Biol. Chem. 265, 3234–3239.

Misteli, T., 2001. The concept of self-organization in cellular architecture. J. Cell Biol. 155, 181–185.

Osley, M.A., Gould, J., Kim, S., Kane, M., Hereford, L., 1986. Identification of sequences in a yeast histone promoter involved in periodic transcription. Cell 45, 537–544.

Prosseda, G., Mazzola, A., Di Martino, M.L., Tielker, D., Micheli, G., Colonna, B., 2010. A Temperature-Induced Narrow DNA Curvature Range Sustains the Maximum Activity of a Bacterial Promoter in Vitro. Biochemistry 49, 2778–2785.

Reis, C.C., Campbell, J.L., 2007. Contribution of Trf4/5 and the nuclear exosome to genome stability through regulation of histone mRNA levels in Saccharomyces cerevisiae. Genetics 175, 993–1010.

Sherman F, Fink GR, Lawrence C (1983). Methods in Yeast Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

Singh, R.K., Kabbaj, M.-H.M., Paik, J., Gunjan, A., 2009. Histone levels are regulated by phosphorylation and ubiquitylation-dependent proteolysis. Nat. Cell Biol. 11, 925–933.

Singh, R.K., Liang, D., Gajjalaiahvari, U.R., Kabbaj, M.-H.M., Paik, J., Gunjan, A., 2010. Excess histone levels mediate cytotoxicity via multiple mechanisms. Cell Cycle 9, 4236–4244.

Spellman, P.T., Sherlock, G., Zhang, M.Q., Iyer, V.R., Anders, K., Eisen, M.B., Brown, P.O., Botstein, D., Futcher, B., 1998. Comprehensive identification of cell cycle–regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization. Molecular biology of the cell 9, 3273–3297.

Stillman, D.J., 2010. Nhp6: A small but powerful effector of chromatin structure in Saccharomyces cerevisiae. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1799, 175–180.

Swygert, S.G., Peterson, C.L., 2014. Chromatin dynamics: Interplay between remodeling enzymes and histone modifications. Biochim. Biophys. Acta 1839, 728–736.

Teste, M.-A., Duquenne, M., François, J.M., Parrou, J.-L., 2009. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in Saccharomyces cerevisiae. BMC Molecular Biology 10, 99.

Verdone, L., Camilloni, G., Mauro, E.D., Caserta, M., 1996. Chromatin remodeling during Saccharomyces cerevisiae ADH2 gene activation. Mol. Cell. Biol. 16, 1978–1988.

Vogelauer, M., Cioci, F., Camilloni, G., 1998. DNA protein-interactions at the Saccharomyces cerevisiae 35 S rRNA promoter and in its surrounding region. Journal of Molecular Biology 275, 197–209.

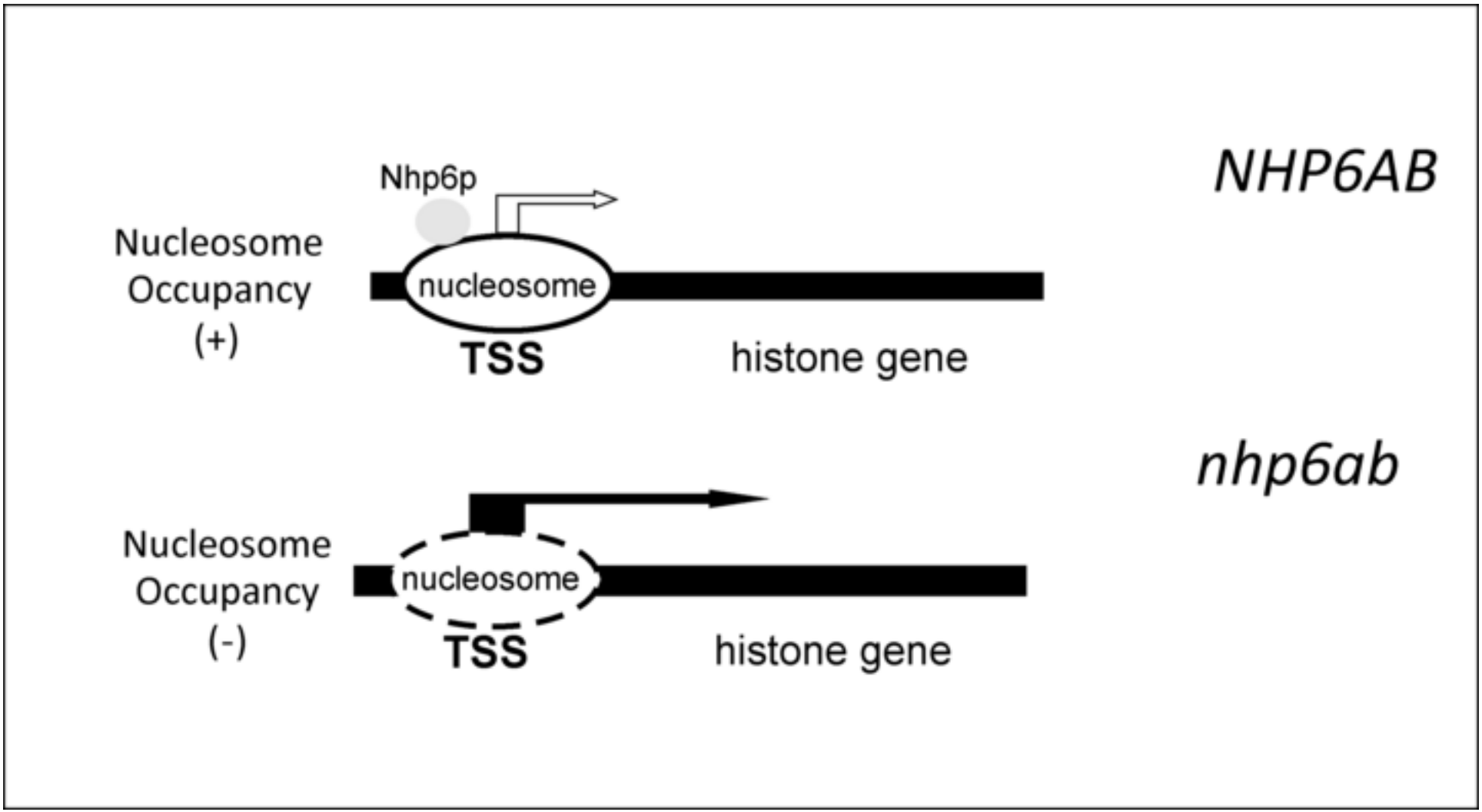
Wu, J., Grunstein, M., 2000. 25 years after the nucleosome model: chromatin modifications. Trends Biochem. Sci. 25, 619–623.

Xue, X., Lehming, N., 2008. Nhp6p and Med3p Regulate Gene Expression by Controlling the Local Subunit Composition of RNA Polymerase II. Journal of Molecular Biology 379, 212–230.

Yang Yu, B., Elbuken, C., Ren C.L., Huissoon J.P., 2011. Image processing and classification algorithm for yeast cell morphology in a microfluidic chip. J. Biomed. Opt. 16:066008

Yen, Y.M., Wong, B., Johnson, R.C., 1998. Determinants of DNA binding and bending by the Saccharomyces cerevisiae high mobility group protein NHP6A that are important for its biological activities. Role of the unique N terminus and putative intercalating methionine. J. Biol. Chem. 273, 4424–4435.

Graphical Abstract



HIGHLIGHTS

- Nhp6 proteins negatively regulate histone genes transcription
- Nhp6 proteins stabilize TSS surrounding nucleosomes
- the absence of Nhp6 proteins leads to reduction of histone translation
- Nhp6 repress histone transcription by stabilizing +1 nucleosomes and act as their regulators

Figure 1

Click here to download high resolution image

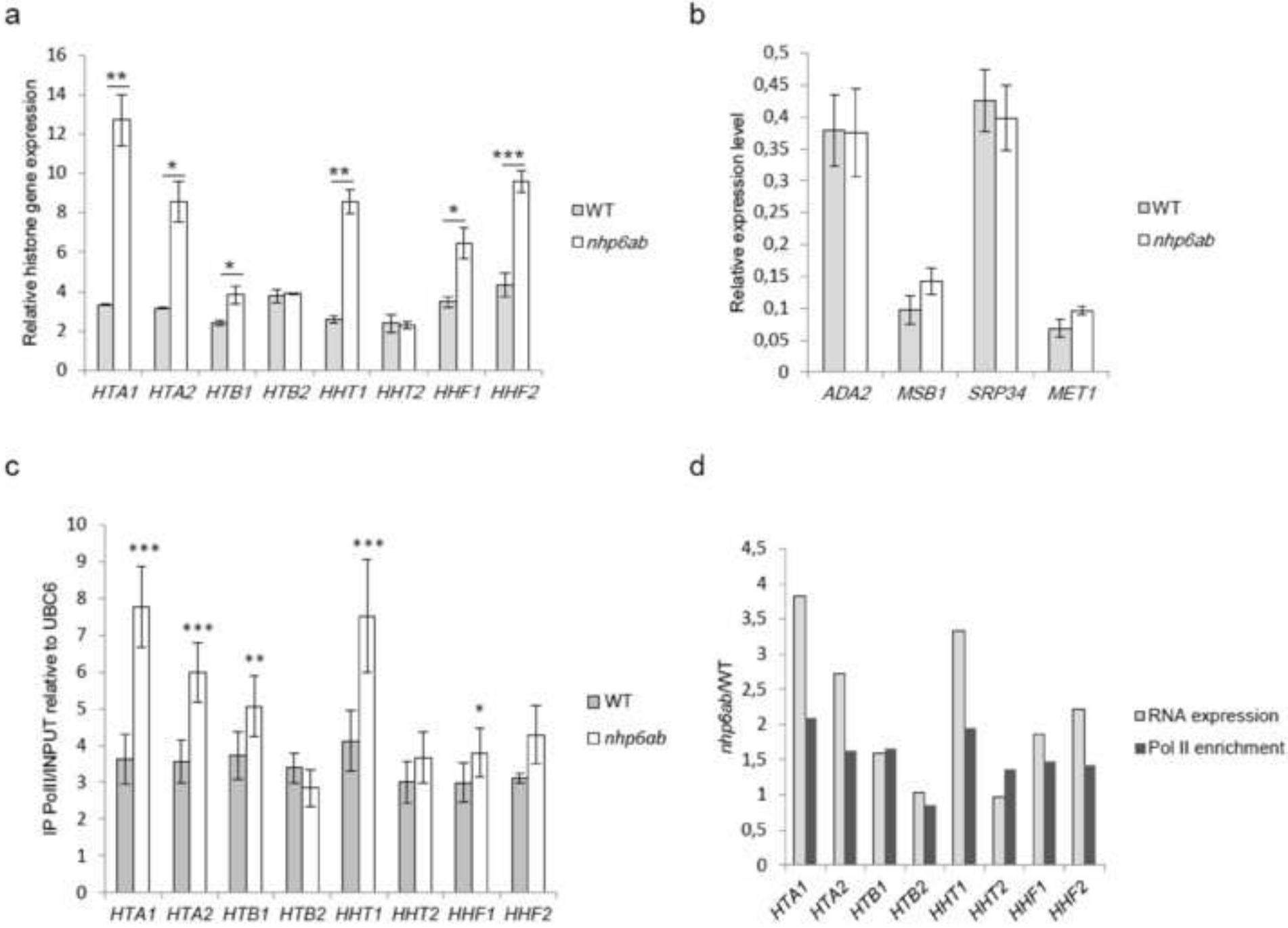


Figure 2

Click here to download high resolution image

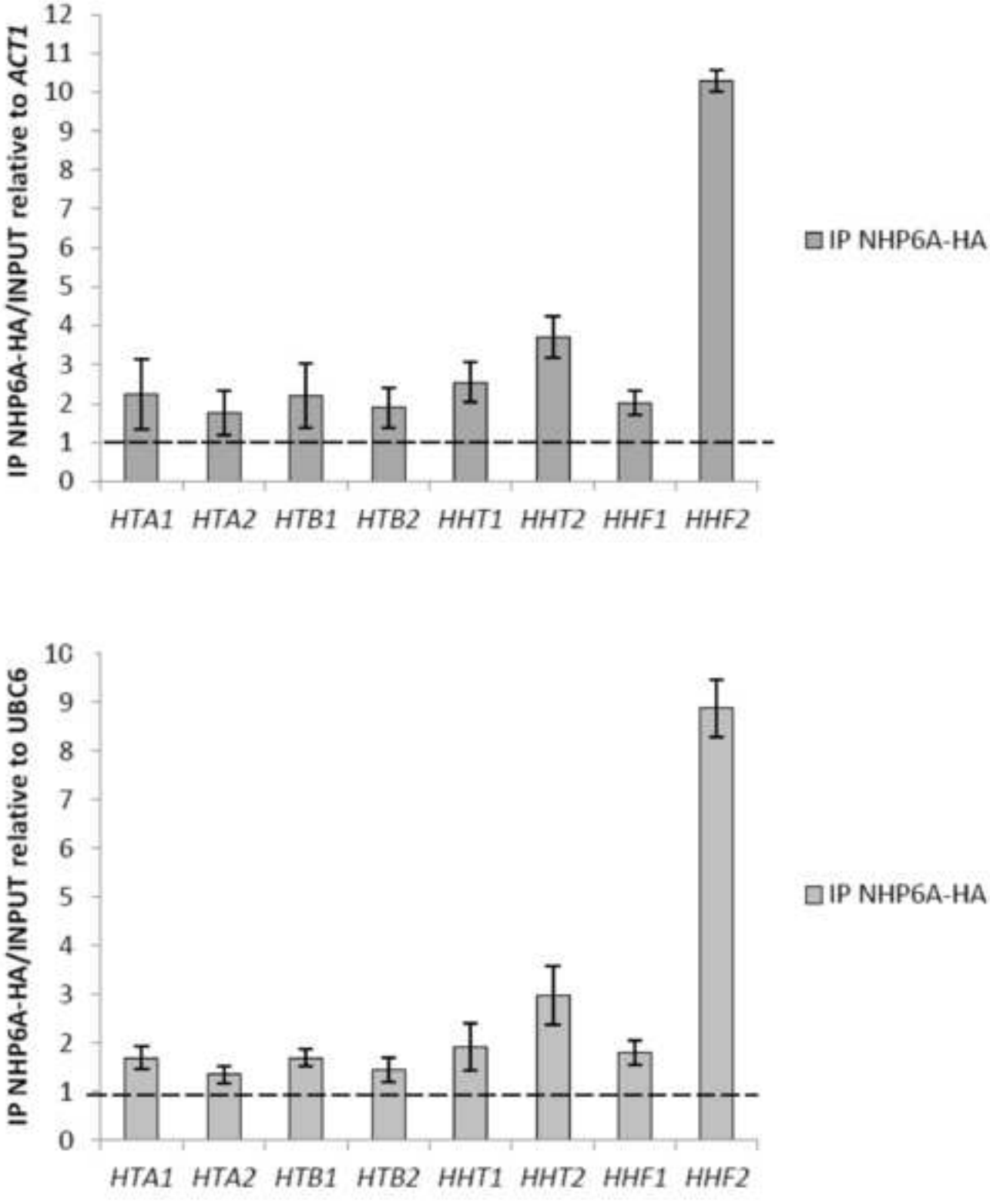


Figure 3

Click here to download high resolution image

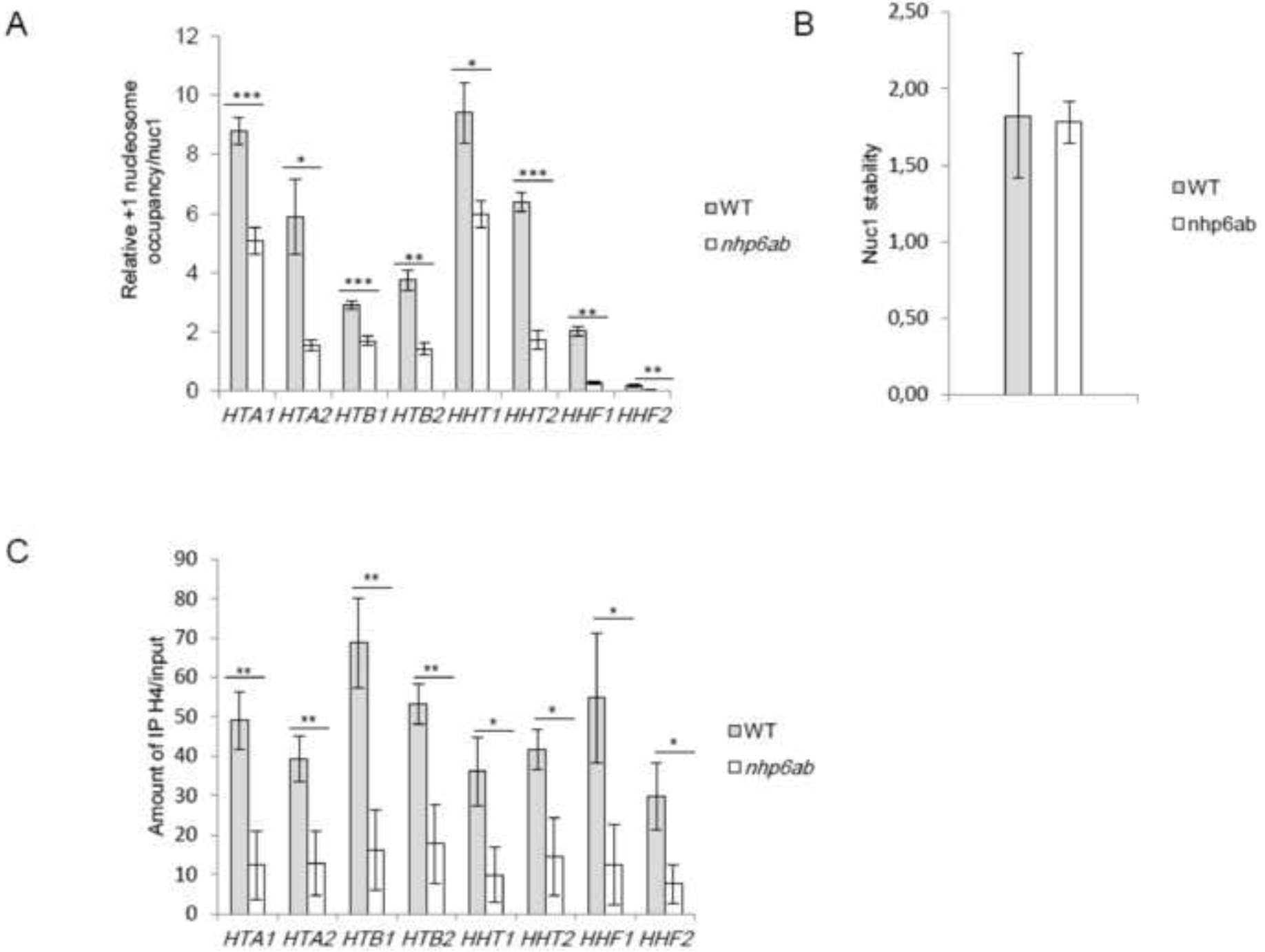
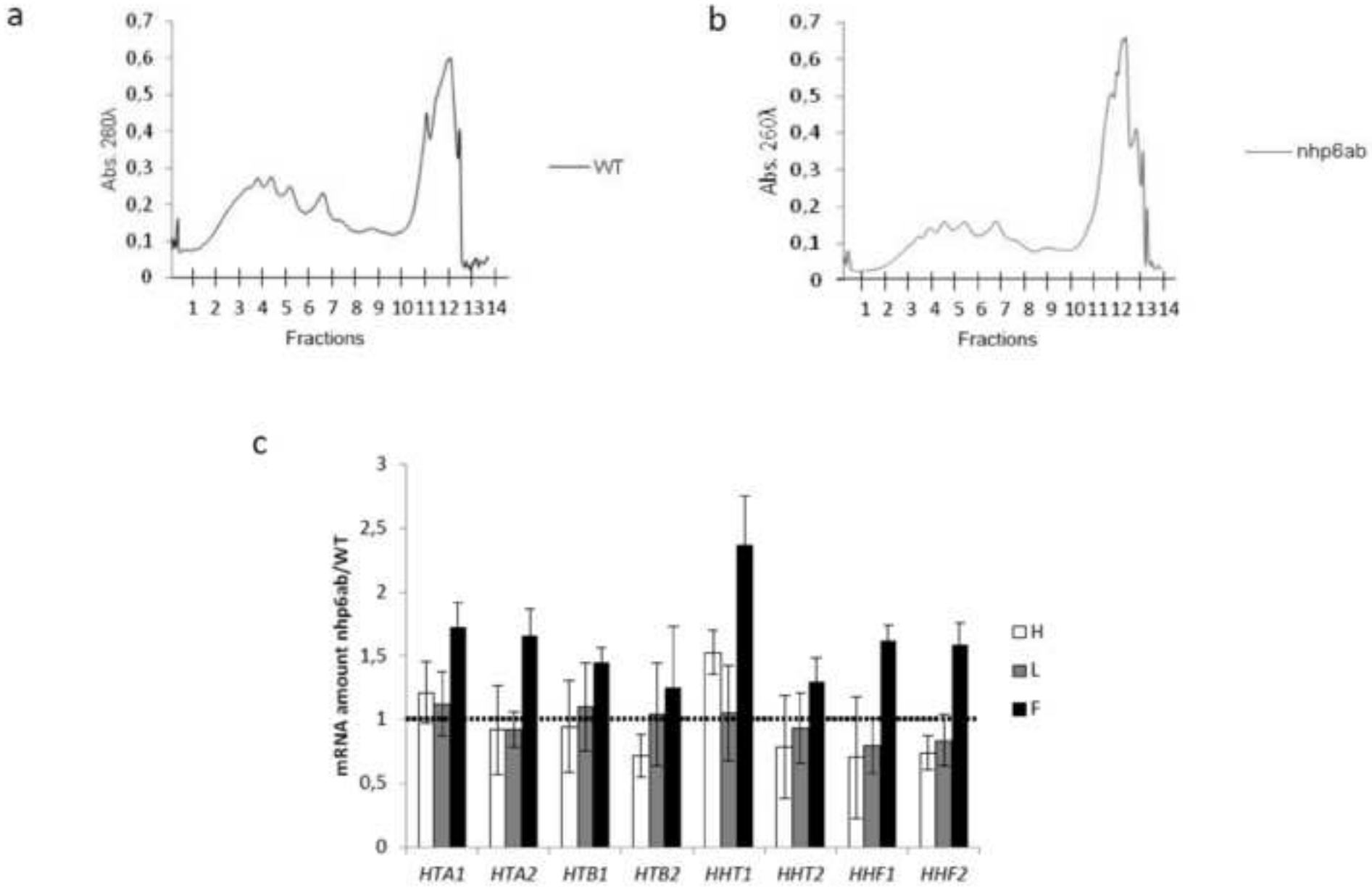


Figure 4

Click here to download high resolution image



TABLES

Table 1. Yeast Strains used in this work

S. cerevisiae strains	Genotypes
WT	*Matα; ura3-52; trp1-289;his3-Δ1; leu2 3,112; gal2; gal10*
nhp6ab (Y869) (§)	*Matα; ura3-52; trp1-289;his3-Δ1; leu2 3,112; gal2; gal10; nhp6a-Δ3::URA3; nhp6b-Δ3::HIS3*
WT	*Matα; his3Δ200; leu2Δ0; met15Δ0; trp1Δ63; ura3Δ0;*
nhp6a-HA(§§)	*Matα; his3Δ200; leu2Δ0; met15Δ0; trp1Δ63; ura3Δ0; NHP6A3xHA::HIS3*

§ kindly provided by M.E. Bianchi

§§ kindly provided by B. Cairns

Table 2. Oligonucleotide list

Oligonucleotide	Sequence (5' to 3')
HTA1 RT-F	GGTTCTGGTGCTCCAGTCTAC
HTA1 RT-R	TCTTCTTGTTATCCCTAGCAGCAT
HTA2 RT-F	AGCTGGTTTAACATTCCCAGTT
HTA2 RT-R	GCAGTTAGATAGACTGGAGCAC
HTB1 RT-F	AGAGAAGCAAGGCTAGAAAGGA
HTB1 RT-R	GGAAATACCAGTGTCAGGGTG
HTB2 RT-F	GATTGATCTTACCTGGTGAATTGGCTAAA

HTB2 RT-R	GGCTTGAGTAGAGGAGGAGTAT
HHT1 RT-F	CTGCCATTCACGCCAAGC
HHT1 RT-R	ATGATCTTTCACCTCTTAATCTTCTAGCC
HHT2 RT-F	CCCCAAGAAAACAATTAGCCTCC
HHT2 RT-R	AAGGCAACAGTACCTGGCTTAT
HHF1 RT-F	AAGAGATAACATCCAAGGTATTACTAAGCC
HHF1 RT-R	CAAACCAGAAATACGCTTGACAC
HHF2 RT-F	ATCAGGGACTCTGTTACTTACACT
HHF2 RT-R	GGTTCTACCTTGTCTCTTCAAAGCATAAA
hht1 for	CATAAATATATAAACGCAAACAATGG
hht1 rev	GGCAGCCTTAGAAGCTAATTG
hhf1 for	ACAATAATGCGACGGAAACC
hhf1rev	CCGCGAATACGGTGGTAAAT
hht2 for	TGTTTTCTTGGGGCTTTACC
hht2 rev	CCAACTGTTCTTCCCCTTTT
hhf2 for	ACCGAGAGTTTCGCATTTGT
hhf2 rev	GCACAAACACGTATGTATCTAGCC
hta1 for	GCTGGTTTGACATTCCCAGT
hta1 rev	CGGCCAAATATTCCAAGACA
htb1 for	GCCTTGCTTCTCTTCTTACCA
htb1 rev	TCCTATATAGACAAGTCAAACCACAAA
hta2 for	CCAACTGGGAATGTTAAACCA
hta2 rev	GGAAAGTACAGAACAAGAGCAAA
htb2 for	CTTACTCTACAACTAATGTCCTCTGC
htb2 rev	AATATAAGAGGAATAGGTCTCCTTTCT
Ubc6F	GATACTTGGAATCCTGGCTGGTCT

Ubc6R	AAGGGTCTTCTGTTTCATCACCTG
MSB1 F	CGCTGATAGGAAATTAGCGG
MSB1 R	GCCGGCAAGTTGTTGATATT
SPC34 F	GTAACCACGACGAGGCGTAT
SPC34 R	CATGGATGATCGGTTTTTCC
ADA2 F	AATACGACTTATGCGTGCCC
ADA2 R	TATCCTGCCAATTACCGAGC
MET1 F	TTACCTGGATTTGGCTACGG
MET1 R	TCACTCAGCTTGTTCATCGG
NUC1-F	GTTTGAATGGTGGTAGTGTA
NUC1-R	CGGAAACGCGCGGGAACATACAA

Table 3. Histone genes chromosomal coordinates

Cluster	Source of sequence § (position in the chromosome)
HTB1-HTA1	Chr IV (914317-915928)
HTA2-HTB2	Chr II (235394-236887)
HHF1-HHT1	Chr II (255373-256741)
HHT2-HHF2	Chr XIV (575640-577038)

§ SGD database