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Sir2 is involved in the transcriptional modulation of *NHP6A* in *Saccharomyces cerevisiae*

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ABSTRACT

The Sir proteins, namely Sir2, 3 and 4, have roles related to heterochromatin, but genome-wide studies have revealed their presence at many euchromatic loci, although the functional meaning of this is still not clear. Nhp6a is an abundant HMG-like protein in yeast, which has a role in transcription by modulating chromatin structure and nucleosome number. Although much is known about its structure and function, information regarding its regulation is scarce. *NHP6A*, among other genes, emerges in ChIP-on chip studies of global Sir proteins binding, suggesting it could be regulated by *SIR*. We have investigated *NHP6A* expression in *sir* deletion mutants as well as in *SIR2* overexpressing conditions. In addition, we have asked if the Sir2 deacetylation activity is involved by using conditions that either inhibit (treatment with nicotinamide) or enhance (calorie restriction conditions) Sir2 activity. We have found that, consistent with previous microarray studies, *NHP6A* expression undergoes a slight increase in *sir* mutant strains, but is strongly repressed when *SIR2* is overexpressed. In a *sir3* mutant strain the gene continues to be transcribed, even in *SIR2* overexpressing conditions. In addition, treating the cells with nicotinamide counteracts the *SIR2* overexpressing effect. Finally, conditions that are known to potentiate Sir2 deacetylation activity seem to mimic the effect of *SIR2* overexpression on *NHP6A*. Our results suggest that Sir2 is involved in the regulation of *NHP6A* promoter, acting more as a specific repressor, rather than a long-range silencer. This effect is specific, and the Sir2 deacetylase activity is required for the Sir2 mediated repression of *NHP6A*. Moreover, the presence of the SIR complex seems required for Sir2 to silence *NHP6A*.

1. Introduction

In *Saccharomyces cerevisiae*, the Sir proteins play relevant roles related to the functions of heterochromatin [1]. The four Sir proteins, Sir1/2/3/4, establish and maintain the transcriptionally silenced status of the cryptic *HM* loci, regulate telomere length and integrity and promote silencing of subtelomeric genes [2]. In addition, Sir2 regulates genome stability and lifespan by binding rDNA and suppressing intrachromosomal recombination [3,4].

In spite of the predominant role of the Sir proteins at silent loci, a number of evidences accumulated in recent years showing the presence of the Sir proteins at euchromatic sites, across the entire genome. Earlier ChIP-on-chip studies, reporting the genomewide mapping of Sir2/3/4 in yeast wild type cells, showed that 12% of Sir2, 15% of Sir3p and 30% of Sir4p binding sites are euchromatic [5]. The data for Sir2 were subsequently confirmed by Tsankov and colleagues that found 24% of actively transcribed genes in yeast cells show interaction with Sir2 [6]. In addition, Sir proteins relocation was observed in mutants where the association of heterochromatin with the nuclear periphery was disrupted [7].

More recently the involvement of the Sir proteins in the modulation of specific genes under particular growth conditions was reported. In fact, Sir2 can partially substitute for the Hst1p deacetylase in the repression of a set of midsporulation genes [8], and can associate with it to downregulate highly transcribed genes during the diauxic shift [9]. Moreover, Sir2 associates with the Fkh1 and 2 transcription factors to regulate the expression of Clb2 cyclin, which drives progression through mitosis [10]. Sir3p was also shown to be recruited at highly expressed genes and at the coding

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regions of inducible genes, such as the GAL genes, under inducing conditions [11].

NHP6A and B are a pair of genes coding for a highly abundant architectural HMGB-like protein [12], known to bind and bend DNA in a sequence-nonspecific fashion. About 50,000–70,000 thousand molecules per haploid cells are present, an average of one molecule for every 1–2 nucleosomes. Nhp6a plays a critical role in both RNA polymerase II and III transcription, seemingly by modulating chromatin structure, through the interaction with chromatin modifying activities (reviewed in 13). Results from our group have shown that yeast cells lacking both Nhp6a and Nhp6b proteins contain a substantially reduced amount of histones and nucleosomes, suggesting a relevant role for this HMG-box protein in controlling nucleosome number in yeast [14]. Although much is known about the structure and function of the Nhp6a protein, information regarding the regulation of its gene is still poor. Nhp6a levels (both protein and RNA) are 3–10 times higher than Nhp6a and decrease strongly following *NHP6B* overexpression, demonstrating that *NHP6* gene expression is regulated by the concentration of Nhp6 proteins or RNA [15].

We chose to study the possible involvement of Sir2 in the regulation of *NHP6A* gene expression, since a survey of the genomewide analysis of Sir proteins' binding by Lieb and coworkers indicated *NHP6A* among many other targets. Since it was shown by microarrays of yeast *sir* mutant strains that, in average, gene transcription undergoes rather small variations [16], we decided to analyze the *NHP6A* transcription in conditions of *SIR2* overexpression. Our results point to a reduction in the level of the steady state transcript of *NHP6A*.

Sir2 is a phylogenetically conserved component of class III NAD+ dependent histone deacetylase, called Sirtuins [17]. The deacetylation reaction can be strongly inhibited by nicotinamide, a product of the reaction itself, which acts as a non competitive inhibitor *in vitro* [18]. On the contrary, moderate calorie restriction (0.5% vs 2% glucose) was reported to promote NAD dependent activation of *SIR2* and extend lifespan [19]. Taking all these in consideration, we further explored the role of Sir2 in the transcriptional modulation of *NHP6A* under conditions that either inhibit (use of nicotinamide) or potentiate (calorie restriction) Sir2 activity.

2. Materials and methods

2.1. Strains and growth conditions

The strains used in this study were: W303-1A (*MATa ade2-1 ura3-1his3-11 trp1-1 leu2-3,112 can1-100*); GCY137 [W303-1A/pAR44] (This study); AYH2.45 (*MATa ade2-12 his3Δ200 leu2-3, 112 lys2-801 trp1-Δ209 ura3-52 thr tyr ADH4:URA3*TELVII-L *sir3:-SIR3HA*/pRS424 (2μ *TRP*), AYH2.8 [AYH2.45 *sir3:LEU2*/pRS424 (2μ *TRP*)], STY30 [AYH2.45 *sir2:TRP1*/pRS424 (2μ *TRP*)], STY36 (AYH2.45 *sir4:TRP1*) [20].

All the strains carrying plasmid pAR44 were grown in SC medium lacking tryptophan and the carbon source used is specified in the text, where needed. The plasmid pAR44 (2μ TRP *GAL10-SIR2*) [21] was used to transform strain W303, AYH2.8 and STY36 for *SIR2* overexpression. Prior to transformation the strains AYH2.8 and STY36 were induced to lose the plasmid pRS424 by sequential subculturing in rich medium. Transformation was performed following standard procedures.

2.2. SIR2++ and NAM treatment

Cells were grown overnight in SC-TRP$^-$/Glu 3% to an A_{600} of 0.5 OD/ml, washed extensively and resuspended in SC-TRP$^-$/Gal 2%. Aliquots were taken from culture at the time points indicated in the text. For NAM experiments 5 mM Nicotinamide (Sigma) was added to the SC-TRP$^-$/Gal 2% cultures and aliquots collected as above.

2.3. Calorie restriction

Cells were grown in either YP plus 3% glucose or YP MOD (0.5% bactopeptone, 0.25% yeast extract, 220 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 70 mM KH_2PO_4) plus two different amounts of glucose: 3% (no CR), 0.5% (moderate CR). Aliquots were taken at the time points indicated in the text.

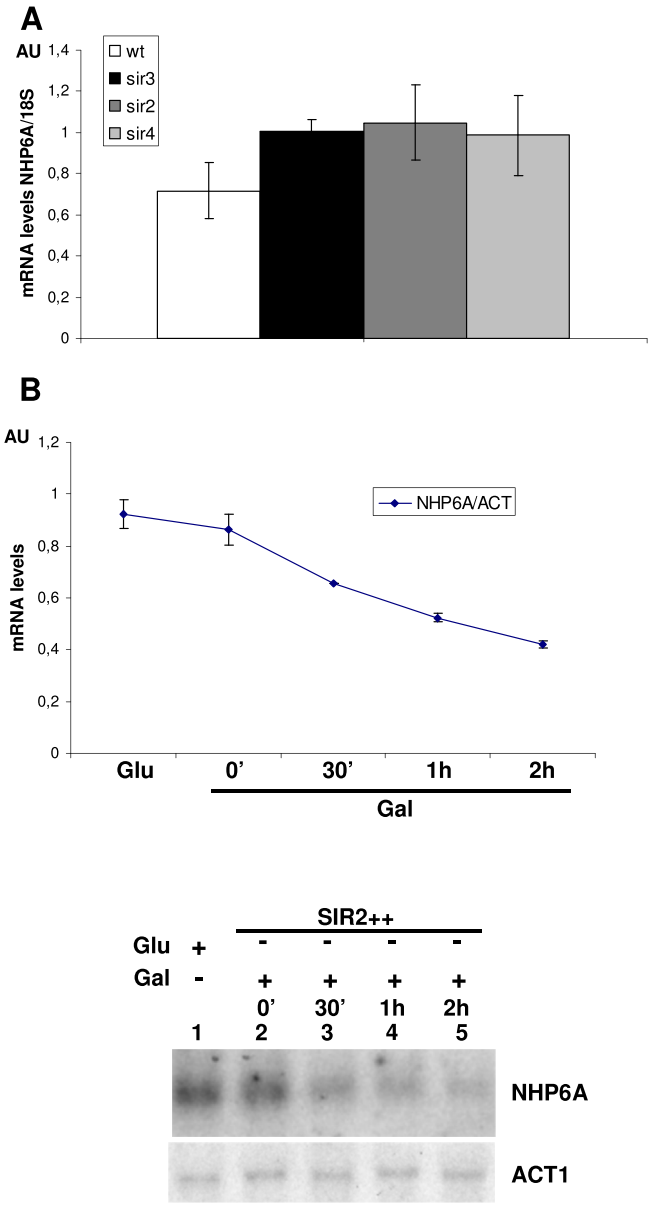

Fig. 1. Overexpression of *SIR2* modulates *NHP6A*. (A) Graphical description of *NHP6A* mRNA from *sir2, 3* and *4* deletion strains. Error bars indicate standard error from three independent experiments. (B) *NHP6A* mRNA extracted from cells containing a *GAL10* controlled episomal copy of *SIR2* (W303a/pAR44), grown in glucose, then shifted to galactose and aliquoted at the indicated time points (*SIR2*++), was analysed by northern blot. Upper panel: The quantitative data represents averages of three independent experiments. The kinetics of *NHP6A* decrease values were normalized to the expression levels of *ACT1*, after rehybridization of the same filter. Error bars indicate standard error. Lower panel: Typical *NHP6A* mRNA profile before and after carbon source shifting.

2.4. Northern analysis

The analysis of *NHP6A* mRNA was conducted using the ^{32}P-labeled oligonucleotide: 5′CCTGGGCCTTGGCCTCGTAAG3′ (*NHP6A*); *NHP6B* mRNA was detected using the ^{32}P-labeled oligonucleotide 5′CTTTGCTTCTTTAGTTGCGGC3′ (*NHP6B*); the control *ACT1* was detected using ^{32}P-random priming labeled specific probes.

3. Results and discussion

3.1. Overexpression of SIR2 modulates NHP6A transcription

ChIP-on-chip studies by Lieb and coworkers [5] first described binding of Sir2, 3 and 4 to many euchromatic loci in *S. cerevisiae*. One of the active genes identified in this screen is *NHP6A*, that codes for the most abundant architectural HMGbox protein in yeast. Although much is known about Nhp6a structure and function [13], its transcriptional regulation was not detailed so far. Given the interest of our laboratory in the chromatin functions of *NHP6A* [14], we decided to investigate the involvement of Sir2 in its expression. Consistent with earlier microarray reports [16], the northern analysis of the steady state levels of *NHP6A* mRNA in *sir2*, *3* and *4* mutant strains shows a moderate increase compared to wild type cells (Fig. 1A). This evidence led us to also examine the modulation of *NHP6A* expression in a Sir2 overproducing system. W303-1A cells were transformed with a pAR44 plasmid, bearing *SIR2* under control of the *GAL10* promoter [21,22]. The cells (W303-1A/pAR44), grown overnight in 3% glucose to the exponential phase, were shifted to a medium containing 2% galactose to allow for *SIR2* overexpression (*SIR2*++) as described [22]. Aliquots were taken at the indicated time points and total mRNA was analyzed by northern (Fig. 1B). The quantitative result drawn in the graph, normalized to *ACT1* mRNA, represents the average of three independent results (Fig. 1B, upper panel). The *NHP6A* steady state mRNA shows a reduction up to 60% starting 30 min after induction, that can be correlated with the overexpression of *SIR2* (a typical pattern is shown in Fig. 1B, lower panel). The gene appears almost completely silenced after 2 h, following the shift. The growth in galactose was not continued for more than 2 h because overexpression of Sir2 was reported to decrease cell viability [21]. The effect we observe is not carbon source sensitive because initial overnight growth in raffinose yields a similar result (Fig. 2, lower panel, lanes 1–5, *NHP6A*), although with a slower kinetics. In addition, Holmes et al. [21] demonstrated that, following GAL induction of *SIR2*: i) there is not a general shut down of Pol II and Pol III transcription, and ii) at least one other gene, *RPL16*, undergoes a twofold reduction. That the decrease of *NHP6A* mRNA is a specific event is supported by the observation that its paralog *NHP6B*, which is not expressed under standard conditions (Fig. 2, lower panel, lane 6, *NHP6B*) and

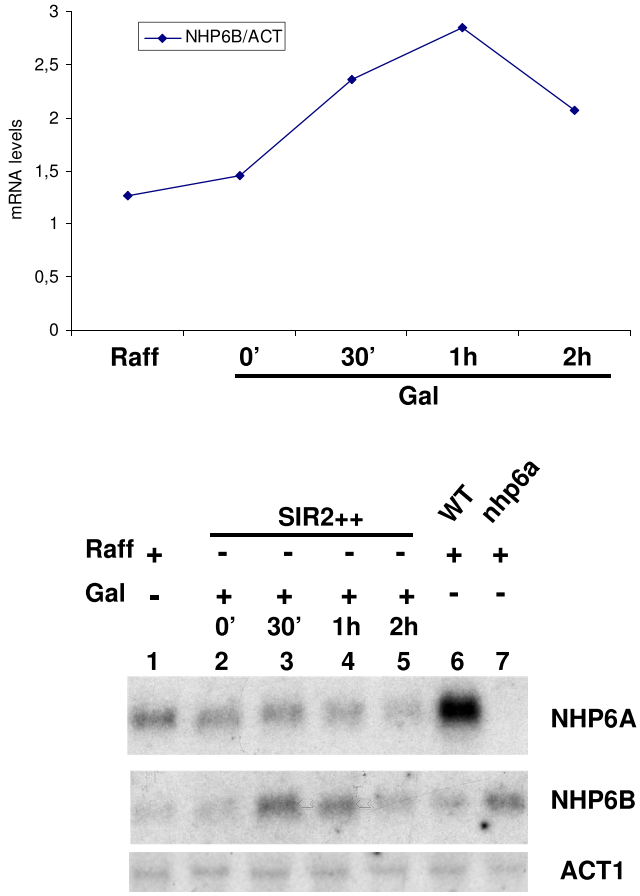

Fig. 2. Overexpression of *SIR2* modulates both *NHP6A* and B. Analysis of *NHP6A/B* mRNA extracted from cells containing a *GAL10* controlled episomal copy of *SIR2* (W303a/pAR44), grown in glucose, then shifted to raffinose, and aliquoted at the indicated time points. Upper panel: Graphical representation of *NHP6A/B* mRNA steady state levels during the shift from raffinose to galactose. Average of two independent experiments. Error bars indicate standard error. Lower panel: Typical northern profile comparison between *NHP6A* and B mRNA steady state levels.

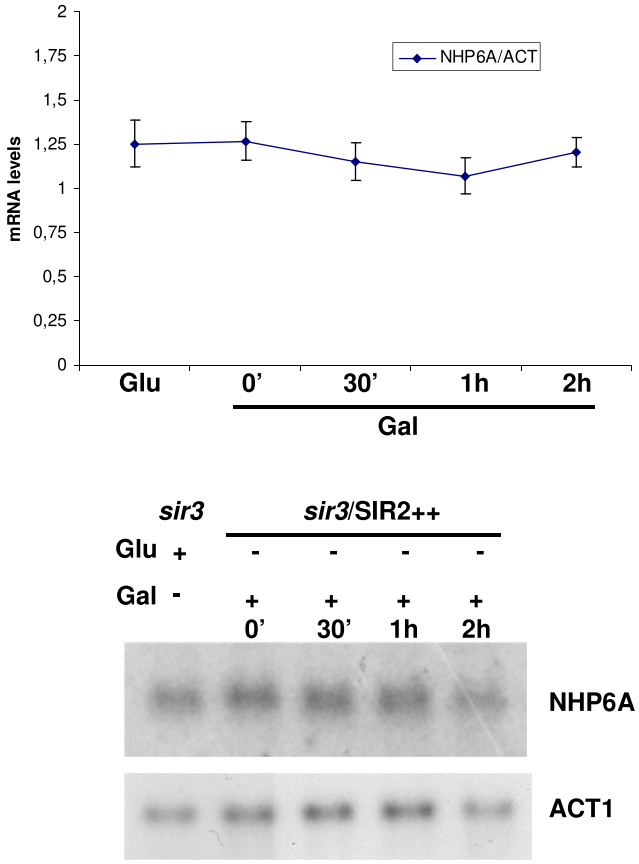

Fig. 3. The SIR complex is required for the modulation of *NHP6A* by Sir2. *NHP6A* mRNA steady state levels of a *sir3* strain transformed with pAR44, before and after shifting the culture from glucose to galactose was examined by northern blot. Upper panel: The graphical quantification shows the averages of three independent experiments and the values are normalized to the levels of *ACT1*, after rehybridization of the same filter. Error bars represent standard error. Lower panel: Typical *NHP6A* mRNA profile before and after the shifting.

becomes activated in a *nhp6a* mutant (Fig. 2, lower panel, lane 7, *NHP6B*), undergoes a significant upregulation starting 30 min after shifting, as shown in the graphical representation (Fig. 2, upper panel). Taken together, and considering that *NHP6A* mRNA increases only moderately in *sir* deletion strains, the results would suggest a role for Sir2 as a promoter-specific repressor, rather than a long-range silencer mediating repression by spreading. It is conceivable that Sir2 modulates transcription, perhaps cooperating with other transcription factors [10] and/or other chromatin modifying enzymes [9].

3.2. The SIR complex is required for the transcriptional modulation of NHP6A by Sir2

Sir2 can form complexes with Sir3/Sir4 at the silenced *HM* loci and telomeres [2], with Net1 on rDNA repeated units [23] and, in *hst1* mutants, with Sum1, a repressor of midsporulation genes [8]. Given the confinement of Net1 to the nucleolus and the specificity of Sum1 for midsporulation genes, we concentrated on the SIR complex to verify if it may be required to mediate the repressive effect of Sir2 on *NHP6A*. To answer this question we transformed the *sir3* and *sir4* deficient strains with the plasmid pAR44, and subjected the cells to the same glucose–galactose shift as for the wt strain, based on the following reasoning: if Sir2 is brought on the *NHP6A* promoter by the SIR complex, loss of Sir3p and/or Sir4p should counteract the effect of *SIR2* overexpression on *NHP6A* transcription. As shown in Fig. 3, the levels of *NHP6A* mRNA for the *sir3*/pAR44 strain (*sir3*/*SIR2*++) remain constant up to 2 h (lower panel), suggesting that Sir3p is required to support this effect. A graphical quantification of the data is reported in the upper panel of the figure, which summarizes the values of three independent experiments, normalizing the *NHP6A* mRNA levels to *ACT1*. This result is not unexpected considering that Sir3p is also recruited at highly expressed genes and at the coding regions of inducible genes, under inducing conditions [11]. A parallel experiment in which pAR44 was inserted in a *sir4* strain, yielded a similar result (not shown). These results suggest that overexpressing Sir2 in *sir3* and *sir4* mutant strain, where the principal partners of Sir2 are lacking, reverts the transcriptional effect on *NHP6A*, and is a clear indication that the whole SIR complex is involved to mediate the repressive effect of *SIR2* overexpression.

3.3. NHP6A modulation depends on SIR2 deacetylase activity

The deacetylation reaction operated by Sir2 is inhibited by nicotinamide [18]. In principle, if the deacetylase activity of Sir2 is directly involved in the observed reduction of *NHP6A* transcription, addition of nicotinamide should counteract the effect of *SIR2* overexpression, and the transcript should continue to accumulate. To test this hypothesis, we added 5 mM nicotinamide to the culture at the time of the glucose to galactose shift and collected aliquots as before (Fig. 4A). We found that the *NHP6A* transcript continues to be produced at all time points. Consistently, actin is made at a constant rate. The graph in Fig. 4A (left panel) summarizes the results of two independent experiments, supporting the hypothesis that the inhibition of *NHP6A* transcription by *SIR2* overexpression is

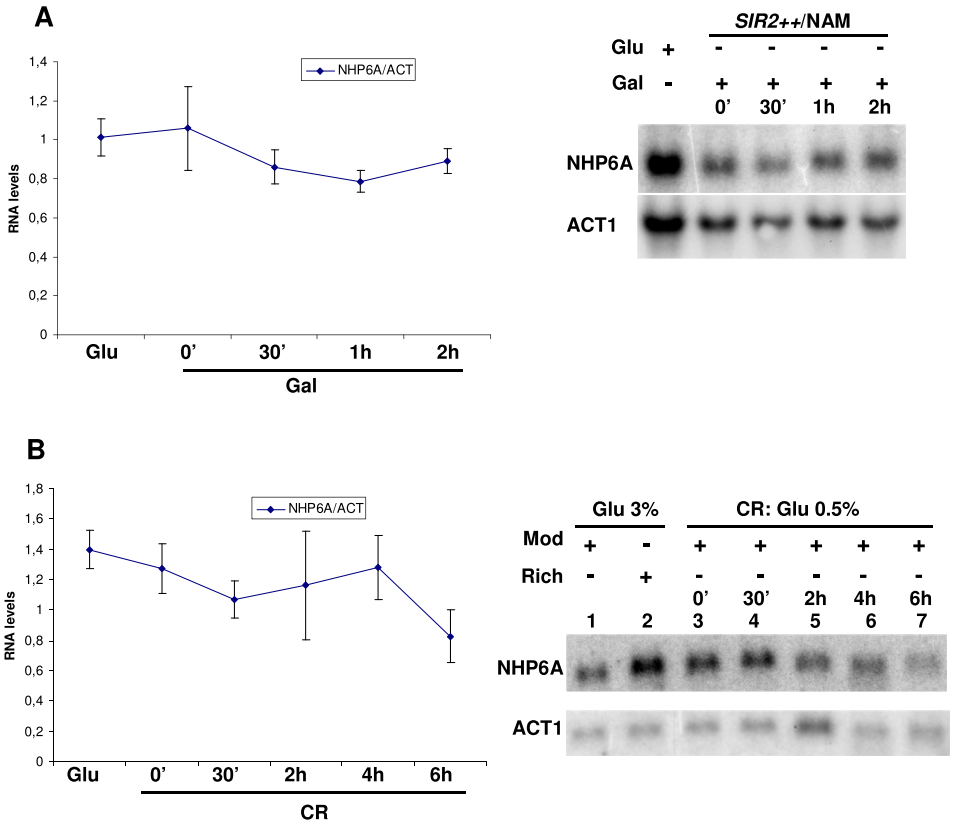

Fig. 4. *NHP6A* modulation by Sir2 is mediated by its deacetylase activity. *NHP6A* mRNA extracted from cells grown under conditions that either inhibit (nicotinamide, NAM) or potentiate (calorie restriction, CR) Sir2 enzymatic activity. (A) Left panel: The quantitative analysis represents the average of two experiments. *NHP6A* mRNA levels are normalized to actin. Right panel: Typical *NHP6A* mRNA profile following addition of 5 mM nicotinamide to the culture. Aliquots time points are indicated. (B) Left panel: Graphical representation of *NHP6A* mRNA accumulation kinetics under calorie restriction conditions. The graph shows the average of two independent experiments. *NHP6A* is normalized to *ACT1*. Right panel: Typical profile of *NHP6A* mRNA from cells subjected to calorie restriction, i.e. growth in modified medium followed by a shift from 3% to 0.05% glucose concentrations.

correlated to the enzyme's deacetylation activity. As opposed to nicotinamide, a condition of moderate calorie restriction is known to potentiate Sir2 enzymatic activity, since it induces stimulation by NAD+, the indispensable cofactor of Sir2 [19]. We, then, asked if calorie restriction could mimic the effect of *SIR2* overexpression on *NHP6A* transcription. W303 cells, grown in rich medium overnight to the exponential phase, were washed and transferred to a modified medium containing a reduced amount of nitrogen source. Moderate calorie restriction is achieved by lowering the glucose to 0.5% (CR: Glu 0.5%; Fig. 4B). Under these conditions *NHP6A* mRNA reduces over time, reaching a minimum at 6 h (Fig. 3D, right panel, lanes 3–7). The graphical scheme in the left panel of Fig. 4B shows that the effect parallels that of *SIR2* overexpression, although the kinetics of this effect appears slower. This difference could be expected considering that in *SIR2* overexpressing conditions the Sir2 activity is potentiated by having more of the enzyme itself, while in calorie restriction an optimization of the endogenous activity through a more favorable NAD+/NADH ratio is most likely occurring. To support our result, *NHP6A* mRNA from a parallel culture, grown in standard conditions is made at a constant rate (not shown). In addition, cells grown in modified medium containing a standard amount of glucose (Fig. 4B, right panel, lane 1: Mod +3% Glu), show decrease of the transcript which, in this case, is probably due to the drastic limitation of other nutrients in the medium. Taken together, all these evidences support the hypothesis that the repressive effect of *SIR2* overexpression on *NHP6A* is linked to the Sir2 deacetylase activity.

The results presented here suggest that Sir2 could act on *NHP6A* as a promoter specific repressor, rather than a long range silencer, and therefore act through different mechanisms. In this light, it is notable that Sir proteins binding at *HM* loci is permissive to pre-initiation complex assembly, but prevents mRNA capping and Pol II elongation [24], indicating that Sir2 may differentially influence accessibility of initiation and capping/elongation factors to chromatin.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

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