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ORIGINAL ARTICLE

Synergy of Hir1, Ssn6, and Snf2 global regulators is the functional determinant of a Mac1 transcriptional switch in *S. cerevisiae* copper homeostasis

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Abstract
To gain insights on the transcriptional switches that modulate proper copper homeostasis in yeast, we have examined in detail functional interactions of the relevant transcriptional activator Mac1. We identified Hir1 transcriptional repressor and histone chaperone as a Mac1-interacting protein. This association directly recruits Hir1 on a Mac1 target, *CTR1* promoter, quantitatively under induction conditions. We also found Hir1 interacting directly with a previously unknown partner, the Ssn6 (Cyc8) co-regulator. On the non-induced *CTR1* promoter, a Hir1 transcriptional activation function was revealed, in the absence of Ssn6, which was dependent on the presence of Snf2 (Swi2) nucleosome remodeler. Moreover, Ssn6 was identified as a Mac1-dependent prominent repressor of *CTR1* transcription, antagonizing Snf2 occupancy. Transcriptional induction by copper depletion was effected by the quantitative recruitment of Snf2 directed mainly by Mac1 and redundantly by the quantitatively accumulated Hir1 and Ssn6 pair. Our analysis showed that the activation-effecting chromatin remodeling of *CTR1* was due to Snf2 and not to the Hir1 histone chaperone activity or ability to regulate histone levels and stoichiometry. Following initiation, Hir1 and Snf2, but not Ssn6, were found to associate also with the actively transcribing *CTR1* coding region, where Hir1 followed the pattern of the elongating RNA polymerase II. Therefore, we have shown that, at the *CTR1* gene, in association with Mac1 DNA-binding transcriptional activator, the distinct and alternate genetic and physical collaboration of three global regulators modulates the transcriptional state of a switch involved in copper homeostasis.

Keywords *Saccharomyces cerevisiae* · Copper homeostasis · Transcriptional switch · Global regulators

Introduction

Regulated gene expression programming in response to different environmental stimuli is a complex phenomenon guided by dedicated transcription factors and a multitude of protein assemblages that activate or repress transcription (Bereketoglu et al. 2017; Grove 2018; Soontorngun 2017). Within this context, individual proteins act in various combinations and execute unique or redundant roles, functioning as switches in the framework of specific promoters or under specific physiological conditions. One such transcriptional program is executed in yeast to ensure copper homeostasis.

Copper is a catalytic cofactor for biological processes, a trace element essential for all organisms but toxic when present in excess, resulting in the production of highly reactive oxygen intermediate species (ROS). Therefore, control of copper homeostasis is a fundamental aspect of cellular metabolism, growth, development, and aging involving many regulatory proteins (Gaggelli et al. 2006; Puig and Thiele 2002; Rees and Thiele 2004; De Freitas et al. 2003; Field et al. 2002; Rustici et al. 2007; Rutherford and Bird 2004; van Bakel et al. 2005; Van Ho et al. 2002; Kusuya et al. 2017). The *Saccharomyces cerevisiae* DNA-binding transcriptional activator Mac1 plays a critical role in sensing

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changes in copper ion concentrations. Under copper deficiency conditions [in the presence of Cu(I)-chelator BCS], the high affinity copper ion uptake system is induced by the transcriptional activation of *CTR1* and *CTR3* copper transporter and *FRE1* and *FRE7* cupric reductase genes (Georgatsou and Alexandraki 1999; Martins et al. 1998; Yamaguchi-Iwai et al. 1997), while in copper replete cells [picomolar Cu(II) concentrations], expression of the uptake system is shut down (Labbe et al. 1997). A key event in both conditions is the state of the copper-binding Mac1 protein (Georgatsou et al. 1997; Graden and Winge 1997) that appears to be in the nucleus under any conditions examined (Jensen and Winge 1998; Keller et al. 2005; Serpe et al. 1999). A copper-induced intramolecular interaction between the Cys-rich motifs in the N- and C-terminal regions of Mac1 by the formation of a polycopper cluster leads to the inhibition of DNA-binding and transactivation functions of the protein (Jensen and Winge 1998).

In addition, several pieces of evidence imply that Mac1 activity is not simply modulated by copper. Mac1 appears bound on its cognate promoter sites prior to induction conditions. First, episomal Mac1 expressed from its own or heterologous promoter activates *CTR1* transcription even in the presence of copper ions (synthetic complete medium) (Voutsina et al. 2001). Second, genome-wide localization assays confirmed Mac1 binding on the *CTR1* and *FRE1* promoters in cells grown under rich medium conditions (containing copper ions) (Lee et al. 2002), and it was also suggested that inhibition of the Mac1 transactivation contributes first and more than attenuation of its DNA binding in copper excess (Keller et al. 2005). These findings suggest that the existence of other transcriptional co-regulators, in addition to copper ions, coordinates the Mac1-dependent transcription.

In this work, we searched for Mac1-interacting proteins potentially possessing repressor functions and we identified Hir1 histone chaperone, a known transcriptional repressor (Amin et al. 2013). We further investigated the role of Hir1 in Mac1-dependent transcription in the context of the *CTR1* gene. We found that Hir1 interacts with the Ssn6 co-repressor (Smith and Johnson 2000; Wong and Struhl 2011) physically and genetically. Together with the Snf2 component of the SWI/SNF nucleosome remodeler (Dimova et al. 1999), they act synergistically and determine the function of the Mac1-dependent transcriptional switch involved in copper homeostasis in *S. cerevisiae*.

Materials and methods

Yeast strains

The yeast strains used in this study are all derivatives of the FT5 (*MAT**a** ura3-52 trp1*Δ*-63 his3*Δ*200 leu2::PET56*) strain. Construction of *mac1*Δ strain (Georgatsou et al. 1997), *ssn6*Δ (*cyc8-*Δ*9::HIS3*) (Tzamarias and Struhl 1995), and *snf2*Δ (*snf2::LEU2*) (Roberts and Winston 1997) was previously described. *snf2*Δ *ssn6*Δ was generated from *snf2*Δ by replacement of the *Pst*I fragments of *SSN6* with *HIS3*. *hir1*Δ, *mac1*Δ *hir1*Δ, *ssn6*Δ *hir1*Δ, and *snf2*Δ *hir1*Δ were generated by replacement of the *Bgl*II fragment of *HIR1* with *Kan*MX4 and confirmed by phenotypic and genomic PCR analysis. C-terminal tagging of the genomic *MAC1, HIR1*, and *SNF2* genes with 9Myc or 3HA epitopes (Knop et al. 1999) was generated in wild-type and deletion mutants and verified by PCR and western blotting. The protease-deficient yeast strain BJ5465 (*MAT***a**, *ura3–52, trp1, leu2*Δ*1, his3*Δ*200, pep4::HIS3, prb1*Δ*1.6R, can1, GAL+*) was also used for preparation of protein extracts (Papamichos-Chronakis et al. 2002). PJ69-4A (*MAT***a** *trp1-901 leu2-3,112, ura3-52 his3-200 gal4*Δ *gal80*Δ UASGAL2-*ADE2 LYS2*::UASGAL1-*HIS3* met2::UASGAL7-*lacZ*), PJ69-4α (*MAT*α) (Georgakopoulos et al. 2001), and L9FT5 (Conlan et al. 1999) were used in yeast one- and two-hybrid experiments. Temperature-sensitive 7864-11-1 (*MAT***a** *trp1 leu2 ura3 his3 spt16-11*), 7973-4-4 (*MAT***a** *trp1 leu2 ura3 his3 pob3-7*), and the parental 4053-5-2 (*MAT***a** *trp1 leu2 ura3 his7*) (Formosa et al. 2002) were also used for deletion of *HIR1*.

Media and growth conditions

Standard bacterial and yeast growth media and methods were used as previously described (Ausubel et al. 1987–2003; Georgatsou et al. 1997). SC (synthetic complete medium) was supplemented with 100 μM bathocuproine disulfonic acid-Na2 salt (BCS) as Cu(I) chelator or 100 μM $CuSO_4$. For all assays described, cells were cultured in SC medium to OD_{550} of 0.6 to 1.0 and aliquots were resuspended to OD_{550} of 0.1 into the desired medium (before cell collection, SC was supplemented with BCS for 3 h or with $CuSO_4$ for 20 min).

Plasmids and primers

Hir1-HA plasmid contains a *Sma*I-*Sal*I fragment from pBTM116 HIR1 (Spector et al. 1997) filled in with Klenow polymerase, cloned in *Eco*RI site of pDB20 filled in with Klenow polymerase. The LexA-FLAG-Hir1 plasmid contains a *Sma*I-*Sal*I fragment from pBTM116 HIR1 filled in with Klenow polymerase, cloned in *Sal*I site of pDB20 filled in with Klenow polymerase, in frame with the LexA-FLAG epitopes cloned in *Eco*RI site of pDB20. FLAG epitope was cloned into the *Xho*I-*Not*I sites of pDB20 (Becker et al. 1991). Plasmids expressing GST-Ssn6, 6His-Ssn6, HA-Ssn6, and GST-Tup1 were previously described (Fragiadakis et al. 2004; Papamichos-Chronakis et al. 2002;

Tzamarias and Struhl 1995). Plasmids expressing GST-N-Hir1 and GST-C-Hir1 contain a *Sma*I-*Sal*I fragment from BTMM116 N-HIR1 and BTMM116 C-HIR1, respectively, filled in with Klenow polymerase and cloned in pGEX-4T digested with *Xma*I and filled in with Klenow polymerase. Mac1 was cloned into the centromeric *LEU2* vector pYX142 (Novagen) to generate Mac1-HA. All Mac1 derivatives used for two-hybrid and in vitro translation were previously described (Voutsina et al. 2001). Synthetic oligonucleotide primers were purchased from the Microchemistry Lab at FORTH (Greece) and MWG-Biotech (Germany). All PCR primer sequences are available on request. The polymerases used for PCR were Vent (New England Biolabs) or *Taq* (MINOTECH Biotechnology).

β-Galactosidase, one- and two-hybrid assays

In all, 5 ml of yeast cultures were grown in SC to an OD_{550} of 1.5, diluted and regrown to an OD_{550} of 0.6–1.0 in SC, and β-galactosidase activity was measured (Ausubel et al. 1987–2003). The yeast two-hybrid screens were performed against a Gal4pAD–yeast genomic library (Bilsland et al. 2004). One- and two-hybrid assays were performed as previously described (Conlan et al. 1999; Georgakopoulos et al. 2001; Voutsina et al. 2001).

RNA analysis

Total RNA from 30-ml cultures of the appropriate yeast strains was extracted using the acid phenol method (Ausubel et al. 1987–2003). RNA samples (40–50 μg) were resolved on formaldehyde-containing 1.5% agarose gels, transferred to nylon membranes and hybridized with [^{32}P]-labeled probes generated by random priming.

In vitro interaction assays

6xHis-tagged proteins were expressed in *E. coli* ER2566 (New England Biolabs) and purified by Ni-NTA chromatography according to the manufacturer's protocol (Qiagen). Bound 6His-tagged proteins were incubated with in vitro translated Mac1 protein derivatives, in the presence of ^{35}[S] Met by the TNT wheat germ system (Promega), for 4 h, at 4 °C, in 200 μl of buffer (75 mM KCl, 20 mM Tris, pH 8.00, 0.01% NP-40, 0.25% BSA) without imidazole. Beads were washed in the same buffer without BSA and retained proteins were eluted in gel loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Alternatively, 6His-tagged proteins expressed in ER2566 were purified by Ni-NTA chromatography, eluted in 75 mM KCl, 20 mM Tris, pH 8.00, 0.01% NP-40, 0.25% BSA, 250 mM imidazole and protease inhibitors and incubated with glutathione Sepharose 4B (Amersham) bead-bound GST or GST-tagged protein for 6 h, at 4 °C, in 200 μl of the above buffer without imidazole. Beads were washed with 'interaction' buffer (without BSA) and retained proteins were eluted in SDS-PAGE gel loading buffer and analyzed by SDS-PAGE and immunoblotting using anti-His [sc-804(G-18), Santa Cruz Biotechnology].

Immunoprecipitation

Cells co-expressing 9Myc- and HA-tagged proteins or HA- and FLAG-tagged proteins, respectively, were grown in SC media to an OD_{550} of 0.8. Proteins were extracted from lysed cells in 50 mM HEPES–KOH, pH 7.5, 5 mM magnesium acetate, 100 mM potassium acetate, 0.1% NP-40, 1 mM NaF, 0.5 mg/ml BSA, 10% glycerol and protease inhibitors. The supernatant was precleared with protein A (or protein G) Sepharose (Amersham) for 1 h at 4 °C, then incubated with anti-c-Myc or anti-HA rabbit polyclonal antibody (sc-789 and sc-805, Santa Cruz Biotechnology) and immunocomplexes were precipitated by incubation with protein A (or G) Sepharose for 6 h at 4 °C. Immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting using polyclonal anti-HA or monoclonal anti-FLAG (F-3165, Sigma), respectively. Secondary antibodies were visualized by an ECL Western blotting detection kit (Pierce) according to the manufacturer's protocol.

GST pull-down assays in yeast cells

Cells co-transformed with plasmids expressing GST- and HA-tagged proteins were grown to an OD_{550} of 0.8, in SC media. Proteins were extracted from lysed cells in 50 mM HEPES–KOH, pH 7.9, 5 mM magnesium acetate, 100 mM potassium acetate, 0.1% NP-40, 0.5 mg/ml BSA, 10% glycerol, and protease inhibitors. The supernatant was incubated with glutathione Sepharose beads for 6 h at 4 °C. Beads were washed four times in the same buffer without BSA. Retained proteins were eluted in gel loading buffer and analyzed by SDS-PAGE and immunoblotting, as described in (Ausubel et al. 1987–2003), using polyclonal anti-HA, anti-GST (GST sc-459, Santa Cruz Biotechnology) and anti-Tup1 (provided by M. Papamichos-Chronakis and D. Tzamarias/IMBB) antibodies.

In vivo nucleosome-remodeling assay

Chromatin analysis of *CTR1* promoter was performed by micrococcal nuclease (MNase) digestion using nystatin-permeabilized spheroplasts followed by indirect end-labeling (Fragiadakis et al. 2004). Secondary digestion was with *Sph*I at position +440 and a ^{32}P-labeled fragment from position +230 to +430 was used as a probe.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed essentially as previously described (Kuo and Allis 1999). Briefly, following formaldehyde cross-linking in living cells, chromatin was purified and subjected to sonication to obtain a mean length of DNA fragments of about 500 base pairs. Sheared chromatin was immunoprecipitated with 5–10 µl of specific antibody. After cross-link reversion and DNA recovery, specific DNA sequences were analyzed by real-time PCR and quantified by a DNA Engine Opticon System (MJ Research) for continuous fluorescence detection.

Results

Mac1 transcriptional activator physically interacts with Hir1 protein in vivo and in vitro

In search of a negative regulator of Mac1 transcriptional activity, we identified Hir1, a transcriptional co-repressor of histone genes outside of the S phase in the cell cycle (Kaufman et al. 1998; Sherwood et al. 1993; Spector et al. 1997), as one of several potentially Mac1-interacting partners in a yeast two-hybrid screen. More specifically, we detected an interaction between a truncated portion of Hir1 (residues 393 to ~800) fused to the Gal4 activation domain and the full-length Mac1 fused to the Gal4 DNA-binding domain. In a two-hybrid assay of the reverse combination using Gal4AD-Mac1 with LexA-Hir1(1–393) or LexA-Hir1(393–840), we verified that the C-terminal and not the N-terminal half of Hir1 interacted with Mac1 (Fig. 1a). Hir1 two halves correspond to separate domains that differently contribute to its co-repressor function (DeSilva et al. 1998). Mac1 protein includes an N-terminal DNA-binding copper-fist domain, two C-half transactivation domains (REPI and REPII) and a C-terminal homodimerization domain (Jensen and Winge 1998; Serpe et al. 1999). To identify the specific Mac1 domain that interacts with Hir1, we tested Mac1 derivatives and found that the N-terminal DNA-binding domain interacted with Hir1 and that this interaction was not abolished by deletion of the C-terminal REPII (transactivation) or the dimerization regions (Fig. 1a). All of the observed interactions were similar in the absence or presence of low or high copper (data not shown), indicating that Hir1 interacted with all Mac1 copper-modulated conformations. The in vivo Mac1–Hir1 interaction was further confirmed by co-immunoprecipitation of epitope-tagged proteins from yeast cellular lysates, either episomal LexA-FLAG-Hir1 and Mac1-HA or chromosomal Mac1-9Myc and Hir1-HA (Fig. 1b). Similar results were obtained when cells were grown in medium with low-copper concentration (SC-0.25 µM $CuSO_4$) or in copper-depleted medium (SCBCS) and implied that Hir1 interacted with transcriptionally active Mac1.

We finally tested in vitro translated Mac1 deletion derivatives for binding to Ni-NTA agarose beads coupled with bacterially produced 6xHis-Hir1 derivatives. Full-length Mac1 was found to interact predominantly with Hir1(393–840) and less with Hir1(1–393). Mac1(1–159) exhibited an interaction pattern similar to the full-length Mac1, while Mac1(189–417) hardly interacted with Hir1 (Fig. 1c).

Therefore, the C-half of Hir1 protein interacts directly with the N-terminal DNA-binding domain of Mac1. This is the first identified direct association between Hir1 and a DNA-binding transcriptional activator in *S. cerevisiae*.

Hir1 is recruited on the *CTR1* promoter in a Mac1-dependent manner

The identified Mac1-Hir1 association suggested that Hir1 might play a role on Mac1-regulated genes and consequently associate with Mac1 target promoters. We have chosen *CTR1* promoter to be tested for Mac1 and Hir1 occupancies, since *CTR1* gene is highly inducible and uniquely regulated by Mac1 (Yamaguchi-Iwai et al. 1997). Chromatin immunoprecipitation (ChIP) assays were performed in cells grown under high copper/repressing (SC-100 µM $CuSO_4$), low-copper/non-inducing (SC-0.25 µM $CuSO_4$) and copper-depletion/inducing conditions (SC-100 µM BCS). The low-copper condition may resemble a 'physiological' state under which multiprotein complexes are formed to mediate a baseline level of expression required for housekeeping copper uptake.

First, we found very low association of endogenous Mac1-9Myc on the *CTR1* promoter in wild-type cells in repressing conditions, higher association in non-inducing and even higher in inducing conditions as compared to background levels of binding on a *PHO5* coding region analyzed in parallel (Fig. 2a, top). Mac1-9Myc cellular protein levels were similar under all three conditions (Fig. 2a, middle), as previously reported for 3-Myc tagged Mac1 in BY4741 strain (Keller et al. 2005) and for overexpressed Mac1 (Jensen and Winge 1998). The functionality of the chimeric protein was verified by the comparable levels of *CTR1* mRNA accumulation in both wild-type and Mac1-9Myc expressing strains examined in parallel under all three conditions (Fig. 2a, bottom).

We subsequently found that endogenous Hir1-9Myc associated with the *CTR1* promoter. Its recruitment was also quantitatively increased from high copper to low copper and copper depletion conditions following Mac1 occupancy and was completely abolished in *mac1*Δ cells (Fig. 2b, top). Conversely, Mac1 recruitment on the *CTR1* promoter was similar in wild-type and *hir1*Δ cells (Fig. 2a, top). Recruitment of Hir1 on the *HTA1-HTB1* promoter,

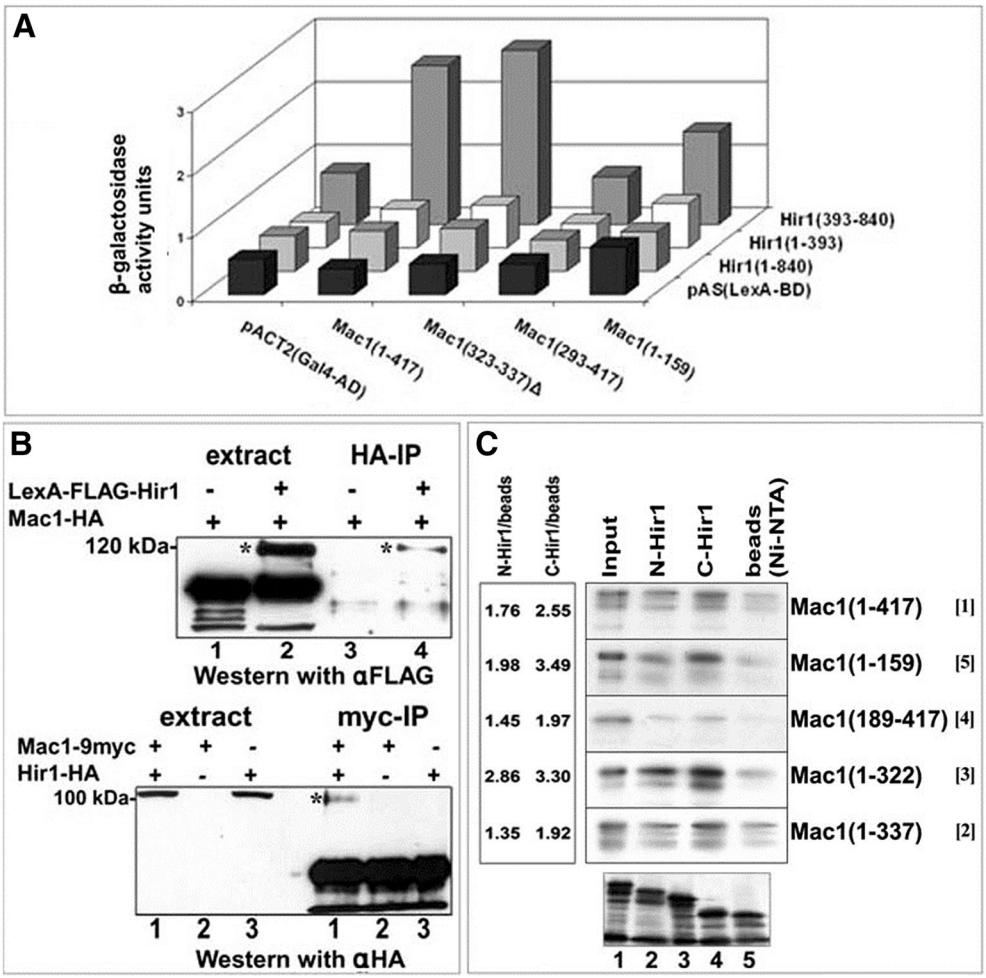

Fig. 1 Physical interaction of Hir1 with Mac1 in vivo and in vitro. **a** β-Galactosidase activity units obtained from L9FT5 yeast cells expressing the indicated pair combinations of hybrid proteins and a *lacZ* reporter in pJK103 vector, shown diagrammatically. **b** Co-immunoprecipitation of Hir1 and Mac1 in yeast cell extracts. Yeast strain BJ5465 was co-transformed with plasmids pDB20-HIR1 and pYX142-MAC1 expressing LexA-FLAG-Hir1 and Mac1-HA, respectively (top, lanes 2 and 4) and with pDB20 and pYX142-MAC1 (top, lanes 1 and 3) alone. Asterisks indicate the position of LexA-FLAG-Hir1 that migrates at a mass of ~120 kDa, detected by western blot analysis with anti-FLAG. Similar immunoprecipitation experiment using yeast FT5 strain with endogenously tagged Mac1-9Myc and Hir1-HA and western blot analysis with anti-HA (bottom, lanes 1–3). Asterisk indicates the position of Hir1-HA that migrates at a mass of ~100 kDa. **c** In vitro interaction of C-Hir1 with N-Mac1. Epitope-tagged Hir1 derivatives, N-half [6His-Hir1(1–393)] and C-half [6His-Hir1(393–840)] were purified from bacterial (ER2566) extracts by Ni-NTA agarose beads. 10 µl/50 µl of each in vitro translation reaction of Mac1 derivative (as indicated) was incubated with the immobilized N-Hir1 or C-Hir1 for 5 h at 4 °C. The material retained on the beads after washing was analyzed on a 10% SDS-PAGE and visualized by autoradiography. 1 µl/50 µl of each in vitro translation reaction was analyzed in parallel as 'Input'. Non-specific interaction of the labeled proteins with the Ni-NTA beads alone is included (lane 'beads'). Resulting bands were quantified using the PhosphorImager and ImageQuant software, and numbers on the left express the indicated ratios. Bottom insert: SDS-PAGE pattern of in vitro synthesized Mac1 derivatives (lanes 1–5)

analyzed in parallel as positive control, was high and at comparable quantities in all three culturing conditions of both wild-type and *mac1*Δ strains (Fig. 2b, bottom). Similarly, we found that Hir1 was also associated with another Mac1 regulated promoter, *FRE1* (data not shown). Therefore, Hir1 protein is recruited on at least two promoters in close and quantitative association with Mac1.

Ssn6 is also recruited on the *CTR1* promoter depending on the presence of Mac1

The association of Hir1 with the *CTR1* and *FRE1* promoters prompted us to investigate its potential collaboration with the Ssn6 co-repressor which is also recruited on *FRE1* promoter (Fragiadakis et al. 2004). Since *FRE1* promoter is regulated by both Mac1 and Aft1 (the iron-responding)

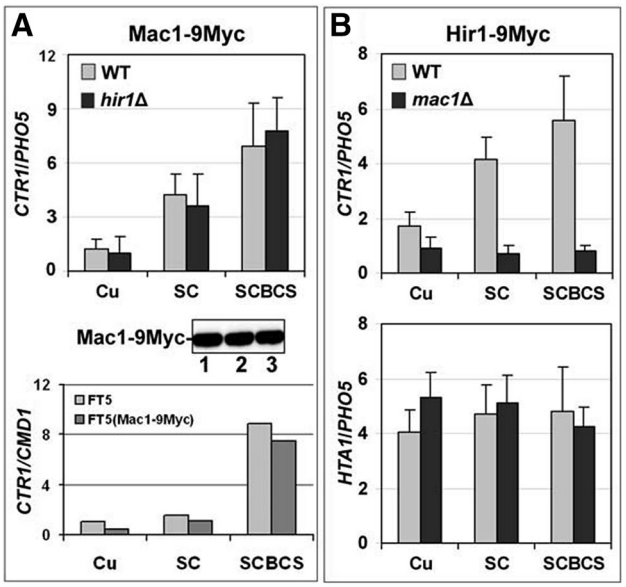

Fig. 2 Mac1-dependent Hir1 localization on the *CTR1* promoter. **a** Mac1 associates with the *CTR1* promoter under both inducing and non-inducing conditions. FT5 Mac1-9Myc cells were grown under non-inducing conditions in SC media or shifted to inducing (SCBCS) or repressing conditions (Cu). Each culture was divided into three parts and subjected to ChIP analysis (top), western blotting (middle) and Northern blotting hybridization (bottom). ChIP assays [in Mac1-9Myc (WT) and *hir1*Δ Mac1-9Myc cells] were performed, as described in "Materials and methods". Sheared chromatin was immunoprecipitated with anti-c-Myc. After cross-link reversion and DNA recovery, the *CTR1* promoter (−475 to −220) and the *PHO5* coding region (+1017 to +1220) DNA sequences were analyzed by real-time PCR. Immunoprecipitate efficiency for *CTR1* DNA relative to the *PHO5* DNA is represented by (*CTR1* immunoprecipitated DNA/input DNA)/(*PHO5* immunoprecipitated DNA/input DNA). Values are the averages of three independent experiments; error bars represent standard error of the mean. Middle panel shows endogenous Mac1-9Myc protein in the three culturing conditions (lanes 1–3) detected by immunoblotting with anti-c-Myc. Bottom panel shows the Northern blot of RNA samples from the FT5 and FT5 Mac1-9Myc cells hybridized with [^{32}P]-labeled probes corresponding to *CTR1* or to *CMD1* that was used as a loading internal control. Bands on blots were quantified using the PhosphorImager and ImageQuant software (Molecular Dynamics). Bars represent the indicated intensity ratios (normalized mRNA levels; *CMD1* mRNA levels were divided by a factor of 10). **b** Mac1-dependent association of Hir1 to *CTR1* promoter. Hir1-9Myc (WT) and *mac1*Δ Hir1-9Myc cells were grown and analyzed by ChIP assays, as described in **a**. After cross-link reversion and DNA recovery, the *CTR1* (−475 to −220) (top panel) or *HTA1* (−442 to −152) promoter-specific (bottom panel) and the *PHO5* coding region-specific (+1017 to +1220) sequences were analyzed by real-time PCR

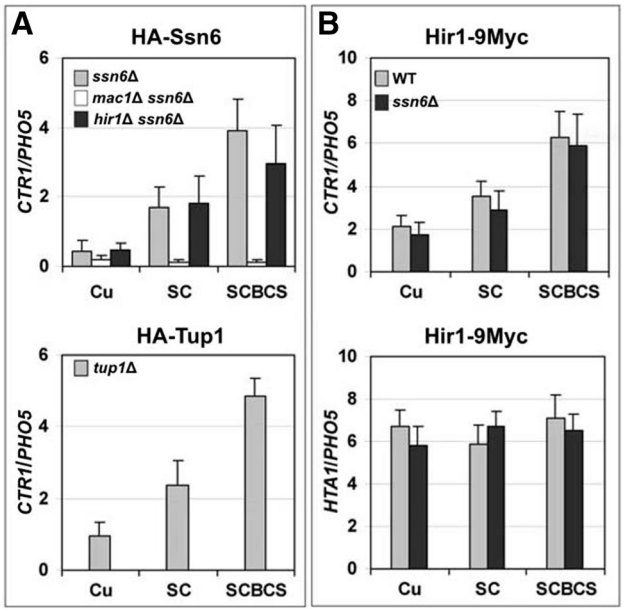

Fig. 3 Mac1-dependent and Hir1-independent Ssn6 localization on the *CTR1* promoter. **a** Indicated yeast strains transformed with either a HA-Ssn6 or a HA-Tup1 expressing plasmid were grown and analyzed by ChIP assays, as described on the legend to Fig. 2 using anti-HA and the *CTR1* promoter- and *PHO5* coding region-specific primers. **b** ChIP assays on the indicated strains with endogenously tagged Hir1-9Myc using anti-c-Myc and the *CTR1* promoter-, *HTA1* promoter- and *PHO5* coding region-specific primers

DNA-binding transcriptional activators, *CTR1* promoter offered the opportunity to investigate this collaboration in a solely Mac1-regulated system. We found that functional HA-tagged Ssn6 was marginally recruited on *CTR1* promoter under non-inducing conditions and occupancies reached maximal levels under inducing conditions, following a parallel pattern with Mac1 occupancy (Fig. 3a, top). Moreover, HA-Ssn6 did not associate with the *CTR1* promoter in a *mac1*Δ strain (Fig. 3a, top). Ssn6 is recruited to different promoters in complex with Tup1 affecting both chromatin structure and the basic transcription machinery (HDACs) (DeRisi et al. 1997; Malave and Dent 2006; Smith and Johnson 2000). We found that HA-tagged Tup1 recruited on *CTR1* promoter following similar patterns to HA-Ssn6 (Fig. 3a, bottom).

We next investigated whether Hir1 was required for Ssn6 association with the promoter. HA-Ssn6 recruitment was independent of the presence of *HIR1* (Fig. 3a, top), and similarly, Hir1-9Myc recruitment was independent of the presence of *SSN6* (Fig. 3b, top). Hir1-9Myc was recruited on *HTA1* promoter, used as an independent control, at similar quantities in both wild-type and *ssn6*Δ strains under all growth conditions (Fig. 3b, bottom). Therefore, Ssn6, similarly to Hir1, is recruited on the *CTR1* promoter only when Mac1 is present and in direct relation to its quantity and transactivation status but independently of the presence of Hir1.

Hir1 and Ssn6 interact in vivo and in vitro

Having detected all these proteins on a single promoter, we then investigated potential physical interactions that could

explain the detected interdependencies and provide functional clues. Since Ssn6 recruitment on the *CTR1* promoter was dependent on the presence of Mac1, we first tested whether these two proteins interacted physically. Although we have obtained variable results using the two-hybrid system, these were not verified by co-immunoprecipitation (data not shown). Similarly, we have not been able to coimmunoprecipitate Tup1 with Mac1 (data not shown). Interestingly enough, Hir1 and Ssn6 do interact both in vivo and in vitro. Full-length Hir1, N-terminal half Hir1 including seven WD repeats, and C-terminal half Hir1 fused to LexA were tested for a two-hybrid interaction with an Ssn6 derivative encoding the ten TPR motifs [known to be involved in distinct protein–protein interactions (Tzamarias and Struhl 1995)] fused to the Gal4AD. The C-half Hir1 interacted with Ssn6 (Fig. 4a) and this was verified by also testing a LexA-Ssn6(10TPR) with the Gal4AD-Hir1(393–800) originally isolated as Mac1 interactant (data not shown). The in vivo Hir1-Ssn6 association was fully confirmed, since episomally expressed Hir1-HA specifically copurified with episomally expressed GST-fused Ssn6 (and not with GST alone) from *ssn6*Δ *hir1*Δ cellular extracts (Fig. 4b, upper panel). The endogenous Tup1, used as positive control of the assay, also copurified as expected (Fig. 4b, lower panel). Copurification assays of bacterially expressed 6xHis-Ssn6(10TPR) protein with GST-fused N-half Hir1 and GST-fused C-half Hir1 proteins revealed a direct interaction between the C-terminal half of Hir1 and the Ssn6(10TPR) domain, while a much weaker interaction was also evident between the N-half Hir1 and Ssn6 (Fig. 4c). Similar results were obtained by testing interactions between episomally expressed 6xHis-Hir1, 6xHis-N-Hir1 and 6xHis-C-Hir1 (in pVTU260) and HA-Ssn6 in the protease minus yeast strain BJ5457 (data not shown). We concluded that Ssn6 and Hir1 have the capacity to interact on *CTR1* promoter, but this interaction is not essential for their mutual recruitment.

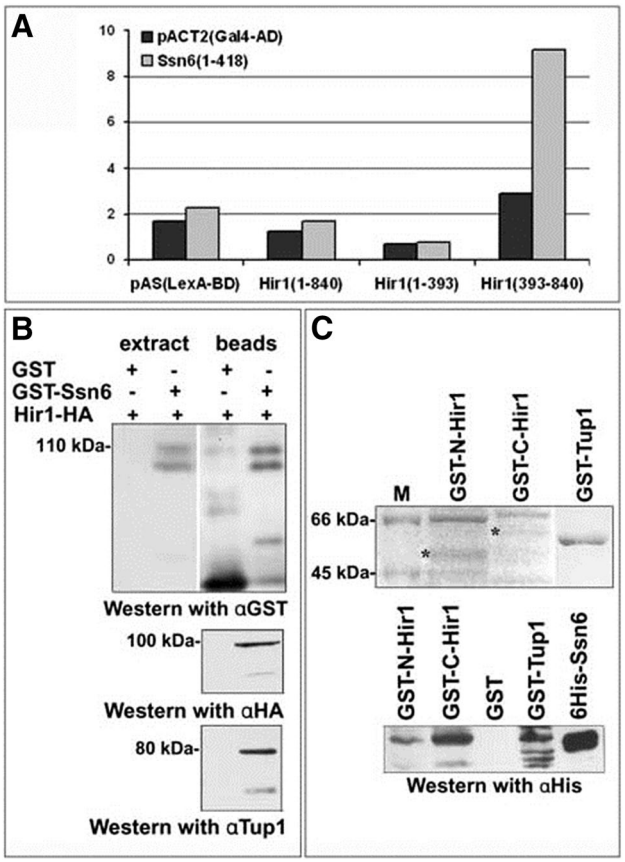

Fig. 4 Physical interaction of Hir1 with Ssn6 in vivo and in vitro. **a** β-galactosidase activity units obtained from L9FT5 yeast cells expressing the indicated pair combinations of hybrid proteins and a *lacZ* reporter in pJK103 vector, shown diagrammatically. **b** Co-immunoprecipitation of Hir1 and Ssn6 in yeast cell extracts. Yeast BJ5465 was co-transformed with plasmids expressing Hir1-HA and GST-Ssn6, respectively, or Hir1-HA and GST alone. Cell lysates were precipitated and analyzed by electrophoresis on 6% SDS-polyacrylamide gels. The presence of GST-Ssn6 (indicated by asterisks) in the immunoprecipitates was detected by western blot analysis, using anti-GST. Panels below, show the immunoprecipitated Hir1-HA using anti-HA and endogenous Tup1 using anti-Tup1, respectively, in GST-Ssn6 pulled extracts. **c** Hir1 interaction with Ssn6 in vitro. 6His-Ssn6(10 TPR) fusion protein purified from *E. coli* ER2566 was incubated with GST-N-Hir1, GST-C-Hir1, GST and GST-Tup1 (also purified from *E. coli*). Hybrid proteins, along with a molecular weight marker (M), were analyzed by SDS-PAGE (top panel). Asterisks indicate the bands corresponding to the expected molecular weight of the indicated proteins. Bound proteins were fractionated by SDS-PAGE, transferred onto nitrocellulose and probed with anti-His (bottom panel). Last lane contains 1/10 of the 6His-Ssn6 sample used for the in vitro interaction

Hir1 and Ssn6 co-regulators act together on *CTR1* expression

We further addressed the functional basis of Hir1 and Ssn6 interaction and recruitment on *CTR1* promoter. We first examined *CTR1* mRNA accumulation under non-inducing conditions, where repressors were expected to act, and found that, compared to wild-type cells, *HIR1* deletion did not significantly affect *CTR1* expression (Fig. 5, WT and *hir1*Δ lanes). Since Hir1 is known to function together with Hir2 protein (Kaufman et al. 1998) as well as the other members of the HIR complex Hir3 and Hpc2 (Amin et al. 2013), we examined the possibility of a functional redundancy on the *CTR1* promoter, but *hir1*Δ *hir2*Δ, *hir1*Δ *hir3*Δ, *hir1-3*Δ or *hir1-3*Δ *hpc2*Δ cells gave similar results to those obtained for *hir1*Δ cells (data not shown). Therefore, Hir1 alone did not satisfy our original requirement to identify a repressor of Mac1 function. On the other hand, deletion of *SSN6* gene showed about tenfold higher than wild-type *CTR1* mRNA levels under non-inducing conditions, indicating a prominent Ssn6 repression function on *CTR1*. Interestingly, the doubly deleted *hir1*Δ *ssn6*Δ strain exhibited drastically reduced

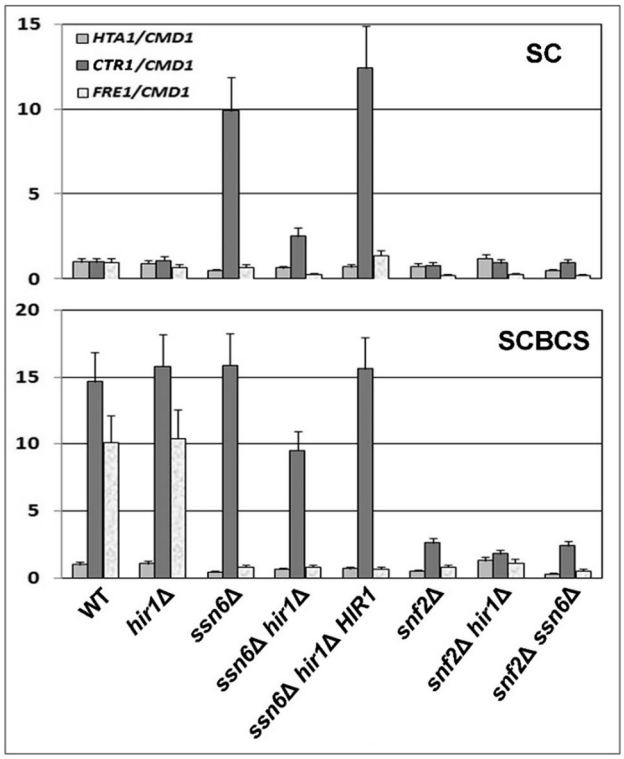

Fig. 5 Distinct and/or synthetic effects of *HIR1, SSN6* and *SNF2* on *CTR1* and *FRE1* mRNA accumulation. Northern blot analysis of total RNA extracted from wild-type (wt) and the indicated deletion strains. All strains were transformed with pDB20 vector and *ssn6*Δ *hir1*Δ *HIR1* with pDB20W-HIR1 (over-expression of LexA-FLAG-Hir1). Cells were grown under non-inducing conditions in SC media (top, SC) or shifted to inducing conditions (bottom, SCBCS). RNA samples were hybridized with [^{32}P]-labeled *CTR1, FRE1, HTA1*, and *CMD1* (internal control) probes. Bands on blots were quantified using the PhosphorImager and ImageQuant software. Bars represent the indicated intensity ratios (normalized mRNA levels; *CMD1* mRNA levels were divided by a factor of 10)

CTR1 expression compared to that of *ssn6*Δ, while over-expression of *HIR1* in the double mutant rescued the *hir1*Δ *ssn6*Δ effect resulting in high levels of *CTR1* mRNA (Fig. 5, lanes in SC). These results clearly indicated a prominent repressor function of Ssn6 under non-inducing conditions but also an essential positive role of Hir1 in the absence of Ssn6. *CTR1* mRNA levels in repressing (high copper) conditions were almost undetectable in all strains (data not shown).

A different picture emerged under inducing conditions. Wild-type maximal *CTR1* expression (16-fold higher than in non-inducing conditions) was not significantly altered in either *ssn6*Δ or *hir1*Δ strains. However, it was reduced by about 40% in *hir1*Δ *ssn6*Δ strain (Fig. 5, lanes in SCBCS). Therefore, under inducing conditions, either Hir1 or Ssn6 is required for maximal *CTR1* mRNA accumulation levels and Ssn6 did not exhibit any overt repression function. We also tested the role of *TUP1* and found *CTR1* expression unaffected in *tup1*Δ cells and somewhat reduced in *hir1*Δ *tup1*Δ strain under all examined conditions (data not shown). All of these effects are *CTR1* promoter context dependent, since Ssn6 is a predominant positive co-regulator of induced *FRE1* expression (Fig. 5 and Fragiadakis et al. 2004) directly assisting Aft1 transcriptional activator on *FRE1* promoter.

Snf2 associates with the *CTR1* promoter in a manner distinctively affected by Hir1 and Ssn6 and is an essential regulator of induced *CTR1* transcription

Our finding that under non-inducing conditions, in the absence of Ssn6, Hir1 conveys activation function on the *CTR1* promoter, prompted us to investigate the possible involvement of SWI/SNF chromatin remodeler in the Mac1-dependent transcriptional activation. Snf2 was previously shown to physically interact with Hir1 for the activation of *HTA1-HTB1* promoter at the G1/S transition of the cell cycle (Dimova et al. 1999). We first asked whether Hir1 was involved in targeting Snf2 protein on *CTR1* promoter. We found the Snf2-9Myc protein recruited on the *CTR1* promoter under all culturing conditions but at much higher quantities under induction. This association was reduced by about 70% in the absence of the *HIR1* gene (Fig. 6a). We used *HTA1-HTB1* promoter as positive control, and found Snf2-9Myc recruited and partly dependent on the presence of *HIR1* [we have not observed an absolute dependence as previously reported for the recruitment of the Snf5 SWI/SNF component (Dimova et al. 1999), either due to the different proteins tested or most probably because those experiments were performed in the absence of both *HIR1* and *HIR2* genes]. Interestingly, we have also observed reduced levels of Hir1-9Myc occupancy in *snf2*Δ cells on *CTR1* promoter (Fig. 6b). Thus, Snf2 and Hir1 are partially reciprocally dependent for their localization on the *CTR1* promoter.

To further investigate the relationships of Snf2 with Hir1 and Ssn6 regulators, we examined the localization of Snf2 on the *CTR1* promoter in the *ssn6*Δ and *hir1*Δ *ssn6*Δ strains (Fig. 6a, right). Snf2-9Myc recruitment was clearly increased in the absence of Ssn6 in both inducing and non-inducing conditions, most obvious under induction when its association is at higher amounts. This implied that Ssn6 mediates its repressor function by interfering at least partly with localization of the Snf2 remodeler. In spite of this negative Ssn6 effect, Snf2-9Myc recruitment on *CTR1* promoter was drastically reduced in *hir1*Δ *ssn6*Δ cells, (much) more than in the absence of *HIR1* alone. Therefore, while Hir1 and Ssn6 have opposite effects on Snf2 association with the *CTR1* promoter, the concomitant absence of both has a drastic negative effect.

To understand these relationships, we further examined the role of Snf2 on *CTR1* expression. Deletion of *SNF2* [that

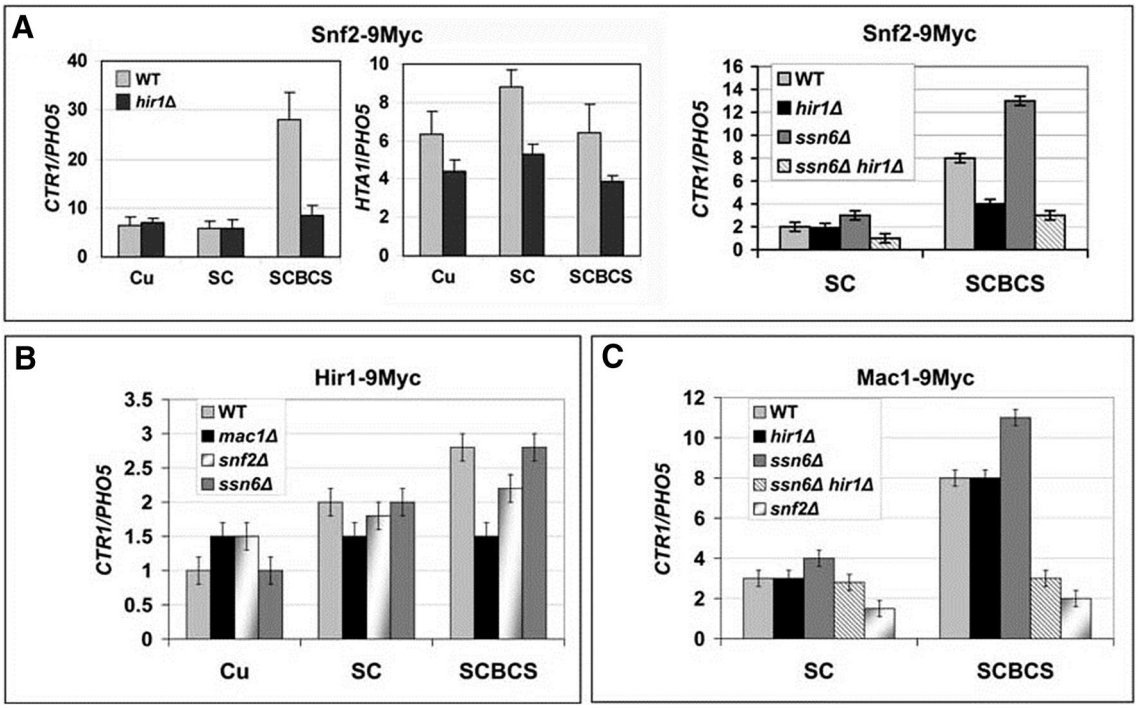

Fig. 6 Interdependencies of Snf2, Hir1, and Mac1 localization on the *CTR1* promoter. **a** Occupancy of endogenously tagged Snf2-9Myc on the *CTR1* promoter in wild type and *hir1*Δ (left and middle panels) and additionally in *ssn6*Δ and *ssn6*Δ *snf2*Δ cells (right panel) grown at the indicated conditions and analyzed by ChIP assays, as described on the legend to Fig. 2, using anti-c-Myc and the *CTR1* promoter-, *HTA1* promoter- and *PHO5* coding region-specific primers. The values shown represent the averages and standard errors from three different experiments. **b** Occupancy of Hir1-9Myc on the *CTR1* promoter in the indicated strains analyzed, as described in **a.** **c** Occupancy of Mac1-9Myc on the *CTR1* promoter in the indicated strains analyzed, as described in **a**

abolishes the primary ATPase activity and the integrity of the SWI/SNF complex (Peterson et al. 1994)], unlike *HIR1* deletion, negatively affected non-induced *CTR1* expression and drastically reduced induced expression (Fig. 5, WT, *hir1*Δ and *snf2*Δ lanes). Deletion of *HIR1* from *snf2*Δ cells did not cause any further change (Fig. 5, *snf2*Δ *hir1*Δ lanes) neither did deletion of *SSN6* (Fig. 5, *snf2*Δ *ssn6*Δ lanes) underscoring the predominant role of Snf2. Therefore, Snf2 has an essential positive role on the induced *CTR1* transcriptional activation. Moreover, even the observed reduced amounts of recruited Snf2 in *hir1*Δ cells seem to be sufficient for maximal transcriptional activation.

Snf2 is required for the activation-effecting chromatin remodeling of the *CTR1* promoter

We next examined whether a specific chromatin organization is involved in the transcriptional activation of *CTR1* gene and affected by *HIR, SSN6*, and *SNF2* deletions. We, therefore, subjected wild-type cells grown under copper-repleted or copper-depleted conditions to micrococcal nuclease digestion followed by indirect end-labeling analysis. We obtained two different patterns of nucleosomal organization on the *CTR1* promoter region corresponding to the repressed or activated state, indicating an activation-effecting chromatin change at this promoter (Fig. 7, lanes 1 and 2). Under repressing conditions, a region including the *CTR1* UAS was protected (Fig. 7, lane 1, bracket), whereas under inducing conditions, it became accessible by nuclease due to a slight nucleosome sliding effect (Fig. 7, lane 1, upper asterisk). The *CTR1* TATA box was accessible under both repressing and inducing conditions (Fig. 7, lines 1 and 2, lower asterisk), consistent with the observed relatively high basal levels of *CTR1* transcription. In *hir1*Δ, *hir1*Δ *hir3*Δ, or *hir1*Δ *ssn6*Δ cells under inducing conditions, as well as in *ssn6*Δ cells under non-inducing conditions, the *CTR1* promoter displayed an induction chromatin organization pattern (Fig. 7, lanes 3, 6, 7 and lane 5, respectively), in accordance with the fact that these mutants were found to support, more or less, *CTR1* transcription in the above conditions. The chromatin analysis results concerning *HIR*, especially, are in consistence with the genome-wide nucleosome occupancy data (Fillingham et al. 2009) which do not include *CTR1* in the set of promoter regions that become nucleosome-free in a *HIR1* deletion strain. On the other hand, in *snf2*Δ cells, the *CTR1* promoter displayed a repression chromatin organization even under inducing conditions (lane 4), consistent with the crucial role of *SNF2* gene in *CTR1* transcription.

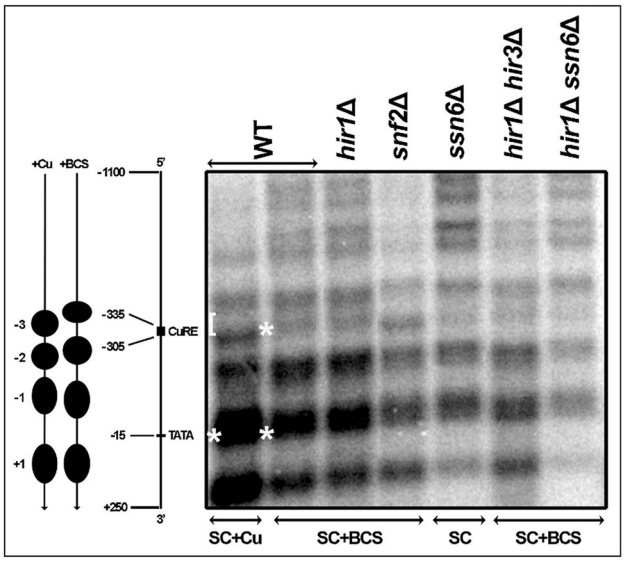

Fig. 7 Low-resolution chromatin analysis of the *CTR1* promoter. Wild-type cells under repression (SC + Cu, SC supplemented with 100 μM $CuSO_4$ 20 min prior to cell collection) as well as wild type, *hir1*Δ, *snf2*Δ, *hir1*Δ *hir3*Δ, and *hir1*Δ *ssn6*Δ under induction (SC + BCS) and *ssn6*Δ under non-inducing conditions (SC) were subjected to MNase digestion followed by indirect end-labeling. On the left, nucleosomes seen as protected areas on both the repressed (+Cu) and induced (+BCS) *CTR1* promoter are depicted as circles or ellipses. The adjacent map indicates the relative positions of the UAS_{Mac1} and the presumptive TATA box. Asterisks and bracket depict nucleosome-free and protected areas, respectively

Taken together, our chromatin analysis results reveal that a change in chromatin structure takes place at a *CTR1* promoter region upon activation due to the *SNF2* gene/protein and not to *HIR* histone chaperone activity or ability to regulate histone levels and stoichiometry.

Mac1 localization on the *CTR1* promoter is affected by the distinct or combined functions of the global regulators Snf2, Hir1 and Ssn6

Since the intracellular copper concentration does not seem to be exclusively responsible for Mac1 functionality on *CTR1* promoter, we tested the specific role of each of the examined co-regulators on the quantitative recruitment of Mac1 on *CTR1* promoter (Fig. 6c). (a) Under both non-inducing and inducing conditions, Mac1-9Myc quantitative recruitment was absolutely dependent on the Snf2 activator as was Snf2-9Myc recruitment upon the presence of Mac1. Namely, there is mutual dependence between Mac1 and Snf2 localization. (b) Mac1-9Myc localization under both conditions appeared partially inhibited by the Ssn6 repressor. (c) While Mac1-9Myc recruitment seemed unaffected in the absence of Hir1, it was significantly reduced by the concomitant absence of Ssn6 and Hir1, most prominent in inducing conditions, closely resembling the situation in the absence of Snf2. A model for the role of each examined factor in the Mac1-specific transcriptional initiation complex on *CTR1* promoter will be presented in the "Discussion".

Hir1 and Snf2 associate with the protein coding region of *CTR1* in distinct patterns-Hir1 association is altered in an *spt16-11* mutant

As deletion of *HIR1* alone did not produce any obvious phenotype on *CTR1* transcription and its role was only revealed by concomitant deletion of *SSN6*, we further investigated the role of Hir1 in *CTR1* transcriptional elongation, taking advantage of the known synthetic lethality phenotypes of *hir1*Δ with two temperature-sensitive mutations of the yFACT subunits, *spt16-11* and *pob3*-7 (Formosa et al. 2002). The heterodimeric yFACT is known to facilitate RNA Polymerase II function in a subset of genes possibly by destabilizing and then reassembling nucleosome structure biochemically acting as histone chaperone (Belotserkovskaya et al. 2004; Mason and Struhl 2003; Singer and Johnston 2004; Svejstrup 2003). *CTR1* mRNA levels were found similar in the single mutants *spt16-11* or *pob3*-7 as well as in the double mutants *hir1*Δ *spt16-11 or hir1*Δ *pob3*-7 compared to the wild type, under inducing conditions and at the non-permissive temperature (data not shown), suggesting that Hir1 is not essential in *spt16-11*or *pob3*-7 cells for the induced *CTR1* transcription.

Although a combined Hir1-yFACT essential function on *CTR1* transcription in wild-type cells was not genetically implied, the fact that Hir1 has been found to associate with transcribing coding regions (Nourani et al. 2006) led us to test several discrete regions spanning *CTR1* gene for Hir1 occupancy. We found Hir1-9Myc localized on *CTR1* coding region under induction conditions and in higher quantities than those detected on *CTR1* promoter. In non-inducing conditions, Hir1-9myc was detected at very low levels throughout the gene, and accordingly, Hir1 association was dependent on the presence of Mac1 (Fig. 8a, top). Furthermore, the observed quantitative association pattern within *CTR1* coding region, showing a peak near the 5′ end and a gradual decrease towards the 3′ end, followed the localization pattern of RNA polymerase II characteristic for that gene (Fig. 8a, bottom). Furthermore, we observed increased Hir1-9Myc occupancy on the *CTR1*-coding region in *spt16*-11 compared to wild-type cells (Fig. 8a, top). Hir1-9Myc recruitment on *CTR1* promoter was at comparable levels in both strains. This suggests that Hir1 and Spt16 may collaborate on *CTR1* gene mostly after transcriptional initiation.

In parallel to Hir1, we also looked for a possible concomitant association in the *CTR1* coding region of the other examined Hir1-interacting factors of the Mac1-specific complex. Under inducing conditions, we found Snf2-9Myc on

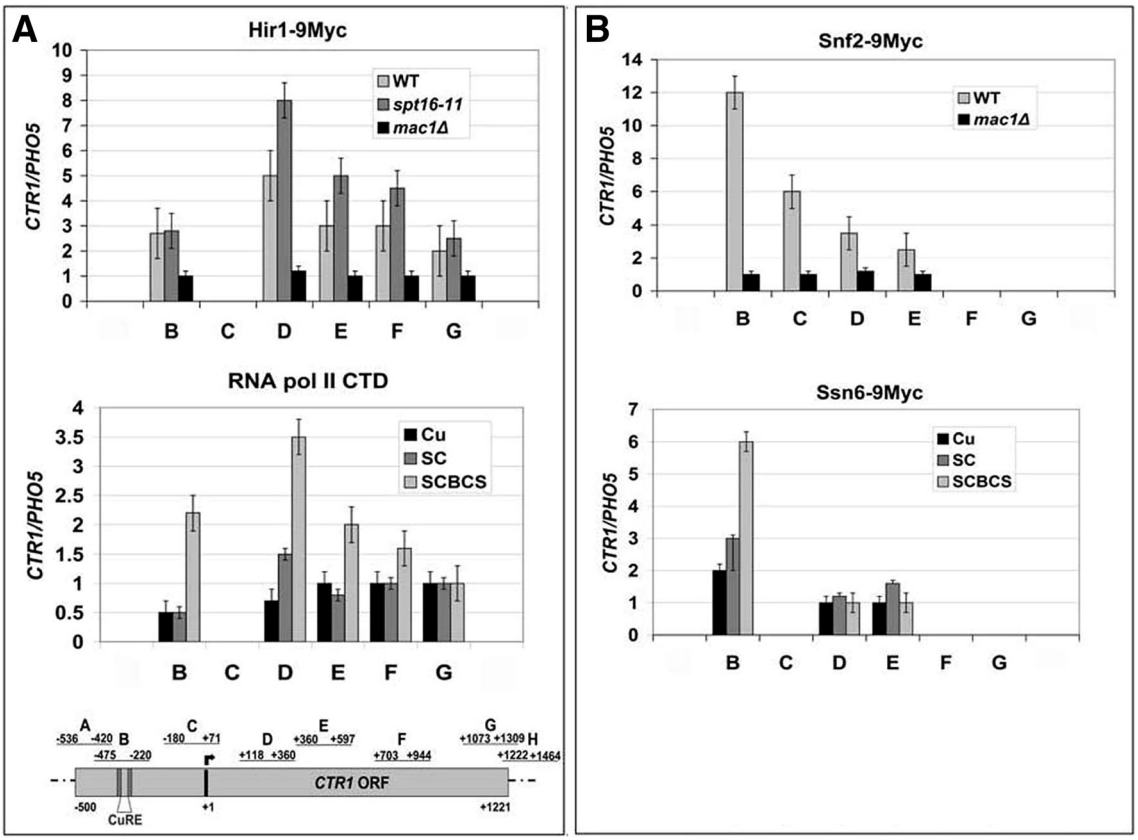

Fig. 8 Hir1 localization on the *CTR1* coding region. **a** Hir1-9Myc association with the *CTR1* promoter and coding region at the indicated strains grown in SCBCS (top) and RNA polymerase II association at three different conditions (middle) subjected to ChIP assays using anti-c-Myc or the CTD region-specific mouse monoclonal 8WG16 antibody (Covance), respectively, analyzed, as described on the legend to Fig. 2. Primers used in the real-time PCR, specific for the promoter and coding region of *CTR1*, are indicated on the diagram at the bottom. **b** Snf2-9Myc association with the *CTR1* promoter and coding region at the indicated strains grown in SCBCS (top) and Ssn6-9Myc association with the *CTR1* promoter and coding region at three different conditions (bottom) subjected to ChIP assays using anti-c-Myc

the coding region, in gradually lower amounts towards the 3′ end, and overall quantitatively less than the amount detected on *CTR1* promoter (Fig. 8b, top). Snf2-9Myc localization pattern was distinct from that of the RNA polymerase II and of Hir1. Thus, while both Snf2 and Hir1 have roles beyond transcriptional initiation, these are not necessarily overlapping. Finally, we examined Ssn6 (Fig. 8b, bottom) and Mac1 (data not shown) localizations and found them exclusively on *CTR1* promoter under all examined conditions. Thus, Hir1 and Snf2 roles in *CTR1* transcriptional elongation are distinct from their function in the initiation complex.

Discussion

Hir1 directly associates with a DNA-binding transcriptional activator in yeast, Mac1, and is specifically and quantitatively recruited on active Mac1-dependent promoters

Hir1, first identified as co-repressor of histone gene transcription and later as co-regulator of silenced loci and component of centromeric chromatin, does not possess intrinsic DNA-binding activity (Sherwood et al. 1993) but represses transcription when artificially recruited to the *HTA1* promoter (Spector and Osley 1993; Spector et al. 1997). Currently, no DNA-binding factor has been identified to specifically associate with the *HTA1-HTB1* locus cis-negative regulatory promoter site, which is known to function in a UAS- and activator-independent manner (Mei et al. 2017; Osley et al. 1986). In this work, we provide the first example in *S. cerevisiae* of a DNA-binding factor associated with

Hir1, which, interestingly, is a transcriptional activator. We have shown that Hir1 interacts with Mac1 in vivo in chromatin of cellular extracts as well as directly in vitro. In fact, this is the first example of a DNA-binding activator protein interacting directly with a histone chaperone in *S. cerevisiae*. Notably, Mac1-regulated promoters were not identified as Hir1 localization targets in a comprehensive yeast genome-wide analysis (Lee et al. 2002), possibly due to low affinity of binding in the culturing conditions used. In agreement with our findings, the mammalian HIRA protein, whose N- and C-terminal regions are most similar to the yeast Hir1 and Hir2 proteins, respectively, interacts with the developmentally regulated DNA-binding homeodomain protein Pax3 (Magnaghi et al. 1998).

Concerning Hir1, we found its least conserved C-half region interacting with the Mac1 DNA-binding region. This suggested that Hir1 could either facilitate or interfere with Mac1 binding. Nevertheless, deletion of *HIR1* alone did not reveal any repressive or essential role of Hir1 on Mac1 function in *CTR1* expression. Neither did the multiple gene deletions in *hir1*Δ *hir2*Δ, *hir1*Δ *hir3*Δ, *hir1-3*Δ or *hir1-3*Δ *hpc2*Δ mutants (our unpublished observations). Notably, we also found no Hir1 distinct involvement in *CTR1* (or *FRE1*) expression at specific phases of the cell cycle in synchronized cultures (our unpublished observations). These results are in agreement with the localization of Hir1 on the *GAL1* coding region, leaving *GAL1* expression unaffected in a *hir1*Δ strain (Nourani et al. 2006). Nonetheless, in contrast to its previously assigned role in transcriptional repression of histone genes, Hir1 interacts with Mac1 and is recruited on actively transcribed genes with no apparent repressor function.

Functional significance of the physical association between Hir1 and Ssn6 on *CTR1* promoter

The Mac1-dependent association of Ssn6 protein with *CTR1* promoter revealed the prominent repressor of the *CTR1* non-induced expression. Indeed, despite the copper-modulated "semi-closed" Mac1 conformation under non-inducing conditions, deletion of the *SSN6* gene resulted in high *CTR1* expression. Moreover, although the *hir1*Δ strain did not disclose any Hir1 function, a potentially activating Hir1 role was revealed in *hir1*Δ *ssn6*Δ cells. This synthetic effect correlated perfectly with the physical association of both proteins on *CTR1* promoter, where both are recruited in a Mac1-dependent manner. Moreover, the detected physical interaction between Hir1 and Ssn6 could be of functional significance. This is supported by the fact that under inducing conditions a synthetic activating function was observed depending on both Hir1 and Ssn6. This function might be modulated by Tup1 which is also recruited on the promoter suggesting that Ssn6 could form alternative complexes. We propose that under *CTR1* activating conditions, the Ssn6 interaction with Hir1 may be favoured over that with Tup1 and this potentiates Hir1 (and Ssn6) activating function. This hypothesis is in agreement with accumulating evidence for dual roles of transcriptional complexes switching co-repressors to coactivators depending on particular interactions on specific promoters and in response to specific metabolic signals. In fact, the Ssn6-Tup1 complex, involved in transcriptional repression of many genes, has also a positive role attributed to the Ssn6 component (Fragiadakis et al. 2004; Kliewe et al. 2017; Papamichos-Chronakis et al. 2002; Proft and Struhl 2002). Alternative interactions of Ssn6, as those hypothesized above, could provide mechanistic clues for such switches.

SWI/SNF is the essential regulator of *CTR1* transcription: interplay with Hir1, Ssn6, and Mac1 proteins

We have demonstrated that Snf2 is localized quantitatively on *CTR1* promoter in a manner primarily dependent on Mac1. Mac1 may not interact directly with Snf2 as other DNA-binding regulators do (Neely et al. 2002), but certainly associates with the SWI/SNF complex (Yoon et al. 2003). This association is of pivotal significance, since in the absence of Snf2, expression of *CTR1* is low under any environmental or genetic condition. Moreover, the observed interdependence of Mac1 and Snf2 for recruitment on *CTR1* promoter indicates the existence of a positive feedback loop that ensures maximal levels of SWI/SNF recruitment under fully induced conditions.

The activating function of Hir1 on the non-induced *CTR1* promoter, observed in the absence of Ssn6, was dependent upon the presence of Snf2, implying a Hir1-Snf2 coactivator relationship in this context. The previously reported in vitro prevention of SWI/SNF remodeling activity by the HIR/HPC complex (Prochasson et al. 2005) on one hand supports our results on the interaction of Hir1 with this complex and on the other emphasizes the importance of specific context-dependent in vivo functions. Moreover, we observed that whereas Hir1 contributes to the recruitment of Snf2 on the *CTR1* promoter, Ssn6 obstructs it, suggesting an antagonistic function. This is in agreement with findings proposing that the Ssn6-Tup1 complex acts by preventing coactivator recruitment necessary for transcriptional activation (Wong and Struhl 2011). Finally, our chromatin analysis experiments clearly showed that the Mac1 binding site on the *CTR1* promoter is not located in a nucleosome-free region but in a region that is remodeled upon activation in a SWI/SNF- (and not HIR- and/or Ssn6-) dependent manner, in consistence with studies supporting the notion that accessibility of the transcription factor binding site precedes nucleosome remodeling (Gutierrez et al. 2007).

Therefore, we propose that under non-inducing conditions, the access of "semi-closed" Mac1 to its binding site is limited, but reinforced by the chromatin remodeling inflicted by the recruited SWI/SNF complex. In addition to SWI/SNF and Hir1, Mac1 recruits the Ssn6/Tup1 complex and Ssn6, in turn, limits the function of the positive feedback loop maintaining a low transcriptional level and, at the same time, poises the promoter for prospective copper shortage. Copper depletion alters the conformation of Mac1 potentiating both its activation and DNA-binding capacities (Jensen and Winge 1998). We postulate that both enhanced functions counteract Ssn6 repressive effect, resulting in increased amounts of recruited SWI/SNF complex. In turn, the highly activated state of the *CTR1* promoter, established in this way, favours accumulation and interaction of Ssn6 with Hir1, a combination that improves the capacity of Mac1 and Hir1 to recruit SWI/SNF. This is in agreement with the observed drastic reduction in Mac1 and Snf2 occupancy on *CTR1* promoter in the *hir1*Δ *ssn6*Δ strain.

A potential role for Hir1 in *CTR1* transcriptional elongation

In addition to its role on the *CTR1* promoter, we also found Hir1 to be recruited on the *CTR1*-coding region under induction conditions. This is in agreement with the finding of Hir1 localization on the transcribing *GAL1*-coding region along with evidence, suggesting that the HIR complex functions together with Spt2 and the PAF complex to inhibit transcription initiation from a cryptic promoter within the *FLO8*-coding region (Nourani et al. 2006). Interestingly, the HIR complex (as well as its associated chaperones Asf1 and Rtt106) was found to localize only to the promoters of histone genes and is excluded from histone gene ORFs (Fillingham et al. 2009), although other elongation factors such as yFACT, Spt5, and Spt6 were seen at the coding regions of *HTA1-HTB1* (Tagami et al. 2004). At *CTR1*, Hir1 localization followed the quantitative localization pattern of the elongating RNA polymerase II implying potential involvement in transcriptional elongation. *HIR1* deletion in combination with the *spt16-1* or *pob3-7* mutations did not significantly affect *CTR1* induced mRNA levels excluding the possibility of an essential functional collaboration of Hir1 with yFACT for *CTR1*-induced transcription. However, a Hir1-yFACT interplay cannot be ruled out, as we found that Hir1 localization on *CTR1*-coding region increased in *spt16-11* cells. This could be due to an altered conformation of the temperature-sensitive spt16-11 protein resulting in increased interaction (directly or indirectly) with Hir1. Nevertheless, the potential Hir1-Spt16 (yFACT) combined role in *CTR1* transcriptional elongation is worthy of further investigation. It may be added to the list of several findings pointing to an apparently redundant function of elongation factors acting cooperatively (Saunders et al. 2006).

Ssn6, unlike Hir1, was not detected in the coding region of *CTR1*, implying that their interaction and combined function concerns only transcriptional initiation. However, Snf2 was found in the coding region of *CTR1*, in agreement with findings implicating SWI/SNF complex in histone eviction during transcriptional elongation (Schwabish and Struhl 2007) and previous results for the mouse hsp70 gene (Corey et al. 2003). Since Snf2 occupancy decreases within *CTR1*-coding region, while Hir1 increases and follows the elongating RNA Polymerase II, we propose that, through its association with Snf2, Hir1 could be delivered to elongating complexes. Therefore, Hir1 collaborates with distinct and alternating partners to affect *CTR1* transcriptional initiation, whereas it could possibly have a role in elongation.

A multitude of associations and functions for Hir1

Hir proteins are conserved histone chaperones involved in chromatin assembly and modulation at various loci, exerting essential or redundant functions (Amin et al. 2013; Loyola and Almouzni 2004; Ricketts and Marmorstein 2017). They interact with the conserved histone H3/H4-binding protein Asf1 directly (Green et al. 2005) and mediate replication-independent chromatin assembly in a conserved eukaryotic pathway for histone replacement throughout the cell cycle (Green et al. 2005; Ray-Gallet et al. 2002; Tagami et al. 2004). Numerous reports implicate them in a variety of cellular processes including transcription, heterochromatin silencing, chromosome segregation, disease, and others (Amin et al. 2013 and references therein).

In *S. cerevisiae*, Hir1 is part of the HIR/HPC complex and is not essential for growth (Green et al. 2005). Physical associations as well as synthetic genetic interactions have identified overlapping, synergistic or redundant functions for Hir1 in functionally distinct complexes (Collins et al. 2007; DeSilva et al. 1998; Dimova et al. 1999; Spector et al. 1997; Tong et al. 2004). The Hir proteins, first identified in the budding yeast as histone gene transcriptional co-repressors (Sherwood et al. 1993; Spector et al. 1997), have been reported to prevent the remodeling activity of the SWI/SNF complex through interaction with nucleosomes (Prochasson et al. 2005). During transcription, they are important for histone deposition mostly on promoters, along with the Asf1 histone chaperone (Kim et al. 2007) or the SWI/SNF complex (Schermer et al. 2005). Moreover, they have been implicated in the assembly and maintenance of heterochromatin in telomeres and mating type loci (Kaufman et al. 1998; Sharp et al. 2001), as well as the formation of kinetochore chromatin (Sharp et al. 2002) and proper chromosome segregation (Ciftci-Yilmaz et al. 2018).

Besides the *S. cerevisiae* histone genes, few other genes or genetic pathways have been reported to be transcriptionally regulated predominantly by the Hir proteins in other organisms. In the fission yeast, the Hir1 homolog has been implicated in the Atf transcription factor-dependent transcriptional regulation of low-dose stress response (Chujo et al. 2012), while the *C. albicans* respective homolog mediates fine tuning of gene transcription that is crucial for driving morphogenetic conversions in this fungal pathogen (Jenull et al. 2017). The N-terminal and C-terminal halves of chicken HIRA have been found to transcriptionally regulate different sets of cell-cycle-related genes that control vertebrate cell growth (Ahmad et al. 2005), whereas in higher eukaryotes, HIRA is bound and recruited to transcriptionally active genes by the DNA-binding protein RPA (replication protein A), where it mediates deposition of newly synthesized H3.3 variant histones necessary for regulation of gene transcription (Zhang et al. 2017).

In this work, we report the copper transporter encoding *CTR1* as a new *S. cerevisiae* gene whose transcription is regulated by the HIR complex. This regulation is exerted through a mechanism dissimilar to that of the histone genes, reviewed by (Eriksson et al. 2012), indicative of an alternative way for HIR functioning in a different DNA context and in response to different stimuli. This mechanism ensures the fine tuning modulation rather than the strict and inflexible on/off regulation of the copper uptake switch in *S. cerevisiae*, given that the cell must be able to rapidly and effectively adjust its copper levels according to the continuously varying environmental metal availability rather than merely respond to conditions of extreme metal depletion or repletion usually imposed in the lab but not often met in real life. In the *CTR1* gene context, Hir1 is the key component accomplishing this particular modulation via its synergy with Ssn6 co-repressor. Hir1 and Ssn6, localizing quantitatively and interacting physically on the *CTR1* promoter, antagonize continuously against each other to promote (Hir1) or prevent (Ssn6) the recruitment of Snf2 nucleosome remodeler. The balance of these two opposing activities regulates the local chromatin structure and determines the extent of transcriptional activation. Therefore, the Hir1/Ssn6 co-regulator complex, together with Mac1 activator, establishes a dynamic interplay on the *CTR1* promoter that senses cellular copper levels and modulates Snf2 recruitment and nucleosome-remodeling activity accordingly. To our knowledge, this DNA-binding regulator-associated Hir1/Ssn6 interplay is novel. Another distinctive point of this mechanism is that, in the particular context, Hir1 is fully committed to the recruitment of Snf2-dependent activity instead of causing nucleosome eviction directly through its chaperone and/or transcriptional activity on histones. This is in contrast to what has been reported for the fission yeast Hir1 homolog (Chujo et al. 2012) and with the findings that the budding yeast HIR complex obstructs the remodeling activity of the SWI/SNF complex by interacting with the nucleosomes in histone gene loci (Prochasson et al. 2005). Finally, a potential role of Hir1 in *CTR1* transcriptional elongation is implied by our observation that it localizes on *CTR1* coding region along with RNA Pol II, in an *spt16-11*-affected manner.

We have, thus, identified new associations and transcriptional functions for Hir1 protein. The interaction with Mac1 suggests a mechanism for Hir1 recruitment on specific promoters and the physical and genetic interactions with Ssn6 reveal a new role for Hir1 as a molecular safety switch operating in conjunction with chromatin-remodeling complexes. The totality of our evidence suggests a simple model depicting the copper availability-dependent *CTR1* transcriptional regulation (Fig. 9). The precise contributions of Hir1 and its alternative partners in different gene contexts and circumstances remain to be studied. Nevertheless, it seems that redundancy and chromatin are the common denominators of all proposed functions for the yeast Hir1. The former reassures robustness in cellular life whereas the latter reveals the capacity of living systems to utilize single functions to effect context-dependent activities.

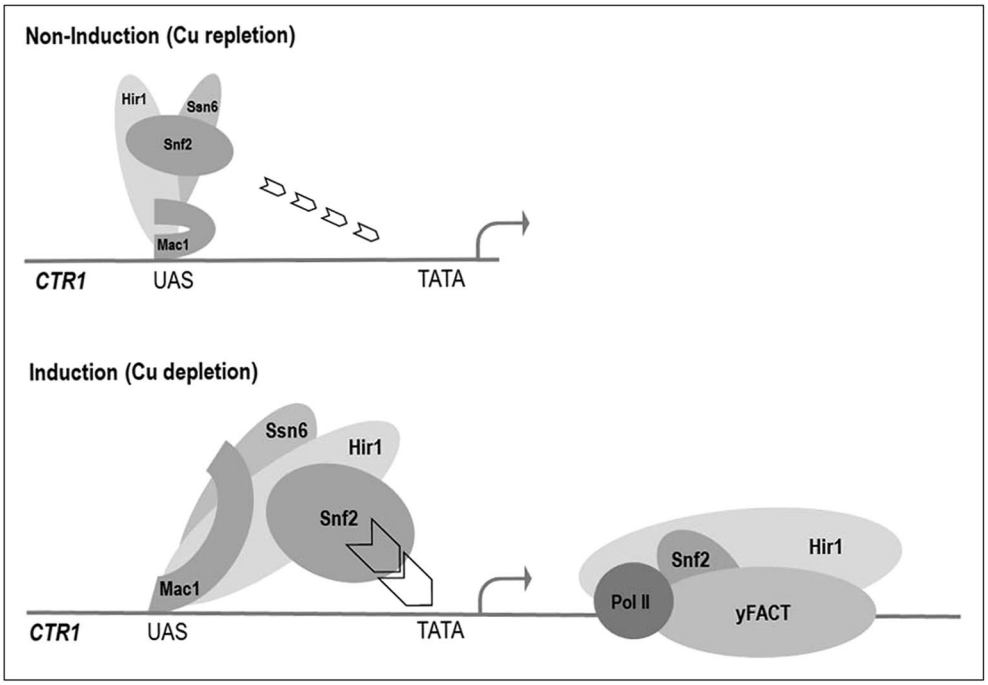

Fig. 9 Model of protein modulation of the Mac1/copper transcriptional switch. The SWI/SNF complex is recruited on the *CTR1* promoter by both Mac1 and Hir1 proteins and this initiates a positive feedback loop that results in further Mac1, Hir1 and SWI/SNF recruitment. In copper repletion (non-induction), this loop is limited by Ssn6, also recruited in a Mac1-dependent manner, and thus transcription is maintained at low levels. In copper depletion (induction), both Mac1 and Hir1 acquire new properties, through the structural alteration of Mac1 as well as the redundant structural/functional modification of the Hir1/Ssn6 complex, resulting in a more efficient antagonism of the Ssn6 function for SWI/SNF recruitment. Hir1 and Snf2 were also found in the coding region of *CTR1*. As Snf2 occupancy decreases within the *CTR1* coding region, while Hir1 increases, following the elongating RNA polymerase II, Hir1 may be delivered to elongating complexes through its association with Snf2. Differences in the relative size of the same protein depicted in the three shown complexes reflect its respective amount

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Compliance with ethical standards

Ethical statement The authors declare that (a) the research presented in this manuscript does not contain any fabricated or manipulated data and has not been submitted to another journal and (b) they have no conflict of interest.

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