Molecular and functional characterization of the only known hemiascomycete ortholog of the carboxyl terminus of Hsc70-interacting protein CHIP in the yeast *Yarrowia lipolytica*

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Abstract The carboxyl terminus of Hsc70-interacting protein (CHIP) is an Hsp70 co-chaperone and a U-box ubiquitin ligase that plays a crucial role in protein quality control in higher eukaryotes. The yeast *Yarrowia lipolytica* is the only known hemiascomycete where a CHIP ortholog is found. Here, we characterize *Y. lipolytica*'s CHIP ortholog (*Yl.Chn1p*) and document its interactions with components of the protein quality control machinery. We show that *Yl.Chn1p* is non-essential unless *Y. lipolytica* is

severely stressed. We sought for genetic interactions among key components of the *Y. lipolytica* protein quality control arsenal, including members of the Ssa-family of Hsp70 molecular chaperones, the *Yl.Bag1p* Hsp70 nucleotide exchange factor, the *Yl.Chn1p* and *Yl.Ufd2p* U-box ubiquitin ligases, the *Yl.Doa10p* and *Yl.Hrd1p* RING-finger ubiquitin ligases, and the *Yl.Hsp104p* disaggregating molecular chaperone. Remarkably, no synthetic phenotypes were observed among null alleles of the corresponding genes in most cases, suggesting that overlapping pathways efficiently act to enable *Y. lipolytica* cells to survive under harsh conditions. *Yl.Chn1p* interacts with mammalian and *Saccharomyces cerevisiae* members of the Hsp70 family in vitro, and these interactions are differently regulated by Hsp70 co-chaperones. We demonstrate notably that *Yl.Chn1p*/Ssa1p interaction is Fes1p-dependent and the formation of an *Yl.Chn1p*/Ssa1p/Sse1p ternary complex. Finally, we show that, similar to Sse1p, *Yl.Chn1p* can act as a “holdase” to prevent the aggregation of a heat-denatured protein.

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Introduction

The maintenance of protein homeostasis within the cell relies on a complex quality control (QC) system that assists the folding of newly synthesized proteins, prevents protein misfolding and aggregation, and targets aberrant polypeptides for degradation (Kostova and Wolf 2003; Kubota 2009). Molecular chaperones of the Hsp70 family, in association with their co-chaperones such as Hsp40s or nucleotide

exchange factors (NEFs), and the ubiquitin–proteasome system (UPS) constitute the main components of this QC system (Kabani 2009; Kabani and Martineau 2008; Buchberger et al. 2010).

When the QC system faces massive protein misfolding and aggregation, for example after a severe heat shock, molecular chaperones may become overburdened despite their upregulation via the induction of the heat shock response (HSR) or the unfolded protein response (UPR) in the cytosol and the endoplasmic reticulum (ER), respectively (Buchberger et al. 2010). Prolonged protein misfolding can lead to protein aggregation and the formation of toxic species that are the hallmarks of a variety of conformational diseases such as Alzheimer's, Parkinson's, or Huntington's diseases (Carrell and Lomas 1997; Gidalevitz et al. 2010). Therefore, it is crucial that molecular triage mechanisms select those misfolded proteins that have a low probability of folding to the native state and target them for degradation or address them to specialized subcellular disposal compartments (Tyedmers et al. 2010). The partitioning of a particular polypeptide into the (re)folding or degradation pathways depends on many parameters, including its folding rate, its aggregation propensity, and its interactions with various molecular chaperones and co-chaperones that will often compete to tip the balance toward one path or the other (Buchberger et al. 2010).

In higher eukaryotes, the carboxyl terminus of Hsc70-interacting protein (CHIP) plays an important role in QC by providing a link between the protein folding pathway and the UPS. On one hand, CHIP is able to interact with the C-terminal EEVD motif of Hsp70 and Hsp90 molecular chaperones via its N-terminal tetratricopeptide repeat (TPR) domain (Ballinger et al. 1999; Meacham et al. 2001; Connell et al. 2001). On the other hand, CHIP has an ubiquitin ligase activity mediated by its C-terminal U-box domain (Murata et al. 2001; Hatakeyama et al. 2001; Demand et al. 2001; Jiang et al. 2001). U-box ubiquitin ligases (e.g., Ufd2p in yeast) constitute a distinct class of ubiquitin ligases that differ from the RING domain (e.g., Hrd1p and Doa10p in yeast) or HECT-domain (e.g., Rsp5p in yeast) containing ubiquitin ligases by their unique ability to catalyze the multi-ubiquitination of client proteins (Hatakeyama and Nakayama 2003). A coiled-coil domain between the TPR and U-box domains mediates the dimerization of CHIP, which is required for its activity (Nikolay et al. 2004; Zhang et al. 2005). CHIP was shown to be capable of multi-ubiquitinating client proteins bound to molecular chaperones and to have an intrinsic heat stress-activated chaperone activity (Cyr et al. 2002; Esser et al. 2004; Rosser et al. 2007). CHIP was also shown to suppress the aggregation and toxicity of expanded polyglutamine proteins (e.g., huntingtin, ataxin-3) and alpha-

synuclein, and in some instances it was also shown to facilitate their ubiquitination and clearance from the cell (Jana et al. 2005; Miller et al. 2005; Shin et al. 2005; Al-Ramahi et al. 2006; Williams et al. 2009; Tetzlaff et al. 2008a, b). The expression of CHIP is induced by a variety of stress conditions, including heat shock (Dikshit and Jana 2007). At the organismal level, CHIP was shown to play important roles in protein quality control, stress resistance, development, and aging in several cellular, animal, or plant models including human cells, mice, *Caenorhabditis elegans*, and *Arabidopsis thaliana* (Dai et al. 2003; Khan and Nukina 2004; Yan et al. 2003; Qian et al. 2006; Min et al. 2008).

While orthologs of CHIP are found in many filamentous fungi such as the *Aspergillus* genera or *Neurospora crassa*, they are absent from all hemiascomycetous yeasts for which complete genome sequences are available to date, with the exception of the yeast *Yarrowia lipolytica* (Kabani and Martineau 2008). The molecular and functional characterization of the *Y. lipolytica* CHIP ortholog constitutes the main focus of the present work and it is, to our knowledge, the first description of the physiological role of CHIP in a lower eukaryote.

Materials and methods

Yeast strains and media

The *Y. lipolytica* strains used in this study are listed in Table 1 and derive from the wild-type *YL136463* strain (*MatB*, *scr1::ADE1*, *leu2-35*, *his-1*, *ura3*; He et al. 1989). Yeast strains were propagated on YPD medium (10 g/L yeast extract, 20 g/L bacto-peptone, 20 g/L glucose) or minimal medium (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose) with appropriate supplements.

Cloning of the *YLCHN1* cDNA by RT-PCR

Total RNA was prepared from 50 mL cultures of a wild-type *Y. lipolytica* strain grown at 28°C to exponential phase using QIAGEN RNeasy kit. RNA preparations were devoid of genomic DNA contamination as standard PCR analysis failed to detect amplicons using the primers and conditions of the reverse transcription polymerase chain reaction (RT-PCR) reactions described below. RT reactions were made using 1 µg of RNA, ready-to-go RT-PCR beads and were primed with an oligo-dT oligonucleotide according to the manufacturer's instructions (Amersham Pharmacia Biotech). PCR amplification was then performed for 35 cycles using an annealing temperature of 55°C and according to the manufacturer's instructions (Amersham Pharmacia Biotech). The primers used for the RT-PCR analysis were

Table 1 *Y. lipolytica* strains used in this study

Strains	Genotype
Wild-type	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302</i>
$\Delta chn1$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1</i>
$\Delta ufd2$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta ufd2</i>
$\Delta hrd1$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta hrd1</i>
$\Delta doa10$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta doa10</i>
$\Delta chn1 \Delta ufd2$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1, \Delta ufd2</i>
$\Delta chn1 \Delta hrd1$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1, \Delta hrd1</i>
$\Delta chn1 \Delta doa10$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1, \Delta doa10</i>
$\Delta ufd2 \Delta hrd1$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta ufd2, \Delta hrd1</i>
$\Delta ufd2 \Delta doa10$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta ufd2, \Delta doa10</i>
$\Delta chn1 \Delta ufd2 \Delta hrd1$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1, \Delta ufd2, \Delta hrd1</i>
$\Delta chn1 \Delta ufd2 \Delta doa10$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1, \Delta ufd2, \Delta doa10</i>
$\Delta ssa5$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta ssa5</i>
$\Delta ssa6$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta ssa6</i>
$\Delta ssa7$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta ssa7</i>
$\Delta ssa8$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta ssa8</i>
$\Delta chn1 \Delta ssa5$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1, \Delta ssa5</i>
$\Delta chn1 \Delta ssa6$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1, \Delta ssa6</i>
$\Delta chn1 \Delta ssa7$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1, \Delta ssa7</i>
$\Delta chn1 \Delta ssa8$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta chn1, \Delta ssa8</i>
$\Delta bag1$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta bag1</i>
$\Delta bag1 \Delta ssa5$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta bag1, \Delta ssa5</i>
$\Delta bag1 \Delta ssa6$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta bag1, \Delta ssa6</i>
$\Delta bag1 \Delta ssa7$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta bag1, \Delta ssa7</i>
$\Delta bag1 \Delta ssa8$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta bag1, \Delta ssa8</i>
$\Delta hsp104$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta hsp104</i>
$\Delta hsp104 \Delta ssa5$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta hsp104, \Delta ssa5</i>
$\Delta hsp104 \Delta ssa6$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta hsp104, \Delta ssa6</i>
$\Delta hsp104 \Delta ssa7$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta hsp104, \Delta ssa7</i>
$\Delta hsp104 \Delta ssa8$	<i>MatB, scr1::ADE1, SCR2, his-1, leu2-35, ura3-302, \Delta hsp104, \Delta ssa8</i>

CHN1-4 (tttgttccgactagctagcctgc) and CHN1-3 (tgtgcaagtggattcatatggctcc) that anneal ~20 bp upstream the start and downstream the stop codons of the *Yl.CHN1* open reading frame, respectively. Several independent RT-PCR reactions were cloned in pBluescript II SK- and sequenced.

Gene disruptions

The *Yl.CHN1* was disrupted using the “pop-in/pop-out” method (Rothstein 1991). A ~500 bp *Bam*HI-*Not*I fragment and a ~1,700 bp *Not*I-*Cla*I fragment corresponding to the regions immediately upstream the ATG codon and downstream the stop codon of the *Yl.CHN1* open reading frame, respectively, were obtained by PCR. These two fragments were digested with *Bam*HI/*Not*I and *Not*I/*Cla*I, respectively, and cloned in the integrative *URA3*-based plasmid pINA300' digested by *Bam*HI and *Cla*I. The resulting plasmid was linearized by *Bgl*III in the terminator region

and transformed into wild-type *Y. lipolytica* cells. The correct integration of the plasmid at the *Yl.CHN1* locus (“pop-in”) was verified by PCR and Southern blotting. Plasmid excision accompanied by the loss of the wild type or the disrupted allele of *Yl.CHN1* occurred by homologous recombination between promoter or terminator regions (“pop-out”) and was selected by plating the cells on 5-FOA-containing medium. The isolated [*ura*⁻] colonies were analyzed by PCR and Southern blotting for the disruption of the *Yl.CHN1* gene.

The *Yl.UFD2*, *Yl.HSP104*, *Yl.SSA5-8*, *Yl.BAG1*, *Yl.HRD1*, and *Yl.DOA10* genes were disrupted using the method described in Fickers et al. (2003). Briefly, ~1 kb fragments of promoter (P) and terminator (T) regions were amplified by PCR. The P and T fragments were annealed via a common I-*Sce*I site, and a ~2 kb P-I-*Sce*I-T fragment was amplified by PCR. This fragment was then cloned as a blunt-ended fragment into *Eco*RV-digested and dephos-

phorylated pBluescript II SK- plasmid, resulting into plasmid pBSK-PT. The *hph* hygromycin resistance gene was recovered from the JMP115 plasmid (Fickers et al. 2003) by *I-SceI* digestion and cloned at the *I-SceI* site of pBSK-PT. The P-*hph*-T disruption cassette was then excised from the plasmid by restriction enzyme digestion and transformed into the appropriate recipient cells. Transformants were selected on hygromycin B-containing YPD plates and analyzed by PCR and Southern blotting. The *hph* hygromycin resistance gene was subsequently removed via Cre-*lox* recombination as described in Fickers et al. (2003). In all cases, at least two independent clones were obtained and analyzed.

Protein purification

To purify *Yl.Chn1p* as a glutathione S-transferase (GST) fusion protein, the *Yl.CHN1* cDNA was cloned into plasmid pGEX-5X-1 (Amersham Pharmacia Biotech). The resulting pGST-CHN1 plasmid was then transformed into *Escherichia coli* BL21(DE3) cells. Fifty milliliters of culture was grown in LB medium containing 100 mg/L of ampicillin overnight at 28°C and diluted in 2 L of the same medium. After 2 h at 28°C, IPTG (isopropyl- β -D-thiogalactopyranoside) was added to a final concentration of 1 mM, and cells were grown for an additional 3 h. Cells were harvested and washed once in water and once in phosphate-buffered saline (PBS, pH 7.4), and the cell pellet was then snap frozen in liquid nitrogen and stored at -80°C until use. The cell pellet was thawed and resuspended in 20 mL of sonication buffer (PBS pH 7.4, 2 mM EDTA, 1 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, antiprotease cocktail; Roche). The cells were sonicated for 30 s six times at a high setting, with 2 min on ice between sonications. The lysate was centrifuged at 30,000 rpm in a Beckman 70 Ti rotor. The cleared lysate was loaded on a 5 mL glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) equilibrated in sonication buffer containing 1% Triton X-100. The column was washed sequentially with 30 mL of (1) sonication buffer; (2) sonication buffer containing 1 M NaCl; (3) 50 mM tris-Cl pH 7.5, 10 mM magnesium acetate, 200 mM potassium acetate, 2 mM adenosine-5'-triphosphate (ATP); and (4) PBS pH 7.4. GST-*Yl.Chn1p* was eluted with 10 mL of elution buffer (50 mM tris-Cl pH 8.0, 50 mM NaCl, 20 mM reduced glutathione, 5% glycerol) and 1 mL aliquots were recovered. Peak fractions were pooled, dialyzed against 10 mM Hepes-OH pH 7.4, 50 mM KCl, 10 mM dithiothreitol, 5% glycerol, snap-frozen in liquid nitrogen, and stored at -80°C.

The *Saccharomyces cerevisiae* proteins Ssa1p, Ydj1p, Sse1p, Fes1p, and Fes1-A79R/R195A were purified as described in Kabani et al. (2002a) and Shomura et al.

(2005), dialyzed against 10 mM Hepes-OH pH 7.4, 50 mM KCl, 10 mM dithiothreitol, 5% glycerol, snap-frozen in liquid nitrogen and stored at -80°C. Purified recombinant human Hsc70 was a generous gift from Samantha Pemberton (Pemberton et al. 2011).

GST pulldown assays

GST pulldown assays were performed essentially as described before (Kabani et al. 2002a; Kabani et al. 2000), with minor modifications. A total of 10 μ g of GST or GST-*Yl.Chn1p* was incubated with 20–100 μ L of glutathione Sepharose beads (Amersham Pharmacia biotech) at 50% slurry in 200–500 μ L binding buffer (Tris-buffered saline, 2% glycerol, 1 mM DTT, 2 mM MgCl₂, 0.1% NP-40, antiprotease cocktail, Roche). The mixture was homogenized on a rotating wheel for 1 h at 4°C. The beads were then washed three times with binding buffer. Purified proteins (5–16 μ g) or cell lysate (500 μ g) were then added to the beads in a total volume of 200–500 μ L binding buffer. When indicated, ATP was added to the mixture at a final concentration of 1 mM and the reaction mixes further incubated on a rotating wheel for 2 h at 4°C. The beads were then washed three times with binding buffer. Proteins bound to the beads were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie blue staining or analyzed by western blotting.

Luciferase aggregation assays

Luciferase (Sigma Aldrich) was diluted to a final concentration of 0.1 μ M in Aggregation Buffer (40 mM Hepes-OH pH 7.4, 50 mM KCl, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM ATP) containing 0.25–8 μ M of GST, GST-*Yl.Chn1p* or Sse1p, as indicated. The reaction mixes were incubated at room temperature for 5 min, then transferred to a 42°C water bath for 15 min. The reaction mixes were then cooled to room temperature, and centrifuged at 16,000 $\times$ g and at 4°C for 15 min. The supernatants were discarded while the pellets were resuspended in Sample Buffer (60 mM tris-Cl pH 6.8, 10% glycerol, 5% β -mercaptoethanol, 2% SDS), heated at 90°C for 10 min, and then analyzed by SDS-PAGE followed by Coomassie blue staining. Protein bands were quantified using the ImageJ software (NIH).

Preparation of cell extracts and western blotting techniques

Total yeast cell extracts for western blot analysis were prepared with the sodium hydroxide/trichloroacetic acid method as described (Sharma et al. 2009). Equal amounts of proteins were electrophoretically resolved using 4–12%

NuPAGE Novex bis–tris polyacrylamide gels (Invitrogen) according to the manufacturer's instructions. Proteins were analyzed by Western blotting using the indicated antibodies, chemiluminescence reagents (Pierce) and a LAS-3000 imager (Fuji). Rabbit polyclonal antibodies against GST–*Yl*.Chn1p and GST–*Yl*.Fes1p were produced according to previously published protocols (Boisrame et al. 1996). Mouse monoclonal anti-Hsp70/Hsc70 (SPA-822) antibodies were purchased from Stressgen Biotechnologies.

For GST pulldown assays, the indicated yeast cells from a 50 mL overnight culture in YPD at 28°C were recovered and suspended in 1 mL binding buffer. Cells were broken by repeated glass beads beating on ice, the cellular debris were removed by centrifugation for 5 min at 1,100×g and at 4°C and the lysate was further clarified by centrifugation for 15 min at 15,000×g and at 4°C. Protein concentrations were determined by the Bradford protein assay (Bio-Rad).

Thermotolerance assays

For thermotolerance assays, cells were grown in YPD at 28°C to exponential phase (OD_{600nm} ~0.6), incubated at 37°C in a water bath for 1 h, and then transferred at 45°C in a water bath. Because the yeast *Y. lipolytica* is strictly aerobic, cells were kept under agitation at all times. Aliquots were taken overtime, and tenfold serial dilutions were plated on YPD plates and incubated at 28°C for 2–3 days to assess cell viability.

Results

The *Y. lipolytica* *Yl*.CHN1 gene encodes an ortholog of CHIP

We previously reported that the proteome of the yeast *Y. lipolytica* contains a protein homologous to the ubiquitin ligase CHIP (Kabani and Martineau 2008). We thus undertook the molecular characterization of the *Y. lipolytica* CHIP protein that we named *Yl*.Chn1p after the *C. elegans* CHIP homologue CHN-1 (Khan and Nukina 2004) and to comply with usual yeast nomenclature. *Yl*.Chn1p is encoded by the *Yl*.CHN1 gene (geneID: YALI0F16753g) that, according to the *Y. lipolytica* genome annotation (<http://cbi.labri.fr/Genolevures/>), contains three introns (Supplementary Fig. S1). To confirm that the *Yl*.CHN1 gene is expressed and its mRNA properly spliced, we isolated total RNA from *Y. lipolytica* cells that were grown to exponential phase at 28°C. The *Yl*.CHN1 cDNA was cloned by RT-PCR as described in “Materials and methods” section, and sequencing analysis revealed that the three predicted introns were spliced as expected. Thus, the *Yl*.CHN1 gene is expressed and encodes a 279-aa protein that displays 28% identity and 50% similarity with human CHIP. As shown in Fig. 1, *Yl*.Chn1p contains an *N*-terminal tetratricopeptide repeat (TPR) domain and a C-terminal U-box domain separated by a mix-charged region. The mix-charged region of human, *Drosophila*, and *C. elegans* CHIP proteins was shown to contain a coiled-coil domain required for the dimerization of the protein (Nikolay et al.

Fig. 1 sequence alignment of CHIP proteins from *Y. lipolytica* (Yl, accession no. XM_505508), *Drosophila melanogaster* (Dm, accession no. AAD33399), *Mus musculus* (Mm, accession no. AAD33401) and

Homo sapiens (Hs, accession no. AAD33400). The alignment was made using ClustalX and GeneDoc

2004). Similarly, a potential coiled-coil domain within the mix-charged region of *Yl.Chn1p* is predicted by the COILS algorithm (Lupas et al. 1991), but with a lower probability score than that obtained for Human CHIP (Supplementary Fig. S2). It should be noted that in some *Yl.CHN1* cDNAs, only the first two introns were spliced (data not shown). This alternate splice variant that retains the third intron could in principle encode an *N*-terminally truncated *Yl.Chn1p* starting from methionine 46 (therefore lacking the first half of the TPR domain). However, because we failed to observe a corresponding band by western blot (see below), it is unlikely that this putative polypeptide is actually translated, at least in standard growth conditions. Neuvéglise and colleagues also reported alternate splice variants for *Yl.CHN1* as well as many other *Y. lipolytica* genes, but their biological significance remains elusive (Mekouar et al. 2010).

Yl.CHN1 is a non-essential gene involved in thermotolerance

In order to investigate the biological role of *Yl.Chn1p*, a $\Delta chn1$ null allele was constructed as described in “Materials and methods” section. The $\Delta chn1$ strain is viable and does not display a significant growth defect compared to the wild-type strain neither at 28°C or 34°C (which is the maximal growth temperature for *Y. lipolytica*), nor in the presence of a variety of stress-inducing agents such as L-canavanine, hygromycin B, dithiothreitol, or heavy metals (Fig. 2, data not shown).

Because CHIP is known for its role in protein quality control and degradation, we asked whether *Yl.Chn1p* is required for cells to survive massive protein misfolding and aggregation upon severe heat shock. Wild-type and $\Delta chn1$ cells were heat shocked for 1 h at 37°C, then shifted to 45°C and cell viability was then assessed over time (Fig. 3a). In these conditions, the viability of $\Delta chn1$ cells

decreased faster than that of the wild-type strain, suggesting that *Yl.Chn1p* is indeed required to cope with heat-induced protein misfolding and aggregation. It should be noted that cell viability was not affected upon incubation at 37°C, even for extended periods of time, whereas shifting the cells directly at 45°C resulted in very rapid loss of cell viability for both the wild-type and $\Delta chn1$ strains (data not shown). As expected, thermotolerance was restored to wild-type levels in a $\Delta chn1$ strain bearing an *Yl.CHN1* expression plasmid (data not shown).

The intracellular levels of *Yl.Chn1p* remained unchanged both after the 37°C and 45°C temperature shifts and during recovery at 28°C, suggesting that the expression of *Yl.CHN1* is not stress-inducible (Fig. 3b), in contrast to what have been described for mammalian CHIP (Dikshit and Jana 2007). As expected, the intracellular levels of Hsp70 in the wild-type and $\Delta chn1$ strains increased approximately twofold after shifting the cells from 28°C to 37°C and remained stable during recovery for 2 h at 28°C (Fig. 3b). The intracellular levels of Hsp70's NEF *Yl.Fes1p* were not affected by heat shock, in agreement with what has been described for its mammalian ortholog HspBP1 (Gottwald et al. 2006) (Fig. 3b). Taken together, these results suggest that while *Yl.Chn1p* plays a significant role in protecting cells from severe heat shock, it does not seem to do so by regulating the levels of Hsp70 as shown in mammalian cells (Qian et al. 2006). The protective role of *Yl.Chn1p* may be to prevent protein aggregation (see below) and to facilitate protein refolding during recovery, as suggested for mammalian CHIP (Rosser et al. 2007).

U-box domain containing ubiquitin ligases are not essential for growth in *Y. lipolytica*

The limited effects we observed on cell growth and stress resistance upon deleting the *Yl.CHN1* gene may be due to the presence of other factors that can functionally replace

Fig. 2 U-box ubiquitin ligases are not essential for growth in *Y. lipolytica*. The indicated strains were grown to exponential phase ($OD_{600nm} \sim 0.6$) and tenfold serial dilutions were spotted onto YPD plates. When present, the final concentration of hygromycin B and L-canavanine was 10 and 0.1 μ g/mL, respectively. Plates were incubated for 2–3 days at the indicated temperatures before being photographed

Fig. 3 Increased thermosensitivity in the Δ chn1 mutant. **a** The wild-type and Δ chn1 strains were grown to exponential phase at 28°C, transferred for 1 h at 37°C and then shifted to 45°C. Aliquots were periodically removed and cell viability was assessed by plating tenfold serial dilutions onto YPD plates. **b** The wild type and Δ chn1 strains

Yl.Chn1p. We identified *Yl.Ufd2p*, encoded by the *Yl.UFD2* gene (geneID: YALI0D12452g), as the only other U-box domain containing protein in the genome of *Y. lipolytica*. *Yl.Ufd2p* is the ortholog of *S. cerevisiae* Ufd2p, *C. elegans* UFD-2 and human Ufd2a and Ufd2b ubiquitin ligases (Supplementary Fig. S3). The *Yl.UFD2* gene was then deleted in wild type and Δ chn1 backgrounds generating the Δ ufd2 and Δ chn1 Δ ufd2 strains. Both strains are viable and display no obvious growth defect at 28°C, 34°C or in the presence of stress-inducing agents such as L-canavanine, hygromycin B, dithiothreitol or heavy metals (Fig. 2, data not shown). Furthermore, the Δ chn1 Δ ufd2 strain did not exhibit enhanced thermotolerance defects compared to Δ chn1 (data not shown).

The protein quality control machinery of *Y. lipolytica* tolerates the loss of multiple components

We then asked whether the simultaneous deletion of *Yl.CHN1* and other genes encoding components of protein quality control machineries would result in synthetic phenotypes. First, we focused on the genes encoding the *Y. lipolytica* orthologs of Doa10p (geneID: YALI0F19360g) and Hrd1p (geneID: YALI0F02981g), two ubiquitin ligases playing important roles in ER-associated degradation (Hampton et al. 1996; Bordallo et al. 1998; Swanson et al. 2001; Boisrame et al. 2006). The *Yl.DOA10* and *Yl.HRD1* genes were disrupted in the wild-type, Δ chn1, Δ ufd2, and Δ chn1 Δ ufd2 genetic backgrounds, but no synthetic phenotypes were observed among these null alleles at 28°C, 34°C, or in the presence of stress-inducing agents (Fig. 2, data not shown).

The *Y. lipolytica* Δ ssa5, Δ ssa6, Δ ssa7, and Δ ssa8 strains, bearing individual disruption of the genes encoding

were grown to exponential phase at 28°C, transferred for 1 h at 37°C shifted to 45°C for 15 min and then allowed to recover at 28°C. Cell extracts were prepared from aliquots removed at each stage and analyzed by SDS-PAGE and western blotting with antibodies against the indicated proteins

Yl.Ssa5p, *Yl.Ssa6p*, *Yl.Ssa7p*, and *Yl.Ssa8p* (Kabani and Martineau 2008; Sharma et al. 2009), respectively, were all viable and did not display obvious growth defects in any condition tested (Fig. 4, data not shown). The Δ chn1 Δ ssa5, Δ chn1 Δ ssa6, Δ chn1 Δ ssa7 and Δ chn1 Δ ssa8 double mutants were also viable without significant growth defects (Fig. 4, data not shown).

Members of three classes of Hsp70 NEFs are found in *Y. lipolytica*: *Yl.Fes1p*, *Yl.Sse1p*, and *Yl.Bag1p* (Supplementary Fig. S4) are the orthologs of Fes1p/HspBP1, Sse1p/Hsp110, and Bag1, respectively, reviewed in Kabani (2009) and Kabani and Martineau (2008). We were not able to obtain null alleles of the *Yl.FES1* and *Yl.SSE1*, suggesting that these genes are essential (Martineau and Kabani, unpublished observations). However, a Δ bag1 mutant was viable but displayed no growth defects in any condition we tested (Fig. 4, Supplementary Fig. S5). The Δ bag1 Δ ssa5, Δ bag1 Δ ssa6, Δ bag1 Δ ssa7 and Δ bag1 Δ ssa8 double mutants were also viable with no increased sensitivity to stress (Fig. 4, data not shown).

Finally, we generated a null allele of the *Yl.HSP104* gene (geneID: YALI0E27962g) encoding the ortholog of *S. cerevisiae* Hsp104p that plays an important role in protein disaggregation, thermotolerance, and yeast prion propagation reviewed in Romanova and Chernoff (2009), Doyle and Wickner (2009) and Haslberger et al. (2010). The Δ hsp104 mutant grew as well as wild-type strain in all the conditions we tested but displayed, as expected, an increased sensitivity to severe heat shock (Supplementary Fig. S5). The Δ hsp104 Δ ssa5, Δ hsp104 Δ ssa6, Δ hsp104 Δ ssa7 and Δ hsp104 Δ ssa8 double mutants grew as well as the wild-type strain and the corresponding single mutants (Fig. 4) and were not more sensitive to a

Fig. 4 The individual or combined deletions of the genes encoding *Yl.Ssa5-8p*, *Yl.Chn1p*, *Yl.Bag1p*, and *Yl.Hsp104p* have limited effects on growth. The indicated strains were grown to exponential phase ($OD_{600nm} \sim 0.6$) and tenfold serial dilutions were spotted onto YPD plates. When present, the final concentration of hygromycin B and L-canavanine was 10 and 0.1 $\mu\text{g/mL}$, respectively. Plates were incubated for 2–3 days at the indicated temperatures before being photographed

severe heat shock than the $\Delta hsp104$ mutant (data not shown).

Taken together, our results clearly demonstrate that *Yl.Chn1p* is not essential under the stress conditions we subjected *Y. lipolytica* cells to. They also suggest that Hsp70 orthologs exhibit a significant level of functional redundancy within *Y. lipolytica*'s protein quality control machinery. The *Y. lipolytica* protein quality control machinery tolerates the loss of many of its constituents without significantly affecting cell viability or survival under harsh conditions, suggesting that different pathways are equally efficient in dealing with protein misfolding and aggregation.

Yl.Chn1p interacts with members of the Hsp70 family

Next, we asked whether *Yl.Chn1p* interacts with members of the Ssa family of Hsp70 molecular chaperones in vitro. The *Yl.Chn1p* protein was expressed in *E. coli* as a GST fusion (GST-*Yl.Chn1p*) and was purified by affinity chromatography as described in “Materials and methods” section. First, we examined the ability of GST-*Yl.Chn1p* to interact with Ssa proteins present in cell lysates prepared

from wild-type or $\Delta chn1$ strains. As shown in Fig. 5a (top panel), a 70-kDa protein efficiently co-purified with GST-*Yl.Chn1p* indicating it specifically binds to *Yl.Chn1p*. The interacting protein was recognized by an anti-Hsp70 antibody (Fig. 5a, bottom panel) indicating that it corresponds to *Yl.Ssa5-8p* (Sharma et al. 2009). Similar results were obtained whether the cell extracts derived from the wild-type or $\Delta chn1$ strains (Fig. 5a). No protein other than Hsp70 co-purified with GST-*Yl.Chn1p* in the pulldown assays, at least not in stoichiometric and detectable amounts (Fig. 5a, top panel), indicating that Hsp70 is the major interacting partner of *Yl.Chn1p* in our experimental conditions. In order to determine whether *Yl.Chn1p* preferentially binds to a specific Ssa family member, we repeated these experiments using cell extracts derived from $\Delta chn1\Delta ssa5$, $\Delta chn1\Delta ssa6$, $\Delta chn1\Delta ssa7$ or $\Delta chn1\Delta ssa8$ cells. As shown in Fig. 5b, similar amounts of Hsp70 protein co-purified with GST-*Yl.Chn1p* in all cases, indicating that *Yl.Chn1p* is able to bind to multiple Hsp70 family members. This is consistent with the finding that CHIP interacts with the highly conserved extreme C-terminus of both Hsp70 and Hsc70 via its N-terminal TPR domain (Ballinger et al. 1999). Accordingly, and in

Fig. 5 *Yl*.Chn1p interacts with *Y. lipolytica* Ssa proteins in vitro. **a** GST or GST-*Yl*.Chn1p (20 µg) were bound to glutathione Sepharose beads as indicated in “Materials and methods” section. Cell extracts (500 µg) prepared from the wild-type or Δ chn1 strains (see “Materials and methods” section) and ATP (1 mM) were added to the pull-down reactions. **b** GST pull-downs between GST or GST-*Yl*.Chn1p and cell extracts (500 µg) prepared from the indicated strains were performed as in **b**. In each case, bound proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The input lanes represent 10 µg of each cell extract. The membrane was stained with amido black (*top panels* in **a** and **b**) and then blotted with anti-Hsp70 antibodies (*bottom panels* in **a** and **b**; see “Materials and methods” section for further details). Asterisks these bands correspond to GST-*Yl*.Chn1p degradation products

agreement with what was described for mammalian CHIP (Ballinger et al. 1999), GST-*Yl*.Chn1p binds efficiently in vitro to purified *S. cerevisiae* Ssa1p and Human Hsc70 proteins in an ATP-independent manner (Fig. 6a). We took advantage of the promiscuous interaction between GST-*Yl*.Chn1p and Ssa1p to examine how this interaction is influenced by Ssa1p co-chaperones (Kabani et al. 2002a; Krzewska and Melki 2006). The interaction between GST-*Yl*.Chn1p (or GST as a negative control) and Ssa1p was examined by GST pulldown assays in the absence or presence of the Hsp40 Ydj1p and the NEFs Fes1p and Sse1p as indicated in Fig. 6b. As expected, neither of these proteins interacted with GST or GST-*Yl*.Chn1p (Fig. 6b). Ydj1p had no influence on the binding of Ssa1p to GST-*Yl*.Chn1p and did not significantly co-purify with the complex (Fig. 6b). In the presence of NEF and Hsp110-family member Sse1p, the amount of Ssa1p that co-purifies with GST-*Yl*.Chn1p decreased, regardless of whether Ydj1p was also present or not (Fig. 6b). Furthermore, a significant amount of Sse1p was found associated with GST-*Yl*.Chn1p-Ssa1p complex (Fig. 6b). We conclude from this observation that GST-*Yl*.Chn1p and Sse1p bind simultaneously to Ssa1p to form a ternary complex given that Sse1p does not interact with GST-*Yl*.Chn1p. In the presence of Fes1p, the amount of Ssa1p that binds GST-*Yl*.Chn1p diminished strongly (Fig. 6b). This further

demonstrates that different types of NEFs act in different pathways (Martineau et al. 2007; Kabani 2009). HspBP1, the mammalian ortholog of Fes1p (Kabani et al. 2002b) was shown to inhibit the activity of CHIP. However, in contrast to our results, CHIP and HspBP1 were shown to interact simultaneously with Hsc70 (Alberti et al. 2004). Whether this difference is due to the heterologous context of *Yl*.Chn1p interacting with *S. cerevisiae* proteins or to a different mode of action between fungal and mammalian proteins is currently not known.

We then showed that the inhibition of GST-*Yl*.Chn1p/Ssa1p interaction by Fes1p is dose-dependent suggesting that Fes1p directly competes with GST-*Yl*.Chn1p for binding to Ssa1p (Fig. 6c). This was confirmed by the finding that Fes1-A79R/R195A variant, which is unable to bind Ssa1p (Shomura et al. 2005), did not compete with GST-*Yl*.Chn1p for binding to Ssa1p (Fig. 6c). Finally, the amount of GST-*Yl*.Chn1p, Ssa1p, and Sse1p ternary complex depended on the concentration of Sse1p (Fig. 6c).

Yl.Chn1p prevents heat-induced aggregation of liferase

Mammalian CHIP has an intrinsic chaperone activity that allows it to prevent protein aggregation (Rosser et al. 2007). To test whether this chaperone activity is conserved in *Yl*.

Fig. 6 The interaction of *YlChn1p* with *S. cerevisiae* Ssa1p is inhibited by Fes1p but not by Sse1p or Ydj1p. **a** GST or GST-*YlChn1p* (10 μ g) were bound to glutathione sepharose beads, and then *S. cerevisiae* Ssa1p (5 μ g) or human Hsc70 (5 μ g) were added to the pull-down reactions. **b** GST or GST-*YlChn1p* (10 μ g) were bound to glutathione Sepharose beads, and then the *S. cerevisiae* Ssa1p, Sse1p, Fes1p, and Ydj1p proteins (5 μ g) were added to the pull-down

reactions as indicated. **c** GST or GST-*YlChn1p* (10 μ g) were bound to glutathione sepharose beads, and then Ssa1p (5 μ g) and increasing amounts (1–16 μ g, as indicated) of Sse1p, Fes1p, and Fes1-A79R/R195A proteins were added to the pull-down reactions. In all cases, bound proteins were analyzed by SDS-PAGE and coomassie blue staining (see “Materials and methods” section for further details)

Chn1p, the ability of GST-*YlChn1p* to prevent the aggregation of heat-denatured luciferase was assayed in vitro. The incubation of luciferase at 42°C leads to the formation of insoluble aggregates that can be recovered in the pellet fraction after centrifugation (Minami et al. 1996; Fig. 7). When increasing amounts of GST-*YlChn1p* were added to the reaction prior to denaturation, the amount of aggregated luciferase decreased in a GST-*YlChn1p* concentration dependent manner by up to ~70% (Fig. 7). As expected, GST was ineffective while Sse1p prevented luciferase aggregation by up to ~70% (Dragovic et al. 2006; Oh et al. 1997, 1999). It should be noted that substantial amounts of GST, GST-*YlChn1p*, and Sse1p were recovered in the pellet fractions regardless of whether luciferase was also present in the reaction or not (Fig. 7). The aggregation of GST-*YlChn1p* and Sse1p during the 42°C incubation may therefore explain the fact that a 10–20-fold molar excess of chaperones is needed for maximal prevention of luciferase aggregation (Fig. 7). Thus, like its mammalian ortholog, *YlChn1p* acts as a “holdase” to prevent protein aggregation after a severe heat shock.

Discussion

In the work presented here, we show that the yeast *Y. lipolytica* contains a functional ortholog of CHIP that plays

Fig. 7 GST-*YlChn1p* and Sse1p prevent the aggregation of heat-denatured luciferase in vitro. Luciferase (0.1 μ M) was incubated at room temperature for 5 min in the presence of increasing concentrations (0.25, 0.5, 1, 2, 4, or 8 μ M) of GST, GST-*YlChn1p*, or Sse1p. The reaction mixes were then transferred to a 42°C water bath for 15 min. The samples were then spun at 16,000 $\times$ g for 15 min at 4°C. The supernatants were discarded, and the proteins present in the pellet fractions analyzed by SDS-PAGE followed by Coomassie blue staining. Control reactions without luciferase contained 4 μ M of GST, GST-*YlChn1p* or Sse1p, and were treated and analyzed similarly

a role in cell survival under severe stress conditions (Fig. 3a). The cytoprotective role of *Yl.Chn1p* after a severe heat shock may be explained by its chaperone activity that allows it to prevent protein aggregation (Fig. 7). While the expression of mammalian and *A. thaliana* CHIP proteins is inducible by a variety of stresses (Dikshit and Jana 2007; Yan et al. 2003), this was not the case for *Yl.Chn1p* (Fig. 3b). We previously showed that the intracellular levels of *Y. lipolytica* Hsp70 mRNAs following heat shock increased by only ~2–5-fold (Sharma et al. 2009), consistent with the modest approximately twofold increase in Hsp70 protein levels we observed after shifting the cells for 1 h at 37°C (Fig. 3b). In contrast, the transcription of the Hsp70-encoding genes was induced by up to 100-fold in mammalian cells and *S. cerevisiae* following analogous heat shock treatments (Mirault et al. 1982; Werner-Washburne et al. 1987; Boorstein and Craig 1990a, b). Furthermore, we previously reported that the UPR was in a constitutive semi-activated state in *Y. lipolytica* cells growing under normal growth conditions, and that treating the cells with tunicamycin resulted in only a ~1.5-fold further induction of the UPR (Babour et al. 2008). One plausible explanation that may account for the ensemble of our observations is that *Y. lipolytica* cells are stressed by the standard laboratory handling techniques that are widely used for *S. cerevisiae* or other yeasts. In such case, many stress-inducible genes, including *Yl.CHN1*, are expected to be expressed at near-maximal levels, preventing us from detecting further upregulation following an additional stress. Such an abnormal level of constitutively activated stress response may shield *Y. lipolytica* against cell insults, in agreement with the limited effects our multiple gene deletions had on cell survival upon exposure to severe heat shock treatments (Fig. 2 and 4). Alternatively, *Y. lipolytica* cells may have not adapted an efficient HSR and UPR induction to face proteotoxic stress during evolution, in agreement with their inability to grow at temperatures beyond 34°C. It is also possible that other molecular chaperones, such as small heat shock proteins, are activated in our strains and experimental conditions, and provide efficient cytoprotection against proteotoxic stresses (Tyedmers et al. 2010). While future investigations are needed to distinguish between these possibilities, our work certainly highlights the remarkable plasticity of the protein QC machinery both within and across species.

We showed that *Yl.Chn1p* binds in vitro to Hsp70 proteins in *Y. lipolytica* lysate or to purified *S. cerevisiae* Ssa1p or human Hsc70 (Fig. 5) in agreement with earlier studies (Ballinger et al. 1999). The effects that co-chaperones had on the interaction between *Yl.Chn1p* and Ssa1p were examined and revealed some differences with what has been reported for mammalian CHIP (Fig. 6;

Alberti et al. 2004). The Hsp40-family member Ydj1p had no effect on the binding of *Yl.Chn1p* with Ssa1p and did not form stable complexes with either protein in the presence of ATP (Fig. 6). Sse1p, the Hsp110-family member and Hsp70 NEF, formed a ternary complex with Ssa1p and *Yl.Chn1p* (Fig. 6). No such complex between mammalian Hsp70, Hsp110, and CHIP proteins has been described. The Fes1p NEF efficiently competed with *Yl.Chn1p* for binding to Ssa1p (Fig. 6), whereas its mammalian homolog HspBP1 inhibited the activity of CHIP by forming a ternary CHIP/Hsc70/HspBP1 complex (Alberti et al. 2004). The differences we report most probably reflect functional changes in co-chaperone activities during evolution. This, together with the functional specificities among Hsp70 family members (Kabani and Martineau 2008; Sharma et al. 2009; Hageman et al. 2011) and the finding that different classes of Hsp70 NEFs exert distinct, sometime opposing, functional roles (Kabani 2009; Martineau et al. 2007) leads to hypothesize that Hsp70/co-chaperone activities are highly specialized within the cells.

Although the role of *Yl.Chn1p* in protein multi-ubiquitination and clearance remains to be established both in vivo and in vitro, we showed that the only *Y. lipolytica* U-box domain containing proteins *Yl.Chn1p* and *Yl.Ufd2p* are not essential for growth or for cell survival following a severe heat shock in *Y. lipolytica*, even in the absence of the *Yl.Hrd1p* or *Yl.Doa10p* RING ubiquitin ligases (Fig. 2). In *C. elegans*, the multi-ubiquitination of a client protein, the myosin chaperone UNC-45, was shown to depend on the joint action of both CHN-1 and UFD-2 as well as CDC-48/p97, all four proteins forming a stable complex (Hoppe et al. 2004; Janiesch et al. 2007). The simultaneous knockdown of CHN-1 and UFD-2 is viable and does not induce the UPR (Janiesch et al. 2007). The fact that both CHN-1 and UFD-2 are required for the multi-ubiquitination of UNC-45 is compatible with our observations that the single or double deletions of *CHN1* and *UFD2* result in comparable phenotypes (Fig. 2). The low impact these multiple gene deletions had on cell viability can be explained by the fact that (1) functionally redundant ubiquitin ligases are equally able to catalyze multiubiquitylation of client proteins; (2) only a subset of client proteins require the multi-ubiquitination activity of *Yl.Chn1p* and *Yl.Ufd2p* for degradation; and/or (3) other protein degradation pathways (e.g., autophagy) are able to rid the cell of aberrant polypeptides. Because it contains several proteins absent from *S. cerevisiae*, such as the orthologs of mammalian CHIP and Bag1 proteins, *Y. lipolytica* appears to be a useful alternate model for further our understanding of the intricate interactions between the components of the cellular protein QC arsenal.

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