Yeast RNA Viruses as Indicators of Exosome Activity: Human Exosome hCsl4p Participates in RNA Degradation in *S. cerevisiae*.

Running Head: Yeast RNA viruses as indicators of human hCsl4p activity

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Abstract

The exosome is an evolutionary conserved 10-mer complex involved in RNA metabolism, located both in the nucleus and in the cytoplasm. The cytoplasmic exosome plays an important role in mRNA turnover through its 3'→5' exonucleolytic activity. The superkiller (SKI) phenotype of yeast was originally identified as an increase of killer toxin production due to elevated levels of the L-A double-stranded RNA (dsRNA) Totivirus and its satellite toxin-encoding M dsRNA. Most *SKI* genes were later on shown to be either components of the exosome or modulators of its activity. Variations in the amount of Totivirus are, thus, good indicators of yeast exosome activity, and can be used to analyze its components. Furthermore, if exosome proteins of higher eukaryotes were functional in *S. cerevisiae*, these viruses would provide a simple tool to analyze their function. In this work, we have found that *hCSL4*, the human ortholog of *SKI4* in the yeast exosome, rescues the null phenotype of the deletion mutant. hCsl4p shares with Ski4p conserved S1 RNA-binding domains, but lacks the N-terminal third of Ski4p. Nevertheless, it interacts with the Dis3p exonuclease of yeast exosome, and partially complements the superkiller phenotype of *ski4-1* mutation. The elimination of the N-terminal third of Ski4p does not affect its activity, indicating that it is dispensable for RNA degradation. We have also identified the point mutation G152E in *hCSL4*, equivalent to the *ski4-1* mutation G253E, which impairs the activity of the protein, thus validating our approach of using yeast RNA virus to analyze higher eukaryotes exosome.

Introduction

A key process in mRNA turnover is the rate of RNA degradation. In *S. cerevisiae* there are two major pathways of RNA decay, both starting with the shortening of the 3' poly(A) tail. The main pathway involves decapping followed by 5'→3' digestion performed by the Ski1p/Xrn1p exonuclease [Deckerd and Parker, 1993; Stevens, 2001; Parker and Song, 2004]. There is a second pathway, which does not require the elimination of cap, carried out by the exosome in the 3'→5' direction [Tomecki *et al.*, 2010]. The exosome is a multimeric complex with exonucleolytic activity located both in the cytoplasm and in the nucleus. Besides its role in mRNA degradation, it participates on

different RNA processes, such as elimination of aberrant RNAs [Bousquet-Antonelli *et al.*, 2000] or snRNA maturation [van Hoof *et al.*, 2000a]. The exact composition of the exosome varies depending on the organism or the subcellular localization, but in all cases it is composed of a ring-shaped core made of three heterodimers (Rrp41p/Rrp45p, Rrp43p/Rrp46p, Rrp42p/Mtr3p) stabilized by the presence of three other proteins (Ski4p, Rrp4p, Rrp40p) [Hernandez *et al.*, 2006; Liu *et al.*, 2006]. In eukaryotic organisms, with the exception of *A. thaliana* [Chekanova *et al.*, 2002], this core has no enzymatic activity. Proteins with endo- and exonucleolytic activity are attached to it. All exosome core components are essential. This complex has been extensively studied in the yeast *S. cerevisiae* in the last few years, and it has been shown that its exonucleolytic activity depends on two different proteins: Dis3p, located both in cytoplasm and nucleus, and Rrp6p, specific to the nuclear exosome [Dziembowski *et al.*, 2007; Greimann and Lima, 2008]. Several factors are also required to modulate the exosome activity. Some of them, like the Ski complex formed by Ski2p, Ski3p and Ski8p [Brown *et al.*, 2000; Synowsky and Heck, 2008], or Ski7p [Araki *et al.*, 2001], play a role in mRNA decay. It has been reported the existence of a genetic separation among different exosome activities. For example, in *SKI4/CSL4*, one of the essential exosome core components, a point mutation named *ski4-1* decreases the mRNA degradation function of the exosome without affecting other processes. In contrast, the *cs14-1* mutation in the same gene impairs RNA processing, but not 3'→5' degradation [van Hoof *et al.*, 2000b].

The superkiller (SKI) phenotype was first described as a more efficient ability to kill sensitive non-killer yeast strains [Vodkin *et al.*, 1974; Toh-E *et al.*, 1978]. *ski* mutants harboured higher amounts of killer virus: the Totivirus L-A and its satellite RNA, M. This superkiller phenotype was caused by overexpression of the killer toxin, encoded by M. Subsequent work showed that, with the exception of *SKII*, the rest of *SKI* gene products were components of the exosome or modulators of its activity. Mutations on *SKI* genes increase the copy number not only of killer elements, but also of other yeast RNA viruses such as L-BC (a Totivirus frequently accompanying L-A in the yeast [Wickner, 1996]) or Narnavirus 20S RNA [Matsumoto *et al.*, 1990]. Thus, variations in the amount of these viruses could be good indicators of the degradation efficiency of the complex, providing a simple method to analyze the role of exosome proteins in mRNA decay. If exosome

components of higher organisms were functional in yeast, this system could also constitute a simple way to analyze the importance of those proteins in RNA degradation. The exosome is highly conserved throughout evolution, being present not only in all eukaryotes, but also in archaea, which have a similar complex also named exosome [Koonin *et al.*, 2001]. Both are related to bacterial PNPs [Sarkar and Fisher, 2006]. In the last years the human exosome has been characterized and all its core components have been identified. Most of them have been named after their yeast counterparts due to the high homology between them. In addition, some of the human proteins have been reported to complement the deletion of the equivalent components of *S. cerevisiae* exosome: hCsl4p [Baker *et al.*, 1998], hRrp4p [Mitchell *et al.*, 1997] and hDis3p [Shiomi *et al.*, 1998].

In this work we have tested the validity of using yeast RNA virus to study exosome activity by expressing *hCSL4*, the human ortholog of *SKI4*, in yeast and measuring its effect on RNA Totivirus and Narnavirus. First, we observed that *hCSL4* complemented the *ski4Δ* deletion, as previously described [Baker *et al.*, 1998], and also interacted with the exosome exonuclease Dis3p, suggesting that the human protein can substitute Ski4p in yeast. The expression of *hCSL4* from plasmids in *ski4Δ* or *ski4-1* mutants decreased L-A, L-BC and M1 Totivirus. Additionally, we found that a point mutation in *hCSL4*, equivalent to *ski4-1*, impairs RNA degradation, suggesting a similar role in the human exosome.

Materials and methods

Strains and media

Strains used in this work are listed in Table 1. Deleted strains *ski2Δ*, *ski3Δ*, *ski7Δ* and *ski8Δ* are derivatives of BY4741 strain from the EUROFAN collection (gently supplied by Dr. J. L. Revuelta). Cells were grown in rich YPAD medium (1% yeast extract, 2% peptone, 0.04% adenine, 2% glucose) supplemented with 0.02% uracil, or in synthetic medium deprived of uracil or leucine, or both. In Narnavirus launching experiments [Esteban and Fujimura, 2003; Esteban and Fujimura, 2005], cells were grown under nitrogen starvation conditions as described previously [Wejksnora and Haber, 1978].

Northern hybridization

Yeast cells were suspended in a buffer containing 50 mM Tris-HCl pH 8.0 and 100 mM NaCl and broken with Glass Beads by vortex mixing (ten pulses of 15 seconds each). Lysates were extracted with phenol followed by phenol:chloroform and precipitated with ethanol. Total RNAs were separated in a native 1.3% agarose gel, denatured in the gel with 50% formamide, 9% formaldehyde, 1 mM EDTA and 10 mM Tris-HCl pH 7.5 at 55 °C for 30 minutes, and transferred to a neutral nylon membrane (GE Healthcare). RNAs on the membrane were detected by hybridization with ³²P-labeled specific probes made by T3 or T7 run-off transcription. *Fosforimager Personal Molecular Imager* (Bio Rad) was used for quantification of the radioactive bands.

Dis3p-TAP purification

TAP-tagged Dis3p was purified from strain BY4741 transformed with a plasmid expressing this protein under the control of *PGK1* promoter. To analyze the interaction of Dis3p and hCsl4p, we co-expressed hCsl4p-HA in the same cells. 50 ml of an overnight culture were suspended in a buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 1× protease inhibitor (GE Healthcare), and broken with Glass Beads in a FastPrep instrument (BIO101 Savant). The lysate was clarified by centrifugation at 5000 r.p.m. for 10 minutes followed by ultracentrifugation at 50000 r.p.m. for 1 hour in a TLA100.2 rotor (Beckman). The supernatant (1 ml) was dialyzed against 100 ml of IPP 150 binding buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl₂ and 0.1% Nonidet P-40) for 1 hour at 4 °C, and incubated another hour with 100 μl calmodulin affinity resin pre-equilibrated with IPP150 binding buffer. The mixture was then transferred to a column, which was washed with 100 ml IPP150 binding buffer. The proteins were eluted stepwise with IPP150 calmodulin elution buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA and 0.1% Nonidet P-40) in six fractions of 150 μl. Eluted proteins were analyzed by SDS-PAGE followed by Western blotting and specific detection with anti-TAP or anti-HA antibodies.

Cytoduction

L-A and M1 viruses together, or L-A virus alone, were introduced into *ski4Δ* strains transformed with different plasmids by cytoduction using as donors strain 1368 or 2404, respectively. These strains carry the karyogamy-deficient *kar1-1* mutation [Conde and Fink, 1976]. Cytoduction was performed as described previously [Wesolowski and Wickner, 1984].

Plasmids expressing yeast or human exosome genes

The *hCSL4* and *hSKI2* open reading frames (ORFs) (0.6 kb and 3.75 kb, respectively) were amplified by PCR from a cDNA library of human fibroblast with oligonucleotides RE480 (5' AATTAGGATCCTCATGGCGCCACCTGTGAGATA3') and RE481 (5' AATTAGGATCCTTAGGTCTGCAAGAATTCGGGT3') for *hCSL4*, and RE388 (5' AATTAGAATTCGGATGATGGAGACAGAGCGACT3') and RE389 (5' AATTACTCGAGTCACTGGGTGTAGAGGCTGGCC3') in the case of *hSKI2*. The ORFs start and stop codons are underlined. The *SKI4* ORF (0.9 kb) was likewise amplified by PCR from strain BY4741 with oligonucleotides RE469 (5' AATTAGGATCCATGGCATGCAATTTTCAGTTTCC3') and RE470 (5' AATTAGGATCCGTAGATAAAGCTGTTACATATCA3'). We also amplified the *SKI4* gene with oligonucleotides RE467 (5' AATTCGGATCCATTCGCGATGTTACACACATTT3') and RE468 (5' AATTAGGATCCAAGCAGTACCGTGTAAGGCAAA3'). This 1.4 kb fragment carries the *SKI4* ORF plus 0.5 kb upstream and 200 bp downstream. The flanking sequences contain promoter and transcription termination and polyadenylation signals for *SKI4*, respectively. The *ski4-1* mutant strain 2375 (Table 1) was also used to amplify the *ski4-1* ORF and *ski4-1* gene with the same two sets of oligonucleotides mentioned above. For overexpression experiments the ORFs (*hCSL4*, *hSKI2*, *SKI4* and *ski4-1*) were cloned under the control of the *PGK1* promoter in a 2μ-derived vector giving rise to plasmids pRE1142, pRE1056, pRE1128 and pRE1129, respectively. DNA fragments containing the genes were cloned and expressed from a centromeric plasmid [Sikorsky and Hieter, 1989]. The *hCSL4* and *hSKI2* ORFs were also cloned in the same centromeric plasmid under the control of the *SKI4* and *SKI2* promoters, respectively.

Plasmid pMR122, that expressed Ski4Ntrp, was constructed introducing two SmaI sites by *in vitro* mutagenesis into the *SKI4* sequence on pRE1128, followed by digestion with SmaI and religation. The resultant plasmid expressed a truncated version of Ski4p without the RPL27 domain that lacked amino acids 14 to 111.

The *DIS3* ORF was amplified from strain BY4741 with oligonucleotides RE484 (5' AATTAGAATTCAATGTCAGTTCCCGCTATCGCCC3') and RE485 (5' AATTAGAATTCTACTGAAGCATCTTCCATCTAT3') and cloned into the same 2 μ -derived plasmid mentioned above, under the *PGK1* promoter. The TAP epitope was fused to its C-terminus to allow purification and detection of the protein (plasmid pMR116). Copurification experiments also required hCsl4p specific detection. The HA epitope was likewise fused to the C-terminus of *hCSL4* in pRE1142, giving rise to plasmid pMR117.

Narnavirus launching plasmids

20S RNA or 23S RNA was launched from vector pRE763 or pMR18, respectively. These plasmids are equivalents to pRE740 and pRE637 described previously [Esteban and Fujimura, 2003; Esteban and Fujimura, 2005], but carry a different auxotrophic marker, *URA3* instead of *TRP1*.

Miscellaneous

Mutations were introduced into *hCSL4* or *SKI4* by oligonucleotide site-directed mutagenesis [Kunkel, 1985] and verified by DNA sequencing. Standard yeast genetic manipulations were performed as previously described [Mortimer and Hawthorne, 1975]. Molecular biology techniques were carried out following [Sambrook et al., 1989].

Results

Yeast RNA viruses increase in mutants with exosome activity reduced

In the course of previous experiments carried out in our laboratory, we have consistently observed that yeast strains mutated in *SKI* genes showed not only increased amounts of Totivirus L-A and L-BC or the toxin-encoding satellite M1 or M2 dsRNA, as expected from their superkiller phenotype, but also higher copy number of Narnavirus 20S

RNA and 23S RNA. This effect was not the same in all *ski* mutants. It has been reported that while Ski1p, the exonuclease responsible for the main degradation pathway in the 5'→3' direction, has no effect on 20S RNA copy number [Esteban *et al.*, 2008], mutations in the *SKI2* or *SKI8* gene, which modulates exosome function, increased the amount of this virus [Matsumoto *et al.*, 1990]. We decided to do a more throughout study using the EUROFAN strain collection deleted of the non-essential *SKI* genes that form the Ski complex (*SKI2*, *SKI3*, *SKI8*) along with *SKI7*. All exosome core components are essential, so they could not be used in this first approach. Strain BY4741, from which the EUROFAN collection derives, carries L-A and L-BC Totivirus, but lacks 20S RNA and 23S RNA. Using our Narnavirus launching vectors [Esteban and Fujimura, 2003; Esteban *et al.*, 2005] we generated both viruses in the four deleted strains. Launching of 20S RNA in *ski2Δ*, *ski3Δ* and *ski8Δ* showed an extremely high copy number of the virus compared with parental strain BY4741 (more than 5-fold). In *ski7Δ* launching efficiency was little lower, but still higher than the wild type strain (Fig. 1B) Similar results were obtained with 23S RNA launching (see Supporting information, figure S1).

A non-lethal mutation in the exosome core component *SKI4* has been described [Toh-E *et al.*, 1978; van Hoof *et al.*, 2000b]. The *ski4-1* mutant strain 1416 from R. Wickner's laboratory carries M1 dsRNA and its helper virus L-A, in addition to L-BC. By Northern hybridization we also observed that it carried 20S RNA (not shown), but lacked 23S RNA. Because the auxotrophic markers of this strain were not appropriate for 23S RNA launching or to be used for complementation studies of the *ski4-1* mutation, we crossed this strain with BY4741. After sporulation and tetrad dissection we obtained strain 969 (Table 1). This strain was later on transformed with the 23S RNA launching plasmid pMR18. Fig. 1C shows the generation efficiency of 23S RNA (panel 1), together with the amounts of endogenous L-A, L-BC and M1 dsRNAs (panels 2 to 4). Again we observed a 2- to 5-fold increase in virus copy numbers as compared to the *SKI4* wild type strain BY4741. All these results together indicate that mutants in the Ski complex, *ski7Δ* or the *ski4-1* mutation showed an increase in the copy number of both Totivirus and Narnavirus. Thus, either type of virus could be used to test yeast exosome activity and to determine which proteins or domains are important for RNA degradation. Besides, because exosome is highly

conserved throughout evolution, presumably exosome components from higher eukaryotes could also be analyzed using these viruses.

Human *hCSL4* interacts with the yeast exosome and decreases RNA viruses copy number

To approach this study we chose two human genes: *hSKI2*, the human ortholog of *SKI2* [Lee *et al.*, 1995; Dangel *et al.*, 1995], an RNA helicase that is part of the Ski complex, and *hCSL4*, the human ortholog of *SKI4*, a component of the exosome core. The ORFs of both genes, amplified by PCR from a cDNA library of human fibroblasts and cloned into yeast expression vectors under the control of the constitutive *PGK1* promoter, were expressed in the corresponding yeast mutants. Expression of *hSKI2* in a *ski2Δ* strain containing endogenous L-A and 20S RNA viruses did not show the decrease in the viral copy number expected if *hSKI2* had complemented the deletion of *SKI2* (see Supporting information, figure S2). Perhaps because as part of a complex *hSKI2* requires, to be fully functional, also the expression of the *SKI3* and *SKI8* human counterparts. So, we decided to focus our work on the *hCSL4* gene.

We first confirmed that expression of the human ortholog from the *PGK1* promoter complemented the deletion of *SKI4* in *S. cerevisiae*, as had been previously reported [Baker *et al.*, 1998]. The expression of *SKI4* or *ski4-1* under the same promoter also complemented the null phenotype of the *ski4Δ* strain (Fig. 2A). In the three cases the growth rate was similar, both at 28 °C and at 37 °C. Cells that expressed *hCSL4* under the *SKI4* promoter in a centromeric vector, however, could not grow. Therefore, in all subsequent experiments we used plasmids that expressed these proteins from the *PGK1* promoter.

The complementation of the lethal *ski4Δ* phenotype by *hCSL4* suggested that the human protein was interacting with the yeast exosome. To analyze this possibility we studied the behaviour of hCsl4p and one of the proteins of the complex: the exonuclease Dis3p. The ORF of this protein, with the TAP epitope fused to its C-terminus, was cloned under the *PGK1* promoter. The TAP epitope has a calmoduline binding peptide, which allows its purification using an affinity resin. To specifically detect hCsl4p throughout the purification steps, it was tagged with the HA epitope. Fractions eluted from the resin were analyzed by Western Blotting and the copurification of Dis3p-TAP and hCsl4p-HA was

detected mainly in fractions 2 and 3 (Fig. 2B). A control experiment carried out with cells that expressed hCsl4p-HA in the absence of Dis3p-TAP showed that retention of the human protein in the column depended on Dis3p-TAP, and that there was no a specific interaction with the resin.

Ski4p is one of the three proteins that stabilize the hexameric ring of the exosome core in *S. cerevisiae* (Fig. 1A). The comparison with its human counterpart shows that it is a larger protein, with additional domains. The 31.5 kDa molecule has an N-terminal RPL27-like domain, an S1 RNA-binding domain and a motif similar to a zinc-binding domain in its C-terminus. It also includes a 38 amino acids linker, rich in glutamate residues, that is absent in most eukaryotes [Schaeffer *et al.*, 2009] (Fig. 3A). The human ortholog hCsl4p is smaller, 21 kDa, and only carries the S1 RNA-binding domain and a small zinc ribbon-like domain. The N-terminal third of Ski4p with the RPL27-like domain is absent in hCsl4p. As mentioned above, however, overexpression of hCsl4p complemented the growth defect of *ski4Δ*. We next analyzed the role of hCsl4p in the yeast exosome by examining the amounts of different viruses in the three strains shown in Fig. 2A. To this aim, Totivirus L-A and M1, or L-A alone were introduced by cytoduction into these cells from strains 1368 or 2404, respectively. RNAs from two colonies of each cytoduction were then analyzed by Northern hybridization. As expected, the amount of L-A was several folds higher in the *ski4-1* cells than in the ones that carried the wild type *SKI4* gene (Fig. 3B). Cells transformed with the plasmid expressing *hCSL4* had a lower amount of L-A as compared to the *ski4-1* strain, but higher than cells expressing *SKI4*, suggesting that hCsl4p is partially complementing the effect of *ski4Δ* deletion on L-A copy number. In cells carrying both viruses (L-A and M1) we observed a similar result in the helper virus L-A (Fig. 3C, EtBr). In these cells the difference of M1 copy number between *ski4-1* and the wild type strain was higher than what we observed with L-A (more than 5-fold). Nevertheless, *hCSL4* expression showed a good complementation of the defect of *ski4Δ*, almost as good as that of *SKI4*. The relative amounts of L-A and M1 in the ethidium bromide-stained gel and the Northern shown in Fig. 3C, however, have to be taken cautiously because their copy number may be affected not only by mutations in the *SKI4* gene but, as it has been described previously, L-A and M1 amounts may vary due to the presence of each other [Field *et al.*, 1982; Ball *et al.*, 1984]. To avoid this so-called

“exclusion phenomenon”, we decided to carry out subsequent experiments on *hCSL4* expression in strains that harbour only the helper virus L-A.

The RPL27-like domain of Ski4p is not involved in RNA degradation

Because hCsl4p lacks the RPL27-like domain of Ski4p (Fig. 3A) we wondered whether the partial effect observed on L-A copy number (Fig. 3B) was dependent on its absence. It has been reported that a truncated Ski4p lacking this fragment could complement the null phenotype of a *ski4-1/dcp1-2* strain [Schaeffer *et al.*, 2009]. However, the effect of this truncation on the amount of RNA or its decay rate was not shown. Thus, we wanted to address directly whether this domain was specifically involved in RNA degradation, so we constructed a vector expressing a truncated version of Ski4p, Ski4Ntrp, that lacked almost the entire RPL27 domain and the adjacent linker (amino acids 14 to 111) (Fig. 4A). First, we generated by vector shuffling a *ski4Δ* strain that carried this plasmid confirming that, similar as described, the N-terminal fragment was not needed for cell viability. Then, L-A virus was introduced by cytoduction from strain 2404 as before and its copy number analyzed by Northern hybridization. We used for comparison the set of deleted strains of Fig. 3B, expressing *SKI4*, *ski4-1* or *hCSL4*. The amount of L-A in cells expressing the truncated Ski4Ntrp was similar to that of those carrying the wild type Ski4p (Fig. 4B). Thus, this result suggests that the RPL27-like domain of Ski4p is not needed for efficient RNA degradation.

G152E mutation impairs *hCSL4* activity

One of the goals of this work was to use *S. cerevisiae* to determine mutations in exosome components that could affect RNA degradation and to try to establish a genetic separation between different exosome functions. The *ski4-1* mutation (G253E) is located in the most conserved amino acid sequence within the S1 domain and decreases the rate of elimination of mRNA without affecting other activities performed by the exosome [van Hoof *et al.*, 2000b] (Fig. 5A). Since the S1 domain is also present in the human ortholog *hCSL4*, we wanted to know if an equivalent mutation in the human gene was involved in RNA degradation. We introduced the G152E modification in the *hCSL4* sequence by *in vitro* mutagenesis. The strain 969 (*ski4-1*) that contained L-BC virus was transformed with

plasmids expressing the wild type genes (*SKI4* or *hCSL4*) or their mutated versions (*ski4-1*, G253E, or *hcsl4*, G152E). Expression of *hCSL4* reduced the amount of this virus, but not as efficiently as *SKI4*, similar to what happened with L-A and M1 Totivirus (Fig. 5B). The strain carrying *hcsl4* (G152E) showed an L-BC copy number similar to that of the *ski4-1* strain, suggesting that the G152 residue in the human ortholog is involved in RNA degradation, like G253 in yeast.

Discussion

In this work we have studied selected exosome-related genes from two organisms (human and the budding yeast *S. cerevisiae*) using two types of RNA virus present in the latter as indicators of the exosome activity. The idea of using Totivirus or Narnavirus copy number in this analysis came from our continuous observations that yeast strains with mutations known to affect the rate of mRNA degradation had increased amounts of RNA viruses. Thus they could be good markers of the exosome efficiency. mRNA degradation is only one of the several tasks accomplished by the exosome, which is also responsible of RNA processing, maturation, etc. We hoped that with this viral approach we might be able to distinguish which exosome components were directly involved in RNA degradation and to establish a genetic separation between different activities.

The exosome is highly conserved throughout evolution and its structure is basically the same in all eukaryotes: a nine-protein ring-shaped core that is catalytically inactive, and to which an exonuclease is attached. The strong similarity among different organisms led us to examine whether ortholog exosome proteins from higher eukaryotes were functional in *S. cerevisiae*. The human exosome has been studied in the last years, although still now little is known about how it works. Several of its components complement lethal exosome gene deletions in *S. cerevisiae*, but its particular effect on yeast RNA degradation has not been studied. We focused our attention on two human genes: *hCSL4*, the ortholog of *SKI4*, and *hSKI2*, the ortholog of the RNA helicase *SKI2* [Lee et al., 1995; Dangel et al., 1995]. In our experiments we did not observe any decrease in Totivirus or 20S RNA Narnavirus amounts in a *ski2-2* or *ski2Δ* strain when we overexpressed *hSKI2*, indicating that the human gene had no activity in *S. cerevisiae*. This lack of effect could be due to the fact that

in yeast Ski2p works as part of a heterotetramer complex (the Ski complex) together with Ski3p and Ski8p [Synowsky and Heck, 2008]. It is likely that the human protein is unable to interact with the other two yeast components and requires the presence of their human counterparts to be fully functional. It has also been reported that Ski7p interacts with the Ski complex in yeast [Wang *et al.*, 2005]. If a similar interaction occurs in human cells, the presence of the human *SKI7* ortholog could also be necessary for the exosome modulator activity of the Ski complex. *hCSL4*, however, not only rescued the null phenotype of *ski4Δ*, but also interacted with the yeast exosome and showed a decrease in the copy number of Totivirus L-A, L-BC and M1, although this effect was slightly different among them. L-A and L-BC amounts decreased to an intermediate level as compared to the expression of *SKI4*, while the amount of M1 was similar in the cells expressing *SKI4* or the human protein *hCSL4*. Because differences in the amounts of M1 between a wild type and a *ski4-1* mutant were larger (more than 5-fold) than those of L-A or L-BC in the same strains (2- to 3-fold), M1 seemed the best choice for a fine analysis of exosome activity. However, M1 needs the helper virus L-A to be maintained in the cell, and it has been described that two Totivirus together in the same host may give rise to mutual exclusion, where each of them is affected by the amount of the other [Field *et al.*, 1982; Ball *et al.*, 1984]. We have observed this phenomenon in several of our experiments, producing somehow unexpected results. For this reason, we decided to use strains with either L-A or L-BC alone. Results of Fig. 3 and 4 show that overexpression of *hCSL4* in yeast decreases Totivirus copy number. Preliminary results pointed that the human gene also affects the amount of 23S RNA Narnavirus (not shown), indicating that this virus could also be used to analyze exosome activity. One example of the data that could be obtained with our approach was the identification of the point mutation G152E in *hCSL4*, equivalent to the G253E modification of *ski4-1*, with a similar effect on L-A copy number. Both mutations are located in the S1 RNA-binding domain, the most conserved region between the two proteins. In the archaeal exosome, it has been reported that the Ski4p counterpart is involved in RNA recognition and confers to the complex some preference for certain substrates [Roppelt *et al.*, 2010]. It is possible that this protein has a similar function in eukaryotes, and that the point mutations analyzed here are eliminating their ability to interact with certain RNAs or to present them to the exosome in a proper way.

When compared with ortholog genes from other organisms, Ski4p shows the highest degree of homology in its central and C-terminal portions, especially in the S1 RNA-binding domain (Fig. 5A). The N-terminal third that contained the RPL27-like motif and the 38 amino acids linker is absent in higher eukaryotes and only present in yeasts. The experiment of Fig. 4 shows that a mutant Ski4Ntrp, which lacked this fragment, had the same effect on L-A copy number than the wild type protein. Therefore, the RPL27-like domain does not seem to be necessary for RNA degradation, though it could be involved in other exosome functions. Further studies would be needed to analyze if this domain is required for activity, or simply represents residual genetic information that has remained in yeast throughout evolution but has lost its function.

Previous works done in other laboratories to study yeast proteins related to the exosome function have often been based on the analysis of the half-life or degradation products of vector-driven RNA transcripts under the control of different promoters: *PGK1*, *GPD*, *GAL*, etc. These transcripts usually contain artificial sequences (e. g. A G-tract that facilitates the identification of degradation products, or different gene reporters) [van Hoof *et al.*, 2000b; He and Parker, 2001; Meaux and van Hoof, 2006]. In our case we have extended these works by analyzing the behaviour of unmodified RNA molecules in their natural environment, the yeast cytoplasm. Even though L-A or L-BC Totivirus have their genomes encapsidated into virions, and thus protected from degradation, their RNA polymerases transcribe them and release the transcripts into the cytoplasm, where they are translated and/or encapsidated to form new virions [Wickner, 1996]. We believe that this cytoplasmic stage is vulnerable to exonuclease degradation and presumably is the step where *hCSL4* works through its interaction within the yeast exosome. Similarly, Narnavirus are also located in the cytoplasm. The genomes with strong secondary structures form ribonucleoprotein complexes with their cognate RNA polymerases [Fujimura and Esteban, 2004; Fujimura and Esteban, 2007], making them quite resistant to degradation by Ski1p. However, as shown here, Narnavirus launching efficiency from vectors or the copy numbers of endogenous 20S RNA and 23S RNA viruses are greatly increased in all exosome mutants tested. Thus, they represent again good indicators of exosome activity.

The use of yeast viruses to study exosome components could provide further information about their role in the complex, either from yeast or from higher eukaryotes.

Human exosome proteins other than the ones analyzed here would be future candidates to increase our knowledge of exosome functions.

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Table 1: List of strains

Strain	Phenotype	Vector	Source
BY4741	<i>MATa his3Δ, leu2Δ, met15Δ, ura3Δ</i> , L-A, L-BC		EUROFAN
Y21980	<i>MATa/α, SKI4/ski4Δ, his3Δ/his3, leu2Δ/leu2Δ, lys2Δ/LYS2, MET15/met15Δ, ura3Δ/ura3Δ</i> , L-A		EUROFAN
969 ¹	<i>MATa ski4-1, ura3Δ, his3Δ</i> , 20S RNA, L-A, L-BC, M1		This work
1368	<i>MATα, kar1-1, his4</i> , L-A, L-BC, M1, 20S RNA		R. B. Wickner ^a
1416	<i>MATα leu1, ski4-1</i> , 20S RNA, L-A, L-BC, M1		R. B. Wickner
2375	<i>MATa ura3Δ, mkt1, ski4-1</i> , 20S RNA, 23S RNA		R. B. Wickner
2404	<i>MATα, kar1-1, his4</i> , L-A, L-BC		R. B. Wickner
1004 ²	<i>MATa, ski4Δ, ura3Δ, his3Δ, leu2</i> , L-A	<i>PGK1-SKI4</i>	This work
1005 ³	<i>MATa, ski4Δ, ura3Δ, his3Δ, leu2</i> , L-A	<i>PGK1-hCSL4</i>	This work
1008 ³	<i>MATa ski4Δ, ura3Δ, his3Δ, leu2</i> , L-A	<i>PGK1-ski4-1</i>	This work
1071 ³	<i>MATa ski4Δ, ura3Δ, his3Δ, leu2</i> , L-A	<i>PGK1-ski4Ntr</i>	This work

¹ Strain 969 was obtained by mating strains 1416 and BY4741, followed by sporulation and tetrad dissection.

² Strain 1004 was obtained by transforming the diploid strain Y29180 with plasmid pRE1128 expressing *SKI4* from the *PGK1* promoter (*PGK1-SKI4*), followed by sporulation and tetrad dissection.

³ Strains 1005, 1008 and 1071 are derivatives of strain 1004 obtained by plasmid shuffling with the vectors indicated.

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Figure legends

Figure 1: The amounts of yeast Narnavirus and Totivirus are affected by mutations in *SKI* genes related to the exosome. (A): Schematic diagram of the yeast exosome core. The three heterodimers (Rrp46p/Rrp43p; Mtr3p/Rrp42p; Rrp41p/ Rrp45p) form a ring-shaped structure stabilized by binding of three other proteins (Ski4p, Rrp4p, Rrp40p). The Ski complex that modulates its activity, together with *SKI7*, is also indicated. (B): Mutant strains deleted of each component of the Ski complex or of *SKI7* were transformed with the 20S RNA launching plasmid pRE763. Total RNAs were prepared from two transformants of each strain, separated in a native 1.3% agarose gel and blotted onto a nylon membrane. The upper panel shows ethidium bromide-staining of the gel (EtBr) with indications of the main RNA species in the samples. The lower panel shows the autoradiogram of the Northern blot in which 20S RNA is detected by hybridization with a specific probe. All *ski* mutants carry higher amounts of L-A dsRNA or 20S RNA than the wild type strain BY4741. (C): Effect of *ski4-1* mutation on Narnavirus and Totivirus copy number. The *ski4-1* mutant strain 969 was transformed with the 23S RNA launching plasmid pMR18 and the virus was detected by Northern hybridization with a specific probe (panel 1). Because this strain also carries L-A and L-BC Totivirus and the killer toxin-encoding M1 dsRNA, we also detected these viruses with specific probes (panels 2-4). As control a wild-type *SKI4* strain (WT) was analyzed.

Figure 2: Expression of *hCSL4* in yeast cells. (A): Growth rate of a *ski4Δ* strain complemented with plasmids expressing *SKI4*, *ski4-1* or *hCSL4* from the *PGK1* promoter. Ten-fold serial dilutions were spotted on YPAD plates and incubated at 28 °C or 37 °C for three days. (B): Interaction of hCsl4p and the exonuclease Dis3p in yeast. TAP-tagged Dis3p was purified with a calmodulin affinity resin from a strain co-expressing hCsl4p-HA. Six fractions were eluted from the resin, and proteins were detected by Western with specific anti-TAP or anti-HA antibodies (upper and middle panels). The lower panel shows a control experiment done in parallel, in which hCsl4p-HA was expressed in the absence of

Dis3p-TAP. We detected no binding of hCsl4p-HA to the resin. E: total extract from the cells. F1-F6: fractions eluted from the resin.

Figure 3: Effect of hCsl4p expression on L-A and M1 Totivirus. (A): Diagram of Ski4p and hCsl4p. The RPL27, S1 and Zn ribbon-like domains of Ski4p are drawn in different grey colours. The 38 amino acids linker rich in glutamate residues is shown in white. hCsl4p only carries the S1 and Zn ribbon-like domains (see text). Numbers under the proteins indicate the amino acid intervals of the domains. (B): L-A Totivirus from strain 2404 was introduced by cytoduction into the *ski4Δ* strains of Fig. 2A. Total RNAs from two colonies of each cytoductant were prepared and hybridized with a specific probe for L-A. Relative virus amounts in each strain are shown under the panel. 1x is the amount of L-A in the strain carrying the wild type *SKI4* gene. (C): L-A and M1 together were cytoduced into the same set of *ski4Δ* cells from strain 1368. Total RNAs were separated in an agarose gel and blotted onto a membrane. L-A and M1 can be visualized by ethidium bromide-staining (EtBr). M1 was also detected with a specific probe.

Figure 4: The N-terminal RPL27-like domain of *SKI4* has no effect on L-A copy number. (A): Diagram of Ski4p and its N-terminal truncated version Ski4Ntrp that lacked amino acids 11-114. hCsl4p is shown below for comparison. (B): L-A virus in the set of *ski4Δ* strains complemented with the genes indicated on the top of the panel were analyzed by Northern hybridization as described in previous figures. An ethidium bromide-staining of the gel and the Northern are shown.

Figure 5: The G152E mutation in the hCsl4p S1 domain impairs RNA degradation activity. (A): Alignment of the amino acid sequences of hCsl4p and Ski4p done with ClustalW (Thompson *et al.*, 1994). Asterisks indicate identical amino acids, two dots conservative changes and one dot semi-conservative changes. S1 RNA binding domains are boxed, and the arrow indicates the position of the G residue modified in the *ski4-1* mutant (G253E). An equivalent mutation (G152E) was introduced in hCsl4p. (B): Strain 969 (*ski4-1*) with endogenous L-BC was transformed with plasmids expressing Ski4p, hCsl4p or their

mutated versions (*ski4-1p* or *hCsl4p, G152E*). The amount of L-BC in two transformants from each plasmid was analyzed as in Figs. 3 and 4.

Figure 1 **Ramírez-Garrastacho M.** **Top**

A

B

Figure 4 Ramírez-Garrastacho M.
Top

A

B

Figure 5 Ramírez-Garrastacho M.
Top

A

Ski4p	MACNFQFPEIAYPGKLICPOYGTEKDGEDIIFNYVPGPG	40
hCsl4p	MAPPVRY---CIPGERLCNLEEGS-----PGSG	25
	** .:: . ** : * . **.*	
Ski4p	TKLIQYEHNGRTLEAITATLVGTVRCEEKKTDQEEEREG	80
hCsl4p	T----YTRHGYIFSSLAGCLMKSEN-----	47
	* * : * : : : * : *	
Ski4p	TDQSTEEKSVDASPNDRVTRRTVKNI	120
hCsl4p	-----GALP-----VVSVVRETES----	61
	. * * : * * : * * : * * : *	
Ski4p	NKYANNDFAANLPKEGDIVLTRVTRLSLQANVEILAVED	160
hCsl4p	-----QLLPDVGAIVTCKVSSINSRFAKVHILYVGS	92
	: * . * * : * : : * : * * * *	
Ski4p	KPSPIDSGIGSNGSGIVAAGGSGAATFSVSQASSDLGET	200
hCsl4p	MP-----LKNS	98
	* * :	
Ski4p	FRGIIRSQDVRSTDRDRVKVIECFKPGDIVRAQVLSLGDG	240
hCsl4p	FRGTIRKEDVRATEKDKVEIYKSFDPGDIVLAKVISLGDA	138
	*** * . : * * : * : : : : : : * : * * * * * * : * : * * * * .	
	↓	
Ski4p	-TNYLTTARNDLGVVFARAANGAGGLMYATDQMMTSPV	279
hCsl4p	QSNYLLTTAENELGVVVAHSE--SGIQMVPISWCEMQCPK	178
	: * * * * * : * * * * : * * * * *	
Ski4p	TGATEKRKCAKPF-----	292
hCsl4p	THTEFRKVARVQPEFLQT-	195
	* : . * * * :	

B

Supplementary figure S1

Supplementary figure S2

Supplementary figure S1: Launching efficiency of 23S RNA from a plasmid is higher in *skiΔ* mutants. Some of the strains used in figure 1, deleted of *SKI2*, *SKI3* or *SKI7* genes, were transformed with the 23S RNA launching plasmid pMR18. As control, strain BY4741 was also transformed. Total RNAs from two transformants of each strain were analyzed as described in figure 1. The upper panel shows an ethidium bromide-staining of the gel, where main RNA species are indicated, and the lower panels show different exposure times of the Northern blot hybridized with a 23S RNA-specific probe. In this native agarose gel the lower amounts of 23S RNA in lanes 1 and 2 do not migrate as a single band, but as a smear along the lane. All *skiΔ* strains showed much higher amounts of the virus.

Supplementary figure S2: *hSKI2* does not complement the *ski2Δ* effect on the amounts of L-A or 20S RNA viruses. (A): The YLR398C strain (*ski2Δ*) derived from the EUROFAN collection was transformed with a plasmid expressing *hSKI2* under the control of *PGK1* promoter. The same strain transformed with the empty vector was used as control. Total RNAs from two colonies of each transformation were prepared and hybridized with a specific probe for L-A. There is no effect of *hSKI2* expression on L-A copy number. (B): Similar results were obtained when the same *ski2Δ* strain was transformed with a centromeric vector expressing *hSKI2* under the *SKI2* promoter or the vector alone. (C): L-A and L-BC Totivirus were eliminated from strain YRL398C by growth at high temperature. Then 20S RNA was launched in these cells from plasmid pRE763. The resultant strain 924 (*MATa*, *his3Δ*, *leu2Δ*, *met15Δ*, *ura3Δ*, 20S RNA) was transformed with the same centromeric plasmid expressing *hSKI2* used in B or the vector alone. Total RNAs from two transformants were prepared, blotted onto a membrane and detected with a specific probe for 20S RNA. The plasmids are indicated in the upper part of the panels with the different promoters expressing *hSKI2* boxed.