

Functional similarities and differences among subunits of the nascent polypeptide-associated complex (NAC) of *Saccharomyces cerevisiae*

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Abstract

Protein factors bind ribosomes near the tunnel exit, facilitating protein trafficking and folding. In eukaryotes, the heterodimeric nascent polypeptide-associated complex (NAC) is the most abundant—equimolar to ribosomes. *Saccharomyces cerevisiae* has a minor β -type subunit (Nac β 2) in addition to abundant Nac β 1, and therefore two NAC heterodimers, α/β 1 and α/β 12. The additional beta NAC gene arose at the time of the whole genome duplication that occurred in the *S. cerevisiae* lineage. Nac β 2 has been implicated in regulating the fate of messenger RNA encoding ribosomal protein Rpl4 during translation *via* its interaction with the Caf130 subunit of the regulatory CCR4-Not complex. We found that Nac β 2 residues just C-terminal to the globular domain are required for its interaction with Caf130 and its negative effect on the growth of cells lacking Acl4, the specialized chaperone for Rpl4. Substitution of these Nac β 2 residues at homologous positions in Nac β 1 results in a chimeric protein that interacts with Caf130 and slows the growth of $\Delta acl4$ cells lacking Nac β 2. Furthermore, alteration of residues in the N-terminus of Nac β 2 or chimeric Nac β 1 previously shown to affect ribosome binding overcomes the growth defect of $\Delta acl4$. Our results are consistent with a model in which Nac β 2's ribosome association *per se* or its precise positioning is necessary for productive recruitment of CCR4-Not *via* its interaction with the Caf130 subunit to drive Rpl4 messenger RNA degradation.

Keywords Ribosomal chaperones · Protein biogenesis factors · Postduplication protein divergence · Protein evolution · NAC-ribosome interaction · CCR4-Not1 complex

Introduction

A number of protein biogenesis factors bind to ribosomes at the tunnel exit, in close proximity to emerging nascent polypeptide chains. The most abundant is the nascent chain-associated complex (NAC). *In vivo* and *in vitro* results, mainly using metazoan systems, indicate that NAC plays important roles in “directing” nascent chains to the appropriate cellular location—by blocking or providing

access to factors such as signal recognition particle or methionine aminopeptidases.^{1–3} NAC also has molecular chaperone activity, for example, preventing aggregation of polypeptide chains.⁴ NAC contains two distantly related subunits, alpha and beta,^{5,6} both of which are less than 20 kDa (Figure 1(a)). Internal segments form the globular core of the α/β heterodimer, which includes antiparallel N-terminal helices followed by 5 beta strands of each subunit. Both subunits also have N-terminal and C-terminal extensions. The N-terminus of the beta subunit is important for NAC's association with the ribosome, through the interaction of positively charged residues with ribosomal RNA (rRNA).^{7,8}

Three genes in the *Saccharomyces cerevisiae* genome encode NAC subunits^{9,10} (Figure 1(a)): *EGD2*, the Nac α subunit; *EGD1*, the abundant Nac β 1 subunit; *BTT1*, the minor Nac β 2 subunit. Nac α and Nac β 1 subunits are at least as abundant as ribosomes, while Nac β 2 is on the order of 20–100 fold less abundant.¹¹ In some

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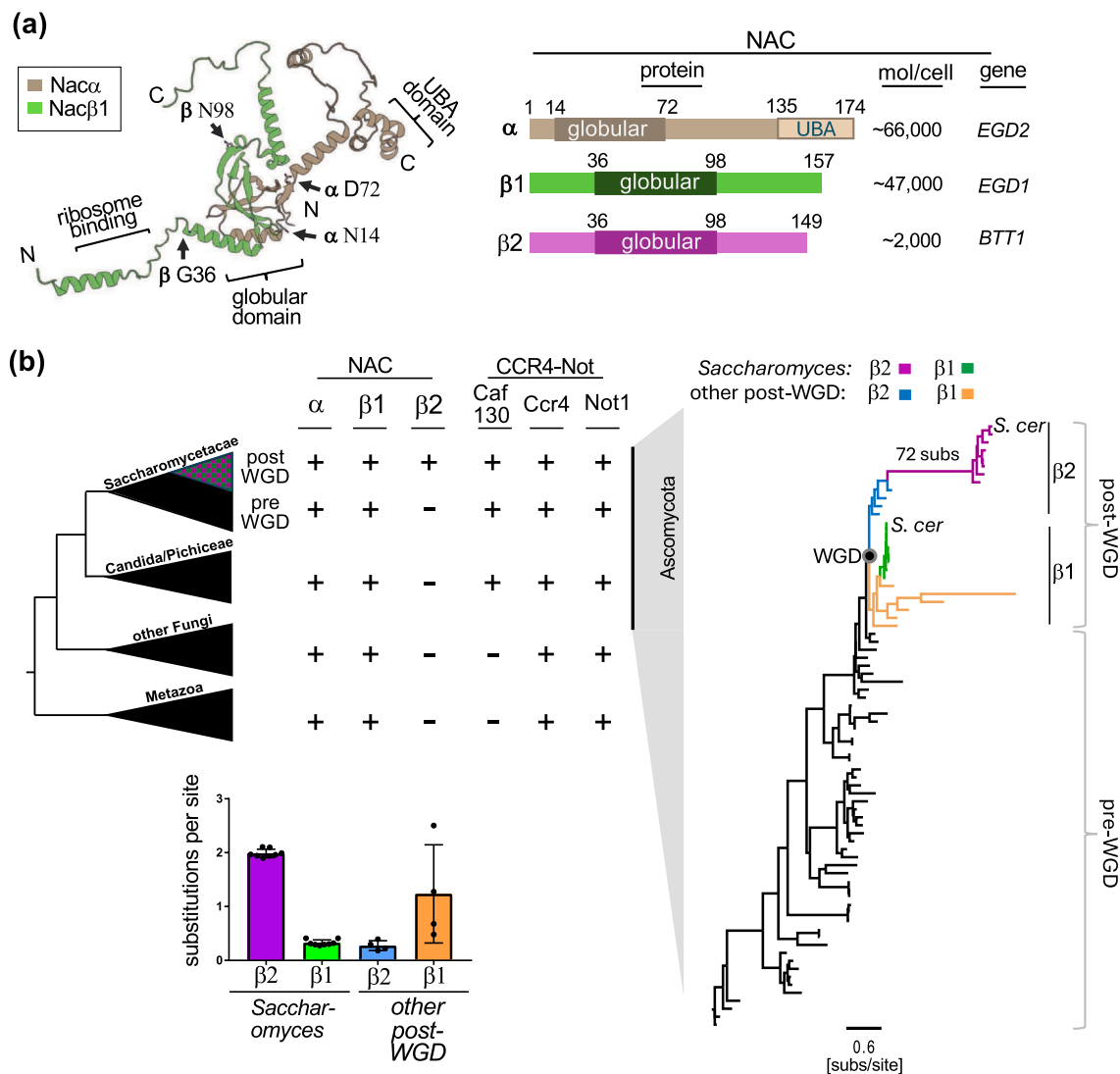


Fig. 1 Evolution of Nacβ1 and Nacβ2 (a) left. Structural model of Nacα/Nacβ1 heterodimer (10.5452/ma-bak-cepc-0495); Nacα (brown); Nacβ1 (green). The bracket designates the globular domain, with its beginning and end indicated by arrows and residue for each subunit. Right. Summary of NAC subunits of *Saccharomyces cerevisiae* with line diagram indicating the globular domains and presence of the UBA domain at the C-terminus of Nacα. Gene names and estimated number of molecules per cell also indicated. (b) Top left. Taxonomic distribution of NAC subunits and selected components of CCR4-Not complex. Saccharomycetaceae pre-WGD and post-WGD clades are indicated. Right: Nacβ protein family evolution in Ascomycota. Phylogeny was reconstructed using beta NAC homologs from 9 pre-WGD and 13 post-WGD Saccharomycetaceae species and 41 homologs from other Ascomycota species using maximum likelihood. The tree topology was constrained such that post-WGD sequences share common ancestry at the WGD node. Nacβ2 (β2): *Saccharomyces* (magenta); other post-WGD species (blue). Nacβ1 (β1): *Saccharomyces* (green); other post-WGD species (orange). Nacβ1 and Nacβ2 from *S. cerevisiae* are indicated. Scale bar—amino acid substitutions per site. Bottom left. Evolutionary distance (substitutions per site) calculated along the branches of the phylogenetic tree from the WGD node to the tips representing beta NAC sequences from post-WGD species. *Saccharomyces* Nacβ2 (magenta) and *Saccharomyces* Nacβ1 (green); other post-WGD Nacβ2 (blue) and other post-WGD Nacβ1 (orange). Abbreviations used: UBA, ubiquitin-associated; WGD, whole genome duplication.

metazoans, NAC has been shown to be essential.¹² In fungi it is not, though its absence causes temperature-sensitive growth in some *S. cerevisiae* strain backgrounds.^{10,13} This lack of essentiality in fungi has been attributed in part to the presence in fungi of the specialized ribosome-associated Hsp70 Ssb.¹⁴ Ssb is the

Hsp70 partner of the specialized ribosome-associated J-domain protein Zuo1 found in all eukaryotes. But in plants and metazoans, Zuo1 functions with the general cytosolic Hsp70s. Ssb and Zuo1 are in very close proximity to NAC at the ribosome tunnel exit, as both can be crosslinked to NAC on the ribosome.¹⁵

Nac β 2, but not Nac β 1, has been implicated in the regulation of the amount of ribosomal protein Rpl4 synthesized.¹⁶ On the practical side, this regulation allows a simple, sensitive readout of Nac β 2 function as its absence allows robust growth of cells having a deletion of the Rpl4-specific chaperone Acl4, which in an otherwise WT background grows very slowly.^{17,18} However, this effect is the result of the action of a conserved complex regulatory system, CCR4–Not, which is involved in global regulation of messenger RNA (mRNA) metabolism both on and off the ribosome.^{19–21} Acl4 chaperone activity is critical because Rpl4 is aggregation-prone when not incorporated into ribosomes.²² When Acl4 is absent or limiting, ribosome-associated Rpl4 mRNA is degraded *via* CCR4–Not, preventing the accumulation of aggregated Rpl4.¹⁶ Evidence indicates that this regulation depends on the recently detected interaction of Nac β 2 (but not Nac β 1) with the Caf130 component of the CCR4–Not complex.^{16,23} The absence of either Nac β 2 or Caf130 suppresses the growth defect of cells lacking Acl4 by alleviating the tight control of mRNA levels.¹⁶ This relief of tight control is thought to allow sufficient expression of Rpl4 to allow more ribosome production, which in turn permits robust growth.

Here, we report the results of our analysis of the specificities and similarities of the two *S. cerevisiae* beta NAC subunits. We found that minimal substitutions outside the globular domain of Nac β 2 disrupt interaction with Caf130 and significantly overcome the growth defect of cells lacking Acl4.

Results and discussion

Evolution of beta NAC subunits and Caf130

As a first step to better understand differences between the two Nac β subunits, we analyzed the evolution of the genes encoding Nac β 1 and Nac β 2. Caf130 was also analyzed because although a CCR4–Not complex is found across both fungi and metazoan species, the CCR4–Not-associated proteins vary among eukaryotes. Interestingly, the presence of both Caf130 and Nac β 2 is restricted to subsets of fungi species. Caf130 is present in the Saccharomycotina clade (Figure 1(b)) but absent in other fungi and in metazoans. Nac β 2 has an even more limited distribution—present in a subset of yeast species that underwent a whole genome duplication (WGD) event.²⁴ Such a distribution suggests that Nac β 2

emerged during the WGD and belongs to a relatively small group of ~450 genes, for which two duplicates were maintained in post-WGD lineages.²⁵

We identified Nac β 1 and Nac β 2 orthologs in post-WGD species based on their position in the genome (i.e., synteny). Among the 13 post-WGD species analyzed, 11 have both Nac β 1 and Nac β 2, while 2 have only Nac β 2. To further analyze the evolutionary relationships among Nac β 1 and Nac β 2 proteins, we reconstructed their phylogeny using the maximum likelihood method (Figures 1(b) and S1). Nac β 2 sequences from *S. cerevisiae* and closely related species (genus *Saccharomyces*) stand out (Figure 1(b), purple). They form a monophyletic group with a very long stem branch, signifying that shared amino acid substitutions accumulated in this lineage—an indication of rapid postduplication divergence. In stark contrast, as indicated by their short branches, paralogous Nac β 1 sequences from the same *Saccharomyces* species accumulated only a few substitutions (Figure 1(b), green). Calculating the number of substitutions along the branches of the tree for Nac β 2 and Nac β 1 sequences from *Saccharomyces* (Figure 1(b)) revealed, on average, six-fold more substitutions for Nac β 2 than for Nac β 1. This substantial accumulation of substitutions in Nac β 2 did not occur in other post-WGD species—in fact, Nac β 1 sequences accumulated on average more substitutions than Nac β 2 (Figure 1(b)). It is also worth noting that, in contrast to other post-WGD species, all analyzed *Saccharomyces* species maintained both Nac β 1 and Nac β 2.

For this report focusing on *S. cerevisiae*, the important point is that our analysis revealed that, in the *Saccharomyces* lineage, Nac β 2 evolved rapidly, accumulating many substitutions, while Nac β 1 evolved slowly, preserving much of its pre-WGD sequence. It is often the case that one duplicate (i.e., Nac β 1) maintains both the original preduplication function(s) and expression level, while the other duplicate (i.e., Nac β 2) evolves a new function and reduced expression level.^{26–30} It is also well documented that a lower expression level correlates with a higher accumulation of neutral and near-neutral substitutions.^{31–33} However, this makes it challenging to tease out sequence differences responsible for functional differences. As discussed below, we turned to a genetic approach to better define the sequences in Nac β 2 specifically important for its function in relation to Acl4 and whether other aspects of NAC functionality held in common with Nac β 1 are important as well.

A Nac β 2 segment C-terminal to the globular domain is required to exacerbate Δ acl4 growth defect

To better define sequences in Nac β 2 required for inhibition of growth of cells lacking Acl4, we made constructs to express variants, concentrating on the regions least conserved between it and Nac β 1 (Figure 2(a)). We first tested a 7 residue segment that is just N-terminal to the α -helix of the globular domain, spanning residues 31–37 (³¹GNLYNNN³⁷). This segment of Nac β 2 was replaced by the analogous segment of Nac β 1 (³¹AGSSAGA³⁷), generating Nac β 2(β 1_{31–37}). Δ acl4 Δ nac β 2 cells expressing Nac β 2(β 1_{31–37}) grew as poorly as those expressing wild-type (WT) Nac β 2 (Figure 2(c)), indicating that it maintained Nac β 2-specific activity. Next, we made Nac β 2 C-terminal truncations as there is

little sequence identity between Nac β 1 and Nac β 2 in this region (Figure 2(b)). Δ acl4 Δ nac β 2 cells expressing Nac β 2_{1–126}, which lacks the 23 most C-terminal residues, grew as poorly as cells expressing full-length Nac β 2. But those expressing Nac β 2_{1–111} grew well (Figure 2(c)), suggesting a loss of Nac β 2 regulatory function and implicating residues between 112 and 126 as functionally important.

To better define important residues, we made two NAC β 2 variants based on comparison with Nac β 1 sequences (Figure 2(a)), beginning with substituting¹: all Nac β 2 residues for those of Nac β 1 between positions 112 and 117 (i.e., ¹¹²SQELEY¹¹⁷ to ¹¹²PEAIQA¹¹⁷)²; residues at positions 120, 123, and 124 (G120Q/H123A/N124Q). The variant with substitutions within the 112–117 interval suppressed the Δ acl4 growth defect, indicating loss of

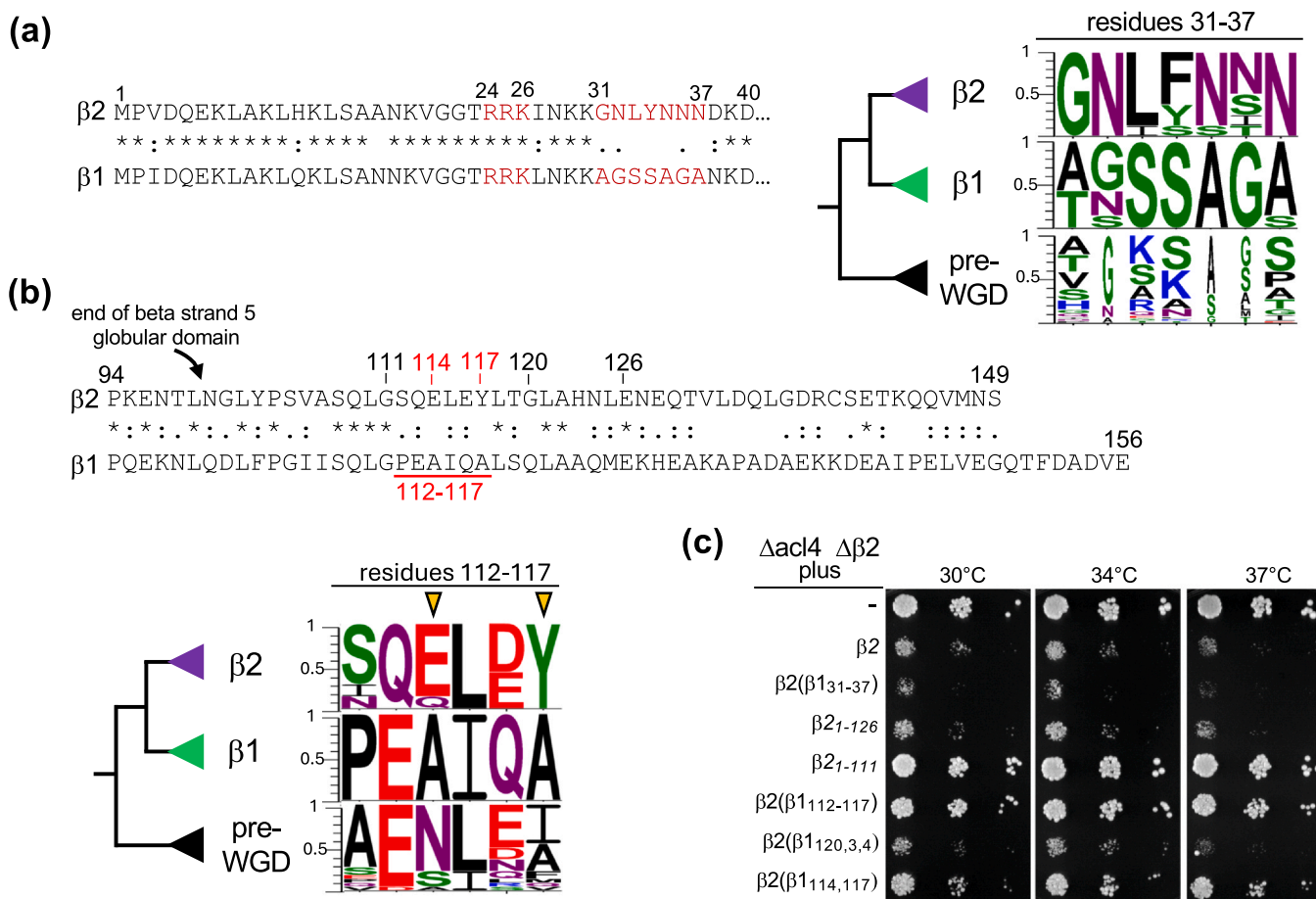


Fig. 2 Sequences C-terminal to the globular domain of Nac β 2 important in inhibiting the growth of cells lacking Acl4. Sequences comparisons of segments N-terminal (a) and C-terminal (b) to the globular domains of Nac β 2 and Nac β 1 ("*" invariant, "." high similarity, "." low similarity). Sequence particularly relevant for genetic analyses underlined/highlighted in red, including RRR (residues 24–26) previously demonstrated to be important for interaction with the ribosome. Sequence logos represent the amino acid frequency of each residue position in the 31–37 and 112–117 segments of Nac β 1 (β 1) and Nac β 2 (β 2) post-WGD, and for Nac β 1 pre-WGD (pre-WGD): positively charged (blue), negatively charged (red), neutral (purple), polar (green), hydrophobic (black); narrow lettering indicates the presence of gaps in the alignment. Position numbering for *S. cerevisiae*. Position of single residue changes made in the 112–117 segment indicated by arrowheads. (c) Ten-fold serial dilutions of Δ acl4 Δ nac β 2 (Δ acl4 Δ β2) cells expressing the indicated Nac β 2 proteins from a plasmid (-, empty vector) were spotted onto plates and incubated at the indicated temperatures for 2 days. Abbreviation used: WGD, whole genome duplication

function. However, the 120/123/124 variant maintained function, that is, $\Delta acl4 \Delta nac\beta 2$ cells grew as poorly as when WT Nac $\beta 2$ was present. Based on conservation in the 112–117 region (Figure 2(b)), we then switched residues at positions 114 and 117 (E114A/Y117A). This double substitution variant has reduced function in regard to $\Delta acl4$, that is, cells having the *ACL4* deletion grew better when expressing Nac $\beta 2_{E114A/Y117A}$ than when expressing WT Nac $\beta 2$ (Figure 2(c)).

The simplest explanation for the observed functional difference between WT Nac $\beta 2$ and the variants is a disruption of its interaction with Caf130. Since results of previous yeast two-hybrid assays defined a region between Nac $\beta 2$ residues 38 and 129 as sufficient for interaction with Caf130,¹⁶ we decided to also employ yeast 2-hybrid assay analysis, which allows *in vivo* detection of protein-protein interaction, to test this idea (Figure 3(a)). As expected, cells grew well under 2-hybrid test conditions when WT Nac $\beta 2$ or Nac $\beta 2_{1-129}$ was used as bait. But when Nac $\beta 1$ or Nac $\beta 2_{1-111}$ was used, growth was not observed. Importantly, cells expressing Nac $\beta 2_{E114A/Y117A}$ also did not grow under test conditions, indicating that the substitutions that suppressed the $\Delta acl4$ growth defect also disrupted Nac $\beta 2$ interaction with Caf130. These

results are consistent with the Caf130-Acl4 interaction being a key distinction between Nac $\beta 1$ and Nac $\beta 2$ in this function.

Because residues critical for the functional interaction with Caf130 (i.e., E114/Y117) are conserved among Nac $\beta 2$ sequences from *Saccharomyces* species (Figure 2(b)), we hypothesized that they emerged in their common ancestor. Indeed, phylogeny-based statistical reconstruction of the ancestral Nac $\beta 2$ sequences revealed that A114E/A117Y substitutions were among the 72 amino acid changes that took place along the long branch leading to the most recent common ancestor of *Saccharomyces* (Figure S1). What triggered the rapid divergence of the Nac $\beta 2$ sequence is unclear. However, many of these substitutions may be neutral, as no growth phenotypes were observed when the Nac $\beta 2$ regions encompassing them were swapped or deleted. These results are consistent with an evolutionary scenario where Nac $\beta 2$ appeared early as a result of the WGD, while substitutions critical for interaction with Caf130 emerged later, in the common ancestor of *Saccharomyces*. Therefore, these substitutions are only present in a subset of post-WGD species closely related to *S. cerevisiae*.

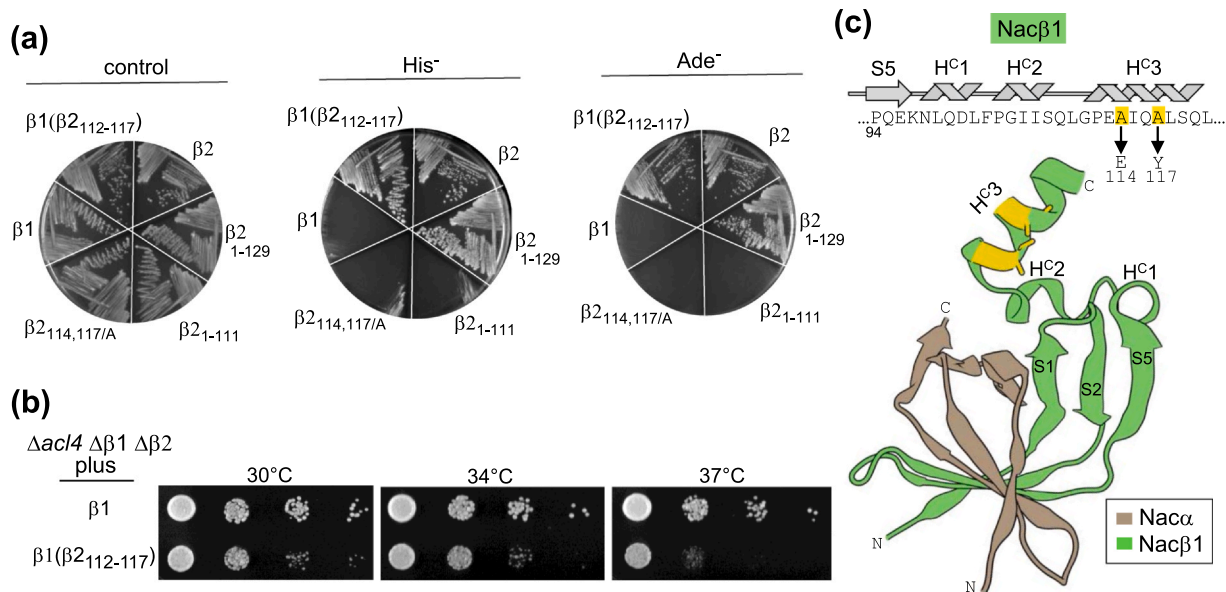


Fig. 3 Nac $\beta 1$ /Nac $\beta 2$ interaction with Caf130. (a) Two-hybrid interaction of Nac $\beta 1$ and Nac $\beta 2$. Plasmids expressing full-length Caf130 fused to the *GAL4* activation domain and the indicated beta NAC wild-type or variants fused to the *GAL4* DNA binding domain were cotransformed into the 2-hybrid readout *S. cerevisiae* strain. Transformants were streaked on media selecting for the plasmids (left), or on media additionally lacking histidine (His, center) or adenine (Ade, right) and incubated at 30 °C for 3 days to monitor interaction. (b) Effect of Nac $\beta 1$ chimera on $\Delta acl4$ phenotype. Ten-fold serial dilutions of $\Delta acl4 \Delta nac\beta 1 \Delta nac\beta 2$ ($\Delta acl4 \Delta \beta 1 \Delta \beta 2$) cells expressing the indicated flag-tagged proteins were plated and grown for 2 days at indicated temperatures. (c) Model of NAC $\alpha/\beta 1$ heterodimer as in Figure 1(a) showing residues Nac α^{29-73} Nac $\beta 1$ (53–121). Alanines at positions 114/117 in stick representation (yellow). Sequence shown above with beta strands 1, 2, 5 (S1, S2, S5) of globular domain and helical regions¹⁻³ of C-terminal extension (H^C1, H^C2, H^C3) indicated.

Substitution of Nac β 2 sequences in Nac β 1 confer Caf130 interaction and inhibition of Δ acl4 growth

Since the results reported above are consistent with the idea that interaction with Caf130 is the sole functional difference between Nac β 2 and Nac β 1 in regard to regulation of Rpl4 levels, we wanted to ask if Nac β 1 could be “converted” to have Nac β 2-like function. We carried out two experiments analogous to those discussed above. First, residues at positions 112–117 of Nac β 1 were changed to those of Nac β 2 (112 PEAIQA 117 to 112 SQELEY 117) generating Nac β 1($\beta_{2_{112-117}}$). Nac β 1($\beta_{2_{112-117}}$) supported growth under both 2-hybrid test conditions (Figure 3(a)), indicating interaction with Caf130. Next, we swapped this Nac β 2 segment into Nac β 1 for expression in yeast to test its effect on the growth of cells having a deletion of *ACL4*. Cells lacking *Acl4*, Nac β 1, and Nac β 2, but expressing Nac β 1($\beta_{2_{112-117}}$), grew more poorly than cells expressing WT Nac β 1, indicating that it had gained Nac β 2 activity (Figure 3(b)). Together, these results support the idea that the ability to interact with Caf130 is the sole difference between NAC β 2 and NAC β 1 needed to exert the “Nac β 2 negative effect” on Δ acl4 growth.

As shown by initial crystal structures of human NAC 5,6 and structural modeling of yeast NAC, 34 an alpha-helical segment is immediately C-terminal to the last beta-strand of the globular domain (S5). This region is composed of two very short helical segments (H c 1 and H c 2), followed by a longer one (H c 3) that was not included in the crystal structures. The residues identified here as important for Caf130 interaction (114/117) are in H c 3 (Figure 3(c)). Their packing against the NAC globular domain in the model makes it unlikely that they directly participate in intermolecular interaction with Caf130. However, the more bulky E114/Y117 residues of Nac β 2 may confer a small difference in positioning of H c 3 that could, without introducing major structural changes, effect changes in conformation that allow or enhance a functionally stable interaction with Caf130. This possibility is especially intriguing considering that their location on H c 3 positions them close to the pivot point of any rotation between H c 3 and the NAC globular domain.

However, since no structural information is available for Caf130, a more detailed analysis of a Nac β 2-Caf130 interaction is problematic. Yet, it is intriguing that even though several functions have been attributed to the globular domain extensions of NAC, $^{35-37}$ to our knowledge, this is the first time the junction between the globular domain and C-terminal extension has been linked to an *in vivo* function. It will be interesting to see in the future whether other functions will be uncovered for this region.

Disturbance of Nac β 2 ribosome association promotes the growth of Δ acl4 cells

Both NAC and the CCR4–Not complex are known to function at the ribosome. To examine whether a defect in ribosome association affects the ability of Nac β 2 to exacerbate the growth defect of Δ acl4 cells, we employed the commonly used variant having alanine substitutions of the conserved sequence previously shown to affect ribosome association— 24 RRK 267 (see Figure 1(a)). Δ acl4 Δ nac β 2 cells expressing Nac β 2 $_{RRK/AAA}$ grew better than those expressing WT Nac β 2 (Figure 4(a)). We also tested the effect when only *Acl4* was absent. Nac β 2 $_{RRK/AAA}$ improved the growth of Δ acl4 cells, even though WT Nac β 2 was present, as did low expression of chimeric Nac β 1 $_{RRK/AAA}$ ($\beta_{2_{112-117}}$). As expected, Nac β 1 $_{RRK/AAA}$ did not (Figure 4(a)). Together, these results indicate a dominant effect of the RRK/AAA mutation in a beta NAC protein that can interact with Caf130.

Together the results presented in these three sections suggest a model (Figure 4(b)) in which Nac β 2’s role in modulating the amount of Rpl4 synthesized is recruitment of the CCR4–Not complex to ribosomes translating Rpl4 mRNA—via its interaction with the Caf130 subunit. Presumably, this places CCR4–Not subunits directly involved in mRNA deadenylation and degradation (such as Not2, Not3, and Not5) in close proximity to the mRNA. 16,38 If Nac β 2 (or chimeric Nac β 1) defective in ribosome association is present, CCR4–Not is titrated away from the ribosome, negating degradation and resulting in an increase in Rpl4 synthesis. This increase in Rpl4 allows the production of more ribosomes, allowing more robust growth.

Cells lacking both beta subunits are temperature-sensitive but rescued by RRK/AAA variant

We noted during our analysis of *Acl4* that, consistent with previous results using strain W303, 10 cells lacking both beta subunits (Δ nac β 1 Δ nac β 2) are temperature sensitive. The double mutant grows poorly at both 34 and 37 °C (Figure 5(a)). The single deletion strains (i.e., Δ nac β or Δ nac β 2) grow like WT. The robust growth phenotype of cells lacking highly expressed Nac β 1 suggests that only a low level of heterodimer is required, as Nac β 2 is not up-regulated in the absence of Nac β 1 (Figure 5(a)). To test whether lower levels of Nac β 1, like Nac β 2, are sufficient for WT growth, we placed it under the control of the Nac β 2 promoter. Expression was substantially reduced, yet cells having this construct grew as well as cells expressing normal levels of Nac β 1 (Figure 5(b)). We conclude that W303 requires only a low level of a NAC

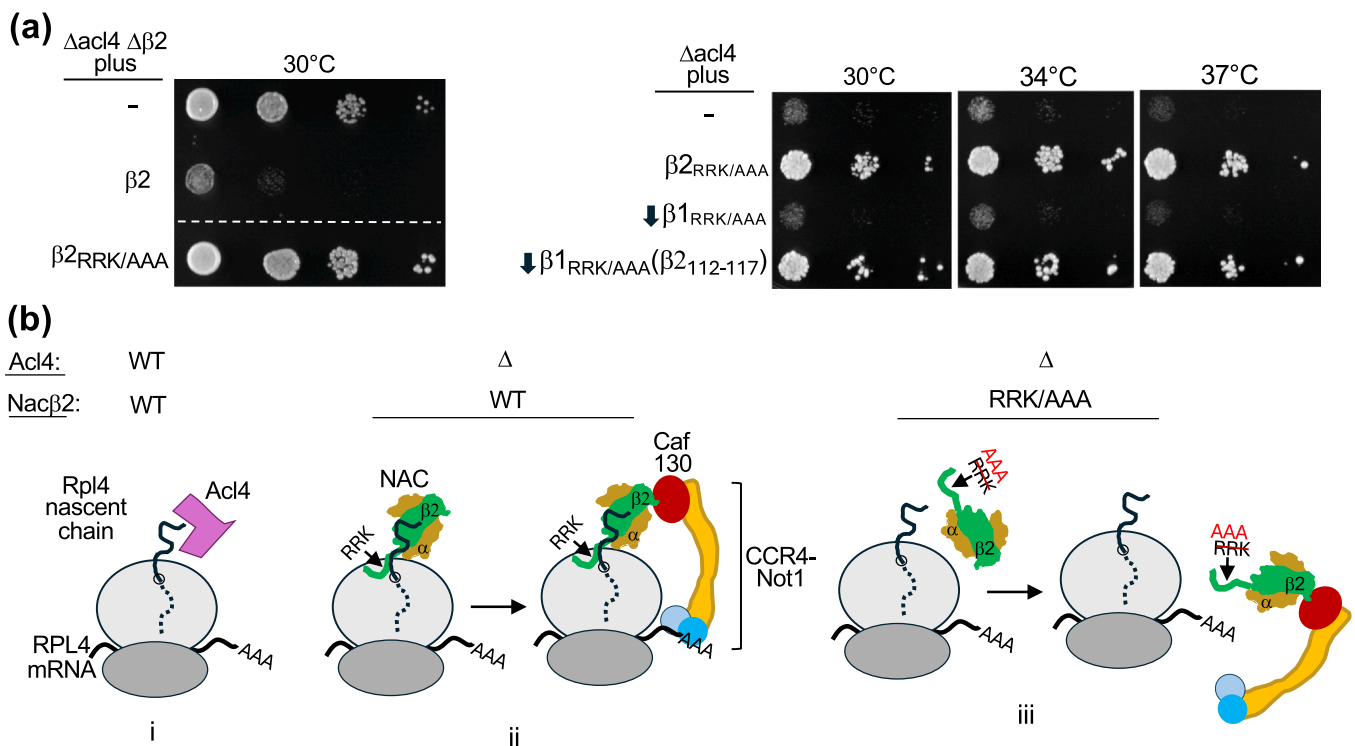


Fig. 4 Effect of RRK/AAA mutation on $\Delta acl4$ cells and model of function in absence of Acl4. (a) Ten-fold serial dilutions of $\Delta acl4 \Delta nac\beta 2$ ($\Delta acl4 \Delta \beta 2$) or $\Delta acl4$ cells (left and right, respectively) expressing the indicated Nac $\beta 2$, Nac $\beta 1$, or chimeric protein from a plasmid (\downarrow indicates wild type or chimeric Nac $\beta 1$ expressed from the Nac $\beta 2$ promoter; -, empty vector) were spotted onto plates and incubated at the indicated temperatures for 2 days. The dotted line on the left indicates splicing out of irrelevant strains from the image of the plate. (b) Model of the effect of NAC and the absence of Acl4 on the regulation of Rpl4 mRNA levels. (i) Normally, Acl4 binds Rpl4 nascent chains, preventing their aggregation. (ii) If Acl4 is absent, Nac α /Nac $\beta 2$ present near the tunnel exit of the 60S ribosomal subunit recruits CCR4-Not1 via interaction with its Caf130 subunit. Other subunits positioned near the mRNA on the 40S initiate the degradation of Rpl4 mRNA. (iii) If association of NAC with the ribosome is disturbed, CCR4-Not complex is not recruited to the ribosome (or positioned nonproductively), resulting in continued translation of mRNA, which allows assembly of sufficient ribosomes for robust growth. mRNA, messenger RNA.

heterodimer for normal growth, and either a Nac α /Nac $\beta 1$ or Nac α /Nac $\beta 2$ heterodimer suffices.

We next tested the ability of Nac $\beta 2$ having the RRK/AAA ribosome association mutation to overcome the temperature-sensitive growth phenotype of cells having neither WT Nac $\beta 1$ nor Nac $\beta 2$. Cells expressing either Nac $\beta 1_{RRK/AAA}$ or Nac $\beta 2_{RRK/AAA}$ as the only beta subunit grew as well as WT cells (Figure 5(c)), as did cells expressing Nac $\beta 1_{RRK/AAA}$ under the control of the Nac $\beta 2$ promoter. This result was unexpected. As recent analyses have tied NAC function closely to the ribosome,^{1,12,39,40} we had anticipated that the RRK/AAA variant would not support growth at 37°C.

The standard test for ribosome association is comigration during high-speed centrifugation through a sucrose cushion to separate ribosomes from soluble proteins.^{7,14} Such an assay requires sustained association after cell lysis. We therefore turned to *in vivo* site-specific crosslinking to assess the effect of the RRK to AAA substitution, because crosslinking prior to cell lysis could help stabilize the interaction through the sucrose cushion centrifugation step. Furthermore, this

method provides an assessment of ribosome interaction in the cell. We made use of the previously detected ability of ribosome-associated Hsp70 Ssb1 to crosslink to NAC.¹⁵ More specifically, Bpa, a photoactivatable non-canonical amino acid, when incorporated at position S563 of Ssb1, crosslinks to NAC $\beta 1$ (Figure 5(d)). A crosslinked product between Ssb1_{S563Bpa} and Nac $\beta 1_{RRK/AAA}$ was also detected in the ribosome pellet fraction. Although less than that observed for WT Nac $\beta 1$, this result indicates that a portion of the RRK/AAA NAC variant is ribosome-associated in the cell.

The crosslinking results indicate that the RRK/AAA variant disturbs but does not completely disrupt NAC's ribosome association. Therefore, whether the temperature-sensitive phenotype of $\Delta nac\beta 1 \Delta nac\beta 2$ is due to a NAC function occurring on or off the ribosome remains unclear. Furthermore, it also leaves open the possibility that the effect of the RRK/AAA mutation on the $\Delta acl4$ phenotype could well be due to altered positioning of CCR4-Not at the ribosome rather than complete displacement from the ribosome. Furthermore, the crosslinked Ssb1-NAC we detected could be an underestimate,

as a change in the position of the globular domain could result in a decrease in crosslinking even though NAC is still present on the ribosome. Unfortunately, designing a variant that completely disrupts ribosome association without disrupting other functions as well is difficult. Other sequences in N-terminal regions,⁸ as well as sequences in the helices of the globular domain,^{41,42} have also been shown to affect NAC's binding to the ribosome.

In the presence or absence of Nac α , Nac β 2 supports growth as well as Nac β 1 when expressed similarly

To carry out the experiments described in the previous section, we constructed a strain carrying a deletion of the Nac α encoding gene. We noted that Δ nac α cells grew moderately slower at 37°C than WT (Figure 6(a))—the only growth phenotype observed for a single NAC deletion.

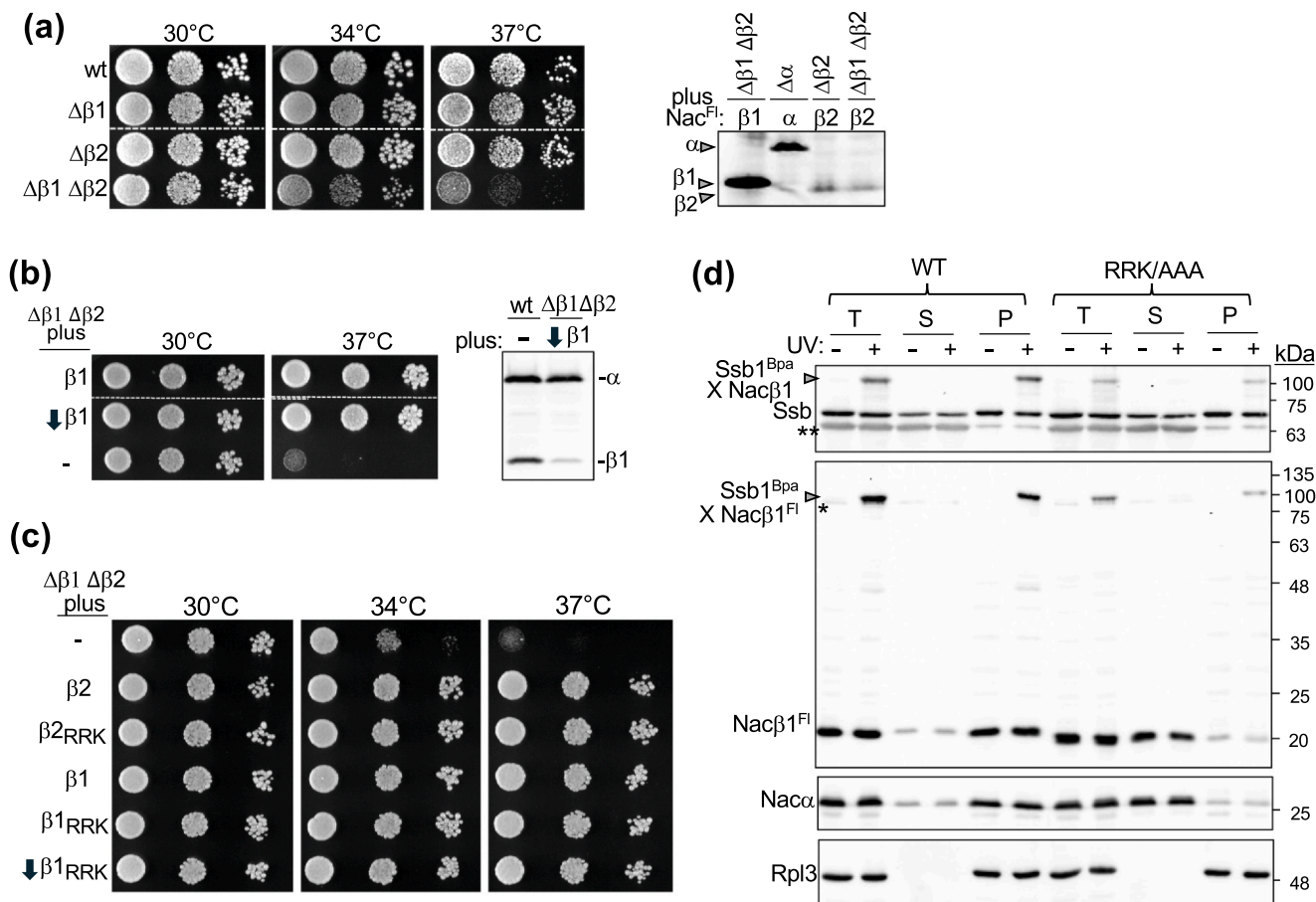


Fig. 5 Growth defect of cells lacking Nac β 1 and Nac β 2 and effect of RRK/AAA mutation. (a) Left. Growth of wild-type (WT) and strains having deletions of genes encoding beta NAC subunits: Nac β 1 ($\Delta\beta$ 1), Nac β 2 ($\Delta\beta$ 2), or both beta subunits ($\Delta\beta$ 1 $\Delta\beta$ 2). Ten-fold serial dilutions were plated on rich media and incubated at indicated temperatures for two days. The dotted line indicates the removal of nonrelevant strains from the plate image. Right. Indicated deletions were transformed with a plasmid carrying a gene encoding an NAC subunit with a Flag tag to allow comparison of expression levels. Immunoblot analysis of cell extracts separated by SDS-PAGE using antibody specific for the Flag tag. (b) Reduced expression of Nac β 1 by placement under the control of Nac β 2 promoter ($\downarrow\beta$ 1). Left. Ten-fold serial dilutions of $\Delta\beta$ 1 $\Delta\beta$ 2 carrying indicated plasmid were plated and incubated for 2 days; empty vector (-). The three strains were plated on the same plate; the dotted line indicates the removal of nonrelevant strains from the plate image. Right. Immunoblot comparing levels of Nac α and Nac β 1 in wt cells and $\Delta\beta$ 1 $\Delta\beta$ 2 cells expressing Nac β 1 under control of Nac β 2 promoter. A mixture of polyclonal antibodies recognizing Nac α and Nac β 1 was used. (c) Growth of $\Delta\beta$ 1 $\Delta\beta$ 2 expressing RRK/AAA (RRK) variants. Ten-fold serial dilutions of $\Delta\beta$ 1 $\Delta\beta$ 2 carrying indicated plasmid were plated and incubated at indicated temperatures for 2 days. (d) Immunoblot analysis of *in vivo* crosslinking on the ribosome between Ssb1 containing Bpa at position 563 and Flag-tagged Nac β 1 or Nac β 1_{RRK/AAA}. After the separation of cell lysates *via* centrifugation through a sucrose cushion, samples of total, supernatant, and pellet were resolved on SDS-PAGE gels. UV-treated (+); untreated (-). Mobility of markers at right; Flag antibody was used to detect Nac β 1^{Fl}; *, indicates background band that is present in total and supernatant fractions; **, truncated Ssb1 caused by incomplete incorporation of Bpa at stop codon.

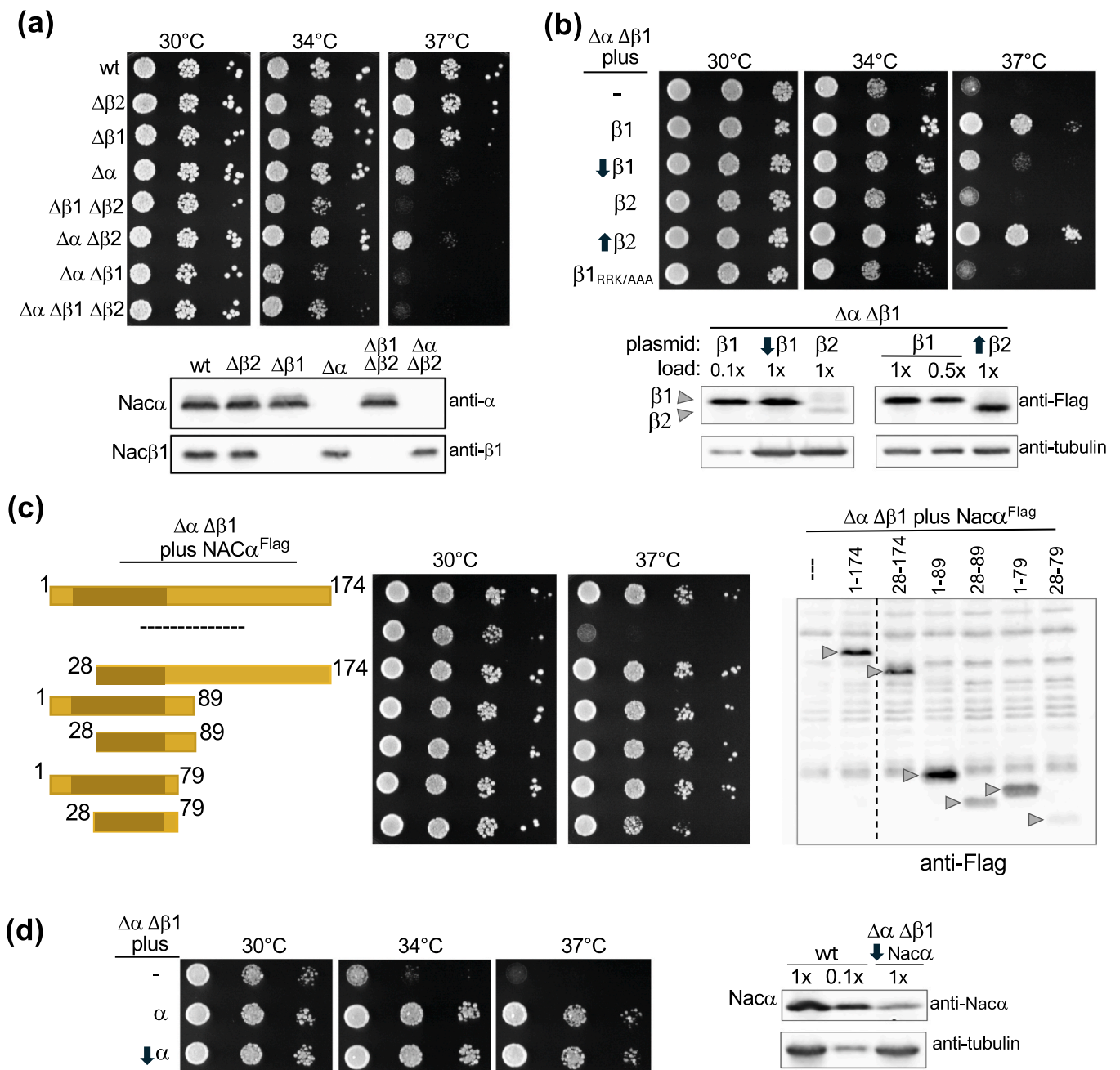


Fig. 6 Growth defects when *Nacα* is absent. (a) Top. Growth of wild-type (WT) and strains with indicated deletions of genes encoding NAC subunits: *Nacα* ($\Delta\alpha$), *Nacβ1* ($\Delta\beta1$), *Nacβ2* ($\Delta\beta2$), or combinations. Ten-fold serial dilutions were plated and incubated at indicated temperatures for 2 days. Bottom. Immunoblot analysis of cell extracts, from indicated deletion strains, after separation by SDS-PAGE, using antibody specific for *Nacα* or *Nacβ1*. (b) Requirement of a beta NAC when *Nacα* is absent. Top. Ten-fold serial dilutions of $\Delta nac\alpha \Delta nac\beta1$ ($\Delta\alpha \Delta\beta1$) cells transformed with plasmids expressing the indicated NAC proteins having a Flag tag were plated and incubated at indicated temperatures for 2 days. Empty vector (-); *Nacβ2* promoter driving expression of *Nacβ1* ($\downarrow\beta1$); the strong *ADHI* promoter driving *Nacβ2* ($\uparrow\beta2$). Bottom. The indicated amount of cell lysates (load) from $\Delta\alpha \Delta\beta1$ cells containing a plasmid expressing the indicated Flag-tagged NAC subunit were subjected to immunoblot analysis using Flag or, as a control, tubulin-specific antibodies. (c) *Nacα* truncations. Left. Ten-fold serial dilutions of $\Delta nac\alpha \Delta nac\beta1$ ($\Delta\alpha \Delta\beta1$) cells transformed with a plasmid expressing the indicated *Nacα* deletion variants having a Flag tag or empty vector (—) were plated and incubated at the indicated temperatures for two days. Right. Immunoblot analysis of cell extracts of indicated deletion strains separated by SDS-PAGE using antibody specific for Flag tag. *Nacα* fragments are indicated by arrowheads. The dotted line indicates that an irrelevant lane of the gel was cropped out of the immunoblot image. (d) Lowered *Nacα* expression. Left. Ten-fold serial dilutions of $\Delta nac\alpha \Delta nac\beta1$ ($\Delta\alpha \Delta\beta1$) cells transformed with plasmids were plated and incubated at the indicated temperatures for 2 days (30 or 34 °C) or 3 days (37 °C). *Nacα* under its native promoter, α ; *Nacα* under the *Nacβ2* promoter, $\downarrow\alpha$; empty vector, -. Right. Immunoblot analysis of lysates from wt and $\Delta\alpha \Delta\beta1$ cells expressing *Nacα* driven by the *Nacβ2* promoter ($\downarrow\alpha$).

We, therefore, compared all combinations of NAC deletions. Of the three double deletion strains, two grew very poorly at 37 ° and more slowly than WT cells at 34 °C— $\Delta nac\alpha \Delta nac\beta 1$ and $\Delta nac\beta 1 \Delta nac\beta 2$, which was discussed above. The triple deletion $\Delta nac\alpha \Delta nac\beta 1 \Delta nac\beta 2$ grew similarly to these two double mutants (Figure 6(a)).

The relatively robust growth of cells expressing Nac β 1 in the absence of Nac α suggests that Nac β 1 is able to, at least to some extent, act independently of Nac α . In other words, a heterodimer is not absolutely required for all NAC-related activity. This result raises the question of whether Nac β 2 could suffice in place of Nac β 1 if present in sufficient amounts. We therefore placed Nac β 2 under the control of the *ADH1* promoter, which drove its expression to approximately the same level as that of NAC β 1 (Figure 6(b)). This construct was able to rescue the temperature-sensitive growth defect caused by the lack of Nac α and Nac β 1 (Figure 6(b)). The previously described construct having Nac β 1 driven by the Nac β 2 promoter, and therefore expressing lower amounts of Nac β 1, did not rescue growth nearly as well as a construct expressing Nac β 1 from its native promoter.

Together these results indicate that, for the function (s) successfully carried out in the absence of Nac α , either Nac β 1 or Nac β 2 suffices—as long as expression is high. They are also consistent with previous indications that beta subunits may well function as homodimers.^{8,43} Whether the requirement for high expression is because only a small amount of functional homodimer is formed or that the sequences in Nac α possess activity important for this function is not clear. Interestingly, Nac β 1 having the RRK/AAA mutation Nac β 1_{RRK/AAA} was not able to rescue the temperature-sensitive phenotype of cells lacking Nac α (Figure 6(b)). Whether this is because Nac α substantially affects ribosome association, partially overcoming the effects of the RRK/AAA mutation remains an open question. It should be noted that the Nac α alpha helix in the globular domain of metazoan NAC has been implicated in ribosome association.⁴²

We next made truncation mutants to test if N or C terminal sequences of Nac α are important for cell growth in the absence of Nac β 1. Deletions removing N-terminal or C-terminal segments that included the ubiquitin association domain supported growth as well as full-length protein (Figure 6(c)). Even a 51 residue 28–79 fragment supported colony formation at 37 °C, though growth was not as robust as when other constructs were used. However, its level of expression was much lower than that of other constructs (Figure 6(c)). We therefore wanted to ask if lower expression of full-length Nac α sufficed. Nac α was placed under the control of the Nac β 2 promoter. Lower levels of Nac α were sufficient to support robust growth (Figure 6(d)). Together, our results suggest that only the core domain of

Nac α suffices to overcome the growth defect of strains expressing only low levels of the beta subunit, that is NAC β 2. This limited requirement is consistent with the possibility that for this phenotype the important function of the alpha subunit is to efficiently form dimers, maintaining the beta subunit in a functional conformation.

Concluding remarks

Our results from combining experimental and evolutionary analyses are consistent with the following scenario regarding the regulatory interaction of Nac β 2 with the Caf130 subunit of the CCR4–Not complex: Caf130 emerged prior to the WGD. After the WGD, Nac β 2 of the *Saccharomyces* lineage underwent extensive divergence. Among the amino acid substitutions, two outside the globular domain were necessary for Nac β 2's interaction with Caf130. As described previously,¹⁶ this positions the CCR4–Not complex such that Rpl4 mRNA is degraded in the absence of its specific chaperone, Acl4.

Our results also indicate that it may be more difficult than generally appreciated to completely disrupt NAC binding to the ribosome without mutating sequences that have other functions. This makes it challenging to formulate definitive conclusions about the relationship between ribosome association and function and also highlights the need for methods to assess associations within the cell, as well as *in vitro* biochemical and structural approaches.

In a broader context, it should be emphasized that *S. cerevisiae* has an unusually complex system of factors at the ribosome tunnel exit—not only the two beta NAC subunits found only in closely related yeast species, the subject of this report, but also the complex tripartite Hsp70 system found in all fungi, including a ribosome-specific Hsp70. Likely because of this, the apparent phenotypes observed upon disruption of NAC in *S. cerevisiae* are not dramatic compared to those seen in metazoans. Yet, as analyses become more nuanced, more phenotypes are becoming more apparent. One example is the Acl4-related phenotype exploited here. But it also has been recently shown that Nac β 1 and Nac β 2 play roles in other important cellular processes—in selective mitochondrial degradation⁴⁴ and mitochondrial protein import,⁴⁵ respectively. Future research will likely uncover additional roles.

Materials and methods

Evolutionary analyses

Orthologs of Nac β 1 and Nac β 2 sequences were retrieved from OMA orthology database HOG_B0614165 group,

KEGG, and Yeast Protein Database (YPD). Nac β 1 and Nac β 2 orthologs in post-WGD species were identified using synteny viewer at Yeast Genome Order Browser. Sequences were aligned using Clustal Omega v1.2.2 with default parameters. Alignment logos were generated using the WebLogo3 server.⁴⁶ Protein phylogeny, with constrained topology such that Nac β 2 and Nac β 1 share common ancestry at the node representing WGD, was inferred using the maximum likelihood method (ML). One thousand ML searches were performed using iQ-TREE⁴⁷ with 1000 rapid bootstrap replicates, under the LG model of amino acid substitution and GAMMA model of rate heterogeneity with four discrete rate categories and the estimate of the proportion of invariable sites (LG + I + F + G4),⁴⁸ which was determined as the best-fit model by Bayesian Information Criterion with ModelFinder.⁴⁹ The constrained tree was validated against unconstrained tree topology using approximately unbiased test⁵⁰ with P -value > 0.05. To map amino acid substitutions that took place on the branches leading to Nac β 2 and Nac β 1 of *Saccharomyces* species, sequences at the nodes connected by these branches were compared. These sequences were inferred based on the phylogeny ancestral sequences reconstruction method using the FastML program based on the ML algorithm for insertions and deletions reconstruction and the Empirical Bayes method using the LG substitution matrix with gamma parameter.⁵¹ Structural visualizations used Blender (<https://www.blender.org/>).

Strains and plasmids

Saccharomyces cerevisiae strains and plasmids used in this study are listed in Tables S1 and Table S2, respectively. Integrated DNA Technologies (Covington, IA) was used for the synthesis of all primers and gBlocks. Codon substitutions, deletions, and insertion mutations were introduced into cloned DNA using the QuikChange protocol (Stratagene, La Jolla, CA). Genomic DNA was isolated using the MasterPure™ yeast DNA purification kit from Lucigen (Middleton, WI). All plasmid constructs underwent sequence analysis by Functional Biosciences (Madison, WI). Yeast was grown in either rich media, YPD (1% yeast extract, 2% peptone [Difco Laboratories, Detroit, MI], 2% dextrose) or selective minimal media (0.67% yeast nitrogen base without amino acids [US Biological, Marblehead, MA], 2% dextrose) supplemented with required amino acids.⁵² All plates shown in this report are minimal media unless stated otherwise. DNA was transformed into yeast using a previously published protocol.⁵³ Cell growth assays were performed by serially diluting cells

for each strain in 10-fold increments starting with 5000 cells in the first position and spotting on plates.

Deletions of genes encoding NAC subunits α (*EGD2*) and β 1 (*EGD1*) were created in the W303 yeast strain background⁵⁴ by CRISPR, as previously described.¹⁵ The gene encoding Nac β 2 (*BTT1*) was similarly deleted using CRISPR. Specifically, a target-specific single-guide RNA (sgRNA) was constructed by cloning a 60-mer bridging primer, which contains a 20-nucleotide target sequence of +385 to +404 for *BTT1*, into *NotI*-digested pXIPHOS vector (accession MG897154, GenBank) using NEBuilder HiFi DNA assembly Master Mix (New England Biolabs). A *BTT1*-specific gBlock was synthesized containing 216 bp of DNA upstream of the ATG and 226 bp of DNA downstream of the stop codon to serve as rescue DNA. The XIPHOS-*BTT1* sgRNA plasmid, along with a 20-fold molar excess of rescue DNA, was transformed into yeast, and deletion candidates were selected for on YPD containing 100 μ g/mL of nourseothricin (Werner BioAgents GmbH, Jena, Germany). Resistant colonies were tested using colony PCR with primers that bind within the upstream and downstream untranslated regions of *BTT1*. A 3x-Flag tag was introduced at the 3' end of *BTT1*'s ORF using CRISPR. The same sgRNA plasmid was used to transform the desired yeast strain along with a gBlock containing 157 bp upstream of the stop codon, a 66 bp flag tag, and 190 bp downstream of the stop codon. The sequence at the target site in the rescue DNA was altered, resulting in a glycine to alanine change at residue 136. Correct addition of the flag tag sequence was confirmed first by colony PCR of nourseothricin-resistant colonies and observing a size increase compared to WT, and second, by sequencing the PCR-generated fragments of the correct size.

To delete *ACL4* by homologous recombination in the W303 background, a DNA fragment containing a KanMX cassette replacement of the *ACL4* ORF was amplified from genomic DNA isolated from the deletion library⁵⁵ by PCR and transformed into the desired yeast strain. Deletion candidates, which grew on YPD with 200 μ g/mL G418 sulfate (VWR Chemicals, LLC Sanbom, NY), were tested by colony PCR with primers specific to KanMX and to the UTR of *ACL4*.

The *BTT1* gene encoding Nac β 2, with a Flag tag, was cloned by amplifying genomic DNA from the strain containing the integrated Flag tag with primers that introduce a *Bam*HI site at the 5' end (at the ATG or 200 bp upstream) and an *Xho*I site at the 3' end (240 bp downstream of stop codon). After restriction digestion of the PCR products, the DNA was ligated into similarly digested plasmid DNAs: pRS314⁵⁶ and p414ADH.⁵⁷ *EGD1* (Nac β 1) and *EGD2* (Nac α) were cloned into

pRS314 and pRS316, respectively, by PCR amplifying the genes from genomic DNA using primers that contain a *Bam*HI site at the 5' end (371 and 344 bp upstream of ATG, respectively) and an *Xho*I site at the 3' end (246 and 239 bp downstream of the stop codon, respectively). A 3x-Flag tag was added to the C-terminus of both Egd1 and Egd2 by subcloning in gBlocks containing the tag sequence using *Hind*III-*Xho*I for *EGD1* and *Bgl*II-*Xho*I for *EGD2*. In order to lower the expression of Egd1 and Egd2, their ORFs were cloned as *Bam*HI-*Xho*I fragments into p414ADH and p416ADH, respectively, in which the *ADH1* promoter was replaced with -200 to -1 of *BTT1*'s 5' UTR. The complete ORF of Caf130 was cloned into the two-hybrid plasmid pGAD-C1⁵⁸ as a *Bam*HI-*Sal*I digested PCR fragment. *BTT1* and *EGD1*, full-length and variants, were cloned into pGBD-C1 as *Bam*HI-*Sal*I digested PCR fragments.

Immunoblot analysis and site-specific crosslinking

To make whole cell lysates, yeast cells were harvested in log-phase growth (OD₆₀₀ 0.8–1.0), treated with 0.2 N NaOH for 5 min, resuspended in sample buffer, and boiled for 5 min⁵⁹. Samples were resolved on sodium dodecyl sulfate polyacrylamide gel using BLUEye-prestained markers (Sigma-Aldrich, St. Louis, MO), transferred electrophoretically to nitrocellulose, and subjected to immunoblot analysis using the Enhanced Chemi-Luminescence System (GE Healthcare) according to the manufacturer's suggestion. Antitubulin (12G10) and Rpl3 (ScRPL3) antibodies were obtained from the monoclonal antibody facility at the University of Iowa (University Heights, IA). Anti-FLAG monoclonal M2 antibody came from Sigma-Aldrich (St. Louis, MO). Antibodies specific for Egd1 (Nac β 1), Egd2 (Nac α),¹⁵ and Ssb⁶⁰ were previously described.

In vivo crosslinking was carried out as described previously.¹⁵ The Δ *ssb1* Δ *ssb2* Δ *nac* β 1 strain was transformed with plasmids able to express Ssb1^{S563TAG} and the indicated Nac β 1^{Flag} construct, as well as a plasmid carrying a suppressor transfer RNA to direct incorporation of p-benzoyl-L-phenylalanine (Bpa) at the TAG stop codon. Log-phase cultures were treated with cycloheximide (0.1 mg/mL final) and exposed to UV light to induce crosslinking prior to glass bead lysis. Cells were lysed by agitation with glass beads for 5 \times 1 min (with 1 min on ice between) at 4 °C in lysis buffer (300 mM sorbitol, 20 mM HEPES-KOH, pH 7.5, 1 mM EGTA, 5 mM MgCl₂, 10 mM KCl, 10% glycerol v/v, 1 mM dithiothreitol, and RNasin RNase Inhibitor [Promega]) at a dilution of 1:1000. Lysates were clarified by centrifugation at 16,100 rcf for 10 min in a microcentrifuge (Eppendorf). A 4 Abs₂₆₀ units of each lysate were applied to a 0.4 mL sucrose cushion in which

0.5 M sucrose replaced 0.3 M sorbitol in the lysis buffer. Samples were centrifuged for 105 min at 108 900 relative centrifugal force in a TLA120.1 rotor (Beckman Coulter) at 4 °C. A 0.15 mL was subsequently removed from the top of the tube to yield the soluble fraction. The ribosomal pellet was resuspended in sample buffer, and 12.5% SDS-PAGE gels were loaded with equivalent portions of lysate (equivalent to 0.2 Abs₂₆₀ units/lane of starting extract), soluble, and pellet fractions.

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Data availability statement Data will be made available on request.

Declarations of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.cstres.2024.10.004](https://doi.org/10.1016/j.cstres.2024.10.004).

References

- Gamerding M, Deuerling E. Cotranslational sorting and processing of newly synthesized proteins in eukaryotes. *Trends Biochem Sci.* 2024;49:105–118. <https://doi.org/10.1016/j.tibs.2023.10.003>
- Wiedmann B, Sakai H, Davis TA, Wiedmann M. A protein complex required for signal-sequence-specific sorting and translocation. *Nature.* 1994;370:434–440. <https://doi.org/10.1038/370434a0>
- Hsieh HH, Shan SO. Fidelity of cotranslational protein targeting to the endoplasmic reticulum. *Int J Mol Sci.* 2021;23:281. <https://doi.org/10.3390/ijms23010281>
- Shen K, Gamerding M, Chan R, et al. Dual role of ribosome-binding domain of NAC as a potent suppressor of protein aggregation and aging-related proteinopathies. *Mol*

- Cell*. 2019;74:729–741.e727. <https://doi.org/10.1016/j.molcel.2019.03.012>
5. Wang L, Zhang W, Wang L, Zhang XC, Li X, Rao Z. Crystal structures of NAC domains of human nascent polypeptide-associated complex (NAC) and its alphaNAC subunit. *Protein Cell*. 2010;1:406–416. <https://doi.org/10.1007/s13238-010-0049-3>
 6. Liu Y, Hu Y, Li X, Niu L, Teng M. The crystal structure of the human nascent polypeptide-associated complex domain reveals a nucleic acid-binding region on the NACA subunit. *Biochemistry*. 2010;49:2890–2896. <https://doi.org/10.1021/bi902050p>
 7. Wegrzyn RD, Hofmann D, Merz F, et al. A conserved motif is prerequisite for the interaction of NAC with ribosomal protein L23 and nascent chains. *J Biol Chem*. 2006;281:2847–2857. <https://doi.org/10.1074/jbc.M511420200>
 8. Pech M, Spreter T, Beckmann R, Beatrix B. Dual binding mode of the nascent polypeptide-associated complex reveals a novel universal adapter site on the ribosome. *J Biol Chem*. 2010;285:19679–19687. <https://doi.org/10.1074/jbc.M109.092536>
 9. Hu GZ, Ronne H. Yeast BTF3 protein is encoded by duplicated genes and inhibits the expression of some genes in vivo. *Nucleic Acids Res*. 1994;22:2740–2743. <https://doi.org/10.1093/nar/22.14.2740>
 10. Reimann B, Bradsher J, Franke J, et al. Initial characterization of the nascent polypeptide-associated complex in yeast. *Yeast*. 1999;15:397–407. [https://doi.org/10.1002/\(SICI\)1097-0061\(19990330\)15:5<397::AID-YEA384>3.0.CO;2-U](https://doi.org/10.1002/(SICI)1097-0061(19990330)15:5<397::AID-YEA384>3.0.CO;2-U)
 11. Wong ED, Miyasato SR, Aleksander S, et al. Saccharomyces genome database update: server architecture, pan-genome nomenclature, and external resources. *Genetics*. 2023;224:14. <https://doi.org/10.1093/genetics/iyac191>
 12. Rospert S, Dubaquié Y, Gautschi M. Nascent-polypeptide-associated complex. *Cell Mol Life Sci*. 2002;59:1632–1639. <https://doi.org/10.1007/pl00012490>
 13. Ott AK, Locher L, Koch M, Deuerling E. Functional dissection of the nascent polypeptide-associated complex in *Saccharomyces cerevisiae*. *PLoS One*. 2015;10:e0143457. <https://doi.org/10.1371/journal.pone.0143457>
 14. Koplín A, Preissler S, Iliná Y, et al. A dual function for chaperones SSB-RAC and the NAC nascent polypeptide-associated complex on ribosomes. *J Cell Biol*. 2010;189:57–68. <https://doi.org/10.1083/jcb.200910074>
 15. Ziegelhoffer T, Verma AK, Delewski W, et al. NAC and Zuo1/Hsp70 chaperone systems coexist at the ribosome tunnel exit in vivo. *Nucleic Acids Res*. 2024;52:3346–3357. <https://doi.org/10.1093/nar/gkae005>
 16. Pillet B, Mendez-Godoy A, Murat G, et al. Dedicated chaperones coordinate co-translational regulation of ribosomal protein production with ribosome assembly to preserve proteostasis. *Elife*. 2022;11. <https://doi.org/10.7554/eLife.74255>
 17. Pillet B, Garcia-Gomez JJ, Pausch P, et al. The dedicated chaperone Acl4 escorts ribosomal protein Rpl4 to its nuclear Pre-60S assembly site. *PLoS Genet*. 2015;11:43. <https://doi.org/10.1371/journal.pgen.1005565>
 18. Stelter P, Huber FM, Kunze R, Flemming D, Hoelz A, Hurt E. Coordinated ribosomal L4 protein assembly into the pre-ribosome is regulated by its eukaryote-specific extension. *Mol Cell*. 2015;58:854–862. <https://doi.org/10.1016/j.molcel.2015.03.029>
 19. Larabee RN. Ccr4-Not as a mediator of environmental signaling: a jack of all trades and master of all. *Curr Genet*. 2021;67:707–713. <https://doi.org/10.1007/s00294-021-01180-5>
 20. Morris C, Cluet D, Ricci EP. Ribosome dynamics and mRNA turnover, a complex relationship under constant cellular scrutiny. *Wiley Interdiscip Rev RNA*. 2021;12:e1658. <https://doi.org/10.1002/wrna.1658>
 21. Collart MA, Audebert L, Bushell M. Roles of the CCR4-Not complex in translation and dynamics of co-translation events. *Wiley Interdiscip Rev RNA*. 2023;15:e1827. <https://doi.org/10.1002/wrna.1827>
 22. Jakel S, Mingot JM, Schwarzmaier P, Hartmann E, Gorlich D. Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. *EMBO J*. 2002;21:377–386. <https://doi.org/10.1093/emboj/21.3.377>
 23. Cui Y, Ramnarain DB, Chiang YC, Ding LH, McMahon JS, Denis CL. Genome wide expression analysis of the CCR4-NOT complex indicates that it consists of three modules with the NOT module controlling SAGA-responsive genes. *Mol Genet Genomics*. 2008;279:323–337. <https://doi.org/10.1007/s00438-007-0314-1>
 24. Wolfe KH, Shields DC. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature*. 1997;387:708–713. <https://doi.org/10.1038/42711>
 25. Kellis M, Birren BW, Lander ES. Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. *Nature*. 2004;428:617–624. <https://doi.org/10.1038/nature02424>
 26. Wagner A. Asymmetric functional divergence of duplicate genes in yeast. *Mol Biol Evol*. 2002;19:1760–1768. <https://doi.org/10.1093/oxfordjournals.molbev.a003998>
 27. Gu X, Zhang Z, Huang W. Rapid evolution of expression and regulatory divergences after yeast gene duplication. *Proc Natl Acad Sci USA*. 2005;102:707–712. <https://doi.org/10.1073/pnas.0409186102>
 28. Kim SH, Yi SV. Correlated asymmetry of sequence and functional divergence between duplicate proteins of *Saccharomyces cerevisiae*. *Mol Biol Evol*. 2006;23:1068–1075. <https://doi.org/10.1093/molbev/msj115>
 29. Soria PS, McGary KL, Rokas A. Functional divergence for every paralog. *Mol Biol Evol*. 2014;31:984–992. <https://doi.org/10.1093/molbev/msu050>
 30. Kuzmin E, VanderSluis B, Nguyen Ba AN, et al. Exploring whole-genome duplicate gene retention with complex genetic interaction analysis. *Science*. 2020;368. <https://doi.org/10.1126/science.aaz5667>
 31. Pal C, Papp B, Hurst LD. Highly expressed genes in yeast evolve slowly. *Genetics*. 2001;158:927–931. <https://doi.org/10.1093/genetics/158.2.927>
 32. Drummond DA, Bloom JD, Adami C, Wilke CO, Arnold FH. Why highly expressed proteins evolve slowly. *Proc Natl Acad Sci USA*. 2005;102:14338–14343. <https://doi.org/10.1073/pnas.0504070102>
 33. Drummond DA, Raval A, Wilke CO. A single determinant dominates the rate of yeast protein evolution. *Mol Biol Evol*. 2006;23:327–337. <https://doi.org/10.1093/molbev/msj038>
 34. Humphreys IR, Pei J, Baek M, et al. Computed structures of core eukaryotic protein complexes. *Science*. 2021;374:eabm4805. <https://doi.org/10.1126/science.abm4805>
 35. Gamberdinger M, Jia M, Schloemer R, et al. NAC controls co-translational N-terminal methionine excision in eukaryotes. *Science*. 2023;380:1238–1243. <https://doi.org/10.1126/science.adg3297>
 36. Gamberdinger M, Kobayashi K, Wallisch A, et al. Early scanning of nascent polypeptides inside the ribosomal tunnel by NAC. *Mol Cell*. 2019;75:996–1006.e1008. <https://doi.org/10.1016/j.molcel.2019.06.030>
 37. Panasencko O, Landrieux E, Feuermann M, Finka A, Paquet N, Collart MA. The yeast Ccr4-Not complex controls

- ubiquitination of the nascent-associated polypeptide (NAC-EGD) complex. *J Biol Chem*. 2006;281:31389–31398. <https://doi.org/10.1074/jbc.M604986200>
38. Alhusaini N, Collier J. The deadenylase components Not2p, Not3p, and Not5p promote mRNA decapping. *RNA*. 2016;22:709–721. <https://doi.org/10.1261/rna.054742.115>
 39. Deuerling E, Gamedinger M, Kreft SG. Chaperone interactions at the ribosome. *Cold Spring Harb Perspect Biol*. 2019;11:a033977. <https://doi.org/10.1101/cshperspect.a033977>
 40. Hsieh HH, Lee JH, Chandrasekar S, Shan SO. A ribosome-associated chaperone enables substrate triage in a cotranslational protein targeting complex. *Nat Commun*. 2020;11:5840. <https://doi.org/10.1038/s41467-020-19548-5>
 41. Jomaa A, Gamedinger M, Hsieh HH, et al. Mechanism of signal sequence handover from NAC to SRP on ribosomes during ER-protein targeting. *Science*. 2022;375:839–844. <https://doi.org/10.1126/science.abc6459>
 42. Muthukumar G, Stevens TA, Inglis AJ, et al. Triage of alpha-helical proteins to the mitochondrial outer membrane by distinct chaperone machinery based on substrate topology. *Mol Cell*. 2024;84:1101–1119.e1109. <https://doi.org/10.1016/j.molcel.2024.01.028>
 43. del Alamo M, Hogan DJ, Pechmann S, Albanese V, Brown PO, Frydman J. Defining the specificity of cotranslationally acting chaperones by systematic analysis of mRNAs associated with ribosome-nascent chain complexes. *PLoS Biol*. 2011;9:e1001100. <https://doi.org/10.1371/journal.pbio.1001100>
 44. Tian Y, Okamoto K. The nascent polypeptide-associated complex subunit Egd1 is required for efficient selective mitochondrial degradation in budding yeast. *Sci Rep*. 2024;14:546. <https://doi.org/10.1038/s41598-023-50245-7>
 45. Avendano-Monsalve MC, Mendoza-Martinez AE, Ponce-Rojas JC, Poot-Hernandez AC, Rincon-Heredia R, Funes S. Positively charged amino acids at the N terminus of select mitochondrial proteins mediate early recognition by import proteins alpha-beta'-NAC and Sam37. *J Biol Chem*. 2022;298:10. <https://doi.org/10.1016/j.jbc.2022.101984>
 46. Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. *Genome Res*. 2004;14:1188–1190. <https://doi.org/10.1101/gr.849004>
 47. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 2015;32:268–274. <https://doi.org/10.1093/molbev/msu300>
 48. Le SQ, Gascuel O. An improved general amino acid replacement matrix. *Mol Biol Evol*. 2008;25:1307–1320. <https://doi.org/10.1093/molbev/msn067>
 49. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods*. 2017;14:587–589. <https://doi.org/10.1038/nmeth.4285>
 50. Shimodaira H. An approximately unbiased test of phylogenetic tree selection. *Syst Biol*. 2002;51:492–508. <https://doi.org/10.1080/10635150290069913>
 51. Ashkenazy H, Penn O, Doron-Faigenboim A, et al. FastML: a web server for probabilistic reconstruction of ancestral sequences. *Nucleic Acids Res*. 2012;40:W580–W584. <https://doi.org/10.1093/nar/gks498>
 52. Sherman F, Fink G, Hicks J. *Methods in Yeast Genetics*. 500 Sunnyside Boulevard, Woodbury, NY: Cold Spring Harbor Press; 1986.11797-2924.
 53. Chen DC, Yang BC, Kuo TT. One-step transformation of yeast in stationary phase. *Curr Genet*. 1992;21:83–84. <https://doi.org/10.1007/BF00318659>
 54. Thomas BJ, Rothstein R. Elevated recombination rates in transcriptionally active. *DNA Cell*. 1989;56:619–630. [https://doi.org/10.1016/0092-8674\(89\)90584-9](https://doi.org/10.1016/0092-8674(89)90584-9)
 55. Giaever G, Chu AM, Ni L, et al. Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature*. 2002;418:387–391. <https://doi.org/10.1038/nature00935>
 56. Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. 1989;122:19–27. <https://doi.org/10.1093/genetics/122.1.19>
 57. Mumberg D, Muller R, Funk M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*. 1995;156:119–122. [https://doi.org/10.1016/0378-1119\(95\)00037-7](https://doi.org/10.1016/0378-1119(95)00037-7)
 58. James P, Halladay J, Craig EA. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics*. 1996;144:1425–1436. <https://doi.org/10.1093/genetics/144.4.1425>
 59. Kushnirov VV. Rapid and reliable protein extraction from yeast. *Yeast*. 2000;16:857–860. [https://doi.org/10.1002/1097-0061\(20000630\)16:9<857::AID-YEA561>3.0.CO;2-B](https://doi.org/10.1002/1097-0061(20000630)16:9<857::AID-YEA561>3.0.CO;2-B)
 60. Lopez-Buesa P, Pfund C, Craig EA. The biochemical properties of the ATPase activity of a 70-kDa heat shock protein (Hsp70) are governed by the C-terminal domains. *Proc Natl Acad Sci USA*. 1998;95:15253–15258. <https://doi.org/10.1073/pnas.95.26.15253>