RESEARCH ARTICLE

Functional Dissection of the Nascent Polypeptide-Associated Complex in Saccharomyces cerevisiae

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Abstract

Both the yeast nascent polypeptide-associated complex (NAC) and the Hsp40/70-based chaperone system RAC-Ssb are systems tethered to the ribosome to assist cotranslational processes such as folding of nascent polypeptides. While loss of NAC does not cause phenotypic changes in yeast, the simultaneous deletion of genes coding for NAC and the chaperone Ssb (nacΔssbΔ) leads to strongly aggravated defects compared to cells lacking only Ssb, including impaired growth on plates containing L-canavanine or hygromycin B, aggregation of newly synthesized proteins and a reduced translational activity due to ribosome biogenesis defects. In this study, we dissected the functional properties of the individual NAC-subunits (α-NAC, β-NAC and β'-NAC) and of different NAC heterodimers found in yeast (αβ-NAC and αβ'-NAC) by analyzing their capability to complement the pleiotropic phenotype of nacΔssbΔ cells. We show that the abundant heterodimer αβ-NAC but not its paralogue αβ'-NAC is able to suppress all phenotypic defects of nacΔssbΔ cells including global protein aggregation as well as translation and growth deficiencies. This suggests that αβ-NAC and αβ'-NAC are functionally distinct from each other. The function of αβ-NAC strictly depends on its ribosome association and on its high level of expression. Expression of individual β-NAC, β'-NAC or α-NAC subunits as well as αβ'-NAC ameliorated protein aggregation in nacΔssbΔ cells to different extents while only β-NAC was able to restore growth defects suggesting chaperoning activities for β-NAC sufficient to decrease the sensitivity of nacΔssbΔ cells against L-canavanine or hygromycin B. Interestingly, deletion of the ubiquitin-associated (UBA)-domain of the α-NAC subunit strongly enhanced the aggregation preventing activity of αβ-NAC pointing to a negative regulatory role of this domain for the NAC chaperone activity in vivo.
Introduction

The folding of newly synthesized proteins requires the assistance of molecular chaperones. At the forefront are ribosome-associated chaperones, which contact nascent polypeptides to control early protein folding processes and to prevent aggregation or degradation of newly synthesized proteins [1, 2]. Yeast ribosomes are transiently associated with two different types of chaperone systems. One is a Hsp70/Hsp40-based chaperone system consisting of the ribosome-associated complex (RAC), a heterodimer formed by Zuotin and Ssz, and Ssb. The second system is the nascent polypeptide-associated complex (NAC). Both systems bind transiently to the large ribosomal subunit for interaction with nascent polypeptides early during protein biogenesis (Fig 1A).

NAC reversibly binds to ribosomes and is described as the first factor that interacts with nascent polypeptides emerging from the ribosome to prevent them from incorrect interactions [3]. The complex is widely conserved from archaea to man, however, archaea only have a homodimeric NAC formed by two α-NAC subunits while yeast and higher eukaryotes mainly form a stable αβ-NAC heterodimer (Fig 1A). Crystal structures of NAC deletion variants from archaea and humans suggest that the two NAC subunits dimerize via their homologous six-stranded β-barrel-like NAC domains (Fig 1B). Only α-NAC contains an additional UBA (ubiquitin-associated) domain with unknown function at its C-terminus (Fig 1B) [4–6]. Ribosome binding of eukaryotic NAC is mediated by the N-terminus of β-NAC involving a conserved ribosome-binding motif (Fig 1B) and probably an adjacent helix element [7–9]. Mutations in this region of β-NAC (βRRK/AAA-NAC, Fig 1C) diminish ribosome binding of the entire complex [9]. Crosslinking data suggest different binding sites for NAC on the ribosome including the ribosomal proteins Rpl31 (eL31) and Rpl25 (uL23), which have been shown to be functional docking sites for other ribosome-attached factors as well, including Zuotin or bacterial Trigger Factor, SRP, and the ER-translocon, respectively [5, 9–11]. Moreover, both subunits of αβ-NAC crosslink to nascent polypeptides [7], suggesting that both can contact substrates, but the substrate binding sites in the individual subunits have not been elucidated yet.

In contrast to other eukaryotic organisms, the Saccharomyces cerevisiae genome encodes three NAC subunits: One α-NAC subunit encoded by the EGD2 gene and two alternative β-NAC paralogns, β-NAC and β’-NAC, encoded by the EGD1 and the BTT1 gene, respectively. Hence, two different types of heterodimers, αβ-NAC and αβ’-NAC, are formed in yeast with potentially different substrate pools and functions [12]. However, β’-NAC (BTT1) is approximately 100-fold less expressed than β-NAC and thus, the heterodimeric αβ-NAC seems to be the dominant species in yeast. Moreover, it is suggested that to a minor extent also homodimers of both subunits exist in vivo [10, 13, 14].

While yeast NAC is not important for growth, NAC is essential in higher eukaryotes and a loss of its function induces early embryonically lethal phenotypes or developmental defects in mice, fruit flies, and C. elegans [15–17]. Recent data show that NAC is a member of the chaperone network in yeast and in C. elegans [12, 18, 19]. The simultaneous deletion of all three NAC genes in yeast does not cause growth defects while the combined deletion of NAC genes together with genes encoding the Ssb chaperone (SSB1 and SSB2) leads to synthetic defects including impaired growth under protein folding stress, enhanced aggregation of newly synthesized proteins and accelerated defects in ribosomal biogenesis and translation [18]. Two recent studies revealed the functions of NAC in metazoans [19, 20]. Depletion of NAC in C. elegans results in enhanced protein aggregation of folding-sensitive polyQ proteins. Moreover, NAC was shown to be associated with heat-induced aggregates of firefly luciferase in transgenic C. elegans strains and loss of NAC prevents the efficient re-solubilization of aggregated luciferase at permissive temperature in these animals [19]. In addition, NAC plays an essential role as a
NAC Functions In Vivo

a)

RAC

Zuo

Ssz

Ssb

NAC

UBA

b)

α-NAC (Egd2)

\[ \begin{array}{ccccccc}
\text{N} & \text{NAC domain} & \text{UBA domain} & \text{C} \\
24 & 78 & 136 & 172 \\
\end{array} \]

β-NAC (Egd1)

\[ \begin{array}{ccccccc}
\text{N} & \text{NAC domain} & \text{C} \\
24 & 78 & 136 & 172 \\
\end{array} \]

β'-NAC (Btt1)

\[ \begin{array}{ccccccc}
\text{N} & \text{NAC domain} & \text{C} \\
24 & 78 & 136 & 172 \\
\end{array} \]

c)

α^UBA-NAC (Egd2)

\[ \begin{array}{ccccccc}
\text{N} & \text{NAC domain} & \text{C} \\
24 & 78 & 136 & 172 \\
\end{array} \]

β^RRK/AAA-NAC (Egd1)

\[ \begin{array}{ccccccc}
\text{N} & \text{NAC domain} & \text{C} \\
24 & 78 & 136 & 172 \\
\end{array} \]
negative regulator in cotranslational protein targeting to the ER in metazoans. Bound to ribosomes, NAC shields the high affinity binding site of ribosomes for the Sec61 translocon and thereby prevents incorrect ribosome-nascent chain complexes from association with the ER-translocon and the erroneous import of incorrect cargo in the ER [20]. However, this function is less well understood in yeast where in vivo studies showed no aberrant translocation phenotype upon NAC deletion [10, 14, 21], perhaps due to the fact that yeast cells use a distinct post-translational ER targeting system in addition.

In this study we set out to better understand the function of NAC and its individual subunits in yeast. To this end, we expressed individual subunits or heterodimeric complexes of NAC in cells lacking NAC and Ssb and investigated which subunits of NAC are potent to complement the phenotypic changes of nacΔssbΔ cells.

Results
The β-subunit of NAC is essential and sufficient to ameliorate growth

The loss of NAC does not result in a growth phenotype, while nacΔssbΔ cells lacking all NAC and Ssb chaperone encoding genes (EGD1Δ, EGD2Δ, BTT1Δ, SSB1Δ, SSB2Δ) show a strong growth deficiency compared to wild type (wt) or ssbΔ cells at 30°C, in particular in the presence of drugs which impair protein synthesis or folding like the arginine analogue L-canaVanine or the translation inhibitor hygromycin B [18]. To understand which NAC subunits are essential for growth, we expressed NAC subunits encoded on centromeric plasmids (Fig 2A) individually or in combinations and tested their ability to complement the growth defects of nacΔssbΔ cells (Fig 2B). Expression of NAC genes was driven by the respective authentic promoter (Fig 2A) and protein levels were probed by Western blotting revealing similar expression levels as in the wt (S2B Fig).

Expression of the αβ-NAC heterodimer ameliorated the growth defect of nacΔssbΔ cells in the presence of L-canaVanine or hygromycin B (Fig 2B, lane 4). This finding is in agreement with an earlier report [18]. Interestingly, also the expression of β-NAC alone as well as in combination with an α-NAC variant lacking the C-terminal 64 amino acid residues including the UBA domain (called hereafter αUBA-NAC, see Fig 1C) ameliorated growth of nacΔssbΔ cells in the presence of both drugs (Fig 2B, lanes 4, 5 and 9). In contrast, the expression of the ribosome-binding mutant βRRK/AAA-NAC alone (Fig 1C) or in combination with α-NAC did not decrease sensitivity to L-canaVanine or hygromycin B compared to the vector control (Fig 2B, lanes 3, 7 and 8). This data suggests that expression of the β-NAC subunit is sufficient to restore growth defects of nacΔssbΔ cells back to the growth properties of ssbΔ cells. Based on the finding that αβRRK/AAA-NAC did not restore growth, we conclude that the α-NAC subunit is not sufficient to promote growth and ribosome-binding of β-NAC is crucial for the activity of αβ-NAC. In contrast to β-NAC, the expression of β'-NAC did not ameliorate growth of nacΔssbΔ cells in presence of L-canaVanine or hygromycin B (Fig 2B, lane 6) implying that the paralogous β'-NAC subunit is either functionally distinct from β-NAC in yeast or, due to its low expression, the level of β'-NAC is not sufficient to support growth under these conditions.
### a) NAC Functions

| Gene     | Description   | pRS316 | αβ-NAC | β-NAC | α-NAC | pRS316 |
|----------|---------------|--------|--------|-------|-------|--------|
| β-NAC    |               |        |        |       |       |        |
| β'-NAC   |               |        |        |       |       |        |
| β^{RRK/AAA}-NAC |          |        |        |       |       |        |

### b) In Vivo

| Condition | Description | SD-Ura | L-canavanine [0.75 μg/ml] | Hygromycin B [10 μg/ml] |
|-----------|-------------|--------|---------------------------|--------------------------|
| wt + ev   |             |        |                           |                          |
| sssΔ + ev |             |        |                           |                          |
| nacΔsssΔ  |             |        |                           |                          |
| + αβ-NAC  |             |        |                           |                          |
| + β'-NAC  |             |        |                           |                          |
| + β-NAC   |             |        |                           |                          |
| + β^{RRK/AAA}-NAC |       |        |                           |                          |
| + α^{RRK/AAA}-NAC |       |        |                           |                          |
| + αURAβ-β-NAC |       |        |                           |                          |

### c) Pβ'-β-NAC

| Gene     | Description   | pRS316 | αβ-NAC | β-NAC | α-NAC | pRS316 |
|----------|---------------|--------|--------|-------|-------|--------|
| Pβ'-β-NAC |             |        |        |       |       |        |
| Pβ'-β'-NAC |            |        |        |       |       |        |
| αβ'-NAC   |               |        |        |       |       |        |
| Pβ'-βα-NAC |            |        |        |       |       |        |
| Pβ'-βα'-NAC |           |        |        |       |       |        |

### d) In Vivo

| Condition | Description | SD-Ura | L-canavanine [0.75 μg/ml] | Hygromycin B [10 μg/ml] |
|-----------|-------------|--------|---------------------------|--------------------------|
| wt + ev   |             |        |                           |                          |
| sssΔ + ev |             |        |                           |                          |
| nacΔsssΔ  |             |        |                           |                          |
| + β-NAC   |             |        |                           |                          |
| + Pβ'-β-NAC |          |        |                           |                          |
| + β'-NAC  |             |        |                           |                          |
| + Pβ'-β'-NAC |         |        |                           |                          |
| + αβ'-NAC |             |        |                           |                          |
| + Pβ'-βα-NAC |        |        |                           |                          |
| + αβ'-NAC |             |        |                           |                          |
| + Pβ'-βα'-NAC |        |        |                           |                          |
Expression levels of β-NAC but not β'-NAC are important for growth

The expression ratio of β-NAC (EGD1) and β'-NAC (BTT1) in yeast cells is about 100:1 [13, 14]. To investigate whether the expression levels of β-NAC and β'-NAC are important for their ability to complement the growth defects of nacΔssbΔ cells, we exchanged the promoter and terminator region of BTT1 with regions from the EGD1 gene and vice versa (Fig 2C) and tested these constructs in nacΔssbΔ cells (Fig 2D).

When the BTT1 promoter and terminator regions were fused to the EGD1 gene, which results in strongly reduced β-NAC mRNA levels (S1A Fig), the growth defect of nacΔssbΔ cells on plates containing translation inhibitory drugs could not be complemented (Fig 2D, compare lane 4 with lane 5). Similarly, low expression of β-NAC in combination with wt levels of α-NAC did not enhance growth under these conditions (Fig 2D, lane 9). We conclude from these results that the high expression level of β-NAC is crucial for enhanced growth of nacΔssbΔ cells on plates containing L-canavanine or hygromycin B.

In turn, expression of β'-NAC under control of the EGD1 promoter and terminator led to significantly higher levels of BTT1 mRNA (S1B Fig). The high level expression of β'-NAC alone or in combination with wt levels of α-NAC enhanced growth of nacΔssbΔ cells on plates containing drugs compared to αβ'-NAC expressing cells (Fig 2D, compare lanes 6 and 7, 10 and 11), however, only slightly compared to cells expressing β-NAC or αβ-NAC (Fig 2D, lanes 4 and 8). It should be mentioned that the expression levels of the β-NAC variants with exchanged promoter and terminator regions were tested on the mRNA level due to the lack of specific antibodies. Thus, we cannot exclude that some variations on the protein level may contribute to the observed effects as well.

In summary, the data suggest that the paralogous ribosome-associated β-NAC and β'-NAC execute distinct functions in vivo even when expressed at similar levels.

Ribosomal defects in nacΔssbΔ cells are suppressed by αβ-NAC

Previous studies revealed that nacΔssbΔ cells show a defect in ribosomal biogenesis leading to the formation of ribosomal halfmers and a reduced translational activity [18]. This defect in ribosome biogenesis can be investigated by separating total cell lysate on a sucrose gradient using ultracentrifugation and subsequent fractionation of the gradient monitoring ribosomal species by measuring the absorption at 254 nm. The peak heights of the absorption traces detected at 254 nm could be used as sensitive indicators for the levels of each ribosomal species because equal absorption units of the samples were loaded. Thereby, the shoulder in the 80S and polysome peaks of the double knockout cells represents the presence of ribosomal halfmers in such fractionation experiments (Fig 3C, arrows). Such halfmers consist of an uncomplexed 40S subunit bound to the mRNA and are typically caused by an impaired balance of 40S and 60S ribosomal subunits due to defects in the assembly of 60S particles [22, 23]. Indeed, higher levels of 40S subunits were detected in nacΔssbΔ cells compared to the wild type. Moreover, the 80S monosome and polysome peaks were significantly reduced and ribosomal halfmers were present in nacΔssbΔ cells compared to wt cells indicating the reduced translational activity.
As reported earlier [18], these ribosomal defects are clearly less pronounced in cells lacking only Ssb (Fig 3B).

To test which subunit(s) of NAC complement(s) the ribosomal defects, ribosome profiles were generated from \( \text{nac}^{-} \text{ssb}^{-} \) cells expressing different NAC variants. We found that in contrast to the growth analysis, only the expression of the \( \alpha \beta \)-NAC heterodimer could restore the ribosome biogenesis defects observed in \( \text{nac}^{-} \text{ssb}^{-} \) cells (Fig 3G). This is demonstrated by a decreased amount of halfmers, a reduced 40S peak and enhanced 80S and polysome peaks resulting in a profile that is similar to \( \text{ssb}^{-} \) cells (Fig 3B, 3C and 3G). Importantly, expression of the ribosome-binding deficient \( \alpha \beta^{\text{RRK/AAA}} \)-NAC version did not suppress these deficiencies in \( \text{nac}^{-} \text{ssb}^{-} \) cells (Fig 3A and 3C).

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ribosome biogenesis and translation (Fig 3I). Moreover, neither expression of β-NAC nor β’-NAC alone cured the ribosomal defects (Fig 3D and 3E). A small reduction in the amount of halfmers could be observed upon expression of αβ’-NAC (Fig 3H). We also investigated whether the expression levels of β-NAC and β’-NAC are crucial for the suppression of ribosomal defects in nacΔssbΔ cells (Fig 4). High level expression of β’-NAC driven by the EGD1 promoter and terminator elements with or without coexpression of α-NAC resulted also in a very mild reduction of ribosomal halfmers confirming again that the β’-NAC subunit and consequently also the αβ’-NAC heterodimer are functionally distinct from β-NAC and αβ-NAC, respectively, even when expressed at similar levels (Fig 4C and 4F). Moreover, a reduced expression of αβ-NAC did not complement the aberrant translation phenotype (Fig 4B and 4E) and also α-NAC itself could not prevent halfmer formation of nacΔssbΔ knockout cells (Fig 4D). Interestingly, the expression of the αUBAβ’-NAC mutant version in nacΔssbΔ cells (Fig 3F) reduced the halfmer formation whereas the 80S and polysome peaks were still reduced compared to ssbΔ cells, suggesting that the UBA domain functionally contributes to the function of αβ-NAC in translation.

Taken together, these data show that, in contrast to the growth analyses where ribosome-bound β-NAC is sufficient for complementation, exclusively the αβ-NAC heterodimer expressed at high levels is able to support proper ribosome biogenesis and translation in yeast cells.

![Fig 4. Expression levels of β-NAC are important for complementation of nacΔssbΔ halfmers. Polysome profiling with wild type (wt) or mutant yeast cells. Absorbance traces at 254 nm are shown. 10 A260 units of lysates of indicated yeast cells were loaded onto 15–45% linear sucrose gradients similar to Fig 3. a) Polysome profile of nacΔssbΔ cells + empty vector control (ev) (in grey). b) and c) Complementation of nacΔssbΔ cells with promoter-swapped β-NAC constructs alone. d) nacΔssbΔ cells expressing α-NAC alone. e) and f) Polysome profiles of nacΔssbΔ cells expressing promoter-swapped β-NAC constructs in combination with α-NAC. Arrows indicate halfmers. The profiles are representative for three independent runs.](https://doi.org/10.1371/journal.pone.0143457.g004)
NAC is transcriptionally not coregulated with components of the translation apparatus

The expression of genes encoding proteins involved in ribosome biogenesis is often coregulated with genes coding for ribosomal proteins [24]. This has been reported also for the ribosome-associated chaperone Ssb [25]. As the ribosomal biogenesis and translation defects of cells lacking NAC and Ssb are more pronounced than in the cells lacking only Ssb, we wondered whether the genes EGD1, BTT1 and EGD2 coding for NAC are also coregulated with ribosomal genes. In a previous study from Albanèse et al. [26] where transcriptional analysis of gene expression in response to environmental stress, e.g. heat shock or nitrogen depletion, was performed, NAC was found to be corepressed together with components of the translational apparatus and ribosome biogenesis chaperones such as Ssb and RAC. To further address this question under non-stress conditions, wt cells were grown in medium containing glycerol as carbon source until they reached an OD₆₀₀ of 0.6 (time point zero). Then the cells were washed and transferred into medium containing glucose because ribosomal genes are upregulated upon carbon upshift from glycerol- to glucose-containing medium. Total RNA was isolated after various time points and followed by quantitative real-time PCR. We found, in agreement with earlier studies [25], that the mRNAs of SSB1 and the ribosomal protein RPL5 as well as the mRNA of the ribosome biogenesis factor JJJ1 were upregulated about 2- to 3.5-fold upon carbon shift (Fig 5A). However, no significantly enhanced transcription of mRNA coding for any of the three NAC subunits was detected. The mRNA levels of EGD1 and EGD2 remained almost constant in comparison to SSB1 or JJJ1 and the mRNA level of BTT1 was even slightly reduced upon carbon shift (Fig 5A). Hence, NAC is not coregulated with ribosomal proteins under these conditions, which is a typical characteristic for ribosomal biogenesis factors and chaperones directly involved in this process, such as Jjj1 or Ssb. It is known that loss of Jjj1 causes a slow growth phenotype and the combined deletion of the SSB1,2 genes and JJJ1 results in synthetic lethality [18]. To further investigate the role of NAC in ribosome biogenesis, we generated jjj1Δ and nacΔjjj1Δ knockout strains to test for a genetic interaction. The nacΔjjj1Δ cells lacking Jjj1 and all three genes encoding NAC showed no synthetic growth phenotype compared to jjj1Δ cells under the conditions tested (Fig 5B). Ribosome profiles of jjj1Δ and nacΔjjj1Δ cells (Fig 5C–5F) revealed that the deletion of JJJ1 resulted in a decrease of 60S subunits and in the appearance of halfmers (Fig 5E), indicating that this strain has a ribosome biogenesis defect as described previously [27, 28]. Loss of NAC in jjj1Δ cells did neither enhance the halfmer formation nor cause a further reduction of 60S, 80S or polysome peaks. This suggests that NAC and Jjj1 do not display overlapping functions in ribosome biogenesis and indicates together with the lack of transcriptional coregulation that NAC supports the activity of the translation apparatus by a mechanism distinct from classical ribosome biogenesis factors.

Suppression of protein aggregation by NAC

The loss of NAC does not provoke protein aggregation while cells lacking the Ssb chaperone activity accumulate misfolded and insoluble proteins. However, defects in protein folding are much more pronounced in ssbΔ cells upon additional loss of NAC suggesting that these two ribosome-associated systems act in overlapping pathways to support the folding of newly synthesized proteins [18]. Therefore, we finally examined if NAC subunits expressed alone or in combination reduce the level of protein aggregation in nacΔssbΔ cells. Mutant cells were grown to exponential phase, harvested and after lysis the insoluble protein material was isolated by centrifugation (Fig 6). Equalized total lysates are shown in S2A Fig and served as a loading control. Three biologically independent experiments were conducted for each NAC variant to test the chaperone activity by analyzing the suppression of protein aggregation in nacΔssbΔ cells.
The data were analyzed using one-way-between-groups ANOVA with post-hoc Tukey test to assess significance. As shown previously, cells lacking Ssb and NAC revealed enhanced levels of insoluble protein material compared to ssbΔ cells (Fig 6A, lanes 1–3). We found that expression of αβ-NAC at wt levels significantly reduced protein aggregation in nacΔssbΔ cells (Fig 6A, lane 8; quantification in Fig 6C and S1 Table (p = 0)) suggesting that this heterodimer is a potent chaperone in vivo. The expression of the single NAC-subunits or of αβ'-NAC ameliorated protein aggregation as well, with some variances compared to the expression of αβ-NAC (Fig 6A, lanes 4, 7, 11 and S1 Table). This suggests that chaperone activity can be displayed by individual subunits and does not necessarily rely on the heterodimeric NAC complex. The chaperone activity of αβ-NAC and β-NAC critically depends on ribosome association as protein aggregation could not be prevented in nacΔssbΔ cells expressing the αβRRK/AAA-NAC or βRRK/AAA-NAC subunit (Fig 6A, lanes 6 and 9, Fig 6C, S1 Table (p = 0.24 and 0.921, respectively)).

Surprisingly, the most potent NAC version in preventing protein aggregation was αΔUBAβ-NAC (Fig 6A, lane 10 and S1 Table, p = 0, highly significant). Fig 6A and 6B show the SDS-PAGES of the isolated and Coomassie-stained aggregated protein species from nacΔssbΔ cells expressing the αβRRK/AAA-NAC or βRRK/AAA-NAC subunit (Fig 6A, lanes 6 and 9, Fig 6C, S1 Table (p = 0.24 and 0.921, respectively)).

Discussion

The eukaryotic conserved heterodimer αβ-NAC was discovered in 1994 by Wiedmann and colleagues [7] and has since then been studied in vitro and in vivo to better understand the diverse roles of this complex e.g. in protein folding and transport [2, 30]. NAC is not essential in yeast but displays a strong genetic and functional interaction with the Ssb chaperone as is evident by the amplification of the pleiotropic defects found in cells lacking Ssb upon additional deletion of NAC encoding genes [18]. In contrast to other eukaryotes, Saccharomyces cerevisiae encodes besides the universally conserved α-NAC and β-NAC subunits that form the stable and abundant αβ-NAC heterodimer, a second paralogous β'-NAC subunit that forms the alternative αβ'-NAC dimer. In this study, we dissected the functions of the individual NAC subunits and the two different heterodimers by their ability to complement the pleiotropic phenotype of nacΔssbΔ cells. In addition, our results demonstrate the importance of ribosome binding of NAC and identify for the first time a potential role for the UBA-domain of α-NAC in regulating the chaperone activity of αβ-NAC.

We found major functional differences between αβ-NAC and αβ'-NAC. Only αβ'-NAC but not αβ-NAC (even when expressed at similar levels as αβ-NAC) can suppress all defects found in nacΔssbΔ cells including the high sensitivity against translation inhibitory drugs, ribosomal deficiencies that result in halfmer formation, reduced amounts of 80S particles and polysomes,
as well as protein aggregation. This suggests that αβ-NAC is functionally most important for yeast vitality. Our results are in agreement with a recent study by Frydman and colleagues analyzing the nascent interactome of NAC [12]. They showed that αβ-NAC has a preference for ribosomes translating metabolic enzymes as well as secretory and membrane proteins while αβ'-NAC preferentially binds to ribosomes translating mitochondrial or ribosomal proteins. This finding implies different substrate pools of αβ-NAC and αβ'-NAC. Both heterodimers are ribosome-associated by the conserved ribosome-binding motif found in β-NAC as well as in β'-NAC (Figs 1B and 7A). In addition, both subunits possess the conserved NAC domain involved in dimerization (Fig 7B). The two different β-subunits display an overall similarity of 64.3% with an identity of 46.5% on their amino acid level. However, β-NAC and β'-NAC obviously reveal strong differences at their C-terminal ends (Fig 7C) as the similarity of this region is only 30.8% with an identity of 10.3%. Both C-termini are predicted to be rather unstructured, however, the C-terminus of β'-NAC is shorter by 8 amino acid residues compared to β-NAC and the last 16 amino acids show no homology to β-NAC at all (Fig 7C). Moreover, β'-NAC has a lower amount of charged amino acids in its C-terminus: 5 negatively charged residues (Asp + Glu) and 2 positively charged residues (Arg + Lys) compared to β-NAC with 11 negatively charged residues (Asp + Glu) and 4 positively charged residues (Arg + Lys). Thus, we speculate that the diverse C-termini of β- and β'-NAC might be involved in substrate selectivity and thus contribute to the functional differences of αβ-NAC and αβ'-NAC. Interestingly, the C-termini of β-NAC subunits from C. elegans and humans also contain a high number of charged residues and are clearly more similar to yeast β-NAC than to β'-NAC (Fig 7C).

Obviously, αβ-NAC can only display its function in vivo when bound to ribosomes and expressed at high levels. This conclusion is strongly supported by the findings that neither αβ<sup>RRK/AAA</sup>-NAC, that forms a stable heterodimer but is deficient in ribosome binding, nor the low expression of αβ-NAC driven by the BTI promoter could complement all phenotypic defects of nac<sub>ΔssbΔ</sub> cells.

Interestingly, expression of ribosome-bound β-NAC but not of β'-NAC was sufficient to restore growth but both versions could lower the aggregation propensity of newly synthesized proteins. This suggests some residual chaperoning activities of ribosome-bound β-NAC and β'-NAC subunits, probably as a homodimer which, in the case of β-NAC, is sufficient to promote growth of nac<sub>ΔssbΔ</sub> cells.

The α-NAC subunit contains a conserved UBA (ubiquitin–associated) domain at its C-terminal end (Fig 1). UBA domains can bind ubiquitin and thus are often found in proteins associated with the ubiquitin-mediated degradation pathway. However, UBA domains can also act as protein–protein interaction or dimerization surfaces by exposing a hydrophobic patch [31]. That far, no ubiquitin binding of the NAC UBA domain could be demonstrated and the role of this domain is enigmatic. In this study, we discovered a potential role for the NAC UBA domain by studying the α<sup>AUBα</sup>β-NAC version, a heterodimer with a wild type (wt) β-NAC subunit and a mutant α-NAC lacking the C-terminal 64 amino acid residues including the complete UBA domain and part of the flexible linker between the UBA and the NAC domain (Fig 1B and 1C). The α<sup>AUBα</sup>β-NAC mutant complemented growth defects of nac<sub>ΔssbΔ</sub> cells similar
to αβ-NAC, but was significantly more potent to suppress protein aggregation in these cells suggesting a higher chaperone activity of αΔUBAβ-NAC compared to αβ-NAC. This led us to conclude that the UBA domain attached via a highly flexible linker to the α-NAC subunit may regulate the chaperone activity of αβ-NAC. How can we envision such a role for the UBA domain? We speculate that the hydrophobic and surface-exposed stretch of the UBA domain may contact and transiently cover a hydrophobic substrate binding site in the αβ-NAC heterodimer. Enhanced exposure of such a binding site in αΔUBAβ-NAC may allow NAC to associate more efficiently with misfolded proteins to prevent their aggregation. However, such a "hyper" chaperone activity might be harmful as well, e.g. by binding too tight or in an unregulated manner to certain substrates. This may explain why the αΔUBAβ-NAC is not as efficient as wt αβ-NAC in suppressing defects in translation. Another possibility would be that the UBA domain contributes to dimerization or oligomerization of αβ-NAC and thereby affects its chaperone activities.

Future in vivo and in vitro experiments are required to evaluate this interesting hypothesis and to shed more light on the chaperone activity of NAC. This study provides the framework to pursue such analyses.
Materials and Methods

Strains, plasmids and growth conditions

The genotypes of the yeast strains are listed in S2 Table. Strains carrying gene deletions were constructed by PCR-based gene disruption [32, 33] and clones were analyzed by PCR. Gene deletions in yeast strains obtained from EUROSCARF were confirmed by growth on YPD-G418 and PCR. All plasmids were generated using standard molecular cloning techniques and are listed in S3 Table. Plasmids encoding wild type (wt) NAC and mutants of NAC were described in [18]. To generate plasmids where the two β-NAC subunits were expressed with the promoter and terminator region originally belonging to the other subunit, the PCR-amplified promoter and terminator regions as well as the coding regions of EGD1 and BTT1 (derived from yeast genomic DNA of BY4741) were cloned into pRS316 or pRS316α-NAC in the desired combinations. All plasmids used are listed in S3 Table. Unless indicated otherwise, yeast cells were grown at 30°C in YPD (1% yeast extract, 2% peptone and 2% dextrose) or defined synthetic complete media (6.7 g/liter YNB, 2 g/liter drop out mix and 2% dextrose). Analysis of cell growth was performed four times.

Density gradient ultracentrifugation and ribosomal profiling

For yeast ribosome profiling 200 ml cultures in –Ura medium were grown at 30°C to an OD_{600} = 1. Cycloheximide was added to a final concentration of 100 μg/ml. Immediately afterwards, the treated cultures were transferred into centrifuge bottles containing 100 g crushed ice and centrifuged at 5000 x g for 5 min. The cell pellets were frozen in liquid nitrogen and stored at -80°C. Cell pellets were resuspended in lysis buffer (20 mM Heps-KOH, pH 7.4, 100 mM KAc, 2 mM MgAc, 100 μg/ml cycloheximide, 0.5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail) and 1 g of acid-washed glass beads was added. The cells were lysed mechanically by glass bead disruption. Triton X-100 and sodium deoxycholate were added to a final concentration of 0.25% each after cell lysis. 10 A_{260} absorption units of each lysate in 500 μl volume were loaded onto an 11 ml linear sucrose gradient (15–45% in lysis buffer) and centrifuged for 2 hours at 4°C at 200,000 x g. The gradients were fractionated from top to bottom with a density gradient fractionator (Teledyne Isco, Inc.) and ribosome profiles were monitored at 254 nm. Data were recorded and processed with PeakTrak V1.1 (Teledyne ISco, Inc.). Experiments were performed four times.

Antibodies and Western Blot analysis

Rabbit polyclonal antibody against the NAC complex was described in [18]; antibodies recognizing PGK1 were obtained from Invitrogen. Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare) by Western blotting (wet blot). Primary antibodies were diluted 1:10,000. Fluorescence-labeled secondary anti-rabbit antibodies (DY-682; Dyomics) were applied and visualized with the FLA-9000 system (Fujifilm). Experiments were repeated at least three times.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from yeast wild type strain using RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol and transcribed into cDNA according to the QuantiTect reverse transcription kit protocol (Qiagen). For quantitative real-time PCR 20 μl triplicate reactions with 1 μl of 1:5 diluted cDNA were used together with 0.2 mM of the primer pair and 1x GoTaq® qPCR Master Mix (Promega). For detection the ABI 7500 Fast Real-Time PCR
System (Applied Biosystems) was used. Data were analyzed using the comparative 2ΔΔCT method and TAF10 as a reference gene. Experiments were repeated at least three times.

Isolation of aggregated proteins

Overnight cultures of yeast cells transformed with the indicated plasmids encoding the different NAC subunits were grown in synthetic complete medium without uracil. The main culture was grown in YPD to the logarithmic stage and 50 OD<sub>600</sub> units were harvested. The cell pellets were resuspended in lysis buffer (20 mM potassium phosphate, pH 6.8, 10 mM DTT, 1 mM EDTA, 0.1% Tween, 1 mM PMSF, protease inhibitor cocktail, 3 mg/ml zymolyase 20T and 25 u/μl DNasel) and incubated at room temperature for 15 minutes. After chilling on ice for 5 minutes, the samples were treated by tip sonication (Branson, eight times at level 4 and duty cycle 50%) and centrifuged at 4°C for 20 minutes and 200 x g. Protein levels were adjusted to identical concentrations and aggregated proteins were pelleted at 16,000 x g for 20 min at 4°C. Aggregated proteins were washed twice with 2% NP-40 (in 20 mM potassium phosphate, pH 6.8, 1 mM PMSF and protease inhibitor cocktail), sonicated six times at level 4 and 50% duty cycle and centrifuged as described above. The final washing step was performed in buffer without NP-40, samples were sonicated for four times at level 2 with 65% duty cycle and subsequently boiled in SDS sample buffer. The total and aggregated proteins were separated by SDS-PAGE and analyzed by Coomassie staining. Experiments were performed three times. Quantification was performed using ImageJ; mean +/- SD is shown.

Statistical analysis

Statistical analysis was performed using one-way-between-groups ANOVA with post-hoc Tukey test [29]. All the data from the aggregation experiment were normalized to the mean raw value of the wt data set. A Levenes test for homogeneity of Variance was performed followed by one-Way ANOVA. Tukeys post-hoc test compared the aggregation level of every strain to the other to check for statistically significant differences.

Supporting Information

S1 Fig. mRNA levels of β-NAC subunits are changed when expressed under different promoters. a) Yeast nacαssbΔ mutant cells transformed with the indicated plasmids were grown to an optical density (OD<sub>600</sub>) of 0.8 and mRNA was isolated. cDNA was obtained by reverse transcription and used for qRT-PCR with EGD1-specific primer pairs. The samples were normalized to an internal control (housekeeping gene) and compared to wild type. b) Experiment performed as in a) with BT11-specific primer pairs.

S2 Fig. Equalized protein levels of lysates used for aggregate isolation. a) 50 OD<sub>600</sub> units of transformed yeast cells in the logarithmic phase were lysed and the aggregated protein material was quantitatively isolated. 15 μg of total lysates were separated by SDS-PAGE and visualized by Coomassie staining. b) Total lysates prepared in a) were used for Western blotting to analyse the expression levels of the different NAC-encoding plasmids. Pgk1 served as loading control. The asterisks mark a degradation product of α-NAC (*) and an unspecific protein band (**).

S1 Table. The levels of aggregated proteins were analysed by one-way-between-groups ANOVA followed by Tukey test. ANOVA Statistics: One-way-between-groups ANOVA assessed a statistically significant divergence of the portions of aggregated proteins between the different strains. Tukey honest significant difference comparison: the levels of isolated
aggregates of each strain were compared with the ones of the respective other strain to test for statistically significant differences.

(S2 Table. Yeast strains used in this study. All yeast strains used in this work were isogenic derivatives of BY4741 and are listed by their genotypes.

(S3 Table. Plasmids used for complementation studies of the nacΔssbΔ phenotypes. All plasmids were generated using standard molecular cloning techniques.

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Author Contributions

Conceived and designed the experiments: A-KO MK LL ED. Performed the experiments: A-KO MK LL. Analyzed the data: A-KO MK LL ED. Contributed reagents/materials/analysis tools: A-KO MK LL. Wrote the paper: A-KO ED.

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