the Yeast TFIID Complex∗

The evolutionarily conserved RNA polymerase II transcription factor D (TFIID) complex is composed of TATA box-binding protein (TBP) and 13 TBP-associated factors (Tafs). The mechanisms by which many Taf subunits contribute to the essential function of TFIID are only poorly understood. To address this gap in knowledge, we present the results of a molecular genetic dissection of the TFIID subunit Taf2. Through systematic site-directed mutagenesis, we have discovered 12 taf2 temperature-sensitive (ts) alleles. Two of these alleles display growth defects that can be strongly suppressed by overexpression of the yeast-specific TFIID subunit TAF14 but not by overexpression of any other TFIID subunit. In Saccharomyces cerevisiae, Taf14 is also a constituent of six other transcription-related complexes, making interpretation of its role in each of these complexes difficult. Although Taf14 is not conserved as a TFIID subunit in metazoans, it is conserved through its chromatin-binding YEATS domain. Based on the Taf2-Taf14 genetic interaction, we demonstrate that Taf2 and Taf14 directly interact and mapped the Taf2-Taf14 interaction domains. We used this information to identify a Taf2 separation-of-function variant (Taf2-ΔC). Although Taf2-ΔC no longer interacts with Taf14 in vivo or in vitro, it stably incorporates into the TFIID complex. In addition, purified Taf2-ΔC mutant TFIID is devoid of Taf14, making this variant a powerful reagent for determining the role of Taf14 in TFIID function. Furthermore, we characterized the mechanism through which Taf14 suppresses taf2ts alleles, shedding light on how Taf2-Taf14 interaction contributes to TFIID complex organization and identifying a potential role for Taf14 in mediating TFIID-chromatin interactions.

RNA polymerase II and the general RNA polymerase II transcription factors (TFIIB, TBP, TAF, and TAF-associated factors) are required for mRNA gene transcription in all eukaryotes (1, 2). These factors assemble at gene promoters to form the preinitiation complex (PIC), a macromolecular assembly required for accurate initiation of transcription. A rate-limiting step for PIC formation is the association of TBP, or its multisubunit chaperones TFIID and SAGA, with the promoter. In the yeast Saccharomyces cerevisiae (Sc), SAGA dominates on gene promoters that respond to stress, whereas TFIID dominates on so-called “housekeeping” genes (3–5). These housekeeping genes often do not contain consensus TATA boxes (so-called “TATA-less” genes, although these genes do contain TATA-like sequences) and instead probably rely on the ability of TFIID to engage with promoter DNA and/or active chromatin to stimulate transcription (6, 7).

The evolutionarily conserved TFIID complex is composed of TBP and 13 TBP-associated factors (Tafs 1–13) (8, 9). Tafs display myriad biochemical activities including binding to promoter-DNA (10–19), binding to or enzymatically modifying chromatin (20–25), binding to gene-specific transactivators (26–29), binding to the general transcription factors (30–34), and binding to Mediator (35). However, few examples exist where the importance of these activities has been genetically and biochemically interrogated to assess the impact of loss-of-function variants on transcription activation (25, 30, 31, 36, 37).

The overall trilobed structure of TFIID is conserved between budding yeast and metazoans (38, 39). In addition, our laboratory has defined the subunit stoichiometry and location of the evolutionarily conserved TFIID subunits using electron microscopy (EM) coupled with difference mapping and immunolabeling (8, 40–42). Consistent with these findings, a reconstituted human eight-Taf complex (Tafs 2, 4, 5, 6, 8, 9, 10, and 12) docks well into the yeast TFIID structure and displays subunit stoichiometry similar to yeast TFIID (43, 44).

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Budding yeast does display some significant differences with regard to TFIID structure and promoter architecture. First, the metazoan Taf1 double bromodomain and the Taf3 plant homeobox domain (PHD) finger, domains that directly interact with modified chromatin, are missing from the yeast homologs (45–47). Second, yeast TFIID contains an additional subunit, Taf14 (8). Third, in metazoan TFIID, Tafs 1 and 2 bind to the initiator (INR) core promoter element (10, 12), and Tafs 6 and 9 bind to the downstream promoter element (11). Neither the INR nor the downstream promoter element has been unambiguously identified in the yeast system (1).

Despite these differences, yeast have evolved mechanisms to achieve similar TFIID promoter-DNA binding and chromatin recognition activities. Instead of having a double bromodomain covalently attached to Taf1, the double bromodomain protein Bd1 (bromodomain factor-1) is a TFIID-associated protein (48, 49), and its occupancy in genome-wide analyses correlates with Taf1 occupancy (7). Although not conserved as a TFIID subunit, Taf14 is conserved through its YEATS domain (50). This domain, which is also present in the super elongation complex proteins AF-9 and ENL (51–53), was recently shown to bind to acetylated and crotonylated lysine 9 of histone H3 (H3K9) (23, 24), marks associated with active transcription (54, 55). In regard to ScTFIID promoter recognition, footprinting analyses have demonstrated that both metazoan and ScTFIID display extended footprints with contacts spanning nearly 100 bp (8, 17, 56). ScTFIID histone fold pairs Taf4/Taf12 and Taf6/Taf9 also display in vitro DNA binding activities, although binding has not been shown to be sequence-specific (15). In addition, structural analyses with ScTFIID-TFIIA-activator in complex with promoter-DNA position DNA in contact with the C terminus of Taf2 (57). Taken together, these observations demonstrate that TFIID promoter-DNA and chromatin interaction activities are maintained in the yeast system. However, how these activities contribute to TFIID transcriptional activation function remains undefined.

In addition to its role in TFIID, Taf14 is also a subunit or associated protein of six other transcription-related complexes (52, 58–60). Although Tafs 1–13 are essential for life (46, 61–65), Taf14-null cells display temperature-sensitive (Ts) growth and defects in expression of galactose (GAL) and DNA repair genes (23, 52). Still, how Taf14 contributes to TFIID structure or function remains poorly understood because mutations in TAF14 could impact the function of all of the transcription-related complexes with which it associates.

To begin to understand TAF14 function in vivo, we need true separation-of-function variants that can specifically dissociate Taf14 from a single complex without disrupting its ability to perform its other functions.

We have minimal understanding of Taf2 function despite the fact that it was the first yeast Taf discovered (10, 46, 61). Our laboratory was the first to discover the location of Taf2 within the TFIID complex (41). In addition to its INR binding function, metazoan Taf2 directly interacts with Taf8, and this interaction is critical for Taf2 to localize to the nucleus (44). For RNA polymerase II to clear the promoter and begin productive elongation, TFIID appears to isomerize in a Taf2-dependent manner to release TFIID from downstream promoter sequences (66, 67). However, none of these biochemical activities have been genetically dissected. In fact, Taf2 has never been subjected to structure-function analysis in any system. While this manuscript was in preparation, a cryo-EM structure of human TFIID allowed Louder et al. (68) to describe the structure of Taf2 in molecular detail. However, this structure was not interrogated genetically or biochemically. Consequently, we have minimal understanding of how Taf2 contributes to TFIID-dependent transcription in vivo.

In this study, we performed a systematic molecular genetic dissection of Taf2 to address this gap in knowledge. Our analyses identified a genetic interaction between TAF2 and TAF14. We demonstrate that these two subunits directly interact and define the interaction domains in both subunits. Despite Taf14 being present in multiple copies per TFIID subunit, mutation in Taf2 can completely disrupt the ability of Taf14 to associate with the TFIID complex. Taf14-less TFIID-containing cells display defects in growth and transcript abundance for the highly transcribed TFIID-dominated ribosomal protein-encoding genes. Furthermore, our data indicate that the Taf14 YEATS domain contributes to TFIID function.

Results

I-TASSER Prediction of Taf2 Structures—To understand the role of Taf2 in TFIID function, we initially pursued classical approaches that have been successfully used to identify TFIID subunit functional domains. First, primary amino acid sequences from S. cerevisiae (yeast), Homo sapiens (human), and Drosophila melanogaster (fly) Taf2 were subjected to ClustalW sequence alignment to identify “hot spots” of amino acid sequence homology that we could target for mutagenesis. These analyses have been successfully applied to the histone fold domain-containing Tafs as well as the conserved TBP C terminus (47, 62, 63, 65, 69). Yeast Taf2, which encodes a 1407-aa protein, only displays weak sequence conservation with human (1199 aa) and fly (1221 aa) Taf2, ~15% sequence identity and ~32% sequence similarity (Fig. 1, B and C). In addition, the amino acids that are conserved do not cluster in hot spots but instead are distributed throughout the amino acid sequence. Second, we performed systematic ~100-amino acid N-terminal, C-terminal, and internal deletion mutagenesis as described previously (28, 36, 70, 71) to generate 49 deletion variants. TAF2 is a single copy essential gene. Thus, to perform genetic complementation assays, we used a pseudodiploid TAF2 wild-type (WT) strain for plasmid shuffle analysis as described previously (28, 32, 36, 47). Forty-eight of these variants were unable to complement a taf2-null strain and failed to stably incorporate into the TFIID complex (summarized in supplemental Table 1). The single variant that could complement a taf2-null strain (Taf2(1–1307)) displayed no discernible adverse growth, steady-state protein levels, or TFIID stable incorporation phenotypes. Third, we created a hydroxylamine-mutagenized TAF2 plasmid mutant library (62) to generate taf2D variants. However, none of the 20 taf2D alleles identified in a plate growth-based screen displayed acute loss of function in liquid culture when shifted to the non-permissive temperature (37 °C) (data not shown) as has been shown to occur for Ts alleles for every essential yeast Taf (4).
Taf2 Molecular Genetic Dissection and Taf14 Interaction

To overcome these technical hurdles, we hypothesized that a structure-guided site-directed mutagenesis strategy would successfully identify Taf2 amino acids critical for Taf2 function. However, at the time of this study, no 3D structural information existed for Taf2. Thus, to generate a putative 3D model for Taf2, we used the 3D structural prediction program I-TASSER to generate models for yeast, human, and Drosophila Taf2 structures (Fig. 1, A, B, and C, respectively) (72). I-TASSER has been successfully used in the gene regulation field to model structures (9). Given the similarities among the yeast, human, and Drosophila Taf2 predicted structures, we concluded that our in silico generated yeast Taf2 structure was a suitable model from which to design a structure-based site-directed mutagenesis screen.

Site-directed Mutagenesis of TAF2—To further genetically interrogate yeast TAF2, we designed two classes of TAF2 site-directed variants comprising 87 mutants. These variants primarily consisted of Ala block substitution mutations but also included charge reversal mutations (see supplemental Table 2).

Class I mutants, designed based on predicted solvent accessibility and proximity to evolutionarily conserved residues, contained 58 variants. Class II mutants, designed based on predicted solvent inaccessibility and to include mutations of groups of conserved amino acids, contained 29 variants. All variants were engineered to contain a three-copy HA tag and SV40 nuclear localization sequence (HA_x3-NLS) (HA) N-terminal tag. Importantly, HA-Taf2 phenocopies untagged Taf2 in genetic complementation assays and can efficiently and stably incorporate into the TFIID complex (Fig. 2, A and B). Every variant was scored for its ability to genetically complement a taf2-null strain at both permissive (25 °C) and non-permissive temperatures (37 °C) as well as by α-HA immunoblotting to ensure each variant accumulated to levels similar to WT Taf2.

Results for these analyses are described in detail in supplemental Table 2. In summary, of the Class I variants, 36% displayed a genetic complementation defect including five that were inviable and eight variants that were both slow growing at the permissive temperature and Ts at the non-permissive temperature (Fig. 2; taf2-ts1 through taf2-ts8). Of the Class II variants, 62% displayed a genetic complementation defect including 10 that were inviable and four variants that were both slow growing at the permissive temperature and Ts at the non-permissive temperature (Fig. 2; taf2-ts9 through taf2-ts12). Of note, none of the variants displayed dominant negative phenotypes. Surprisingly, none of the variants displayed drastic reduction in steady-state protein levels (>2×), even variants that could not complement the taf2-null strain.

To assess the mechanism by which the loss-of-function Taf2 variants fail to complement the taf2-null strain, inviable and taf2 variants were subjected to α-HA co-immunoprecipitation (co-IP) (Fig. 2B and supplemental Table 2). A strain expressing untagged WT Taf2 was used as a negative control. Despite similar steady-state protein levels and IP efficiency, none of the loss-of-function taf2 mutant variants tested could co-precipitate either the TFIID-specific subunits Taf7 and Taf8 or Taf14 as efficiently as HA-Taf2. These results suggest that these mutations disrupt Taf2-TFIID subunit interactions, contributing to growth defects.

TAF14 Overexpression Suppresses Select taf2 Growths—Considering that all of the loss-of-function taf2 variants display defects in stable incorporation into the TFIID complex, likely as a result of disruption of specific Taf2-TFIID subunit interactions, we hypothesized that artificially driving Taf2-TFIID stable incorporation would suppress the taf2-associated growth defects. Ideally, if a taf2 mutant variant has a reduction in binding affinity to a TFIID subunit, increasing concentration of that subunit could drive complex formation and rescue the ability of Taf2 to stably associate with TFIID. To this end, we individually overexpressed every TFIID subunit...
Taf2 Molecular Genetic Dissection and Taf14 Interaction

A.

| TAF2* | Pre-Shuffle | (taf2ts, TAF2, TAF2*) | Post-Shuffle | (taf2ts, –, TAF2*, 28°) | (taf2ts, –, TAF2*, 37°) |
|-------|-------------|------------------------|-------------|------------------------|------------------------|
|      | TAF2        |                        |             |                        |                        |
|      | HA-TAF2     |                        |             |                        |                        |
|      | HA-taf2-ts1 |                        |             |                        |                        |
|      | HA-taf2-ts2 |                        |             |                        |                        |
|      | HA-taf2-ts3 |                        |             |                        |                        |
|      | HA-taf2-ts4 |                        |             |                        |                        |
|      | HA-taf2-ts5 |                        |             |                        |                        |
|      | HA-taf2-ts6 |                        |             |                        |                        |
|      | HA-taf2-ts7 |                        |             |                        |                        |
|      | HA-taf2-ts8 |                        |             |                        |                        |
|      | HA-taf2-ts9 |                        |             |                        |                        |
|      | HA-taf2-ts10|                        |             |                        |                        |
|      | HA-taf2-ts11|                        |             |                        |                        |
|      | HA-taf2-ts12|                        |             |                        |                        |

Allele Key:

| taf2ts Variants | I-TASSER Structural Predictions |
|-----------------|---------------------------------|
| taf2-ts1 - Ala 29-30, 32 | Solvent Exposed |
| taf2-ts2 - Ala 462-466 | Solvent Exposed |
| taf2-ts3 - Ala 640-644 | Internal/Buried |
| taf2-ts4 - Ala 671-672, 675 | Internal/Buried |
| taf2-ts5 - Ala 693-696 | Internal/Buried |
| taf2-ts6 - D848K, E850K | Internal/Buried |
| taf2-ts7 - Ala 1105-1107, 1112 | Internal/Buried |
| taf2-ts8 - Ala 1143-1144, 1146-1149 | Internal/Buried |
| taf2-ts9 - Ala 225-229 | Internal/Buried |
| taf2-ts10 - Ala 375, 377-380 | Internal/Buried |
| taf2-ts11 - Ala 909-913 | Internal/Buried |
| taf2-ts12 - Ala 914-918 | Internal/Buried |

B.

FIGURE 2. Identification of taf2ts alleles using structural prediction and amino acid conservation-guided site-directed mutagenesis. A, a genetic complementation assay. taf2-null cells harboring both URA3-marked TAF2 and LEU2-marked TAF2* plasmids were serially diluted 1:4 from left to right and spotted with a pinning tool onto either SC – Leu (Pre-Shuffle) or SC – Leu + 5-FOA to select for cells that have spontaneously lost the URA3-marked TAF2 plasmid (Post-Shuffle). Plates were grown for 72 h at 37 °C or 96 h at 25 °C prior to imaging. TAF2*, test gene (either no ORF (--)), TAF2, HAx3NLS-TAF2, or various HAx3NLS-tagged taf2 Ts alleles (HAx3NLS-taf2-ts1 through -ts12). The variant amino acids in the taf2ts alleles are shown in the Allele Key to the right (taf2ts variants). Representative plate images from at least two biological replicates are presented. HAx3NLS tag is labeled as HA in all figures. B, Taf2-TFIID co-IP. Whole cell extracts derived from the strains containing TAF2* (TAF2, HAx3NLS-TAF2, and -taf2-ts1 through -ts12) described in A were used for immunoprecipitation with the anti-HA mAb 12CA5 IgG. One percent of the lysate (Input) and 33% of the pellet (Immunoprecipitate) were separated via SDS-PAGE, blotted to PVDF membrane, and probed with anti-HA (α-HA[Taf2]) and the indicated anti-Tf IgGs (α-Taf7, α-Taf8, and α-Taf14). Representative immunoblots from at least two biological replicates are presented.

(Fig. 3A) in strains harboring the taf2ts variants. We have previously shown that overexpressed TFIID subunits TAF4 and TAF11 have displayed positive genetic interactions with TOA2, a protein that directly interacts with the TFIID complex, demonstrating that overexpression of individual TFIID subunits is a viable method for identifying genetic interactions (32).

Overexpression of TAF1, TAF3, TAF7, TAF8, and TAF14 resulted in dominant negative growth phenotypes, consistent with published results (75). As expected, overexpression of TAF2 could complement growth for all taf2ts variants (Fig. 3B and data not shown). In addition, we observed strong taf2ts allele-specific suppression when we overexpressed TAF14 but not for any of the other non-TAF2 TFIID subunits (Fig. 3B and C, see taf2-ts6 versus taf2-ts7 and ts8). TAF14 overexpression suppression of the taf2-ts7-associated growth phenotype was particularly potent, rescuing growth to near WT levels at both permissive and non-permissive temperatures.

Although five of the 12 taf2ts variants displayed mild growth improvement, TAF14 overexpression suppression of taf2-ts7 and -ts8 significantly improved growth at all temperatures tested. The amino acids mutated in the taf2-ts7 and -ts8 variants map to adjacent α-helices in the yeast Taf2 predicted structure, whereas taf2-ts6 is predicted to reside on the opposite side of the molecule (Fig. 3D). These results suggest that amino acids mutated in the taf2-ts7 and -ts8 form a domain that is likely involved in Taf2-TFIID subunit physical interactions, potentially Taf2-Taf14 interaction. Because of the potency of the genetic interaction between TAF14 and taf2-ts7, further molecular and genetic analyses were performed with taf2-ts7.

TAF14 Overexpression Suppresses Defects in Ribosomal Protein Gene Transcript Abundance Associated with taf2-ts7—We hypothesized that strong taf2-ts7 growth defects likely result in a reduction in abundance of ribosomal protein-encoding gene (RPG) transcripts, a class of genes that is TFIID-dominated (3–7). To assess RPG transcripts, log phase growing yeast strains, harboring either TAF2 or taf2-ts7 and containing either an empty overexpression vector or a TAF14 overexpression vector, were abruptly shifted to the non-permissive temperature for 2 h followed by RNA extraction and quantitative reverse transcription-PCR (qRT-PCR). We have assessed RPG
transcript abundance using this temperature shift paradigm using Ts variants for TAF1–TAF13, TOA1, and TOA2 (28, 32, 36).

We found that taf2-ts7 without TAF14 overexpression displays a statistically significant ~3-fold reduction compared with TAF2 in TFIID-dominated RPG transcripts (RPS3, RPS5, RPS8A, and RPS9B; Fig. 4A) without a concomitant significant decrease in SAGA-dominated transcripts (PGK1 and PYK1; Fig. 4A), RNA polymerase I transcripts (RDN58; Fig. 4B), or RNA polymerase III transcripts (SNR6; Fig. 4C) (54). The RPG transcript defects were ameliorated when TAF14 was overexpressed in the taf2-ts7 strain. These data suggest that the ability of taf2-ts7 to appropriately regulate RPG transcript abundance is aided by elevated TAF14 levels.

TAF2 and TAF14 Directly Interact in Vitro—The simplest model for the genetic interaction between Tafl2 and TAF14 is that these two proteins directly interact. Consistent with this hypothesis, Tafl2 was identified as a TAF14-interacting protein in a genome-wide yeast two-hybrid screen but was not authenticated as a direct interaction (59). To determine whether Tafl2 and TAF14 directly interact, Tafl1-TAP-purified TFIID, maltose-binding protein (MBP), and MBP-Tafl2, all purified from yeast (see “Materials and Methods”), were subjected to Far-Western blotting with and without purified recombinant His<sub>6</sub>-Tafl2 (Fig. 5A). His<sub>6</sub>-Tafl2-bound proteins were detected with antigen affinity-purified anti-Tafl2 IgG (60). When His<sub>6</sub>-Tafl2 was omitted from the Far-Western blotting, the only signals observed were for purified Tafl2 from the TFIID complex as well as trace amounts of Tafl2 that co-purify with the yeast-generated MBP-Tafl2. Upon overlay with His<sub>6</sub>-Tafl2, His<sub>6</sub>-TAF14 bound to both Tafl2 from TFIID and MBP-Tafl2 but not MBP or other TFIID subunits. To extend these analyses to solution binding assays, increasing concentrations of MBP-Tafl2 were mixed with either purified recombinant His<sub>6</sub>-glutathione S-transferase (GST) or His<sub>6</sub>-GST-TAF14 in the presence of BSA as a nonspecific competitor and subjected to GST pulldown. His<sub>6</sub>-GST-TAF14 specifically bound MBP-Tafl2 in a dose-dependent and saturable manner (Fig. 5B). Thus, we have shown through two independent methods that Tafl2 and TAF14 specifically and directly interact in vitro.

The Tafl2 C Terminus Is Necessary and Sufficient for Binding Tafl14 in Vitro and in Vivo—Using the Far-Western blotting assay, we determined the domain of Tafl2 where Tafl2 and TAF14 directly interact (Fig. 6). To this end, purified MBP-Tafl2 (lane 1), His<sub>6</sub>-Tafl2(1–407) (lane 2), His<sub>6</sub>-Tafl2(401–1007) (lane 3), His<sub>6</sub>-Tafl2(1001–1407) (lane 4), His<sub>6</sub>-TAF14(1001–1207) (lane 5), and His<sub>6</sub>-TAF14(1201–1407) (lane 6) were subjected to Far Western blotting as described above (Tafl2-purified forms in Fig. 6A, left; constructs diagrammed in Fig. 6B). When His<sub>6</sub>-Tafl2 was omitted from the overlay, the only signal present was the co-purifying TAF14 in the MBP-Tafl2 sample. Upon His<sub>6</sub>-Tafl2 overlay, His<sub>6</sub>-TAF14 bound to MBP-Tafl2 (lane 1), His<sub>6</sub>-TAF14(1001–1407) (lane 4), and His<sub>6</sub>-TAF14(1201–1407) (lane 6). These results suggest that the TAF14 binding domain resides in TAF14(1201–1407).

As described above, our systematic 100-aa TAF2 truncation analysis showed that Tafl2(1–1307) could complement a taf2-null strain and stably incorporate into the TFIID complex,
whereas Taf2(1–1207) could do neither (supplemental Table 1). Because these C-terminal amino acids contain the Taf14 binding domain, we hypothesized that a finer truncation analysis of the Taf2 C terminus may define the amino acids necessary for Taf14 binding in vivo. Taf2 was subjected to 10-aa serial truncations of its C terminus. These variants were analyzed for their ability to complement a taf2-ts7–null strain, had reduced steady-state protein levels, and the ability to co-immunoprecipitate TFIID subunits (Fig. 6, C and D). We found that Taf2(1–1250) could not complement a taf2-null strain, had reduced steady-state protein levels compared with HA-Taf2, and could not co-immunoprecipitate TFIID subunits Taf4, Taf7, Taf8, Taf9, and Taf14. Smaller truncations (Taf2 aa 1–1260 to 1300) still maintained their ability to complement the taf2-null strain, displayed elevated steady-state protein levels compared with HA-Taf2, and could co-immunoprecipitate TFIID subunits Taf4, Taf7, Taf8, and Taf9. In regard to the Taf14 co-IP, Taf2(1–1280), Taf2(1–1290), and Taf2(1–1300) all maintained the ability to co-precipitate Taf14 at levels similar to WT. However, Taf2(1–1270) showed a mild reduction in the ability to co-precipitate Taf14 and Taf2(1–1260) (hereafter referred to as Taf2-ΔC) completely lost the ability to co-immunoprecipitate Taf14. Based on these data, we conclude that Taf2(1261–1407) is necessary for Taf2-Taf14 interaction in vivo.

Purified MBP-Taf2-ΔC was tested for its ability to directly interact with Taf14 in vitro. MBP-Taf2-ΔC was subjected to both Far Western blotting (Fig. 6A, lane 7) and GST pulldowns (Fig. 6E) as described above. MBP-Taf2-ΔC displayed no observable binding to His$_6$-Taf14 in the Far Western blot or His$_6$-GST-Taf14 in the GST pulldown. Thus, Taf2(1261–1407) is necessary for Taf14 binding in vitro.

To assess whether the Taf2 C terminus is sufficient for binding to Taf14 in vivo, untagged Taf2, HA-Taf2, HA-Taf2-ΔC, and HA-Taf2 C-terminal fragments (aa 1261–1407, 1271–1407, 1281–1407, 1291–1407, and 1301–1407) were subjected to α-HA co-IP as described above (Fig. 6F). As expected, HA-Taf2 could co-immunoprecipitate Taf7, Taf8, and Taf14, whereas HA-Taf2-ΔC could co-immunoprecipitate Taf7 and Taf8 but could not co-immunoprecipitate Taf14. Surprisingly, all of the Taf2 C-terminal fragments displayed the ability to co-immunoprecipitate Taf14. Despite Taf2(1261–1407) being necessary for binding to Taf14 in vivo, aa 1261–1407 and smaller C-terminal fragments are sufficient for binding to Taf14 in vivo. Considering that TFIID contains multiple copies of Taf1 per TFIID molecule (8), these data allowed us to hypothesize that the Taf2 C terminus contains multiple Taf14 binding domains that are independently capable of promoting Taf14-TFIID association.

**Defining the Minimal Taf2 Interaction Domain in Taf14**—In parallel to our analyses to map the Taf14 interaction domain in Taf2, Taf14 was also subjected to systematic truncation mutagenesis to identify the Taf14 domain operative in suppressing the taf2-ts7 Ts phenotype. Previous Taf14 studies have shown that the N terminus contains the YEATS domain, whereas the Taf14 C terminus can both complement the growth deficiencies associated with a taf14-null strain and associate with transcription-related complexes such as TFIID and TFIIF in vivo (76). In addition, C-terminally tagged Taf14 variants display defects in growth, likely because the tag negatively impacts the ability of Taf14 to interact with transcription-related complexes (59).

In strains harboring taf2-ts7, Taf14 full length (aa 1–244), the Taf14 N terminus (aa 1–123), and the Taf14 C terminus (aa 124–244) were overexpressed to determine whether these fragments could suppress the Ts phenotype (Fig. 7A). All variants were expressed with a two-copy FLAG tag with an SV40 nuclear localization sequence (FLAG$_x$$_x$NLS) N-terminal tag. Although Taf14(1–244) displayed the most robust suppression of the taf2-ts7 Ts phenotype, Taf14(124–244) also suppressed the taf2-ts7 Ts phenotype. The N-terminal YEATS domain-containing fragment (aa 1–123) could not suppress the taf2-ts7 Ts phenotype. We further dissected the Taf14 C terminus in this overexpression suppression assay by performing systematic N- and C-terminal truncations of Taf14(124–244) (Fig. 7A and data not shown). These analyses identified that the domain minimally required for suppression of the taf2-ts7 Ts phenotype lies within Taf14(164–244).

Again, the simplest model for the ability of the Taf14 C terminus to suppress the taf2-ts7 Ts phenotype is that the C ter-
minus of Taf14 directly interacts with Taf2. To test this hypothesis, purified recombinant His$_6$-GST, His$_6$-GST-Taf14(1–244), and His$_6$-GST-Taf14(124–244) were mixed with purified Taf2 and, in the presence of BSA, subjected to GST pulldown as described above (Fig. 7B). His$_6$-GST-Taf14(1–244) and His$_6$-GST-Taf14(124–244) were able to pull down purified Taf2 in a dose-dependent manner, whereas His$_6$-GST and His$_6$-GST-Taf14(1–123) did not pull down any detectable Taf2. These data confirm that the Taf14 C terminus, the domain required for suppression of the taf2-ts7 Ts phenotype, is necessary and sufficient for direct interaction with Taf2 in vitro.

To identify point mutants that disrupt the TAF2-TAF14 genetic interaction, we performed three-Ala scanning mutagenesis with 1-aa overlap of Taf14(164–244). These variants were overexpressed in the context of full-length Taf14 to determine whether they could suppress the taf2-ts7 Ts growth phenotype. Of the 39 Ala variants generated, we identified 11 that displayed defects in the ability to suppress the taf2-ts7-associated Ts phenotype at 37 °C. The results of the suppression analyses for these 11 taf14-ala variants are summarized in Fig. 7C. A particularly sensitive hot spot was identified between Taf14 aa 218 and 230 where every mutant variant (m5–m10) displayed a defect in taf2-ts7 Ts growth suppression. These amino acids are likely critical for the function of the Taf14 C terminus.

Fine Mapping of the Taf2 C Terminus Reveals Two Taf14 Interaction Domains—A previous study reported that the Taf2 C terminus is insoluble when expressed in Escherichia coli (59). Our studies confirm this observation. E. coli expressed Taf2(1301–1407)-His$_6$ is largely insoluble and refractory to native purification with Ni$^{2+}$-NTA-agarose (Fig. 8C, lane 1). We have previously shown that co-expression of insoluble Tafs with their cognate binding partner results in solubilization (47). Therefore, we hypothesized that co-expression of the Taf2 C terminus with either full-length Taf14 or Taf14(164–244) would result in solubilization of the Taf2 C-terminal fragment. To test this, we co-expressed Taf2(1301–1407)-His$_6$ with either Taf14(1–244) or Taf14(164–244) and subjected these complexes to Ni$^{2+}$-NTA-agarose purification. Using this strategy, we could generate soluble Taf2-Taf14 complexes in milligram quantities/liter of E. coli culture (Fig. 8A and B, respectively). We also attempted to co-express full-length Taf2 with Taf14(164–244) in E. coli, but these attempts were unsuccessful (data not shown).

We then used this co-purification assay to fine-map the Taf14 binding domain within Taf2(1301–1407). N- and C-terminal truncations of Taf2(1301–1407)-His$_6$ were co-expressed with Taf14(164–244) in E. coli and subjected to Ni$^{2+}$-NTA-agarose purification (Fig. 8C; diagrammed in Fig. 8D). These analyses revealed that Taf2(1381–1407) were sufficient for Taf14 co-purification, whereas Taf2(1362–1407) were necessary for Taf14 co-purification. Henceforth, we will refer to Taf2(1363–1407) as Domain 1. Surprisingly, Domain 1 not only binds to Taf14 but also contributes to the insolubility of the Taf2(1301–1407) fragment because fragments deleted for Domain 1 can be purified from E. coli without co-purifying Taf14.

To assess the relevance of Taf2 Domain 1 to binding to Taf14 in vivo, we performed α-HA co-IPs with a series of Taf2 C-terminal deletion variants as described above (Fig. 8E). Considering that Taf2-ΔC (aa 1–1260) fails to interact with Taf14 in vivo and in vitro and that the Taf2(1301–1362)-His$_6$ fragment fails to co-purify Taf14, we reasoned that a second Taf14 binding domain likely resides within Taf2(1261–1300) (hereafter Domain 2). Deletion of either Domain 1 (Δ1) or Domain 2 (Δ2) had no impact on the ability of Taf2 to co-precipitate TFIIID subunits Taf7, Taf8, and Taf14. However, a Taf2 double deletion variant (Δ1 + Δ2) could co-precipitate Taf7 and Taf8 but failed to co-precipitate Taf14. Furthermore, successively smaller deletion within Domain 2 (Δ1261–1291, Δ1261–1281, and Δ1261–1271), when combined with Δ1, displayed strong
**Figure 6.** The Taf2 C terminus is necessary and sufficient for binding to Taf14 in vitro and in vivo. A, far Western blotting. Purified Taf2 forms were analyzed in triplicate as in Fig. 5 with one replicate analyzed by SYPRO Ruby gel staining (shown in A, left), whereas the other two were analyzed by anti-Taf14 far-Western blotting with and without His6-Taf14 overlay (shown in A, right). Two technical replicates were performed. * indicates common E. coli protein contaminant. B, schematic of Taf2 forms used in A; numbers refer to the proteins loaded in A, lanes 1–7. C and D, plasmid shuffle complementation and Taf2-TFIID co-IP. Taf2 C-terminal truncation variants (TAF2*: no ORF (−), TAF2 full length aa 1–1407, HA-NLS-TAF2 full length, and successive HA-NLS-TAF2 truncation variants ranging from aa 1–1251 to aa 1–1300) were analyzed for their ability to complement a taf2-null strain via plasmid shuffle (D) and to co-immunoprecipitate TFIID subunits as described in Fig. 2. Additional anti-Taf IgGs were used in these analyses (α-Taf4 and α-Taf9). E, GST pulldowns. Similar to Fig. S8 except 5 pmol of His6-GST-Taf14 were incubated with 0.1 mg/ml BSA and between 1 and 32 pmol of MBP-Taf2 or MBP-Taf2-ΔC. Two independent binding assays were performed, once with 2 pmol of His6-GST-Taf14 and once with 5 pmol of His6-GST-Taf14, both displaying high affinity saturable binding to MBP-Taf2 and a lack of specific binding to MBP-Taf2-ΔC. One picomole of purified MBP-Taf2 and 1 pmol of purified MBP-Taf2-ΔC were loaded to demonstrate purity of input material. F, Taf2-TFIID co-IP performed as described in Fig. 2. The black line depicts non-contiguous lanes from the same SDS-polyacrylamide gel and from the same film exposures. One technical replicate was performed. ΔC, Taf2(1–1260).

**Figure 7.** The Taf14 Binding Domains in Taf2 Are Necessary for TAF14 Overexpression Suppression of the taf2-ts7 Growth Defect—Our overarching hypothesis has been that **TAF14** overexpression suppression of the **taf2-ts7** Ts phenotype occurs via a Taf2-Taf14 direct interaction. By extension, if Taf2 and Taf14 could no longer physically interact, then **TAF14** overexpres-
Suppression would no longer be able to suppress the *taf2-ts7* Ts phenotype.

To test this hypothesis, we performed genetic complementation assays with *TAF2*, *taf2-DΔC*, *taf2-ts7*, and *taf2-ts7-DΔC* with or without *TAF14* overexpression (Fig. 9A). As shown before, overexpression of *TAF14* did not have a strong impact on growth of strains harboring *TAF2*. However, overexpression of *TAF14* in strains harboring *taf2-DΔC* resulted in a synthetic slow growth phenotype. Similarly, although *TAF14* overexpression suppressed the *taf2-ts7* Ts phenotype, it did not suppress the *taf2-ts7-DΔC* phenotype but instead caused synthetic lethality. Consistent with our hypothesis, the Taf14 binding domains in the Taf2 C terminus are required for suppression of the *taf2-ts7* Ts phenotype.

Furthermore, our hypothesis also predicts that *TAF14* overexpression drives Taf2-Taf14 complex formation, resulting in stable incorporation into the TFIIID complex. To test this hypothesis, we performed α-HA co-IP analysis as described above to determine the impact of *TAF14* overexpression on the ability of Taf2, Taf2-DΔC, Taf2-ts7, and Taf2-ts7-DΔC to co-precipitate TFIIID subunits (Fig. 9B). The strains used for these analyses were pseudodiploid for both *TAF2*, containing WT and a test *TAF2* allele (*TAF2*), and *TAF14*, containing genomically encoded WT *TAF14* and either an empty overexpression plasmid or an expression plasmid containing FLAG<sub>5</sub>-NLS-tagged *TAF14*. *TAF14* overexpression had no impact on the ability of Taf2 to co-immunoprecipitate Taf7 and Taf8; however, Taf2 co-precipitated elevated levels of Taf14 compared with the no-*TAF14* overexpression strain. Consistent with the synthetic sick growth phenotype, Taf2-DΔC reproducibly displayed a modest reduction in the ability to co-immunoprecipitate Taf7 and Taf8 in strains that overexpressed *TAF14*. Validating our hypothesis, *TAF14* overexpression rescued the ability of Taf2-ts7 to co-immunoprecipitate TFIIID subunits Taf7, Taf8, and Taf14. However, Taf2-ts7-DΔC was not responsive to *TAF14* overexpression and still failed to efficiently co-immunoprecipitate TFIIID subunits Taf7, Taf8, and Taf14.

Replacing the Taf14 Binding Domain in Taf2 with Taf14 via Gene Fusion Partially Suppresses the *taf2-ts7*-associated Growth Defects—Our data suggest that the domain necessary for Taf2-Taf14 interaction resides within Taf2(1261–1407),...
FIGURE 8. Taf2 amino acids 1261–1407 contain two domains that contribute to Taf14 binding. A and B, co-purification of Taf2 and Taf14. Taf2(1301–1407)-His, was co-expressed in E. coli with either Taf14(1–244) or Taf14(164–244) and purified using Ni²⁺-NTA-agarose. Purified Taf2(1301–1407)/Taf14(1–244) (shown in A) and Taf2(1301–1407)/Taf14(164–244) (shown in B) were separated via SDS-PAGE and stained with Coomassie Blue. C and D, fine mapping of the Taf14 interaction domain in Taf2(1301–1407). C-terminally hexahistidine-tagged Taf2(1301–1407) and N- and C-terminal truncation variants of Taf2(1301–1407) (diagrammed in D) were co-expressed with Taf14(164–244) in E. coli. As a negative control, C-terminally hexahistidine-tagged Taf2(1301–1407) was expressed alone. Denatured whole cell extracts or Ni²⁺-NTA-agarose-purified complexes were separated on SDS-PAGE and stained with Coomassie Blue (C) or Coomassie Blue and stained with Coomassie Blue (D). Samples that co-purify Taf14(164–244) are shown in black, whereas samples that fail to co-purify Taf14(164–244) are shown in red. A representative image of two technical replicates is shown. E, Taf2-TFIID co-IP performed as described in Fig. 2. All deletion variants contain an HA₆-NLS tag. Black lines separate non-contiguous lanes from the same SDS-polyacrylamide gel and from the same film exposures. One technical replicate was performed. ΔC, deletion of Taf2(1261–1407); Δ1, deletion of Taf2(1363–1407); Δ2, deletion of Taf2(1261–1300).

To distinguish between these two models, we constructed Taf2-Taf14 chimeras where taf2-ΔC and taf2-ts7-ΔC were fused to the TAF14 ORF. Ideally, these chimeric fusions would bypass the need for TAF14 overexpression to achieve saturable binding to the Taf2 C terminus because these chimeras contain covalently attached Taf14. These chimeras were tested to see whether they could complement a taf2-null strain (Fig. 10A). A “WT” chimera (taf2-ΔC-Taf14) supported growth at a level similar to Taf2, suggesting the chimera does not negatively impact Taf2 function. This taf2-ts7-ΔC-TAF14 chimera suppressed the slow growth phenotype at 30 °C associated with taf2-ts7 and taf2-ts7-ΔC.

We then sought to determine whether this suppression was Taf14-dependent and, if so, which domain(s) was involved. To
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A.

| TAF2* | T14 | Unshuffled | HAx3NLS-TAF2 | TAF2* TAF14 |
|-------|-----|------------|-------------|-------------|
| -     | +   | -          | -           | -           |
| +     | -   | -          | -           | -           |

B.

| TAF14 OE | α-HA (Taf2) | α-Tafl | α-Taf8 | α-Taf14 |
|----------|-------------|--------|--------|--------|
| 185      | 80         | 40     | 25     |

**FIGURE 9.** The Taf14 binding domain in Taf2 is necessary for TAF14 overexpression-mediated suppression of taf2-ts7. A, intragenic TAF14 synthetic genetics. Plasmid shuffle complementation assays were performed as described in Fig. 3C. Cells were grown for 72 h at 25°C prior to imaging. Representative images from at least two biological replicates are presented (TAF2*, no ORF (-/-)); TAF2, HANLS-TAF2, HANLS-TAF2-D C, HANLS-taf2-ts7, and HANLS-taf2-ts7-ΔC). B, Taf2-TFIID co-immunoprecipitation performed as described in Fig. 2 except that strains also contained either an empty HIS3-marked overexpression (OE) plasmid or a FLAG-NLS-TAF2 cDNA containing HIS3-marked overexpression plasmid. A representative image of at least two biological replicates is shown (TAF2*, no ORF (-/-)); TAF2, HANLS-TAF2, HANLS-TAF2-D C, HANLS-taf2-ts7, and HANLS-taf2-ts7-ΔC). Taf14 WT is labeled 14; FLAG-NLS-Taf14 is labeled F14.

This is the end, we made taf2-ts7-ΔC-taf14 chimeras with mutations either in the Taf2 binding domain of Taf14 (taf14-m3; L186A,T187A,K188A) or in the Taf14 YEATS domain that disrupt its ability to bind to acetylated or crotonylated H3K9 (taf14-W81A, taf14-G80K) (23, 24). Mutations in the Taf14 C terminus completely abrogated the ability of the chimera to suppress the growth defect associated with taf2-ts7-ΔC. In addition, whereas both YEATS domain mutant fusions displayed some reduction in the ability to suppress the growth defects associated with taf2-ts7-ΔC, the G80K mutation displayed a significant loss in the ability to suppress. The loss of suppression cannot be attributed to a reduction in steady-state protein levels because all constructs were expressed at least as well as WT Taf2 (Fig. 10B).

**TAF14-less TFIID Mutant Cells Display a Slow Growth Phenotype and Defects in RPG Transcript Abundance**—Our results suggest that the Taf2 C terminus is not only required for Taf2-Taf14 interaction but is also required for association of Taf14 with the TFIID complex. To test this hypothesis, we engineered two strains: one that genomically encodes an HAα1-tag at the N terminus of Taf1 for anti-HA immunopurification and one that encodes both an HAα1-Taf1 and genomic deletion of Taf2(1261–1407). TFIID was purified from these two strains as described previously (8) and then subjected to SDS-PAGE and gel staining to score TFIID subunit composition and stoichiometry (Fig. 11A). When comparing the TFIID variants, two differences were apparent. First, in the Taf2-ΔC TFIID, the size of Taf2 was reduced, reflecting the genomic Taf2 C-terminal deletion. However, Taf2-ΔC is maintained at an apparent 1:1 Taf2-TFIID stoichiometric ratio relative to Taf1, similar to HAα1-Taf1 TFIID, indicating that the deletion does not negatively impact the stability of Taf2 in the TFIID complex. Second, in the Taf2-ΔC TFIID, Taf14 is completely absent, consistent with our Taf2 C terminus truncation analyses. Quantitation of these TFIID preparations demonstrates that the stoichiometry for the rest of the TFIID subunits is similar between the two TFIID forms. Based on these data, we can conclude that Taf2(1261–1407) is necessary for Taf14 stable incorporation into the TFIID complex and that strains lacking Taf2(1261–1407) have TFIID devoid of Taf14 (Taf14-less TFIID).

When we streaked these TFIID purification strains onto rich medium to isolate single colonies, we found that the Taf14-less TFIID strain displayed a reduced growth rate as measured by colony size (data not shown). To determine whether this phenotype was directly attributable to the Taf2-ΔC variant, growth curves were performed in yeast strains that contain only HAα1-TAF2 or HA-raf2-ΔC. These growth curves revealed that strains harboring HA-TAF2-ΔC displayed a 14.5-min (15.6%) slower growth rate during log phase at 30°C (Fig. 11B).

We then assessed steady-state transcript abundance for these two strains. RNA was extracted from mid-log phase cells growing at 25°C and analyzed using qRT-PCR. Despite no reduction in transcript abundance for RNA polymerase I-transcribed RDN58 (Fig. 11D) and RNA polymerase III-transcribed SRN6 (Fig. 11E), we reproducibly observed a statistically significant ~2-fold reduction in RPG transcript abundance (RPP5, RPS5B, RPS8A, and RPS3) (Fig. 11C). In addition, we observed a moderate (~25%), although not statistically significant for both genes, reduction in steady-state transcript abundance for the SAGA-dominated glycolytic PGK1 and PYK1 genes. Although the glycolytic genes are not considered TFIID-dependent, our data are consistent with previously observed modest reductions in PGK1 steady-state transcripts in taf4 variants (32).

**Discussion**

Multiple structural and biochemical studies have attributed specific Tafs with promoter-DNA or modified chromatin binding capabilities (10–12, 15, 16, 18, 19, 21, 23, 24, 37, 56). These activities provide convenient mechanisms through which TFIID can engage with genes to facilitate PIC formation. Indeed, an activator-TFIID-TFIIA promoter-DNA quaternary complex with distinct TFIID-DNA interactions displays a locked DNA conformational state that likely serves as a platform for general transcription factor and Pol II binding (57). However, these many biochemical activities have rarely been interrogated genetically to establish their importance in vivo (37). This lack of knowledge was the impetus for the work reported here.

Specifically, the functional role of Taf2 in vivo was largely unknown despite longstanding evidence for its in vitro INR binding activity (12, 18, 19). A lack of Taf2 molecular genetic
studies is likely a result of technical challenges. In addition to being essential for life, Taf2 is a large protein and particularly labile in ScTFIID purifications (41, 61). As our study demonstrates, systematic deletion analyses largely disrupt Taf2 protein stability and the ability of Taf2 to stably incorporate into the TFIID complex, precluding conventional methods of genetically interrogating large proteins (42). Our site-directed mutagenesis approach generated part of the Taf2 C terminus as a Taf14-binding function of Taf2 (68). One of the site-directed mutants analyzed in this study, taf2-m32, targeted a subset of these residues but displayed only a mild slow growth phenotype and was not further pursued (supplemental Table 2). Genetic interrogation of this putative INR binding domain will likely shed insights into the role of INR binding in TFIID transcriptional activation.

Our key finding in this report is the discovery of a genetic interaction between TAF2 and TAF14. Individual overexpression of each TFIID subunit identified TAF14 overexpression as a mechanism to achieve suppression of select taf2-ts alleles (taf2-ts7 and -ts8). The location of the residues mutated in these Taf2 variants is suggestive of a functional domain. Furthermore, we demonstrate that Taf2 and Taf14 directly interact. Molecular genetic dissection of both Taf2 and Taf14 led to the identification of the domains responsible for physical and functional interaction in vivo and in vitro.

Our previous structural and biochemical characterization of the TFIID complex identified the stoichiometry and location of all of the TFIID subunits with the exception of Taf14 (8, 40–42). Purified TFIID displays a stoichiometry of at least two copies of Taf14 per TFIID molecule. In addition, Taf14 self-associates in vivo. However, gel staining of purified SWI/SNF and TFIIF show one copy per complex so this multicopy per complex phenotype is likely to be specific to TFIID (52, 58). Although deletion of the Taf2 C terminus completely disrupts association of Taf14 with the TFIID complex, fine mapping of the Taf2 C terminus identified two domains that can independently facilitate Taf14 incorporation into the TFIID complex. These data are consistent with a genome-wide two-hybrid screen, which identified part of the Taf2 C terminus as a Taf14-interacting protein (59). However, Taf2 variants that fail to stably incorporate into the TFIID complex as well as Taf2 C-terminal fragments display reductions in Taf14 co-precipitation relative to WT Taf2. This observation suggests that when Taf2 incorporates into the TFIID complex Taf14 binding is enhanced, potentially through a multivalent binding site between Taf2 and another TFIID subunit(s).
Interestingly, the Taf2 domain identified to directly interact with Taf14 does not contain the amino acids mutated in taf2-ts7 or -ts8 despite this domain being required for TAF14 overexpression suppression of these variants. To test the possibility of a multivalent Taf14 binding site between Taf2 and another TFIID subunit, we fused TAF14 to a taf2Δ variant deleted for the Taf14 binding domain. This chimeric fusion improved growth in a TAF14-dependent manner consistent with the existence of a multivalent binding site. The TFIID subunit responsible is likely to be Taf8. Human Taf2 directly interacts with Taf8 (44). Taf1-TAP purifications of TFIID result in substoichiometric levels of Taf2, Taf8, and Taf14 (41). Furthermore, specific deletions in Taf1 result in dissociation of Taf2, Taf8, and Taf14 from the TFIID complex, suggesting that these three subunits form a subcomplex (70). As with human TFIID, the association of Taf2 and Taf8, along with Taf14, with the TFIID core may stimulate a structural transition in the TFIID assembly pathway (43).

The function of Taf14 in transcription regulation has remained enigmatic. Functional interpretation of TAF14 mutant variants is limited because of its presence in multiple transcription-related complex assemblies (52, 58–60). Specifically, molecular defects in taf14-null strains or strains harboring taf14 mutant variants unable to bind modified H3K9 could...
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be attributed to TFIID-promoter interactions, the role of TFIIF in PIC or elongation function, ATP-dependent chromatin remodeling, or the myriad transcription-related functions associated with Mediator. Our study begins to decipher the role of Taf14 in transcription regulation through the identification of taf2-D, a separation-of-function Taf2 variant that can stably incorporate into the TFIID complex but precludes incorporation of Taf14. The existence of this variant is consistent with the model presented by Kabani et al. (59) that Taf14 has a particular entry point protein in each complex with which it is associated. Thus, it may be possible to generate genetic reagents that specifically dissociate Taf14 from the TFIIF, Ino80 (inositol-requiring complex), Swi/Snf, NuA3 (nucleosome acetyltransferase complex), Mediator, and RSC without perturbing complex integrity or the other functions of the complex. taf2-D mutant cells display a modest slow growth phenotype as well as a reduction in transcript abundance for the TFIID-dominated RPGs. In addition, purification of TFIID from strains harboring taf2-D yields a Taf14-less TFIID complex. Structural analyses of this complex in contrast with WT TFIID may yield important insights into the location of Taf14 in TFIID function as well as the mechanism by which two copies of Taf14 can associate with a single TFIID molecule.

Our data suggest that taf2-D is a true separation-of-function variant whose cellular and molecular phenotypes reflect the contribution of Taf14 to TFIID function (Fig. 12). However, the mechanism by which Taf14 contributes to TFIID transcription activation mechanism remains speculative. The Taf14 YEATS domain responsible for binding to modified chromatin (23, 24) may enhance TFIID occupancy of active genes by increasing the number of contact points between TFIID and gene promoters. In addition, our Taf2-Taf14 chimeric fusion analyses suggest that the YEATS domain does contribute to TFIID function, likely through its ability to interact with modified chromatin. This hypothesis is consistent with the observation that the Taf3 PHD finger H3K4me3 binding activity stimulates transcription, especially in the context of a mutant TATA box (37). Similar in vitro transcription experiments with chromatin templates need to be performed with WT and Taf14-less TFIID to assess the validity of this model for hTAFII considering that the Taf3 PHD finger is not present in the yeast system.

In contrast, the Taf14 YEATS domain is non-essential and displays minimal slow growth defects when deleted, whereas deletion of the Taf14 C-terminal domain phenocopies the taf14-null strain (76). Considering the Taf14 C-terminal domain is responsible for the interaction with Taf14-associated complexes, this domain could mediate interactions among these complexes that have thus far not been explored. We do not know whether a single Taf14 molecule can bind to multiple transcription-related complexes at the same time or whether binding of Taf14 to TFIID or TFIIF, for example, is mutually exclusive. If these interactions are not mutually exclusive, Taf14 could serve as a bridge between the transcription machinery that could play a key role in the transcription process.

In summary, through systematic mutagenesis of the TFIID subunit Taf2, we have uncovered important physical and functional interactions between Taf2 and Taf14. These discoveries have shed light on the role Taf14 plays in TFIID function including a putative role in TFIID-chromatin interaction. We believe our study could provide a model for disambiguating the role Taf14 plays in gene regulatory mechanisms.

Materials and Methods

Bacterial Strains and Cloning—E. coli DH5α was used for all cloning and propagation of plasmids. Recombinant proteins were expressed in E. coli Rosetta2 (DE3) strains (Novagen). All cloning was performed using restriction enzyme-based methods. Appropriate restriction ends were added to all cloned sequences using PCR with either Pyrococcus woesei DNA polymerase as described (78) or Q5 DNA polymerase according to the manufacturer’s instructions (New England Biolabs). Site-directed mutagenesis was performed using gene splicing
by overlap extension PCR (79). All constructs were sequence-verified.

**Yeast Manipulations**—Strains were grown in YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) or synthetic complete medium (SC) (0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) dextrose or 1% (w/v) raffinose where indicated), 0.2% (w/v) amino acid dropout mixture) without (-) leucine (Leu), histidine (His), or uracil (Ura) or a combination of the three where appropriate as indicated. All transformations were performed using the lithium acetate/PEG/salmon sperm carrier DNA transformation protocol (80). For plasmid shuffle assays, cells were grown on SC –Leu, –His, or both including 0.1% 5-fluoro-orotic acid (5-FOA) (81).

All strains generated in this study were derivatives of BY4741 (82). Yeast expression vectors were derivatives of shuttle vectors described by Mumberg et al. (83, 84) A taf2-null strain (JFTAF2del) was generated by co-transforming BY4741 with a p416ADH TAF2 covering plasmid and a linearized TAF2 deletion cassette. The TAF2 deletion cassette replaced –233 to +4224 relative to the start codon of the TAF2 ORF with the hgymyc B resistance cassette from pAG32 (85). Co-transformants were sequentially plated on SC –Ura followed by replica plating to YPD + 300 μg/ml hygromycin B. Hygromycin B+ and Ura+ clonal isolates were confirmed as taf2-null using plasmid shuffle genetic complementation. An HAx3-TAF1 strain (JFHTAF1) was generated using ends-in integration of an HAx3-TAF1 N-terminal tagging cassette into the TAF1 locus using a similar strategy described previously for Mot1 (86). The resulting strain harbors a G418K cassette and an N-terminal MYPYDVPDYAGVE tag (HA epitope underlined) at the site-directed mutants, deletion mutants, and site-directed TAF14 interaction.

**I-TASSER Structural Prediction and TAF2 Site-directed Mutagenesis**—Primary amino acid sequences for *S. cerevisiae* (yeast) Taf2, *Drosophila melanogaster* (fly) Taf2, and *Homo sapiens* (human) Taf2 were submitted to the I-TASSER server for 3D structure prediction (72). Resulting 3D models of yeast, fly, and human were imported in PyMOL, displayed in cartoon format and colored based on secondary structure. The first of five models generated for yeast Taf2 was used to define predicted solvent-exposed residues.

To assess amino acid conservation, yeast, fly, and human Taf2 primary sequences were aligned in MacVector using ClustalW. Fifty-eight mutants were designed based on solvent accessibility and proximity to amino acids that are either similar or identical among yeast, fly, and human Taf2. Twenty-nine additional mutants were designed based on amino acids predicted to be solvent-inaccessible but highly conserved among yeast, fly, and human Taf2. All mutations were arbitrarily limited to a maximum of 8 contiguous amino acids. A list of the mutants is provided (supplemental Table 2).

**Plasmid Shuffle and Overexpression Suppression**—All TAF2 site-directed mutants, deletion mutants, and TAF2-TAF14 chimeras were expressed from p415ADH with an N-terminal HAx3-NLS (additional details are available upon request). For plasmid shuffle analyses, JFTAF2del was transformed with an empty p415ADH plasmid, a p415ADH-TAF2 plasmid, a p415ADH-HAx3-NLS-TAF2 plasmid, or a p415ADH-HAx3-NLS-TAF2 mutant plasmid, and transformants were grown on SC –Leu plates. Leu+ colonies were grown to saturation in SC –Leu at 30 °C, serially diluted 1:4 in sterile H2O, spotted to SC –Leu or SC –Leu + 5-FOA 15-cm plates using a pinning tool, and grown at various temperatures (20, 25, 30, 34, and 37 °C) to assess temperature-sensitive growth. Duration of growth ranged from 48 to 96 h as indicated.

For overexpression suppression screening of all TFIID subunits, the ORFs of TAF1–TAF14 and SPT15 (TBP) were cloned into p413GPD using varied restriction ends (details are available upon request). Plasmid shuffle analyses were performed essentially as above with the following exception. JFTAF2del was co-transformed with LEU2-marked TAF2 plasmids and an empty p413GPD or p413GPD containing the ORF for each of the TFIID subunits and grown on SC –His, –Leu plates. For each LEU2-marked TAF2 and p413GPD TFIID subunit combination, two His+ Leu+ colonies were spotted undiluted to SC –His, –Leu or SC –His, –Leu + 5-FOA. TFIID subunits that scored positive for overexpression suppression displayed uniformly improved growth for both colonies tested.

For directed overexpression suppression studies with TAF14, both WT and mutant TAF14 variants were expressed with an N-terminal FLAGx3-NLS.

**Immunoblotting and Co-immunoprecipitation**—For steady-state protein immunoblotting, protein was extracted from approximately 1.2 × 10⁷ cells of early to mid-log phase-grown culture using sodium hydroxide-based lysis (87). For co-immunoprecipitations, JFTAF2del strains were grown as pseudodiploids containing both a wild-type URA3-marked TAF2 gene and a WT or mutant LEU2-marked TAF2 gene. In these analyses, 50 ml of yeast cells were grown at 30 °C to early to mid-log phase (approximately 2.4–3.6 × 10⁷ cells/ml), harvested by centrifugation, and lysed in co-IP buffer (20 mM HEPES-KOH, pH 7.9, 200 mM potassium acetate, 10% glycerol, 0.1% Nonidet P-40 substitute (Sigma-Aldrich), 1 mM DTT, 1 μg protease inhibitors (0.1 mM PMSF, 1 mM benzamidine HCl, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 1 μg/ml pepstatin A)) using glass bead lysis. Soluble protein was separated from insoluble material by centrifugation at 20,800 × g for 15 min. Protein concentrations were determined using a BSA standard curve with the Bio-Rad Protein Assay. 2 mg of soluble protein extract were incubated with 2.5 μg of anti-HA 12CA5 mAb and 50 ng/μl ethidium bromide in a total volume of 412 μl at 4 °C for 2 h. Immune complexes were captured with 10 μl of protein A-Sepharose beads (Life Technologies) for 30 min with mixing at 4 °C. Captured protein complexes were washed twice with 1 ml of ice-cold co-IP buffer and eluted with 2× lithium dodecyl sulfate NuPAGE Sample Buffer (Life Technologies) and heating at 75 °C for 10 min. Proteins for both steady-state immunoblotting and co-immunoprecipitations were separated via SDS-PAGE using 4–12% NuPAGE Bis-Tris gradient gels (Life Technologies) run with 1× MOPS running buffer and electrotransferred to PVDF membranes using a wet transfer system. Polyclonal anti-Taf and anti-TBP antibodies were used...
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as described previously (60, 70). Anti-actin (mAb8224) and anti-HA HRP conjugate (3F10) antibodies were procured from Abcam and Roche Applied Science, respectively. Goat anti-rabbit (Fc) HRP conjugate and horse anti-mouse IgG HRP conjugate were procured from Thermo Fisher and Cell Signaling Technology, respectively. Protein signal was detected using Amersham Biosciences ECL Western blotting detection reagent (GE Healthcare) and exposed to Blue Basic double emulsion autoradiography film (GeneMate). For immunoblotting loading controls, the blot was stained with Ponceau S (0.5% (w/v) Ponceau-S in 1% (v/v) glacial acetic acid) following the electrotransfer and destained with H₂O prior to imaging.

Protein Overexpression and Purification—All His₆-tagged proteins were expressed from pET28a (Novagen). All His₆-GST-tagged proteins were plasmid-generated from pBG101 in Rosetta2 (DE3) E. coli. His₆-Taf14 and Taf2 fragments were N-terminally tagged with MGSSHHHHHHSSGLVPSHH-MAS (bold indicates the hexahistidine tag). E. coli expression strains were grown at 37 °C with shaking at 250 rpm to an optical density at 600 nm (OD₆₀₀) of 0.6 – 0.9, shifted to 30 °C, and induced for 4 h with 1 mM isopropyl β-D-1 thiogalactopyranoside. His₆-Taf14, His₆-GST, His₆-GST-Taf14(1–244), His₆-GST-Taf14(1–123), and His₆-GST-Taf14(124–244) were purified in the following manner. 4 g of E. coli pellet were lysed in 40 ml of E. coli lysis buffer (20 mM Tris-HCl, pH 7.9, 200 mM NaCl, 20 mM imidazole, 0.1% Triton X-100, 10% glycerol, 1 mM DTT, 1× protease inhibitors) in the presence of approximately 100 μg/ml lysozyme. Lysates were sonicated, and insoluble material was pelleted by spinning at 27,000 × g in a Sorvall RC 5C Plus centrifuge with an SS-34 rotor for 15 min. Soluble lysate was then bound in batch to 2 ml of Ni²⁺-NTA-agarose (Qiagen) for 2 h at 4 °C with mixing. Ni²⁺-NTA-agarose and bound proteins were transferred to disposable 20-ml chromatography columns (Bio-Rad) and washed in column format with >10 column volumes of E. coli lysis buffer. Proteins were eluted with elution buffer (lysis buffer without chicken egg white lysozyme but with 200 mM imidazole), and peak fractions were collected. His₆-Taf2 fragments were purified as described above with the following modifications for denaturing purification. Approximately 200 mg of E. coli pellet were lysed in 6 ml of denaturing lysis buffer (20 mM Tris-HCl, pH 7.9, 1 × PBS, 6 M guanidinium HCl, 10 mM imidazole). Denatured cell extract was mixed with 200 μl of Ni²⁺-NTA-agarose for 2 h at 20 °C. Ni²⁺-NTA-agarose and bound proteins were washed with >10 column volumes of freshly made denaturing wash buffer (20 mM Tris-HCl, pH 7.9, 1 × PBS, 7 M urea, 10 mM imidazole). His₆-Taf2 fragments were eluted with freshly made denaturing elution buffer (denaturing wash buffer except 200 μM imidazole). For all purified proteins, protein concentration was determined via in-gel quantitation using a BSA standard curve.

Purification of MBP-Taf2 from S. cerevisiae—MBP-3C cleavage site (MBP-3C) was derived from pLM302, a derivative of pET27 (Novagen) engineered to contain MBP (derived from pMAL (New England Biolabs)) followed by the PreScission protease cleavage site LEVLFQ↓GP and a multiple cloning site. MBP-3C and Taf2 or Taf2-ΔC fragments were sequentially cloned in p425GAL1 (84). An MBP-3C-only plasmid was also generated. MBP-3C, MBP-Taf2, and MBP-Taf2-ΔC expression plasmids were transformed in BY4741. Leu⁺ colonies were grown at 30 °C with shaking at 250 rpm in SC – Leu with 1% (w/v) raffinose as the sole carbon source until the cultures reached a cell density of approximately 1.8 × 10³ cells/ml. Solid galactose was added to the culture to reach a concentration of 2% (w/v), and the cells were allowed to grow for 3 h at 30 °C. Cells were harvested by centrifugation and stored at −80 °C until purification. For purification, all manipulations were performed at 4 °C or on ice unless specified. Typically, 30 g of yeast cell pellet were lysed in 30 ml of Taf2 purification buffer (20 mM HEPES-KOH, 500 mM potassium acetate, 0.5% Nonidet P-40 substitute, 10% glycerol, 2 mM DTT, and 2× protease inhibitors) using glass bead lysis. Lysate was then centrifuged at 27,000 × g in a Sorvall RC 5C Plus centrifuge with an SS-34 rotor for 15 min, and soluble cell extract was mixed with 5 ml of DE-52 resin equilibrated in Taf2 purification buffer for 10 min with mixing at 20 °C. DE-52 flow-through was then diluted with 20 mM HEPES-KOH, pH 7.9, 10% glycerol, 1 mM DTT, 1× protease inhibitors to reduce the potassium acetate and the Nonidet P-40 substitute concentration to 200 mM and 0.2%, respectively, and bound to 5 ml of amylose resin (New England Biolabs) in batch with mixing for 2 h. The amylose resin-bound proteins were transferred to a disposable chromatography column and washed with >10 column volumes amylose wash buffer (20 mM HEPES-KOH, pH 7.9, 200 mM potassium acetate, 0.1% Nonidet P-40 substitute, 10% glycerol, 1 mM DTT, 1× protease inhibitors). MBP-3C, MBP-Taf2, and MBP-Taf2-ΔC were eluted with wash buffer + 10 mM maltose, and peak fractions were collected. Alternatively, Taf2 was eluted with laboratory-generated 3C protease at 100 ng/ml concentration at 4 °C for 16 h. MBP-3C was subsequently dialyzed extensively against dialysis buffer (20 mM HEPES-KOH, 100 mM potassium acetate, 10% glycerol, 1 mM DTT, 1× protease inhibitors). MBP-Taf2, MBP-Taf2-ΔC, or free Taf2 was further purified using a Mono Q column (GE Healthcare). Peak eluate fractions from the amylose resin purification were loaded onto Mono Q with BA200 (BA = 20 mM HEPES-KOH, pH 7.9, 10% glycerol, 1 mM DTT, 1× protease inhibitors with variable concentrations of potassium acetate; e.g. BA200 contains 200 mM potassium acetate). Proteins were eluted with a linear gradient of BA200 to BA1500. Peak MBP-Taf2, MBP-Taf2-ΔC, and Taf2 fractions eluted at approximately BA1200. Peak fractions were pooled and dialyzed extensively against dialysis buffer.

Taf2/Taf14 Co-expression Solubilization Analyses—For Taf2 and Taf14 co-expression, a bicistronic expression plasmid was generated by cloning in order either full-length Taf14 or Taf14(164–244), an internal ribosome binding site sequence (88), and Taf2 C-terminal fragments (aa 1301–1407) into pET28a. Taf2 fragments contained a C-terminal KLAALAE-HHHHH(stop) tag. Taf2/Taf14 co-expression solubilization assays were performed by growing 6 ml of E. coli expression strains at 37 °C with shaking at 250 rpm to an OD₆₀₀ of 0.6 – 0.9, shifted to 30 °C, and induced for 2 h with 1 mM isopropyl β-D-1 thiogalactopyranoside. One-sixth of the cell pellet was lysed in 1 ml of E. coli lysis buffer with lysozyme as described above. Soluble protein was mixed with 7.5 μl of Q Sepharose to remove nucleic acids for 30 min at 4 °C.
The flow-through was mixed with 20 μl of Ni²⁺-NTA-agarose for 2 h at 4°C. Bound proteins were washed two times with 500 μl of E. coli lysis buffer and eluted with 200 μl of E. coli elution buffer. Total cellular protein and purified Taf2-Taf14 complexes were separated via SDS-PAGE as described above except running with 1X MES buffer (Life Technologies) to separate smaller protein species. The proteins were visualized using Coomassie Brilliant Blue.

**TFIID Purification**—HAx₁-Taf1 TFIID and HAx₁-Taf1-Taf2-ΔC TFIID were purified essentially as described (8, 46) with the following modifications. Extracts prepared according to Woonter et al. (89) were dialyzed against 20 mM HEPES-KOH, pH 7.9, 50 mM potassium acetate, 20% glycerol, 5 mM DTT, 1X protease inhibitor mixture until the dialysate reached a conductivity equivalent to BA300. Dialyzed extract derived from a maximum of 300 g of yeast cell pellet was chromatographed over a 200-ml Bio-Rex70 100–200-mesh column. For immunopurification, 10% Surfact-Amps Nonidet P-40 was added to the Bio-Rex70 1 m fraction to a final concentration of 0.2%. The Bio-Rex70 1 m fraction was subsequently diluted 1:2 in BA0, ethidium bromide was added to a final concentration of 50 μg/ml, and the fraction was subjected to anti-HA affinity chromatography with 10 mg of anti-HA 12CA5 mAb covalently coupled to 2.5 ml of protein A-Sepharose beads (Life Technologies) at 4°C for 16 h with mixing. Bound proteins were transferred to a 10-ml disposable chromatography column, washed with >5 column volumes of BA300 with 0.1% Surfact-Amps Nonidet P-40, >5 column volumes of BA300 with 0.01% Surfact-Amps Nonidet P-40, and >5 column volumes of BA300 with 0.001% Surfact-Amps Nonidet P-40. TFIID was eluted from the column two times with 2.5 ml of elution buffer (BA300 with 0.001% Surfact-Amps Nonidet P-40 plus 2 mg/ml HAx₁ peptide) for 30 min at 20°C with mixing. The HAx₁ peptide eluate was immediately subjected to ion exchange chromatography on a 1.2-ml UnoS column (Bio-Rad). Following binding of the HAx₁ peptide eluate, the UnoS column was washed with >5 column volumes of BA300 and then subjected to a linear gradient of BA300 to BA1000. TFIID elutes at approximately BA650.

Taf1-TAP TFIID was purified according to a modified tandem affinity purification protocol. 1 kg of YLSTAF1 (41) pellet was lysed in 500 ml of 3x TAP buffer (0.45 m Tris acetate, pH 7.8, 0.15 m potassium acetate, 60% glycerol, 3 mM EDTA, 3X protease inhibitors) using glass bead lysis. Then the salt concentration of the lysate was adjusted with 5 m potassium acetate to the conductivity equivalent of 300 mS potassium acetate and centrifuged at 205,000 × g in a Beckman Optima LE-80K ultracentrifuge with a Ti-45 rotor for 90 min. The supernatant was collected, avoiding the turbid material at the bottom of the centrifugation tube, and then bound to 100 ml of IgG-Sepharose (GE Healthcare)/liter of protein extract in batch at 4°C for 2 h with mixing. Following binding, the IgG-Sepharose and bound protein were washed extensively in IgG-Sepharose binding buffer (20 m Tris acetate, pH 7.8, 300 mS potassium acetate, 10% glycerol, 0.5 mM EDTA, 1 mM DTT, 1X protease inhibitors). TFIID was eluted from the IgG-Sepharose in 100 ml of IgG-Sepharose binding buffer plus tobacco etch virus protease at 250 ng/ml for 2 h at 4°C. Following tobacco etch virus protease elution from IgG-Sepharose, the eluate was immediately subjected to ion exchange chromatography on a 1.2-ml UnoS column as described above. TFIID elutes at approximately BA650.

**Far-Western Blotting**—0.5 pmol of Taf1-TAP TFIID, 5 pmol of purified MBP-Tc, 1 pmol of MBP-Taf2, and between 1 and 5 pmol of Taf2 truncation variants were subjected to Far-Western blotting analysis with a His₆-Taf14 overlay essentially as described (28) with the following modifications. For all analyses, proteins samples were subjected to SDS-PAGE in triplicate: one gel for SYPRO Ruby gel staining, one mock overlay control, and one His₆-Taf14 binding assay. During the blotting process, all binding steps and washes were performed in renaturation buffer (20 mM HEPES-KOH, pH 7.6, 75 mM potassium chloride, 2.5 mM magnesium chloride, 0.25 mM EDTA, 0.05% Triton X-100) with 1 mM DTT freshly added. The overlay was performed with 7 mM His₆-Taf14 with 1% BSA as a nonspecific competitor or with just the BSA competitor for the mock control. Bound His₆-Taf14 was detected using a standard immunoblotting protocol (primary antibody, antigen affinity-purified anti-Taf14 polyclonal rabbit IgG at a concentration of 0.1 ng/ml; secondary antibody, goat anti-rabbit Fc-HRP used according to the manufacturer’s instructions). Prior to treatment with ECL reagent and exposure to film, the blots were washed once with Tris-buffered saline (25 mM Tris-HCl, pH 7.5, 150 mM NaCl).

**GST Pulldown Assays**—Typically, binding reactions were performed in a total volume of 200 μl in the following reaction buffer: 20 mM HEPES-KOH, pH 7.9, 300 mM potassium acetate, 10% glycerol, 1 mM DTT, 0.1% Nonidet P-40 substitute, 0.1 mg/ml BSA. MBP-Taf2 and MBP-Taf2-ΔC GST pulldowns were performed twice, either with 2 pmol of His₆-GST-Taf14 or 5 pmol of His₆-GST-Taf14 and twice that amount with His₆-GST-only pulldowns. Between 1 and 32 pmol of MBP-Taf2 or MBP-Taf2-ΔC were used in the binding assays. For Taf2 binding assays, 16 pmol of His₆-GST, 8 pmol of His₆-GST-Taf14, 12 pmol of His₆-GST-Taf14(124–244) were used. These proteins were mixed either with no Taf2 or with between 0.78 and 6.25 pmol of Taf2. Binding reactions were allowed to proceed at 20°C for 1 h followed by 30-min capture at 20°C with 10 μl of a 1:1 slurry of glutathione-agarose Fast Flow (GE Healthcare) equilibrated in reaction buffer. Glutathione-agarose-bound complexes were pelleted by centrifugation, and the supernatant was discarded. The pellet was washed with 500 μl of binding buffer without BSA and pelleted again, and the wash buffer was discarded. Bound proteins were eluted with 2X lithium dodecyl sulfate sample buffer with 100 mM DTT, heating at 75°C for 10 min. Proteins were separated with 4–12% NuPAGE Bis-Tris gradient gels run with 1X MOPS running buffer and stained with SYPRO Ruby according to the manufacturer’s instructions. Proteins were imaged with a PhorosFX scanner (Bio-Rad).

**qRT-PCR**—For all RNA analyses, JFTAF2del was used as the parent strain. For temperature shift experiments, this strain was co-transformed with p415ADH-HAₓ₃-NLS-TAF2 and p415ADH-HAₓ₃-NLS-taf2-ts7 and either p413GPΔ or p413GPΔ-FLAGₓ₃-NLS-TAF14. Uniq “His” colonies were sub-
jected to plasmid shuffle on SC−His,−Leu+5-FOA. For each co-transformed shuffled strain, two independent colonies were grown in SC−His,−Leu at 25°C until they reached a cell density of ~1.2 × 10^7 cells/ml, then abruptly shifted to 37°C by adding an equal volume of 50°C heated SC−His,−Leu, and then grown at 37°C for 2 h. Cells were harvested by centrifugation, and pellets were stored at −80°C. For steady-state RNA experiments, JFTAF2del was transformed with either p415ADH-HA_x-NLS-TAF2 or p415ADH-HA_x-NLS-taf2-ΔC. Leu+ colonies were subjected to plasmid shuffle by growth on SC−Leu+5-FOA. For each shuffled strain, four independent colonies were grown in YPD until the cells reached ~2.4 × 10^7 cells/ml and harvested by filtration.

For all samples, RNA was extracted using hot acidic phenol as described (90). Reverse transcription was performed with 1 μg of total RNA using SuperScript III according to the manufacturer’s instructions. cDNA was generated using oligo(dT)16 and 1 pmol each of gene-specific reverse primers for U3, RDN58, and SNR6. The primer sequences used in these analyses are as follows: U3-F, CAAAGAGCCACCTGATCCACCTTGGG; U3-R, GTACCCACCATAGAGCCTATACCC; RDN58-F, AAGGGTACTCTGGTTCTCG; RDN58-R, GTTCGTCTCAAGATTGGATG; SNR6-F, CGAGATACCCCTTCGTGAC; SNR6-R, TCACTCCTATGCAAGGGGAAC (54); RPS3-F, TACGGTGTGTCATAGACGG; RPS3-R, GACACAGTGTGCAAGAAACC; RPS5-F, GGAAGAGTGCCTTGCAAG; RPS5-R, TCTTGGAGATACCTTCCTT; RPS8A-F, AAAGATCCGCTACGAGTAACCCTTCGTGGAC; SNR6-R, TCATCCTTATGCAAGGGGAAC (54); RPS3-F, TACGGTGTGTCATAGACGG; RPS3-R, GACACAGTGTGCAAGAAACC; RPS5-F, GGAAGAGTGCCTTGCAAG; RPS5-R, TCTTGGAGATACCTTCCTT; RPS8A-F, AAAGATCCGCTACGAGTAACCCTTCGTGGAC; SNR6-R, TCATCCTTATGCAAGGGGAAC (54); RPS3-F, TACGGTGTGTCATAGACGG; RPS3-R, GACACAGTGTGCAAGAAACC; RPS5-F, GGAAGAGTGCCTTGCAAG; RPS5-R, TCTTGGAGATACCTTCCTT; RPS8A-F, AAAGATCCGCTACGAGTAACCCTTCGTGGAC; SNR6-R, TCATCCTTATGCAAGGGGAAC (54).

Quantitative PCR was performed using SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions. Samples were quantified using the relative standard curve method, normalized to U3, and expressed as a percentage of the average of the HA−TAF2 strain. The standard curve was generated by mixing equal amounts of RNA from each of the samples tested prior to reverse transcription. This RNA mixture was used both as the standard curve and as the no-RT control. Three values were used for each standard curve based on the amplification traces displayed apparent aberrations in amplification efficiency.

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