IV. Genetic Analysis of TAF12 Isoforms in *C. albicans*

We carried out extensive genetic analyses in *C. albicans* to gain further insights into the functional roles of the two TAF12 proteins. Because the TAF12 gene is essential in *S. cerevisiae* (Moqtaderi et al., 1996), we thought it likely that at least one of the two TAF12-like genes might also be essential for viability of *C. albicans*. Therefore, at the outset, we placed the two genes under the regulation of a conditional promoter to study the effect of promoter shut-off on growth of *C. albicans* strains.

IV.1. Construction of $P_{MAL2}$-regulatable TAF12a and TAF12b strains

We inserted a maltose-regulatable promoter upstream of the TAF12a and TAF12b ORFs using the pHAH1-$P_{MAL2}$ construct (gift from Dr. K. Ganesan, IMTECH). The construct contains an HAH marker, which has an ARG4 gene flanked by non-functional his$IΔ5'$ and his$IΔ3'$ gene fragments followed by the $P_{MAL2}$ promoter (Fig. IV.1.A). The $P_{MAL2}$ promoter is induced by maltose and repressed by glucose, thus providing a straightforward means to check the effect of depletion of a gene by growing the strains in the presence of either of the two sugars as the carbon source.

The pHAH1-$P_{MAL2}$ plasmid was used as a template to generate gene-specific promoter replacement construct employing a split-marker strategy. It involved the PCR amplification of the cassette in two fragments, the up-split and down-split with ~1.0 kb overlapping region within the ARG4 gene. Upon introduction into *C. albicans* cells, the up-split and the down-split fragments would recombine to recreate a functional ARG4 gene conferring an Arg$^+$ phenotype. The two HIS1 gene segments flanking the ARG4 gene also contain 354bp direct repeat regions that can recombine to generate a functional HIS1 gene, excising the ARG4 gene in the process. However, spontaneous gene conversion or mitotic recombination events can cause the HAH cassette from one allele to be integrated into the second. Subsequently, the recombination of the his$IΔ5'$ and his$IΔ3'$ fragments at one of the two alleles would
give rise to Arg\(^+\) His\(^+\) strains (Fig. IV.1.B). This approach allows the genetic modification of both the alleles of the gene of interest by a single transformation event as originally described (Enloe et al., 2000).

We used long primers with homology to upstream and downstream regions of the transcription start site of \textit{TAF12a} and \textit{TAF12b} in combination with cassette-specific primers for amplification using Phusion DNA polymerase. The up-split forward primers contained an 18 bp homology to the vector sequence and were designed to include a 60 bp sequence upstream of the ATG codon into the 5' end of the cassette. We used the primer pairs ONC117-ONC115 for the \textit{TAF12a} and ONC119-ONC115 for \textit{TAF12b} up-split fragments. The down-split reverse primers annealed to the last 28bp of the \textit{P}_{\text{MAL2}}\textit{ promoter and were designed to include a 60 bp sequence immediately downstream of the ATG into the 3'end of the down-split amplicon. This ensured the integration of the promoter replacement construct directly upstream of the transcription start site and the fusion of the \textit{P}_{\text{MAL2}}\textit{ promoter (containing the ATG codon) with the target ORF. We first amplified the down-flank fragments of \textit{TAF12a} and \textit{TAF12b} using the primers ONC118-ONC126 and ONC120-ONC127 respectively. These amplicons, encompassing +3 to +160 bp of \textit{TAF12a} ORF and +3 to +150 bp of \textit{TAF12b} ORF, also contained an extra 28 bp sequence complementary to the \textit{P}_{\text{MAL2}}\textit{ promoter at their 5' end. We then carried out a mutually primed PCR of the down-flank amplicons with the linearized HAH1-\textit{P}_{\text{MAL2}}\textit{ to generate the \textit{TAF12a} and \textit{TAF12b} down-split marker fragments using ONC114-ONC126 and ONC114-ONC127 respectively.
Fig. IV.1. Schematic of the HAH1-PMAL2 promoter replacement strategy.

(A) The HAH-PMAL2 cassette consists of the ARG4 gene (1960 bp) flanked by non-functional HIS1 truncated fragments his1Δ5' (871bp) and his1Δ3' (854bp), which share a 355bp homology region (hisR). This His-Arg-His (HAH) cassette is followed by the MAL2 promoter (537bp). Recombination between the two hisR direct repeat regions excises the ARG4 gene to generate a functional HIS1 gene.

(B) The HAH1-PMAL2 system works by replacing the native promoter of the target gene with that of the MAL2 promoter by double replacement and selection using a single transformation event. First, the promoter of one allele is replaced and transformants selected on the basis of an Arg+ phenotype. These transformants yield spontaneous segregants in which the HAH1-PMAL2 insertion allele becomes homozygous and undergoes recombinational excision of the ARG4 gene at one of the alleles. This yields an Arg+ His+ phenotype with both the alleles of the gene of interest under the regulatable promoter.
Fig. IV.2. Strategy for generating the up- and down-split markers. The up-split marker was amplified as a single fragment using gene-specific forward primer and ONC115. The down-split marker was amplified in two steps, first the down-flank was amplified using gene-specific forward and reverse primers. Then, the gene-specific down-flank amplicons was used along with linearized pHAH-PMAL2, ONC114 and the gene-specific reverse primer in a mutually primed PCR reaction to generate the down-split marker.

Finally, the up-split and down-split amplicons were purified by phenol extraction and ethanol precipitation and quantitated on an agarose gel by comparing with known amounts of the 1Kb ladder (MBI Fermentas). The corresponding up- and down-split fragments for each gene were mixed in equimolar amounts, equivalent to ~1 µg total DNA, and transformed into the Arg⁺ His⁺ strain SN95 (Noble and Johnson, 2005) in two separate reactions as described in Section II.7. Transformants were selected for Arg⁺ phenotype on minimal medium (SD medium) lacking arginine and screened for the correct integration of the cassette by PCR. Genomic DNA was isolated from sixteen independent TAF12a and TAF12b transformants each as described in Section II.8. and ~10ng used as templates in two separate PCR screens (see Section II.9) using Taq DNA polymerase (MBI Fermentas). First, we used a gene-specific forward primer located upstream of the site of insertion (ONC124 for
TAF12a and ONC125 for TAF12b) along with a cassette-specific reverse primer ONC115. Four out of sixteen clones were positive for correct integration at the TAF12a locus showing a band of 2.7 Kb in the PCR (Fig. IV.3.A, lanes 1-2), while six of the TAF12b clones showed the expected PCR amplicons of 2.8 Kb (Fig. IV.3.A, lanes 4-5). The positive clones were further tested by another PCR using gene-specific reverse primers located within the ORF but downstream of the site of integration (ONC94 for TAF12a and ONC98 for TAF12b) along with a cassette-specific forward primer located within P_{MAL2} (ONC116). All the TAF12a clones tested showed the presence of the expected 2.5 Kb band (Fig. IV.3.B, lanes 2-3), in addition to a non-specific 2 Kb band (lanes 1-3, marked with an asterisk) that was also amplified in the control wild-type genomic DNA (Fig. IV.3.B, lane 1). The TAF12b clones also amplified a specific 1.8 Kb band as expected (Fig. IV.3.B, lanes 6-7). We therefore obtained independent strains bearing one allele of TAF12a or TAF12b under the P_{MAL2} promoter, named ISC9 and ISC10 respectively.

We then selected spontaneous segregants that underwent inter-chromosomal gene conversion to replace the promoter of the second allele for Arg+ His+ prototrophy. Two independent Arg+ clones each of ISC9 (P_{MAL2}-TAF12a/TAF12a) and ISC10 (P_{MAL2}-TAF12b/TAF12b) were grown in rich medium to saturation and plated out on SD media lacking both arginine and histidine but containing 2% maltose as carbon source instead of glucose. The Arg+ His+ derivatives from each of the Arg+ parents were screened by diagnostic PCR to confirm the promoter replacement at both the alleles. Genomic DNA was isolated from sixteen Arg+ His+ clones, eight each from two independent Arg+ parents, for both TAF12a and TAF12b transformants and used as template for PCR using Taq DNA polymerase. We used gene-specific upstream and downstream primers (ONC124-ONC126 for TAF12a and ONC125-ONC127 for TAF12b) which would confirm the presence of the HIS1-marked P_{MAL2}
Fig. IV.3. (A) PCR screen to confirm the insertion of the HAH1-\(P_{\text{MAL}2}\) cassette in Arg\(^+\) transformants using gene specific upstream and cassette specific reverse primers. Lanes 1, 2: two positive clones of ISC9 \((P_{\text{MAL}2}-TAF12a/TAF12a)\), Lane 3: 1 Kb ladder, Lanes 4, 5: two positive clones of ISC10 \((P_{\text{MAL}2}-TAF12b/TAF12b)\). (B) PCR screen of Arg\(^+\) transformants using \(P_{\text{MAL}2}\) specific forward and gene specific reverse primers. Lane 1: \(TAF12a\) specific PCR with control genomic DNA, Lanes 2-3: ISC9, Lane 4: 1 Kb ladder, Lane 5: \(TAF12b\) specific PCR with control genomic DNA, Lanes 6, 7: ISC10. (C) PCR screen of Arg\(^+\) His\(^+\) transformant using gene specific upstream and downstream primers. Lanes 1-2: ISC11 \((P_{\text{MAL}2}-TAF12a/P_{\text{MAL}2}-TAF12a)\), Lane 3: 1 Kb ladder, Lane 4: \(TAF12a\) specific PCR with control genomic DNA, Lanes 5-6: two positive clones of ISC12 \((P_{\text{MAL}2}-TAF12b/P_{\text{MAL}2}-TAF12b)\), Lane 7: 1 Kb ladder, Lane 8: \(TAF12b\) specific PCR with control genomic DNA.
cassette. The same primers would also amplify a smaller band corresponding to the native ORF if present. The control PCRs with wild-type genomic DNA did not amplify the marker-specific band but showed the presence of the native ORF band for both TAF12a and TAF12b (Fig IV.3.C. lanes 4 and 8 respectively). We also obtained several clones that were positive for the marker HIS1-specific 2.7 Kb band in TAF12a (Fig IV.3.C. lanes 1-2) and 2.8 Kb in TAF12b strains (Fig IV.3.C. lanes 5-6). We therefore obtained two independent strains ISC11 and ISC12 expressing the TAF12a and TAF12b genes respectively under the P MAL2 promoter.

IV.2. Construction of taf12aΔ and taf12bΔ null mutants

To construct TAF12a and TAF12b deletion strains we decided to employ sequential deletion of both alleles using the deletion cassette from pSFS2a (Reuss et al., 2004). The pSFS2a construct consists of a SATJ gene that acts as a dominant selection marker by conferring resistance to the drug nourseothricin (Fig. IV.4.A.). In addition, it also contains a C. albicans-adapted PLP gene, encoding a site-specific recombinase under the expression of maltose inducible promoter. The cassette is flanked by 34 base pair direct repeats of the PLP target sequence that allows for the excision of the entire cassette, thus making it possible to use the same cassette for the next round of transformation (Fig. IV.4.A.). We used long primers with homology to the region upstream of the ATG and downstream of the stop codon to PCR amplify the deletion construct for targeted deletion of the gene of interest (Fig. IV.4.B.). We amplified the cassette in two fragments, the up-split and down-split with a ~1.4 kb overlapping region within the cassette using Phusion DNA polymerase. Both the fragments would recombine in vivo after transformation to recreate a functional SATJ gene, deleting one allele of the gene of interest and giving a nourseothricin-resistant (Nou8) phenotype. Following excision of the SATJ flipper cassette to obtain Nou8 strains, the
same cassette would be used to re-transform the heterozygous mutants to delete the second allele of the gene of interest (Fig. IV.4.B.)

Towards this end, we used ONC139-ONC140 and ONC141-ONC142 to amplify the \textit{TAF12a} specific up-split and down-split fragments. Similarly, ONC161-ONC140 and ONC141-ONC162 were used for the \textit{TAF12b} up- and down-split marker segments. The amplicons were purified by phenol extraction and ethanol precipitation and quantitated on an agarose gel as discussed above. The corresponding up- and down-split fragments for each gene were mixed in equimolar amounts equivalent to \(0.5 \mu g\) total DNA and transformed into the \textit{Arg}^+ \textit{His}^+\ strain SN95 (Noble and Johnson, 2005) in two separate reactions. The transformants were selected for \textit{Nou}^R by plating on YPD plates containing \(200 \mu g/ml\) of the drug and screened for the correct integration in two separate PCR reactions. First, we used gene-specific upstream primers ONC124 (\textit{TAF12a}) or ONC125 (\textit{TAF12b}) with cassette-specific reverse primer ONC141. Of sixteen clones screened we obtained four positive clones that amplified a 2.8 Kb band specific to the \textit{TAF12a} locus (Fig IV.5.A, lanes 1-2). Four out of sixteen \textit{TAF12b} transformants also showed the locus-specific 2.9 Kb band (Fig IV.5.A, lanes 5-6). Next we checked the positive transformants using gene-specific reverse primers located downstream of the \textit{TAF12a} or \textit{TAF12b} ORFs (ONC96 or ONC100) along with a cassette-specific forward primer ONC140. We found that all the transformants produced the expected 2.9 Kb amplicons in both the TAF12a (Fig.IV.5.B, lanes 1-2) and TAF12b (Fig.IV.5.B, lanes 4-5) transformants. We thus obtained \textit{Nou}^R heterozygous deletion strains ISC13 (\textit{taf12a}Δ::\textit{SAT1 FLP/TAF12a}) and ISC37 (\textit{taf12b}Δ::\textit{SAT1 FLP/TAF12b}).

Next we used two independent transformants of ISC13 and of ISC37 to obtain \textit{Nou}^S segregants. Since the \textit{MAL2} promoter driving \textit{FLP} gene in the flipper cassette is reported to be leaky (Backen et al., 2000), we passaged cells from the
Fig. IV.4. (A) The SAT1 flipper cassette. (B) Schematic showing the strategy for deletion of TAF12a and TAF12b ORFs using the SAT1 flipper cassette.
abovementioned strains in YPD medium to allow for FLP-mediated excision of the SAT1 flipper cassette. After 6-8 hrs, cells were diluted and ~100 cells plated out on YPD containing 25μg/ml nourseothricin. At this concentration, the NeuS cells form small colonies that can be distinguished from the fast growing, and large NeuR colonies. The resulting strains were verified for the excision of the cassette by PCR using gene-specific upstream primers ONC124 (TAF12a) or ONC125 (TAF12b) with cassette-specific reverse primer ONC141. The NeuS transformants screened were unable to amplify the cassette-specific band while the a NeuR clones used as positive control showed a 2.8 Kb band in the TAF12a specific PCR (Fig.IV.5.C., compare lanes 1-2 with lane 4) and a 2.9 Kb band in the TAF12b specific PCR (Fig.IV.5.C compare lanes 5-6 with lane 8). The strains thus obtained were NeuS and had one of the two alleles of TAF12a or TAF12b replaced by a single FRT site and were named ISC35 (tafl2a∆::FRT/TAF12a) and ISC38 (tafl2b∆::FRT/TAF12b) respectively.

To delete the second TAF12a and TAF12b allele in ISC35 and ISC38 respectively, we again transformed the same deletion cassettes into two independent derivatives of ISC35 and ISC38 and selected the transformants on YPD plates containing 200μg/ml nourseothricin. Most ISC35 transformant colonies were smooth and fast growing but a few had rough morphology and were slow growing. We analyzed representative colonies of the two categories for the deletion of the TAF12a ORF by PCR using the gene-specific upstream primer ONC124 and cassette-specific reverse primer ONC141. We observed the amplification of the specific 2.8 Kb band in both the rough (Fig. IV.5.D, lanes 3-4) as well as the smooth colonies (lanes 5-6) but not in the control NeuS parent (lane 2). To confirm the knockout of both the alleles of TAF12a, we carried out a test PCR using an ORF specific forward primer ONC93 with a downstream reverse primer ONC96. We observed that all the smooth colonies showed the presence of an ORF specific band of 670bp (data not shown).

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indicating that the deletion cassette may have integrated into the same locus again while the second allele remained undisturbed. The rough colonies however did not amplify the ORF specific band indicating that both the alleles of TAF12a in these clones were knocked out (data not shown). Thus we obtained viable taf12aΔ strain ISC36 that exhibited a rough colony morphology and slow growth defect.

In the TAF12b transformation also, we obtained a large number of smooth, fast-growing colonies and a few very tiny rough colonies. The rough colonies upon streakout, however, did not appear to be stable and reverted to smooth, fast-growing colonies. Nevertheless, we tested representative colonies of both the kinds by PCR. First, we tested the correct integration of the cassette using gene-specific upstream primer ONC125 with ONC140 and gene-specific downstream primer ONC100 with ONC141. Only three out of twenty-four rough colonies were positive for PCR with ONC125-ONC140. However, all the three were negative for PCR with ONC100-ONC141 primer pair (Fig.IV.5.E, compare lanes 3-4 versus 9-10) indicating that the cassette was not properly integrated into the locus. On the other hand, five out of the seven smooth colonies tested showed correct integration of the cassette in both the PCRs (Fig.IV.5.E, lanes 5-6 and 11-12). To confirm the complete deletion of the TAF12b ORF, we carried out another PCR test using ORF specific forward primer ONC97 with the downstream reverse primer ONC100. All the transformants, irrespective of their rough or smooth colony phenotype, showed the presence of the TAF12b ORF (Fig.IV.5.F, lanes 3-6). We were therefore unable to obtain viable taf12b null mutants using this strategy.

IV.3. TAF12b but not TAF12a is essential for viability of C. albicans

We tested the growth phenotypes of strains ISC11 (Pmal2-TAF12a) and ISC12 (Pmal2-TAF12b) in maltose and glucose media. The strains were grown to saturation in
Fig. IV.5. (A) PCR screen to confirm heterozygous deletion of TAF12a and TAF12b with the SAT1 flipper cassette in NouR transformants using gene specific upstream and cassette specific reverse primers. Lanes 1-2: two positive clones of ISC13 (taf12a::SAT1/TAF12a), lanes 3-4: 1 Kb ladder, lanes 5-6: two positive clones of ISC37 (taf12b::SAT1/TAF12b). (B) PCR screen of NouR heterozygous deletion transformants using cassette specific forward and gene specific reverse primers. Lanes 1-2: ISC13, lanes 4-5: ISC37, Lanes 3,6: 1 Kb ladder (C) PCR screen of NouS heterozygous mutants using gene specific upstream and cassette specific reverse primers. Lanes 1-2: ISC35 (taf12a::FRT/TAF12a), lane 4: TAF12a positive control PCR with NouR strain ISC13, lane 3, 7: 1 Kb ladder, lanes 5-6: two positive clones of ISC38 (taf12b::FRT/TAF12b), Lane 8: TAF12b positive control PCR with NouR strain ISC37. (D) PCR screen of second round NouR transformants of ISC35 using gene specific upstream and cassette specific reverse primers. Lane 2: TAF12a control PCR with NouS strain, lanes 3-4: two representative rough colonies, lanes 5-6: two representatives of smooth colonies, lanes 1, 7: 1 Kb ladder (E) PCR screen of second round NouR transformants of ISC37 using gene specific upstream with cassette specific reverse primers and gene specific reverse with cassette specific upstream primers. (F) PCR screen to test for the presence of ORF using gene specific upstream and downstream primers. Lane 2: TAF12a control PCR with WT strain, lanes 3-4: two representative rough colonies, lanes 5-6: two representatives of smooth colonies, lane 1: 1 Kb ladder
permissive medium (YPD), serial dilutions spotted onto YPD and YPM plates and incubated at three different temperatures 25°, 30° and 37°C. The \(PMAL2-TAF12b\) strain showed a very severe growth defect on YPD media as compared to the same strain grown in YPM or the wild-type strain (Fig. IV.6.A). The \(PMAL2-TAF12a\) strain, on the other hand, showed only a mild growth defect as compared to the wild type strain. These results suggested that \(TAF12b\), and not \(TAF12a\), plays an essential role in \(C. albicans\).

Next, we examined the protein levels by western blot analysis using rabbit TAF12a and TAF12b polyclonal antibodies (see Section II.11 and II.12.). Strains SN95, ISC11 and ISC12 were pre-grown for 16-18h in YPM and each culture was diluted to a starting OD\(_{600}\) ~0.15 into fresh YPM as well as YPD media. The cultures were grown to mid log phase and harvested after 6 hours. Protein extracts were prepared as described in Section II.14 and 100μg each were resolved in SDS-PAGE, blotted to Hybond-ECL membrane and detected by western blot using α-TAF12a and α-TAF12b antibodies as described in Section II.14. There was no difference in the expression levels of the TAF12a and TAF12b proteins in the wild-type strain grown in maltose versus glucose media (Fig IV.6.B, compare lanes 1-2 and 5-6). However, we observed that the expression of both proteins was undetectable within 6h of growth in glucose containing media, while significant levels were maintained in the same strains grown in maltose (Fig IV.6.B, compare lanes 3-4 and 7-8). Thus the western blot data confirmed that the depletion of TAF12b (as well as TAF12a) protein levels indicating that impaired growth of ISC12 was due to TAF12b depletion.

We then used another strategy to determine the essentiality of \(TAF12b\) using the \(HIS1-ARG4-HIS1\) deletion cassette pHAH1 (a kind gift from Dr. Ganesan, IMTECH). This plasmid has the same marker cassette as the pHAH-\(PMAL2\) described in Section IV.1. We used long primers with homology to the regions upstream of
ATG and downstream of the stop codon of the \textit{TAF12b} gene to PCR amplify the deletion cassette in two fragments. Linearized pH AH1 was used as a template in PCR reactions to amplify the up-split and the down-split fragments using ONC119-ONC115 and ONC114-ONC324 with Phusion DNA polymerase. The amplicons were purified, quantitated and mixed in equimolar amounts and ∼0.5μg used to transform the \textit{C. albicans} SN95 strain auxotrophic for histidine and arginine. The Arg\textsuperscript{+} colonies with one of the \textit{TAF12b} alleles deleted were selected on SD-Arg plate and confirmed for the integration of the cassette by PCR. First, we used ONC125, the gene-specific forward primer located upstream to the site of insertion along with a cassette-specific reverse primer ONC115. The positive clones were further tested by another PCR using gene-specific reverse primer ONC100 downstream of the ORF along with a cassette-specific forward primer ONC114. Eleven of the sixteen clones tested showed the presence of the expected 2.8 Kb band in the up-check PCR and ∼2.6 kb band in the down-check PCR (Fig. IV.6.C). We therefore obtained strain ISC45 with one of the alleles of \textit{TAF12b} replaced by the HAH cassette.

Four independent clones of ISC45 were plated out at a high density on SC-His-Arg media to select spontaneous His\textsuperscript{+} Arg\textsuperscript{+} segregants (as described in Section IV.1) to delete the second \textit{TAF12b} allele. However, we were unable to obtain any His\textsuperscript{+} Arg\textsuperscript{+} colonies, despite screening several independent Arg\textsuperscript{+} clones. This suggested that deletion of both alleles of \textit{TAF12b} was lethal. The failure to obtain \textit{TAF12b} null mutants using two independent strategies, and the severe growth defect upon promoter shutoff indicated that \textit{TAF12b} is indispensable for \textit{C. albicans} growth and survival.

\textbf{IV.4. \textit{TAF12b} depletion leads to altered cellular morphology}

Since the \textit{TAF12b} shutoff strains were unable to grow on solid media, we decided to examine their cellular morphology in liquid cultures. The strains SN95 (WT) and
Fig. IV.6. TAF12b but not TAF12a is essential for \textit{C. albicans} growth. (A) Effect of TAF12a and TAF12b shutoff on growth. Strains SN95 (WT), ISC11 (\textit{P}_{\text{MAL2}}-\text{TAF12a}) and ISC12 (\textit{P}_{\text{MAL2}}-\text{TAF12b}) were grown to saturation in YPM, serial dilutions spotted onto YPM and YPD plates and incubated at 25°C, 30°C and 37°C. (B) Western blot of WT, ISC11 and ISC12 strains grown in YPM and YPD for 6h to check for depletion of the TAF12a and TAF12b proteins. Lanes 1, 5: WT grown in YPM, lanes 2, 6: WT grown in YPD, lane 3: ISC11 grown in YPM, lane 4: ISC11 grown in YPD, lane 7: ISC12 grown in YPM, lane 8: ISC12 grown in YPD. Lanes 1-4 were probed with \textalpha-TAF12a rabbit polyclonal antibody and lanes 5-8 were probed with \textalpha-TAF12b rabbit polyclonal antibody. (C) PCR screen of heterozygous deletion strain for TAF12b (ISC45) using HAH disruption. Lanes 1-2: PCR using up check and cassette specific reverse primer, lanes 4-5: PCR using down check and cassette specific forward primer. Lanes 3, 6: 1 Kb ladder.
ISC12 were pre-grown for 16-18h in YPM and each culture was diluted to a starting OD₆₀₀ of ~0.1 into fresh YPM as well as YPD media. The cultures were allowed to grow for 6 hours at 30°C to ensure depletion of the TAF12b protein. Cells were fixed and stained with DAPI and Calcofluor as described in Section II.10 and imaged under bright field (DIC) or fluorescence modes in a confocal microscope. We observed that the wild-type cells grown in YPD showed cells at various stages of budding, typical of exponentially growing population of yeast cells (Fig IV.7.A.). The microscopy results also showed no difference in the cellular morphology between wild-type SN95 cells grown in glucose versus maltose (data not shown). The ISC12 (P_{MAL2}-TAF12b) cells grown in the presence of maltose also showed the normal budding yeast pattern identical to the wild-type cells indicating that the expression of TAF12b from the P_{MAL2} promoter did not affect the cellular morphology (data not shown).

However, we observed that the P_{MAL2}-TAF12b cells cultured in glucose formed heterogeneous and altered cell morphologies (Fig IV.7.A.). To obtain quantitative estimates of the different cell types we counted cells and classified them based on their characteristic cell sizes and shapes. Of the 400 cells counted, only 59% showed normal budding yeast-like morphology (Fig IV.7.B, Type 1). Of the remaining 41% cells, we were able to discern at least two distinct sub-populations of cells based on their altered morphology. The most abundant (~26%) were pseudohyphal (Berman, 2006) cells that exhibited either unipolar budding with axial branching or bipolar budding without branching. However, these branched chains were usually small, with 3 to 5 cells in each unit (Fig IV.7.B, Types 2 and 3). Nuclear staining with DAPI indicated that there was no significant defect in nuclear division as each cell in a cluster appeared to have a single nucleus (Fig IV.7.A). The cell wall staining with Calcofluor showed that the mother and daughter cells were joined together at the septa to form the chain-like units characteristic of pseudohyphal cells (Berman, 2006).
There was also a small population of cells with longer chain lengths, 5 to 10 enlarged cells per unit (Fig IV.7.B, Type 4).

The other prominent population of cells (~10%) appeared to have enlarged, rounded mother cells with small tubular projections similar to germ tubes (Fig IV.7.B, Type 5). DAPI staining indicated that many of these structures possessed a single nucleus within the germ tube-like daughter cell while the mother cell had no nucleus (Fig IV.7.A.). Calcofluor staining indicated that the septa between the mother cell and the germ tube were not completely formed (Fig IV.7.A.). The germ tubes-like cells, however, did not grow further even at later time points (data not shown) and there are reports that this is characteristic of cells that have undergone a cell cycle arrest (Berman, 2006; Cote et al., 2009; Sellam et al., 2010). Interestingly, several TAFs including TAF1, TAF5 and TAF12 have been shown to be important for normal cell cycle progression in S. cerevisiae (Apone et al., 1996; Reese and Green, 2001; Walker et al., 1997). Therefore it seems likely that TAF12b might be essential for normal cell cycle progression in C. albicans.

IV.5. Loss of TAF12a leads to pseudohyphal cellular morphology

We also checked the cellular morphology of C. albicans strains SN95 (WT), ISC11 (Pmal2-TAF12a) and ISC36 (taf12aΔ) cultured in the same way as described in Section IV.5. The stains were pre-grown in YPD medium for 16-18 hours, inoculated into fresh YPD medium, grown for 6 hours and cells were fixed in formaldehyde. The ISC36 cells were subjected to mild sonication to disperse the aggregates. All samples were stained with DAPI and Calcofluor (see Section II.10) and imaged in bright field (DIC) as well as fluorescence mode in a confocal microscope.

The TAF12a depleted cells also showed pseudohyphal morphology with long, branched chain of varying lengths (Fig IV.8.). The pattern of budding appeared to be
Fig. IV.7. Cellular morphology of TAF12b-depleted cells. (A) WT or ISCl2 were cultured overnight in YPM and diluted to fresh YPD and cultured for 6h at 30°C. Cells were harvested, fixed with formaldehyde and stained with DAPI plus calcofluor white for imaging with confocal microscope Olympus FluoView FV1000 using 100x objective (oil immersion). The boxes in the DIC panel (left) show the area zoomed-in (2x digital) and are displayed in the Center and Right panels. The different categories of cellular morphologies identified are marked in the DAPI+CFW panel. (B) Summary of the cell counts from the different categories of $P_{MAL2}$ TAF12b cellular morphologies. A total of 411 cells were counted in different fields and categorized into each of the five types as represented.
A.

WT

No. of cells counted (Total = 411)

Percentage

DIC

DAPI + CFW

P_{MALZ}^- TAF12b

Cellular Morphology

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B.
Fig. IV.8. Cellular morphology of $P_{MAL2}^{+}TAF12a$ cells. WT or ISC11 ($P_{MAL2}^{+}TAF12a$) strains were cultured overnight in YPM, diluted to fresh YPD and cultured for 6h at 30°C. Cells were harvested, fixed with formaldehyde and stained with DAPI plus calcofluor white. Imaging was done with Olympus Fluoview FV1000 confocal microscope using 100x objective (oil immersion). The boxes in the DIC panel (Left) show the area zoomed-in (2x digital) and are displayed in the Center and Right panels. The different categories of cellular morphologies identified are marked in the DAPI plus CFW panel.
**Fig. IV.9. Cellular morphology of taf12aΔ cells.** WT or ISC36 (taf12aΔ) strains were cultured overnight in YPM, diluted to fresh YPD and cultured for 6h at 30°C. Cells were harvested, fixed with formaldehyde, sonicated to disperse the clumps and stained with DAPI plus calcofluor white. Imaging was done with Olympus Fluoview FV1000 confocal microscope using 100x objective (oil immersion). The boxes in the DIC panel (Left) show the area zoomed-in (2x digital) and are displayed in the Center and Right panels.
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<tr>
<td>Description</td>
<td>Normal</td>
<td>Elongated, 1-2 cells</td>
<td>3-4 cells</td>
<td>5-6 cells</td>
<td>6-10 cells</td>
<td>&gt;10 cells</td>
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**Cellular Morphology**

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<td>$P_{MAL2}^{-}$TAF12a</td>
<td>138</td>
<td>72</td>
<td>69</td>
<td>42</td>
<td>44</td>
<td>44</td>
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<tr>
<td>Percentage</td>
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<td>17.6</td>
<td>16.9</td>
<td>10.3</td>
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<tr>
<td>taf12aΔ/taf12aΔ</td>
<td>0</td>
<td>84</td>
<td>13</td>
<td>13</td>
<td>34</td>
<td>69</td>
</tr>
<tr>
<td>Percentage</td>
<td>0</td>
<td>39.4</td>
<td>6.1</td>
<td>6.1</td>
<td>16.0</td>
<td>32.4</td>
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Fig. IV.10. Summary of the cell counts from the different categories of $P_{MAL2}^{-}$TAF12a and taf12aΔ morphologies. A total of 409 cells were counted for $P_{MAL2}^{-}$TAF12a and 213 for taf12aΔ in different fields and categorised into each of the six types as represented.
unipolar with axial branching. DAPI staining indicated that there was no defect in nuclear division or distribution, as almost every cell in the chain appeared to contain a nucleus. We examined ~400 pseudohyphal units in the DIC and DAPI/calcoflour images, counted and grouped the population based on the number of cells in each pseudohypha. The data showed that normal budding pattern constituted ~34% of the total cells (Fig IV.10, Type 1), whereas the rest of the population was pseudohyphal. We classified the pseudohyphal cells into five categories based on the number of cells in each pseudohyphal unit. Nearly 34% contained chains of 2 to 4 elongated cells (Fig. IV.10, Types 2 and 3) and the rest 32% contained 5 or more cells (Types 4 and 5). Strikingly, nearly 10% of the total cells counted formed profusely branched clusters comprised of more than 10 cells each (Type 5). Calcofluor staining showed that cells within each cluster were physically attached through the septa (Fig.IV.8). This branched cluster morphology has been previously reported to be characteristic of a cell separation defect (Esteban et al., 2005; Firon et al., 2007; Kelly et al., 2004; Sellam et al., 2010).

The taf12aΔ strain also exhibited a similar cellular morphology with unipolar axially budding chains of unseparated cells. However, the chains were much longer and branched more profusely in the null mutant, forming clumps and aggregates in liquid culture (Fig. IV.9). We counted ~200 units and found that the constitutively pseudohyphal morphology comprised of ~32% (Fig. IV.10, Type 5) with more than 10 unseparated cells and another 28% with 3 to 10 cells per unit (Types 2, 3 and 4). DAPI staining showed a normal distribution of nuclei in the deletion mutant as well, indicating that TAF12a played a role in cell separation but not cell division.

**IV.6. TAF12a loss results in altered colony morphology**

Since strains lacking TAF12a exhibited pseudohyphal cellular morphology, we tested
their colony morphology on pseudohyphae-inducing media. For this, strains SN95 and ISC11 were grown in YPM for 16-18 hrs, while ISC36 was grown in YPD and subjected to mild sonication to break up the aggregates. Dilutions of each of the cultures were counted on a hemocytometer and 20-25 cells were plated onto YPD, Yeast Carbon Base (YCB) and Synthetic-Low-Ammonium-Dextrose (SLAD) media. In addition, the SN95 and ISC11 strains were also plated on YPM and Synthetic-Low-Ammonium-Maltose (SLAM) media as controls. The plated cells were allowed to grow and form colonies at two different temperatures, 30°C and 37°C and imaged using a stereomicroscope. The three strains were able to grow on all the media but exhibited different phenotypes. The wild-type (WT) strain produced round and smooth edged colonies on YPD at 30°C but when incubated at 37°C, they formed wrinkled colonies (Fig IV.11.A and B). The TAF12a depleted strain ISC11, however, formed marginally smaller, but smooth round colonies on YPD both at 30°C and 37°C but the colonies did not have a crenulated appearance at 37°C (Fig IV.11.A and B). On YCB and SLAD media, the colonies formed by the wild-type strain SN95 produced fuzzy colonies at both 30°C and 37°C with filamentous structures at their boundaries (Fig IV.11, panels C-J). Closer observation of the colonies under transmitted light showed that the filamentous structures invaded the agar substratum (Fig IV.11, panels G and J). Colonies formed by ISC11 however, were greatly diminished in filamentation as well as agar invasion on both YCB and SLAD media (Fig IV.11.), but exhibited the wild-type pattern of colony morphology on SLAM media (data not shown). The taf12aΔ strain ISC36 on the other hand, formed constitutively wrinkled colonies with rough edges on all the media tested and did not show any agar invasion or filamentous projections (Fig. IV.11.).

We also tested the colony morphology of the above strains under true hyphae
Fig. IV.11. Colony morphology of SN95 (WT), ISC11 (P_{MALZ}TAF12a) and ISC36 (taf12aΔ) on pseudohyphae-inducing media. The above strains were grown on the indicated plates and isolated colonies were imaged using a Nikon stereozoom microscope SMZ1500 with a Nikon digital camera DXM1200C at the indicated days. (A) YPD, 37°C (1x, day3); (B) YPD, 30°C (1x, day3); (C) YCB, 37°C (1x, day5); (D) YCB, 37°C (3x, day5); (E) YCB, 30°C (1x, day5); (F) YCB, 30°C (3x, day5); (G) YCB, 30°C, transmitted light (day7); (H) SLAD, 37°C (2x, day5); (I) SLAD, 30°C (2x, day5); (J) SLAD, 30°C, transmitted light (day7). The WT colony boundaries in YCB (panel G) and SLAD media (panel J) are marked with white arrows. 1x and 3x refer to the objective magnifications.
Fig. IV.12. Colony morphology of SN95 (WT), ISC11 (P\textsubscript{MAL2}\textsuperscript{-}TAF12a) and ISC36 (taf12a\textDelta) on true hyphae-inducing media. The above strains were grown on the indicated plates and isolated colonies were imaged using a Nikon stereozoom microscope SMZ1500 with a Nikon digital camera DXM1200C at the indicated days. (A) YPD, 30°C (day3); (B) GlcNAc, 37°C (day3); (C) Serum, 37°C (day6). All images were taken at 1x objective magnification.
inducing conditions by plating them on YPD media containing 10% Serum or 0.25mM N-acetyl glucosamine (GlcNAc) at 37°C. The wild-type strain formed fuzzy colonies with filamentous projections in the presence of GlcNAc and highly wrinkled colonies in serum-containing plate as expected (Fig. IV.12, panels B and C). The ISC11 strain also formed fuzzy colonies on the GlcNAc plate and the filamentous projections, though quite dense, were shorter than the wild type and did not spread into the agar to the same extent. This strain also exhibited the characteristic wrinkled pattern on the serum plate, although it was reduced as compared to the wild-type (Fig. IV.12) The \( \text{taf}12a\Delta \) strain ISC36 produced extensively wrinkled colonies on both GlcNAc as well as serum plate, characteristic of cells capable of true hyphae formation IV.12). These results indicate that the TAF12a depleted cells are capable of producing true hyphae under the appropriate inducing conditions. Moreover, though the \( \text{taf}12a\Delta \) strain constitutively produces pseudohyphal cells, it is defective in producing filamentous projections for agar invasion on solid media under both true hyphae-inducing as well as pseudohyphae-inducing conditions.

**IV.7. TAF12a is required for growth under oxidative stress**

In order to gain further insights into the function of TAF12a, we also tested the TAF12a mutant strains for growth under various stress conditions. Strains SN95 and ISC11 were grown to saturation in YPM and ISC36 in YPD and serial dilutions of each strain spotted onto YPM and YPD plates containing different stress inducing agents. We used various compounds such as heavy metals (cadmium, nickel), oxidative stress-inducing agents (H\(_2\)O\(_2\), menadione) and compounds that induce DNA metabolism defects such as hydroxy urea (HU), methyl-methane sulfonate (MMS) or transcriptional elongation defects (mycophenolic acid, 6-aza-uracil). The various plates were incubated at 30°C and images acquired. We observed that all strains were
able to grow well on mycophenolic acid as well as 6-aza-uracil containing media (data not shown) indicating that depletion or deletion of TAF12a did not result in transcription elongation defect.

Next, we tested the strains for resistance to heavy metal stress. The TAF12a depleted strain was sensitive to growth on media containing 0.05 mM cadmium, (Fig. IV.12) and the severity of the growth defect increased at higher concentrations (data not shown). This result was further confirmed by the phenotype of the taf12aΔ strain that showed a severe growth defect on 0.05 mM cadmium (Fig. IV.12) and was completely unable to grow on higher concentrations (data not shown). However, growth of both the ISC11 and ISC36 strains was comparable to control YPD plates in media containing 2mM nickel. This indicated that the sensitivity of the mutant strains to cadmium was not due to general heavy metal stress.

Previous reports show that cadmium toxicity can be a result of inhibition of DNA repair that can lead to accumulation of errors during DNA replication (Serero et al., 2008). We therefore tested the two TAF12a mutant strains for sensitivity to DNA damaging agents MMS and HU and found slight growth defect in the taf12aΔ strain but not in TAF12a depleted strain in the presence of these compounds (Fig. IV.12). We also tested the strains for sensitivity H2O2 and menadione because cadmium has also been shown to cause oxidative stress (Brennan and Schiestl, 1996). The taf12aΔ strain showed a severe growth defect in the presence of the two oxidative stress agents. The PMal2-TAF12a strain also showed a slow growth phenotype under oxidative stress on YPD media but not on YPM, confirming that the growth defect was a result of TAF12a depletion (Fig. IV.12). Because TAF12a elimination caused growth impairment to Cd2+ as well as H2O2 and menadione, but not to HU and MMS,
Fig. IV.13. TAF12a depletion causes growth defect under oxidative stress. WT, ISC11 (P_{MAL2}·TAF12a) and ISC36 strains (taf12aΔ) were grown overnight in YPM, serial dilutions spotted onto YPD plates containing the indicated stress agents and incubated at 30°C. Images were captured at the indicated days using the Alphalnnotech gel documentation system.
we conclude that TAF12a is required for oxidative stress response but not for DNA damage response.

IV.8. Role of TAF12a and TAF12b in virulence

We wished to study if TAF12a and TAF12b have a role in pathogenicity of *C. albicans*. Strains SN95, ISC11 (*P_{MAL2}\text{-TAF12a})*, ISC12 (*P_{MAL2}\text{-TAF12b})* were grown in YPM for four hours (OD600 to ~1.0 from 0.1), harvested and washed in sterile saline water, counted and ~1x10^6 cells were injected into lateral tail vein of female BALB/c mice (*n* = 8). We assumed that in the absence of glucose within the mice, the expression of the TAF12 proteins would be shutoff and the effect of their depletion on virulence could be monitored. The ISC36 (*tafl2a\Delta)* strain was also allowed to grow for an equal duration in rich media. Cells were harvested, washed with sterile saline and subjected to mild sonication to disperse the aggregates into smaller units which were counted. 1x10^6 and 5x10^6 cells were then injected into BALB/c mice (*n* = 8). The animals were monitored for disease progression twice a day, moribund mice were sacrificed humanely and fungal burden from kidneys were examined. Statistical analysis of the survival curves was done using a Kaplan-Meier plot in the MedCalc software (p-value <0.05 was considered significant).

Mice injected with the wild type strain developed infection as expected and mortality was 100% by day 8 with a median survival of 5.5 days (Fig. IV.14.). The ISC12 strain showed a delay in the onset of virulence (p-value 0.0221) with a median survival of 7.25 days but mortality of this group of mice was not compromised. It is conceivable that depletion of TAF12b causes slow growth of the *Candida* cells in vivo as well and therefore, the fungal burden might take more time to build up causing a delay in manifesting the full virulence potential.
The ISC11 strain did not show any significant difference in mortality as compared to the wild type (p-value 0.9020; Fig. IV.14.). On the other hand, taf12a deletion strains completely lost their ability to cause infection in BALB/c mice (Fig. IV.14.) and indeed all mice survived up to day 30 (data not shown). It is not clear why we observed a disparity in the virulence potentials of \( P_{MAL2-TAF12a} \) and the taf12a\( \Delta \) strains even though the phenotypes of TAF12a depletion were similar to that of the deletion strain under laboratory conditions. It is possible that the pseudohyphal phenotype of the null mutant might affect the colonization simply due to poor dissemination in vivo. Thus the role of TAF12a in virulence might be indirect. Alternately, TAF12a might still have an important role to play in virulence but the \( P_{MAL2-TAF12a} \) strain does not mirror the null phenotype because of a possible leaky expression from the MAL2 promoter, sufficient to sustain wild-type levels of virulence.

IV.9. Role of CaTAF12 isoforms in genome-wide transcription

To examine the role of each CaTAF12 isoform in genome-wide transcription, we carried out transcriptome profiling as described previously (Bowtell and Sambrook, 2003; Natarajan et al., 2007) using \textit{C. albicans} whole genome microarray (Brown et al., 2006). The \textit{C. albicans} microarray contained 6,346 ORFs, where each ORF is represented by a specific 70-mer oligonucleotide, and spotted in three replicates per slide. We compared the transcriptional profiles of wild type versus \( P_{MAL2-TAF12a} \) (ISC11) or \( P_{MAL2-TAF12b} \) (ISC12) cells cultured in synthetic complete media containing glucose as the sole carbon source for 6h. The MAL2 promoter would be repressed with consequent depletion of the TAF12 proteins. Total RNA was isolated from the cells, amplified amino allyl RNA was prepared using the MessageAmpII aminoallyl kit (Ambion) and wild type labeled with HyPer5, and \( P_{MAL2-TAF12a} \) or
Fig. IV.14. Survival curve of mice challenged with *C. albicans* strains. Survival data was analysed by Kaplan Meier test using MedCalc software. The median survival and significance of % survival probability with respect to wild-type SN95 strain (p-value) was calculated for the indicated strains.
with Cy3, combined and hybridized to \textit{C. albicans} microarray slides at 42\(^\circ\)C for 16 hrs. The slides were washed, images scanned and spots with equal average signal intensity in the 635nm and 532nm channels were further quantified using GenePix Pro 6.0 software (Molecular Devices). The data was filtered to retain good spots based on the GenePix criteria as described previously (Yatskou et al., 2008). The data was further processed by normalization and log\(_2\) ratio of wild-type was calculated for each spot and replicate spot values were assessed for concordance. A positive log\(_2\) ratio of a gene indicates that it is down-regulated in the mutant as compared to the wild-type.

As expected from the genotypes of SN95 and ISC11/ISC12, the log\(_2\) ratio for \textit{ARG4} and \textit{HIS1} was \(\leq -2.0\) in the WT/\(P\_MAL2\)-\textit{TAF12a} and WT/\(P\_MAL2\)-\textit{TAF12b} comparisons. Of the total 4932 genes that passed the quality metrics, 216 genes showed \(\geq 4\)-fold (log\(_2\) ratio \(\geq 2.0\)) change in a Ca\textit{TAF12a}-dependent manner that included 109 upregulated genes and 107 downregulated genes. In the case of Ca\textit{TAF12b}, we obtained 133 genes (out of 4651 genes) showing \(\geq 4\)-fold change inclusive of 73 genes that were upregulated and 60 genes that were downregulated as compared to wild-type. Thus about 4.4\% and 2.9\% of \textit{C. albicans} genes require Ca\textit{TAF12a} and Ca\textit{TAF12b} respectively for high level transcription. Additional microarray hybridizations would help to confirm the above results.

To examine the overlap in the transcript profiles upon Ca\textit{TAF12a} and Ca\textit{TAF12b} shut-off, we plotted Venn diagrams comparing the same genes in the two data sets, separately for upregulated and downregulated categories (Fig. IV.15.A.). We took all genes whose log\(_2\) expression ratio was \(\geq 1.0\) in the two data sets and identified 170 commonly up-regulated genes comprised of 22\% of the TAF12a-dependent, and 32\% of the TAF12b-dependent genes (Fig. IV.15.A. Top). We also
identified 99 commonly down-regulated genes made up of 13% of the TAF12a dependent genes, and 21% of the TAF12b dependent genes (Fig. IV.15.A. Top). This indicated that a significant fraction of the transcriptome changes upon depletion of either of the TAF12 proteins. Interestingly, the genes with high fold-change (log2 ratio ≥ 2.0 or ≤ -2.0) showed very poor overlap in the two datasets. It is unclear how both the TAF12 proteins might cause 2-4 fold changes in the expression of a common set of genes.

Among the 69 genes exclusively down-regulated in the TAF12b depleted cells are 15 RPS and 20 RPL genes encoding ribosomal protein subunits (Fig. IV.15.B.). It has been reported previously in *S. cerevisiae* that RPS and RPL genes are coordinately regulated by IFH1/IFH2 (Rudra et al., 2005; Schwalder et al., 2004; Wade et al., 2004) and RAP1 (Garbett et al., 2007; Mencia et al., 2002) transcriptional regulators. Moreover, the RPS and RPL genes are also highly dependent on TAFs in the TFIID complex (Huisinga and Pugh, 2004; Kuras et al., 2000; Li et al., 2000). The microarray data also showed that 12 RPS and 16 RPL genes also showed low magnitude (fold-change between 2 and 4) down-regulation in TAF12a-depleted cells. This behavior of both TAF12a- and TAF12b-dependence is unexpected. But it is believed that ribosomal protein genes are subjected to growth stage regulation. Given that the TAF12a and TAF12b cells showed altered growth patterns, part of the regulation could be attributed to the growth differences between wild-type and depleted cells. Additional experiments involving chromatin immunoprecipitation assays would be required to examine TAF12a and TAF12b occupancies to understand this regulation.

For the 105 down-regulated genes that showed exclusive TAF12a dependence, 34 genes were of unknown function. *WH11* (orf19.3548.1) was very
Fig. IV.15. Transcriptional profiling of TAF12a and TAF12b dependent genes. 

(A) Venn diagram showing overlap between WT/P_{MAL2-TAF12a} (Red) and WT/P_{MAL2-TAF12b} (Yellow) for Up-regulated (log$_2$ ratio $\geq$ 1.0 or $\geq$ 2.0, Left) or Down regulated (log$_2$ ratio $\leq$ -1.0 or $\leq$ -2.0, Right) genes. The Orange sector indicates the overlapping genes in the two datasets and the number of genes in each category is indicated. 

(B) Heat map of the log$_2$ ratio values of ribosomal protein genes RPS (left) and RPL (right) from the WT/P_{MAL2-TAF12a} and WT/P_{MAL2-TAF12b} datasets. Color scale indicates the range of log$_2$ ratio values. Gray color in the heat map represents missing data and black denotes log$_2$ ratio values $\approx$-0.1.
A.

Up regulated

TAF12a TAF12b

Genes with \( \log_2 \) ratio \( \geq 1.0 \)

\( 617 \) 170 370

Genes with \( \log_2 \) ratio \( \leq -1.0 \)

\( 691 \) 99 378

Down regulated

TAF12a TAF12b

Genes with \( \log_2 \) ratio \( \geq 2.0 \)

105 4 69

Genes with \( \log_2 \) ratio \( \leq -2.0 \)

98 9 51

B.

\begin{align*}
\text{orf19.5341}_R \text{or19.2994}_1 & \text{or19.3325}_3 \\
\text{or19.1700}_0 & \text{or19.4375}_1 \\
\text{or19.4660}_6 & \text{or19.8381}_9 \\
\text{or19.6312}_2 & \text{or19.6673}_8 \\
\text{or19.7048}_1 & \text{or19.2329}_7 \\
\text{or19.5996}_1 & \text{or19.5466}_4 \\
\text{or19.1470}_5 & \text{or19.6253}_3 \\
\text{or19.7018}_0 & \text{or19.4193}_1 \\
\text{or19.6286}_2 & \text{or19.5627}_1 \\
\text{or19.6265}_0 & \text{or19.4336}_4 \\
\text{or19.2179}_2 & \text{or19.6375}_0 \\
\text{or19.3002}_3 & \text{or19.6265}_1 \\
\text{or19.6663}_2 & \text{or19.4131}_1 \\
\text{orf19.6677}_1 & \text{or19.5964}_2 \\
\text{orf19.8271}_8 & \text{or19.4632}_1 \\
\text{orf19.1607}_7 & \text{or19.4684}_0 \\
\text{orf19.5982}_7 & \text{or19.3446}_4 \\
\text{orf19.4932}_4 & \text{or19.4490}_0 \\
\text{orf19.3942}_1 & \text{or19.3234}_3 \\
\text{orf19.7217}_1 & \text{or19.4940}_0 \\
\text{orf19.6085}_1 & \text{or19.3942}_1 \\
\text{orf19.3003}_0 & \text{or19.2179}_2 \\
\text{orf19.2935}_9 & \text{or19.1636}_0 \\
\text{orf19.1470}_6 & \text{or19.4336}_2 \\
\text{orf19.6265}_0 & \text{or19.4336}_4 \\
\text{orf19.3002}_3 & \text{or19.6265}_1 \\
\text{orf19.6663}_2 & \text{or19.4131}_1 \\
\text{orf19.6677}_1 & \text{or19.5964}_2 \\
\text{orf19.8271}_8 & \text{or19.4632}_1 \\
\text{orf19.1607}_7 & \text{or19.4684}_0 \\
\text{orf19.5982}_7 & \text{or19.3446}_4 \\
\text{orf19.4932}_4 & \text{or19.4490}_0 \\
\end{align*}
strongly down-regulated (log₂ ratio ≥ 4.6) in a TAF12a-dependent manner and it is interesting that *WH11* has been annotated as a yeast-phase specific gene. Thus the down-regulation of *WH11* in TAF12a-depleted cells is consistent with the pseudohyphal morphology exhibited by these cells. The data also showed TAF12a-dependent down-regulation of *CSP37* (orf19.2531), *BUL1* (orf19.5094), *PES1* (orf19.4093) and *DBF2* (orf19.1223) that are annotated as being either yeast-form specific or induced in pseudohyphae, all of which could lead to a pseudohyphal phenotype. The down-regulation of *DBF2* in TAF12a-depleted cells has interesting consequences, because this gene has been annotated as an essential protein kinase involved in septum formation, spindle formation and cytokinesis. As concluded in Section IV.6, the TAF12a depleted cells showed cell separation defect (Fig.IV.8). Additional experiments would be directed to explore the connection between *TAF12a* and *DBF2*. We also identified five genes encoding GPI-anchored proteins which were also down-regulated in TAF12a depleted cells and their significance needs to be understood.

Sixty seven of the 98 genes exclusively upregulated upon TAF12a depletion are unnamed genes, but a few of them are putative transcriptional regulators. Because both TAF12a depletion or *tafl2a* deletion both impaired growth of *C. albicans* cells in H₂O₂ and menadione we looked for altered expression of genes related to oxidative stress response. We noted that *GTT11*/orf19.6947 and *GTT11*/orf19.6998 encoding glutathione-S-transferase and *GST3* (orf19.720; peroxide-induced) showed upregulation in TAF12a depleted cells but not in TAF12b depleted cells. This is an unexpected behavior because TAF12a dependent expression of oxidative stress resistance genes should have been impaired in TAF12a depleted cells. It is conceivable that TAF12a dependent expression of *GTT1*, *GTT11* and *GST3* would be
better understood by analyzing the RNA from cells treated with oxidative stress agents \( \text{H}_2\text{O}_2 \) and menadione.

Among the 51 genes exclusively upregulated in TAF12b-depleted cells are TBP and seven genes encoding GPI-anchored cell wall proteins. Recently 115 putative GPI-anchored proteins have been catalogued from \( \text{C. albicans} \) genome sequence, but the regulation of most of this large family of genes is not understood (Richard-Plaine, EC2006). Interestingly the \( \text{PGA} \) genes differentially expressed in TAF12a and TAF12b depleted cells are different. Moreover 30 of the 51 genes upregulated in the TAF12b-depleted cells are unnamed genes and therefore their significance is not understood.

To validate the microarray data we carried out quantitative real-time PCR analysis of \( \text{TAF12a, TAF12b, TBP and ACT1} \) mRNA levels. \( \text{C. albicans} \) strains SN95, ISC11 and ISC12 were pregrown for 22-24 hrs in SC plus 2% maltose and the culture was diluted to a starting \( \text{OD}_{600} \) of 0.2 into fresh SC media containing 2% glucose. Cells were grown for 6h, harvested and flash frozen in liquid nitrogen. Total RNA was extracted using hot phenol method (as described in Section II.16), purified using the RNAeasy purification kit (Qiagen) and treated with RNase-free DNasel. We used 150 ng of DNase I-treated RNA sample in 7.5\( \mu \)l cDNA synthesis reaction. A control mock reaction was also set up where no enzyme was added. Primers for PCR were designed using the Primer Express software (ABI). \( \text{SCRI} \), a putative \( \text{C. albicans} \) ortholog of \( \text{S. cerevisiae SCRI} \) and transcribed by RNA pol III (Marck et al., 2006) was used as an endogenous control.

The differential expression analysis of the qRT-PCR data showed that \( \text{TAF12a} \) mRNA level in ISC11 (\( \text{P}_{\text{MAL2-TAF12a}} \)) and ISC12 (\( \text{P}_{\text{MAL2-TAF12b}} \)) cells grown for 6h in glucose containing medium was \( \sim 2.5 \) fold below the level in the wild-type SN95
**Fig. IV.16. qRT-PCR analysis.** Total RNA from \( P_{\text{MAL}} TAF12a \) and \( P_{\text{MAL}} TAF12b \) strains cultured in glucose (promoter repressing medium) for 6h was used for cDNA synthesis. DNase I-treated first strand cDNA was used for qRT-PCR using SYBR Green chemistry (ABI). Primer pairs for the indicated genes were used in PCR in a ABI 7500 instrument and \( C_r \) values determined. Relative expression values were calculated as per \( 2^{-\Delta C_T} \) method.
cells cultured in parallel (Fig. IV.16.). The TAF12b mRNA level, however, was reduced about 17-fold in ISC12 (P_{MAL2}-TAF12b) cells consistent with strong repression of TAF12b in this strain. In ISC11 (P_{MAL2}-TAF12a) cells, the TAF12b mRNA level was increased about 2-fold compared to the wild-type level. As discussed previously (see Section IV.3), the levels of TAF12a protein in ISC11 and TAF12b protein in ISC12 cells were almost undetectable. Thus the TAF12b mRNA and protein levels showed consistent behavior, whereas the TAF12a mRNA levels do not appear to be reduced to the extent the TAF12a protein level was reduced (Fig. IV.16.). The microarray data showed that the relative level of ACT1 mRNA, encoding β-actin, was reduced two-fold in both TAF12a and TAF12b depleted cells. However, qRT-PCR analysis showed that ACT1 mRNA was not significantly altered in either sample. It seems likely that this difference may be due to the normalization control used in the qRT-PCR analysis viz., SCR1 RNA, which is a pol III transcript.

The microarray data showed that TBP expression was strongly upregulated in TAF12b-depleted cells. Therefore we validated this result using qRT-PCR assay. The results showed that indeed TBP mRNA level was 4-fold upregulated in TAF12b-depleted cells but not in TAF12a-depleted cells (Fig. IV.16.). It would be interesting to investigate in details the mechanism and the consequences of TBP upregulation in TAF12b depleted cells.

**IV.10. Summary**

To carry out genetic analysis, we attempted to knock-out TAF12a and TAF12b genes in *C. albicans*. We successfully obtained taf12a null mutants but were unable to obtain viable taf12b null mutants indicating that TAF12b is essential for *C. albicans* growth. Therefore we used strains bearing TAF12a and TAF12b genes under MAL2 promoter to study the effects of depletion of these proteins in *C. albicans* biology.
Western blot analysis showed that when the \textit{MAL2} promoter-regulated TAF12a and TAF12b were cultured for 6h in glucose, there were little or no detectable levels of the two proteins, indicating that the \textit{MAL2} promoter regulated alleles can be effectively used for genetic analysis of TAF12 proteins in \textit{C. albicans}.

The TAF12a and TAF12b depleted cells gave different cellular, colony and stress response phenotypes. Both strains yielded a mixture of different morphologies including normal yeast-form, as well as chain of cells comprised of mother and daughter cells joined together at the septa that could be broadly classified as pseudohyphal-type morphology. More than half of the TAF12b-depleted cells were normal yeast-form and about 30\% were pseudohyphal and exhibited unipolar or bipolar axial budding pattern. It is likely that at least some proportion of the yeast-form cells originated from these pseudohyphal chains. In addition, about 10\% of cells were enlarged with rounded mother cells bearing small tubular projections similar to germ tubes that had incomplete septa between mother and daughter cells. A single nucleus was found in the evaginated daughter cells but no nucleus was found within the mother cell. This phenotype has been described as cell cycle arrest phenotype. Thus it appears that TAF12b depletion induces defects in cell cycle progression. Further experiments would be directed to understand the cell cycle progression in the TAF12b mutant cells.

The TAF12a depleted strain was distinct in that the cells were long and formed branched chain of varying lengths. The pattern of budding appeared to be unipolar with axial branching and indicative of a cell separation defect. The \textit{taf12aΔ} strain also exhibited a similar cellular morphology with unipolar axially budding chains of unseparated cells. However, the chains were much longer and branched more profusely in the null mutant. The \(P_{\textit{MAL2}}\text{-TAF12a}\) colonies were severely
impaired for pseudohyphae formation and agar invasion in solid YCB medium and SLAD medium. However, the colonies of $P_{MAL2-TAF12a}$ strain retained the ability to form true hyphae but filamentation was less profuse compared to the wild-type control strain. The $taf12a\Delta$ mutant also formed wrinkled colonies like the wild-type strain but was defective in forming filamentous projections at the colony boundaries. The $MAL2p-TAF12a$ and $taf12a\Delta$ strains were found to be impaired for growth under oxidative stress imposed by $H_2O_2$, menadione and cadmium. The TAF12a and TAF12b depleted cells and $taf12a\Delta$ strains were examined for their virulence potential in mice model of candidiasis. We found that the onset of virulence was delayed with high statistical significance in mice injected with $P_{MAL2-TAF12b}$ strain, while the $taf12a\Delta$ strain was completely avirulent.

Our microarray study revealed that the expression of over 3% and 4% of the genes analyzed are regulated $\geq 4$-fold in a TAF12a and TAF12b dependent manner respectively. The data showed little or no overlap in the highly regulated $TAF12a$ and $TAF12b$ dependent genes, but sizable overlap in the genes regulated between 2 and 4-fold. It would be important to examine more datasets and carry out time-course of mRNA expression analysis to establish the full scope of the transcriptome regulated by TAF12a and TAF12b proteins.