

FULL REPRESSION OF RNA POLYMERASE III TRANSCRIPTION REQUIRES INTERACTION BETWEEN TWO DOMAINS OF ITS NEGATIVE REGULATOR MAF1

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Maf1, first identified in yeast *Saccharomyces cerevisiae*, is general negative regulator of RNA polymerase III (Pol III). Transcription regulation by Maf1 is important under stress conditions and during the switch between fermentation and respiration. Maf1 is composed of two domains conserved during evolution. We report here that these two domains of human Maf1 are resistant to mild proteolysis and interact together as shown by pull-down and size exclusion chromatography and that the comparable domains of yeast Maf1 interact in a two-hybrid assay. Additionally, in yeast, a mutation in the N-terminal domain is compensated by mutations in the C-terminal domain. Integrity of both domains and their direct interaction is necessary for Maf1 dephosphorylation and subsequent inhibition of RNA polymerase III transcription on a nonfermentable carbon source. These data are the report relating Maf1 structure to RNA polymerase III transcription inhibition.

In eukaryotic cells, RNA polymerases (Pol) I and III are responsible for the synthesis of RNA species involved in ribosome biogenesis and the translation process. RNA synthesis by Pol I and Pol III represents more than 80% of all yeast nuclear transcription activity and is controlled in a coordinated way in response to various cellular and environmental conditions (1, 2, 3).

Pol III is responsible for the transcription of ~300 different genes in yeast (class III genes), mostly tRNA genes (4, 5, 6). Analyses of the Pol III transcription system in yeast have revealed a series of protein-DNA and protein-protein interactions leading to the recruitment of Pol III to its target tRNA genes:

binding of the six-subunit TFIIC factor to the intragenic promoter, TFIIC-directed recruitment and assembly of the three components of TFIIB (TBP, Brf1 and Bdp1), and subsequent recruitment of the seventeen-subunit Pol III enzyme (7). Whereas the essential factors and the basal mechanisms of class III gene transcription are well defined, much less is known about the molecular mechanisms of Pol III regulation.

The unique global negative regulator of Pol III transcription in yeast is the Maf1 protein that mediates several signaling pathways, but is not essential (8, 9). In addition to the down-regulation that normally occurs in the stationary phase and in response to various drugs, also DNA damage, oxidative stress, secretory defects and respiratory growth require Maf1 to achieve Pol III repression (8, 10, 11, 12). The activity of Maf1 is regulated by its phosphorylation, which occurs in favorable conditions. Apart from decreasing direct Maf1 binding to Pol III (13), this phosphorylation acts both to relocate the nuclear pool of Maf1 to the cytoplasm (14) and to prevent import of cytoplasmic Maf1 to the nucleus (15). Diverse unfavorable conditions lead to rapid Maf1 dephosphorylation and its nuclear accumulation, physical association of the dephosphorylated Maf1 with Pol III, and genome-wide Maf1 targeting to Pol III-transcribed genes (13, 16).

The Pol III machinery is remarkably conserved between yeast and human. The most conserved components are those involved in transcription complex assembly: the τ 131 subunit of TFIIC and two components of TFIIB (TBP, Brf1). The five Pol III-specific subunits in yeast (C31, C34, C37, C53 and C82), all have structural and functional homologs in human cells (17). Also Maf1 is conserved across

eukaryotic organisms from yeast to man (9). This conservation is of particular interest considering that misregulation of Pol III in human has been linked to malignant transformation. Excessive activation of Pol III-directed transcription can lead to tumorigenesis (18, 19, 20, 21) and, in line with this observation, two mammalian tumor suppressors, Rb and p53, have been shown to act as global repressors of Pol III transcription (22). Recent results of several groups report Maf1-mediated repression of Pol III transcription in human implicating HsMaf1 ortholog as a new class of mammalian Pol III regulators (23, 24, 25, 26). The involvement of HsMaf1 in the aberrant control of Pol III transcription in cancer cells remains to be studied. In the light of the high evolutionary conservation of the Pol III machinery including Maf1, insights into Pol III (mis)regulation by Maf1 gained by studying model organisms, such as yeast, should provide some insight into the role of HsMaf1 in cancer.

Our current interest concerns the relation between Maf1 structure and activity. All members of the Maf1 family have three fairly conserved segments (9) which, however, show no significant homology with protein domains of known function resulting in the striking lack of information on the functional significance of those regions. Point mutations have only highlighted the importance of several serine residues (mostly not phylogenetically conserved) and two nuclear localization sequences (15, 16, 27). We describe here identification of two conserved domains in HsMaf1 and show that the corresponding regions in yeast Maf1 interact. This interaction is crucial for the regulation of Maf1 activity by phosphorylation. Our data provide the first insight into the Maf1 structure in relation to Pol III regulation.

Experimental Procedures

Expression and purification of HsMaf1 protein - Human full-length Maf1 (HsMaf1)(aa 1-256) was expressed as a C-terminal 6His-tagged protein (HsMaf1-CHis) in insect cells. The protein was purified using cobalt affinity resin (Clontech). As a final purification step, the protein was applied to size-exclusion chromatography equilibrated in buffer I (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT).

HsMaf1 fragments aa 1-45, aa 1-59, aa 1-63, aa 1-74 and aa 85-210 were co-expressed for 15 h at 18°C in *E. coli* BL21 Gold (DE3) cells (Stratagene) using expression vector pETMCN-

His (C. Romier, IGBMC) coding for TEV-cleavable N-terminal His-tagged HsMaf1 aa 1-45, aa 1-59, aa 1-63, aa 1-74 and expression vector pETMCN (as above) coding for non-tagged Maf1 aa 85-210. The complex was purified by Ni-affinity chromatography (NiNTA, Qiagen) followed by TEV protease cleavage and a second Ni-affinity chromatography to remove the His-Tag. The complex was further purified by anion-exchange chromatography (MonoQ 10/100, GE Healthcare) and size exclusion chromatography.

HsMaf1 fragment aa 82-236 was expressed from pETM11 (EMBL) as a potentially TEV-cleavable, N-terminal His-tagged protein which was purified by nickel affinity chromatography (Chelating Sepharose, GE Healthcare). However the purified tagged protein HsMaf1 82-236 aa could not be cleaved by TEV protease - most likely due to its aggregated state. The tagged protein was further purified by anion-exchange chromatography (5 ml HiTrap Q-Sepharose HP, GE Healthcare) and size-exclusion chromatography (S200 10/300, GE Healthcare).

Limited proteolysis of HsMaf1 protein - hMaf1-CHis protein at a concentration of 1 mg/ml was digested with trypsin for 30 min. at 4°C in buffer I using a protease:protein ratio of 1:150 (w:w). The reaction was stopped by adding PMSF to a final concentration of 1 mM. The proteolysis product was concentrated to 2 mg/ml using an Amicon MWCO 3000 concentrator (Millipore) and subsequently purified using two, consecutive Superdex 200 10/300 columns (GE Healthcare) to improve resolution in buffer I. Purified HsMaf1 fragments were unambiguously identified using a combination of Edman degradation and Mass Spec (Q-Tof).

Yeast S. cerevisiae strains and media - The yeast strains used in this study included wild type YPH500 (*MAT α* , *ade2-101*, *his3- Δ 200*, *leu2- Δ 1*, *lys2-801*, *trp1- Δ 63*, *ura3-52*), *maf1- Δ* , a derivative of YPH500 (13) and the two-hybrid reporter strain Y190 (*MAT α* , *gal4-542*, *gal80-538*, *his3*, *trp1-901*, *ade2-101*, *ura3-52*, *leu2-3*, *112*, *URA3::GAL1-lacZ*, *LYS2::GAL1(UAS)::HIS3*, *cyh^R*) (28). Rich media contained 1% yeast extract, 2% peptone and 2% glucose (YPD) or 2% glycerol (YPGly). The minimal medium (SC) contained 2% glucose and 0.67% yeast nitrogen base without amino acids (29). Solid media contained 2% agar. All reagents were from Difco.

Construction of plasmids to express fragments of yeast Maf1 protein for two-hybrid

study - DNA encoding fragments aa 1-12, 1-16 and 1-23 of domain A of yeast Maf1 were synthesized as oligonucleotides. The larger DNA sequences, encoding aa 1-34, 1-39 and 1-42 fragments were amplified using forward primer 5'TCATCGGGATCCGAATGAAATTTATTGATGAGCTAGATATAGAGAGAGTG3' and reverse primers: 5'TCATCGCTCGAGTTTCTATCTGATGCAACCGC3', 5'TCATCGCTCGAGTGATGCAACCGCCTTTGTTGTG3' and 5'TCATCGCTCGAGTGTGTGAAAATATCGCAACTGCC3', respectively. The intron sequence of the *MAF1* gene (localized between bp 7 and 87) was excluded. DNA encoding fragment aa 196-349 fragment of BC domain was amplified with primers: 5'TCATCGGGATCCGATCTGGTACAGCAACCAACAATG3' and 5'TCATCGCTCGAGTTCGCCTGTA~~CTCGAATTTAG3~~'.

All *MAF1* parts were amplified with *Bam*HI and *Xho*I termini fragments, and inserted into MATCHMAKER GAL4 Two-Hybrid Vectors (Clontech) – either the pACT2 plasmid carrying the activation domain of Gal4 or the pAS2 plasmid carrying the binding domain of Gal4. The resulting plasmids were named pACT2-Maf1-A(1-12), pACT2-Maf1-A(1-16), pACT2-Maf1-A(1-23), pACT2-Maf1-A(1-34), pACT2-Maf1-A(1-39), pACT2-Maf1-A(1-42) and pAS2-Maf1-BC(196-349). Expression of fusion proteins involving HA-tagged truncated versions of A domain was verified by Western blotting. Each of the derivatives of pACT2 and the single derivative of pAS2, were co-expressed pair wise in the two-hybrid reporter strain Y190. Cells containing these two plasmids were patched on SC medium lacking leucine and tryptophan. The patches were then examined for β -galactosidase activity using an overlay plate assay (30). The intensity of the coloration was calibrated by comparison with a pair of known interactors (τ 95/ τ 55, two TFIIIC subunits) for which the β -galactosidase activity had been measured previously (31). For a β -galactosidase liquid assay cell lysates were prepared and the activity was measured colorimetrically as nmoles of ONPG (o-nitrophenyl- β -D-galactopyranoside) hydrolyzed per minute per mg of protein. Conversion $0.0045 \times A_{420} = 1$ nmole ONPG cleaved was used (30).

Generation of yeast S. cerevisiae Maf1 mutant strains - *MAF1* gene was cut from pFL44-MAF1 (32) subcloned in pRS315 (LEU2, CEN) plasmid (33) resulting in pRS315-MAF1. The pAG70, pLM11 and pLM12 plasmids were derived from pRS315-MAF1 using a rapid

method for localized mutagenesis (34). For this purpose *MAF1* fragment encoding aa 1-180 was PCR-amplified from pRS315-MAF1 under mutagenic conditions using 5'CGAGTTGCTTGTC AATCAGG3' and 5'CTGCTACTGCTCCTTCTTCT3' primers and Diversify PCR Random Mutagenesis Kit (Clontech). The product of the low-fidelity PCR was transformed together with gapped linear plasmid pRS315-MAF1 (digested with *Bcl*II and *Bsg*I) into the YPH500 *maf1*- Δ strain (13). Transformants, selected on minimal medium lacking leucine, were subsequently tested for Maf1 activity by replica-plating on YPGly and incubation at 37°C for three days; Among 38 independent mutants, pAG70 (*maf1*-K35E) was selected from colonies that showed defective growth. To isolate pML11 (*maf1*-K35E-D250E) and pML12 (*maf1*-K35E-V260D-N344I) plasmids carrying suppressor mutations, pRS315 plasmid pAG70 (carrying the previously isolated *maf1* allele with the K35E mutation) was digested with *Bsa*BI and *Bsu*36I and introduced into the YPH500 *maf1*- Δ strain together with a *MAF1* fragment encoding BC domain (aa 174-375) PCR-amplified under mutagenic conditions using primers: 5'AGAAGAAGGAGCAGTAGCAG3' and 5'CGTATTCTCCTTCGTATTCA3'. The obtained library of potential suppressor mutants was screened for overcoming the thermosensitivity on YPGly medium caused by the K35E mutation. This screen resulted in identification of pLM11 and pLM12 suppressor mutations in BC domain of Maf1.

To generate mutations in the two-hybrid plasmids carrying fragments encoding Maf1 domains, the *Bam*HI-*Xho*I fragment encoding aa 1-42 of Maf1 in the pACT2-Maf1-A(1-42) plasmid was substituted with a PCR-amplified fragment of the *maf1* allele K35E from pAG70. Similarly the mutations found in pML11 and pML12 were introduced in pAS2-Maf1-BC(196-349). The N344I mutation found in the pML12 plasmid was introduced to pAS2-Maf1-BC(196-349) by using a modified reverse primer 3'TCATCGCTCGAGTTCGCCTGTA~~CTCGAAATTAGACGCGAGC5~~' with a mutation leading to the desired amino acid substitution.

QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to introduce D250E, N344I, V260D and V260D-N344I to pRS315-MAF1. The sequences of primers used are available upon request.

Northern analysis - Cells (50 ml of liquid culture, A_{600} of about 0.8) were harvested by

centrifugation and resuspended in 50 mM Na acetate, pH 5.3, 10 mM EDTA. Total RNA was isolated by heating and freezing the cells in the presence of SDS and phenol as described previously (12,35). RNA (5 µg per sample) was resolved by electrophoresis in 10% PAGE with 8 M urea, transferred to Hybond-N+ membrane (Amersham) by electroblotting in 0.5 x TBE and crosslinked by UV radiation (1200 mJ/cm²). The membrane was prehybridized in 7% SDS; 0.5 M sodium phosphate pH 7.2; 1 mM EDTA pH 7.0; 1% BSA and hybridized at 37°C in the same solution with oligonucleotide probes labeled with [γ -³²P]-ATP and T4 polynucleotide kinase (New England Biolabs). The probes were: 5'TATTCC CACAGTAACTGCGG3' for tRNA^{Leu}(CAA), 5'CCTCCAGATGACTTGACCG3' for tRNA^{Phe}(GAA) and 5'GGATTGCGGACCAAGCTAA3' for U3 snoRNA. After hybridization the blots were washed 2 x 10 min. with 1×SSC, 1% SDS and 3 x 10 min. with 0.5×SSC, 0.1% SDS at 37°C and exposed to an X-ray film or a phosphoimager screen (Molecular Dynamics).

Protein Extraction and Immunoblotting - To avoid action of endogenous kinases or phosphatases during cell harvesting and protein extraction, yeast cells were rapidly harvested by centrifugation at 4°C, and 20% trichloroacetic acid (TCA) was added to the cell pellet as described earlier (12, 13). Cells were broken with acid-washed glass beads, the supernatant was retained and TCA-precipitated proteins were pelleted by centrifugation. The pellet was resuspended in sample buffer (pH 8.8) and boiled for 5 min. Protein extracts were separated on SDS-PAGE using a modified acrylamide: bisacrylamide ratio (33.5:0.3). One lane was loaded with protein from 1 OD of cell culture (10–20 µg). The membrane was blocked for 30 min. in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) containing 5% fat-free dry milk and then incubated for 1 h with Maf1-specific antibody at 1:10 000 dilution (12). The membrane was incubated with secondary anti-rabbit antibody coupled to horseradish peroxidase (DAKO) which was then visualized by chemiluminescence using the ECL detection kit (Milipore).

RESULTS

Two domains of Maf1 do interact. The yeast *MAF1* gene encodes a hydrophilic protein of 395 amino acids rich in serine and asparagine residues, with a predicted molecular mass of 44.7

kDa. Screening of multiple databases with the yeast Maf1 sequence revealed numerous orthologs in other eukaryotes: one in human, 50 in animals, 28 in plants and 27 in lower eukaryotes, but none in prokaryotes. Maf1 proteins contain three phylogenetically conserved sequence regions, labeled A, B and C (9). The similarity of the yeast and human Maf1 sequences is presented in figure 1 and more Maf1 proteins are aligned in figure S1. Since the distance between the B and C segments of approximately 10 aa is constant in evolution, with the exception of *Aspergillus nidulans* (insertion of 15 aa), we consider this region could be a single domain named here as BC. In contrast, the space between regions A and B largely varies between species. The A and BC domains are fused in *Encephalitozoon cuniculi* whereas in the yeast *Saccharomyces cerevisiae* and *Candida glabrata* they are separated by a long linker of 182 aa and 174 aa, respectively. Within the BC domain signature sequences for the Maf1 protein family can be identified (PDYDFS and LWSFnYFFYNKkIKR, Supplemental Figure S1) (9). These sequence "motifs" are not reported in the PROSITE database. Interestingly, in the majority of Maf1 orthologs, the second motif includes a nuclear targeting signal, which was proved to be functional in *S. cerevisiae* Maf1 (15).

To experimentally characterize the structure organization of human Maf1 (HsMaf1), we carried out limited proteolysis experiments in combination with size-exclusion chromatography as shown in figure 2. Proteolytically stable fragments are considered to be structurally well defined, whereas protease-sensitive sites often correlate with disordered regions of the proteins. Using limited proteolysis with trypsin, HsMaf1 protein (256 aa) was digested into two major stable fragments that were identified as HsMaf1 aa 1-45 and Maf1 aa 75-234 using a combination of N-terminal sequencing and mass spectrometry (Q-Tof). The HsMaf1 linker region between those fragments (aa 46-74) and the C-terminal acidic tail (aa 235-256) were degraded and thus presumably unstructured (Figure 2a, lane 2). Both fragments were further analyzed by size-exclusion chromatography. Surprisingly, the two HsMaf1 fragments, although of substantially different molecular masses, co-eluted in an apparent 1:1 stoichiometry, suggesting an intramolecular interaction between them (Figure 2B, red profile).

Taking into account the limited proteolysis and secondary structure prediction results (data not shown), we designed N- and C-terminal constructs of HsMaf1 for co-expression in bacteria. The N-terminal construct aa 1-45 corresponds to the minimal domain defined by proteolysis while fragment aa 85-210 was designed slightly shorter than the initial proteolytic fragment. During purification His-tag containing recombinant HsMaf1 fragments aa 1-45 but also slightly larger constructs aa 1-59, aa 1-63 and aa 1-74 co-precipitate the un-tagged C-terminal construct aa 85-210 in an apparent 1:1 stoichiometry suggesting a direct interaction between the N- and C-terminal fragments (Supplemental Figure S2).

Analysis by size exclusion chromatography supports the results obtained for the proteolytic fragments. Constructs aa 1-45 and aa 85-210 co-elute as a single peak at a volume corresponding to the expected molecular weight of ~20 kDa (Figure 2B). These results provide further support for a direct interaction between A and BC domains of HsMaf1. Interestingly, the co-expressed complex is considerably more compact compared to the full-length protein, presumably because it is lacking the C-terminal acidic tail. In contrast, an additional construct aa 82-236 that includes the C-terminal acidic tail eluted as soluble aggregate (Figure 2, bottom panel) when expressed in the absence of the N-terminal fragment aa 1-45. We also tried to express the N-terminal fragment aa 1-45 as GST fusion protein, but only obtained minimal amounts presumably because the protein aggregates after TEV cleavage (data not shown). Apparently, N- and C-terminal domains of HsMaf1 are both required for the soluble expression of HsMaf1 and co-elute during size-exclusion chromatography indicating a direct interaction between them.

To investigate if the interaction between domains of Maf1 is conserved through evolution, we analyzed the proposed interaction in yeast, an organism more amenable to study structure-function relationships of Maf1 using the two-hybrid system.

The putative BC domain of *S. cerevisiae* Maf1 (aa 196-349) was fused to the DNA-binding domain of Gal4 and co-expressed with various Maf1 A domain constructs fused to the Gal4 activation domain in the yeast reporter strain Y190. Interactions between fusion proteins should result in activation of the β -galactosidase reporter gene. Using this approach we observed a

physical interaction between the BC domain of Maf1 encoded by plasmid pAS2-Maf1-BC(196-349) and fragments of domain A of Maf1 encoded by plasmids pACT2-Maf1-A(1-42), pACT2-Maf1-A(1-39) and pACT2-Maf1-A(1-34) (Figure 3). Domain BC failed to interact with shorter fragments of domain A encoded by pACT2-Maf1-A(1-12), pACT2-Maf1-A(1-16) or pACT2-Maf1-A(1-23). Cells containing the pair pAS2-Maf1-BC and empty pACT2 had no detectable β -galactosidase activity (data not shown in the figure). These results demonstrate the specificity of the two-hybrid interaction between Maf1 domains and define aa 1-34 as the smallest Maf1 A domain still able to bind the BC domain. Reciprocal interactions were impossible to study because the presence of pAS-Maf1-A activates reporter gene in the absence of a pACT2 fusion. Importantly pAS2-Maf1-BC was also negative tested with pACT2 encoding fusions of several genes unrelated to Maf1 and encoding components of Pol III complex.

Taken together, our results as described above suggest a strong, direct interaction of the A and BC domains of human and yeast Maf1.

Interaction between A and BC domains is important for the function of Maf1. Limited proteolysis and two-hybrid results show that the A and BC domains of Maf1 together form a stable complex, possibly reflecting an active conformation of Maf1. To evaluate the physiological significance of the interaction between the Maf1 domains, we screened for mutants impaired in Maf1 function that were located in domain A and presumably compromised in domain BC binding. *MAF1* fragment encoding aa 1-180 was PCR-amplified under mutagenic conditions and transformed into *maf1- Δ* cells together with a gaped single copy plasmid encoding Maf1. Transformants were selected for poor growth at 37°C on glycerol medium suggesting defect of Maf1 function in Pol III repression (12). Sequencing of plasmid pAG70 encoding mutant Maf1 isolated in this manner revealed mutation K35E located in domain A.

To inspect the effect of K35E mutation in Maf1 on Pol III activity, RNA isolated from cells grown in the presence of glucose and transferred to glycerol medium at 37°C was analyzed by Northern blotting using probes for pre-tRNA^{Leu} and tRNA^{Phe} (Figure 4A). Following transfer to the medium with the nonfermentable carbon source, pre-tRNA levels were decreased in the wild type but not in *maf1- Δ* cells (Figure 4A,

compare lanes 1, 5 and 6, 10). Similarly to *maf1-Δ*, the K35E mutant was defective in its ability to repress pre-tRNA transcription upon transfer to glycerol medium (Figure 4A, lanes 2 and 7). Thus, the single missense mutation within the Maf1 A domain precluded Pol III repression in *maf1-K35E* strain.

One approach to confirm that the K35E mutation negatively affects the interaction with the BC domain of Maf1 is to identify second-site mutations that compensate for the observed defects. We looked therefore for second-site suppressor mutations within the BC-encoding region of *MAF1* that allowed *maf1 K35E* yeast cells to grow on glycerol medium at 37°C. DNA encoding aa 174-375 of Maf1 was randomly mutagenized by PCR and the mutant pool was co-transformed with the pAG70 plasmid containing the primary K35E mutation and gapped within the region of the BC domain. Sequencing of *in vivo* reconstituted *MAF1* from six transformants showing reversion of the original defect identified two plasmids with suppressor mutations: pLM11 (*maf1-K35E-D250E*) and pLM12 (*maf1-K35E-V260D-N344I*). The remaining transformants were either back revertants of K35E mutation or contained suppressor mutations outside the *MAF1* gene. Both isolated suppressors of K35E mutation in A domain, single D250E and combined V260D-N344I, were located in the BC domain, thus supporting the interaction between these domains.

Phenotypic characterization of the two suppressor strains showed that they compensate for the defect of growth on glycerol medium at 37°C of the primary K35E mutation (Figure 4B). The increased growth capacity of the suppressors on glycerol medium was due to a compensation of the defect in Pol III regulation observed for the K35E mutation. As determined by Northern blotting, the suppressor mutations restored the ability to repress pre-tRNA transcription upon transfer from glucose to glycerol medium (Figure 4A, lanes 3, 4 and 8, 9). These results indicate that the detected genetic interaction between the two Maf1 domains is a functional one.

To further characterize the association of A and BC domains, we sought to verify the effects of K35E, D250E, V260D and N344I mutations, identified in the mutant and suppressor strains, on the interaction of Maf1 domains in the two-hybrid system. The respective mutations were generated in the Maf1 A domain fragment fused to the Gal4 activation domain in the pACT2-

Maf1-A(1-42) plasmid or the Maf1-BC domain fused to the DNA-binding domain of Gal4 in pAS2-Maf1-BC(196-349). Various combinations of plasmids, one encoding a wild type Maf1 domain and the other with a mutated domain, both fused to the respective Gal domains, were co-expressed in the yeast reporter strain followed by determination of β-galactosidase activity (Figure 5). The K35E mutation of domain A reduced the two-hybrid interaction with wild type BC domain by ca. 40 % (Figure 5, compare lanes 2 and 1). Similarly, both the single D250E and the double V260D-N344I mutations in BC domain decreased the interaction with wild type A domain by 22 and 62 %, respectively (Figure 5, compare lanes 7 and 8 with lane 1). Moreover, the double V260D-N344I mutant showed a defect in regulation of tRNA transcription and temperature sensitive growth in glycerol medium (Supplemental Figure S3).

Confirming the genetic suppressor results, a combination of mutated Maf1 domains restored their interaction (Figure 5, compare lanes 3 and 4 with lane 1). Rather unexpectedly, the strongest level of two-hybrid interaction, 80% over the wild type one, was observed for the combination of K35E domain A and domain BC with a single V260D mutation (Figure 5, compare lane 5 with lane 1). This indicated that in the double suppressor mutation V260D-N344I, identified in genetic screen, the former one is in fact sufficient to restore the interaction with K35E domain A.

Domain interaction supports Maf1 dephosphorylation upon transfer cells to a nonfermentable source. As shown before, phosphorylation of Maf1 precluded Pol III repression when yeast cells were grown on glucose medium (14). Transfer of yeast cells to a nonfermentable carbon source resulted in Maf1 dephosphorylation, import of Maf1 into the nucleus and inhibition of tRNA synthesis (12). Since, we showed that the interaction between the Maf1 A and BC domains is crucial for its regulatory action on Pol III, we now asked if this interaction is also required for efficient regulation of Maf1 activity.

To investigate whether the altered domain interactions in the Maf1 mutant proteins were correlated with changes in the phosphorylation of Maf1, differentially phosphorylated forms of Maf1 were resolved by SDS-PAGE and identified by immunoblotting at various times after culture transfer from glucose to glycerol medium (Figure 6). As reported previously, wild type Maf1 was quickly dephosphorylated upon

this transition. Remarkably, the K35E mutation in domain A, preventing its interaction with the BC domain, appeared to preclude the dephosphorylation of Maf1. Significantly, restoring the domain interaction with D250E (pLM11) and V260D-N344I (pLM12) suppressor mutations re-established rapid dephosphorylation of Maf1 following transfer from glucose to a nonfermentable carbon source. Maf1 dephosphorylation in the suppressor mutants was only a little slower than in the wild type strain (Supplemental Table S1). Interestingly, double V260D-N344I mutation in context of wild type A domain precluded interaction between domains (Figure 5) and slowed the rate of dephosphorylation of Maf1 upon transfer of yeast to glycerol medium (Supplemental Figure. S4).

Altogether, these results suggest that the interaction of A and BC domain greatly facilitates the dephosphorylation of Maf1.

DISCUSSION

In this paper we describe the importance of the interaction between two Maf1 domains for its activity as a repressor of Pol III transcription.

Limited proteolysis of HsMaf1 resulted in two stable fragments (aa 1-45 and aa 75-234) corresponding to the evolutionarily conserved A and BC domains. Size-exclusion chromatography of the proteolytic fragments corresponding to the two domains showed their co-elution. Similar fragments were co-expressed in bacteria, where they co-purified and co-migrated during size exclusion chromatography as soluble complex. In contrast, both fragments behaved poorly when individually expressed. Our results suggest that Maf1 A and BC domains form modules that do not fold independently but rather need to be co-expressed to form a stable and soluble entity. In contrast, the connecting linker (aa 46-74) and the C-terminal acidic tail (aa 235-256) appear solvent exposed and unstructured as suggested by HCA analysis (Supplemental Figure S6).

Considering the high conservation of Maf1 in eukaryotes, the domain interaction and its functional role was probed in the model organism yeast *Saccharomyces cerevisiae*. Using the yeast two-hybrid system we confirmed the physical interaction between A and BC domains of Maf1 in yeast and identified the first 34 residues as the shortest fragment of the A domain sufficient for interaction with the BC domain.

Lack of interaction between further truncated fragments of the A domain (aa 1-12, 1-16 or 1-23) with the BC domain shows the specificity of the method used and emphasizes the importance of structural features of the A domain for its interaction with the BC domain.

To further investigate the role of domain A we isolated single-point mutant K35E (on plasmid pAG70). In accordance with its growth characteristics, this mutant failed to repress Pol III activity. Moreover, as shown by the two-hybrid system, the K35E mutation reduced the interaction between the A and BC Maf1 domains by 40%. In support of structural and/or functional interactions between the Maf1 domains, we isolated second-site suppressor mutations within the BC domain that compensated for the defect caused by the K35E mutation in domain A. We found two such suppressors: D250E and V260D-N344I. Further phenotypic characterization of the suppressor strains revealed that these additional mutations in BC domain restored not only the ability of the K35E-mutated Maf1 to grow on glycerol medium at 37°C, but also its ability to repress Pol III activity. Interestingly, residue 260 in domain BC seems to be crucial for the interaction with domain A. While the V260D mutant BC domain interacted with K35E mutant domain A over 80% stronger as did wild type domain BC, its interaction with the wild type domain A was decreased by almost 60% (Figure 5, lanes 5 and 9). All these results indicated that the genetic interaction identified between Maf1 domains corresponds to a physical interaction indispensable for the Maf1 function.

Bioinformatic analysis using the protein structure prediction server (PSIPRED v 2.6) (36, 37) provides further support for our biochemical analysis. In the yeast and human Maf1 A domain, two α -helices (aa 7-16 and aa 40-56) separated by two adjacent β -strands (aa 24-27 and 29-35) are predicted, while for the BC domain five α -helices and concomitant four β -strands are predicted (Supplemental Figure S5). No secondary structure elements are predicted for the linker region. Notably, the K35E mutation is located at the end of the second β -strand of the A domain. Our attempts to minimize the Maf1 A domain identified aa 1-34 as the smallest fragment still enabling interaction with the domains. We hypothesize that the interaction between domains A and BC requires the first α -helix and two adjacent β -strands. We also applied Hydrophobic Cluster Analysis

(HCA), a tool to investigate protein stability and folding that uses two-dimensional (2D) helical representation of protein sequences to identify possible hydrophobic cores formed by several residues (38). The HCA analysis revealed the presence of two regions rich in hydrophobic cores corresponding to the A and BC domains of *S. cerevisiae* Maf1 (Supplemental Figure S6). Between them a region poor in hydrophobic residues was found which, according to the alignment of Maf1 eukaryotic sequences, corresponds to the linker region between the A and BC domains. Similar analysis in human Maf1 revealed the same organization of two clusters of hydrophobic cores separated by a short region free of hydrophobic clusters consistent with our limited proteolysis results.

We found the interaction between the A and BC domains, facilitating Maf1 dephosphorylation, to be necessary for the full repression of Pol III activity. One might hypothesize that in the same time the interaction between domains of Maf1 is influenced by its phosphorylation state. Accordingly, different forms of Maf1 observed on polyacrylamide gels, interpreted as phosphorylated and subsequently dephosphorylated form of Maf1, might represent different conformational states depending on the interaction between A and BC domains. Note that the S90A, S101A and S177A, S178A mutations in the linker were found to change the proportion between slow- and fast-migrating Maf1 forms in favor of the former, without

strongly affecting the phosphorylation status of Maf1 thus indicating that residues in the linker could strongly modify the Maf1 shape (39).

Considering that Maf1 contains two conserved domains, we propose the unstructured linker to become phosphorylated and thus modulating the conformational state of Maf1. To further investigate this question we used the NetPhos 2.0 server (40) to predict possible phosphorylation sites in four Maf1 orthologs containing linkers of different lengths. Predicted phosphorylation sites are frequently found in the long linkers between the Maf1 A and BC domains of *S. cerevisiae* and *C. glabrata* relatively, while they are more uniformly distributed along the Maf1 sequences of *C. elegans* and *H. sapiens*, although they are also present in their shorter linkers, (Supplemental Figure S7). We therefore speculate that phosphorylation and dephosphorylation of the exposed linker evokes a specific conformation of the protein that changes the distance between domains modulating their interaction and Pol III repression (Supplemental Figure S8). The interaction of the domains, mediated by dephosphorylation of the linker may affect both, the import of Maf1 into the nucleus and its interaction with Pol III. This conjecture is supported by data of (15) showing that PKA-mediated phosphorylation of six residues located in linker inhibited nuclear import of Maf1 and Pol III repression.

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FOOTNOTES

Mass Spectrum analysis was carried out at the Proteomics Core Facility at EMBL Heidelberg. We are grateful to Andre Sentenac, Joël Acker, Christine Conesa, Danuta Oficjalska-Pham, Raphaël Guerois and Isabelle Callebaut for stimulating discussions. We also thank Gudrun von Scheven for technical assistance. This work was supported by the Ministry of Science and Higher Education, Poland (grant N301 023 32/1117), by grant ANR-07-BLAN-0039–01 from the French National Research Agency and by grant 1078 from the Association pour la Recherche contre le Cancer.

FIGURE LEGENDS

Fig. 1. Similarity of *S. cerevisiae* (Sc) and *H. sapiens* (Hs) Maf1 sequences. Alignment of conserved A and BC domains. A and BC domains are boxed and conserved amino acids are highlighted. Stars indicate position of K35E, D250E and V260D mutations.

Fig. 2. Domain structure of human Maf1. A. Limited Proteolysis and resulting proteolytic fragments of HsMaf1. PAGE of full-length human HsMaf1 protein (1, black) that is digested into two stable fragments aa 1-45 and aa 75-234 using trypsin (2, red). Bacterially co-expressed and co-purified HsMaf1 domains aa 1-45 and aa 85-210 (4, green). HsMaf1 aa 82-236 (3, blue) lacking the N-terminal 45 residues was expressed as a control. B. Size-exclusion chromatography profiles. Samples presented in (A) were separated on single or tandem Superdex 200 10/300 size-exclusion columns (GE Healthcare). Only HsMaf1 construct 82-236 eluates in the void volume of the Superdex 200 size-exclusion column, while all other samples are monodisperse and elute approximately at volumes corresponding to monomers. The elution profile of different molecular weight standards is shown as a dashed line. Peaks I, II, III and IV correspond to ovalbumin (Mr=44000), carbonic-anhydrase (Mr=29000), ribonuclease A (Mr=13700) and aprotinin (Mr=6500), respectively. Fractions corresponding to the red and green elution profiles were analyzed by denaturing gel electrophoresis and shown as insets. Fragment 1-45 co-elutes with proteolytic fragments 75-234 and 75-262 (red profile) and with the recombinant fragment 85-210 (green profile).

Fig. 3. Two-hybrid interaction of Maf1 domains. pACT2-Maf1-A(1-12), pACT2-Maf1-A(1-16), pACT2-Maf1-A(1-23), pACT2-Maf1-A(1-34), pACT2-Maf1-A(1-39) and pACT2-Maf1-A(1-42) plasmids were transformed individually together with pAS2 (control plasmid) or pAS2-Maf1-BC(196-349) plasmids into yeast strain Y190. Transformants were assayed for β -galactosidase expression using overlay plate assay.

Fig. 4. Regulation of Pol III transcription and growth is impaired by the K35E mutation in region encoding A domain and restored by second-site suppressor mutations in BC domain of *MAF1*. *maf1- Δ* , *maf1-K35E*, *maf1-K35E-D250E* and *maf1-K35E-V260D-N344I* mutants and isogenic wild type strain YPH500 (WT) were used. A. Cells were grown to exponential phase in glucose medium (YPD) at 30°C, then transferred to glycerol medium (YPGly) and incubated at 37°C for 1.5 h. Total RNA isolated from cells was tested by Northern blotting with pre-tRNA^{Leu} and tRNA^{Phe} probes. B. Ten-fold serial dilutions of cells grown to exponential phase in glucose medium were plated on glucose medium (YPD) and incubated at 30°C or on glycerol medium (YPGly) and incubated at 37°C for 2-3 days.

Fig. 5. Two-hybrid interaction of Maf1 domains is impaired by primary mutations in these domains and restored by second-site suppressor mutations. A. pACT2-Maf1-A(1-42) plasmid and its mutated version were transformed individually with pAS2-Maf1-BC(196-349) plasmid or its mutated versions in yeast strain Y190. Transformants were assayed for β -galactosidase expression. Presented profile compares following two-hybrid interactions: wild type A and BC domains of Maf1 (1), mutated A domain K35E with wild type BC domain (2); mutated A domain K35E with four versions of mutated BC domain: D250E (3), V260D-N344I (4), V260D (5), N344I (6), and wild type A domain with mutated BC domain containing single D250E, V260D or N344I and double N344I-V260D mutations (7-10). B. Scheme of Maf1 domain interaction.

Fig. 6. Mutations altering interaction of Maf1 domains affect kinetics of Maf1 dephosphorylation upon transfer of yeast from glucose to medium with nonfermentable carbon source. *maf1*-K35E, *maf1*-K35E-D250E and *maf1*-K35E-V260D-N344I mutant strains and isogenic wild type strain YPH500 (WT) were grown to exponential phase in YPD glucose medium (Exp), then transferred to glycerol YPGly medium, incubated at 37°C and harvested as indicated. Protein extracts of lysed cells were analyzed by Western blotting with polyclonal anti-Maf1 antibodies.

Fig. 1

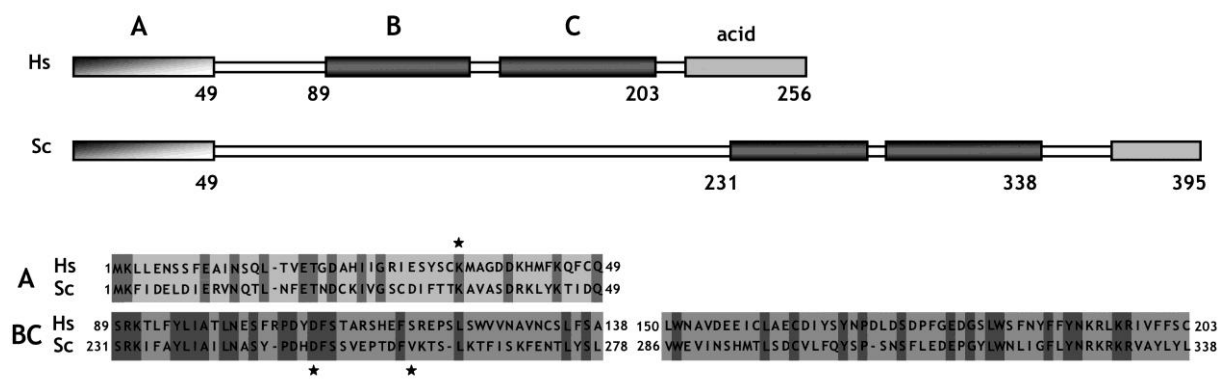


Fig. 2

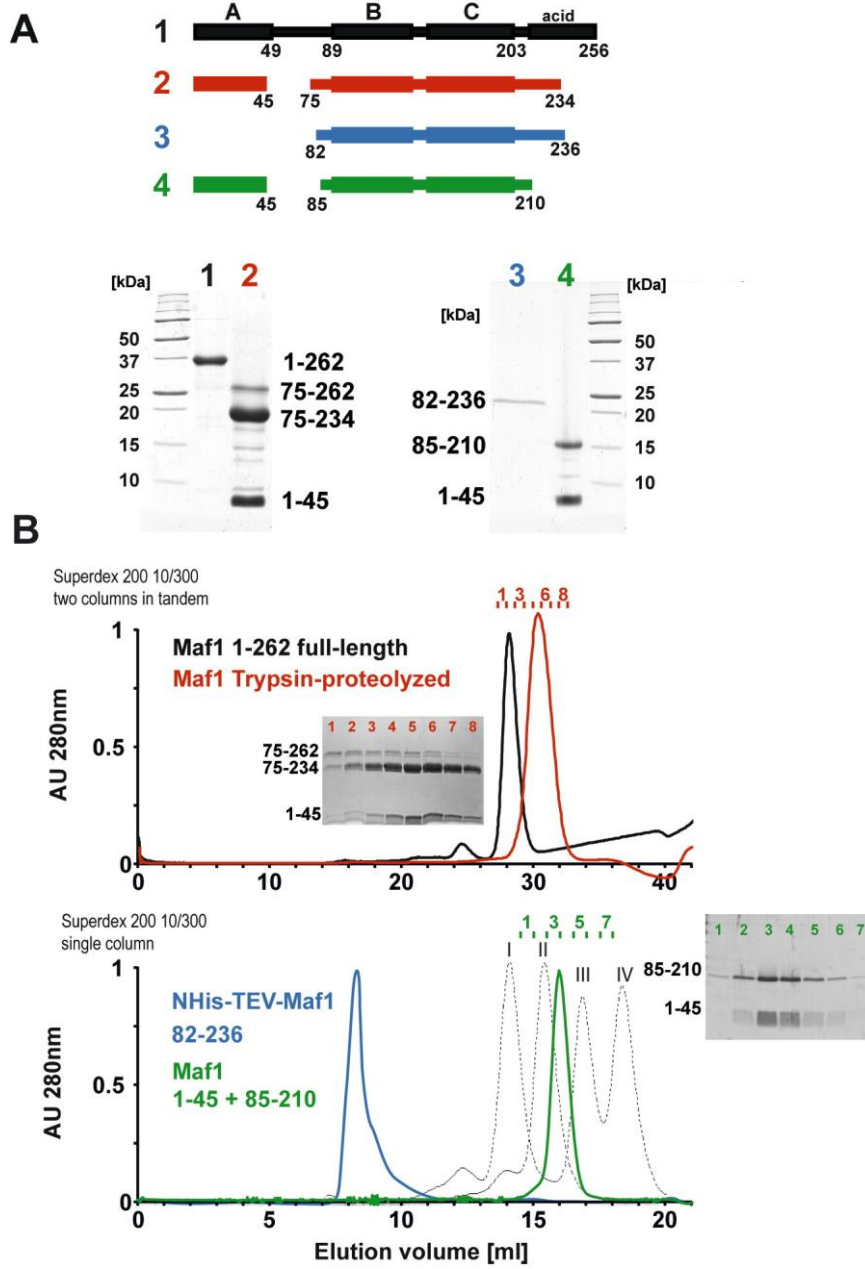


Fig. 3

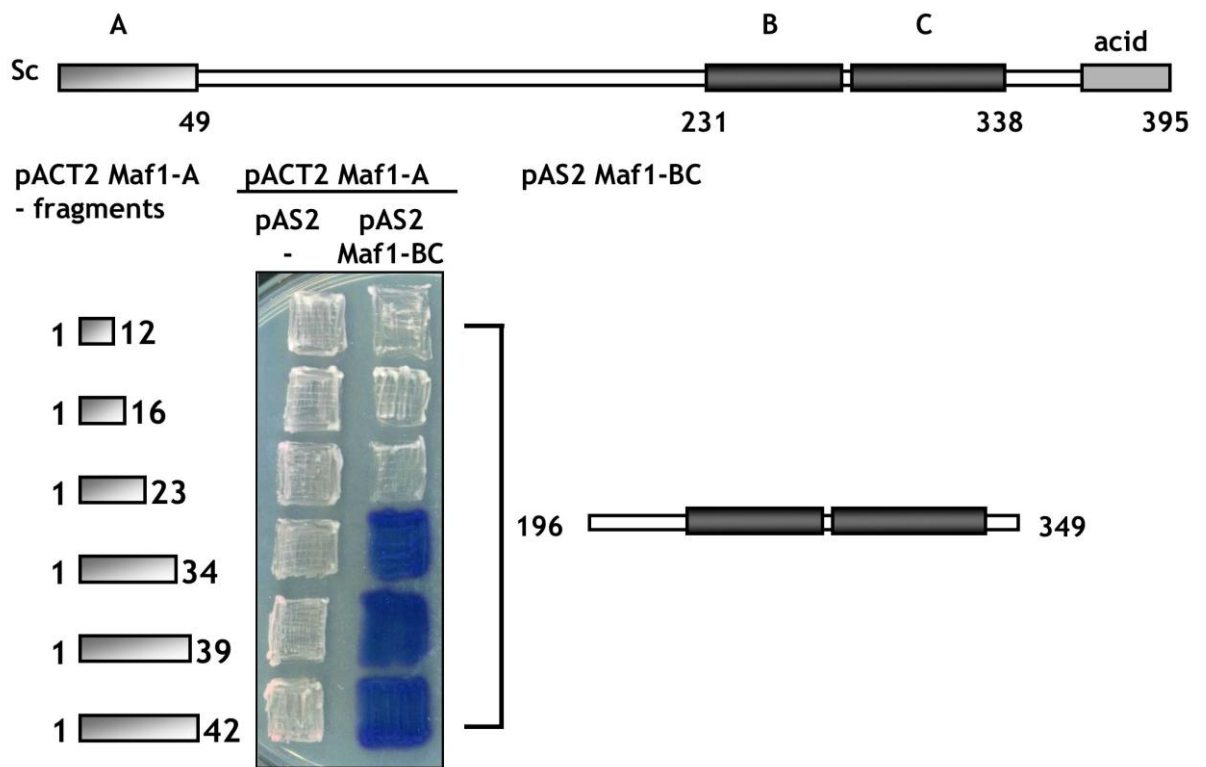


Fig. 4

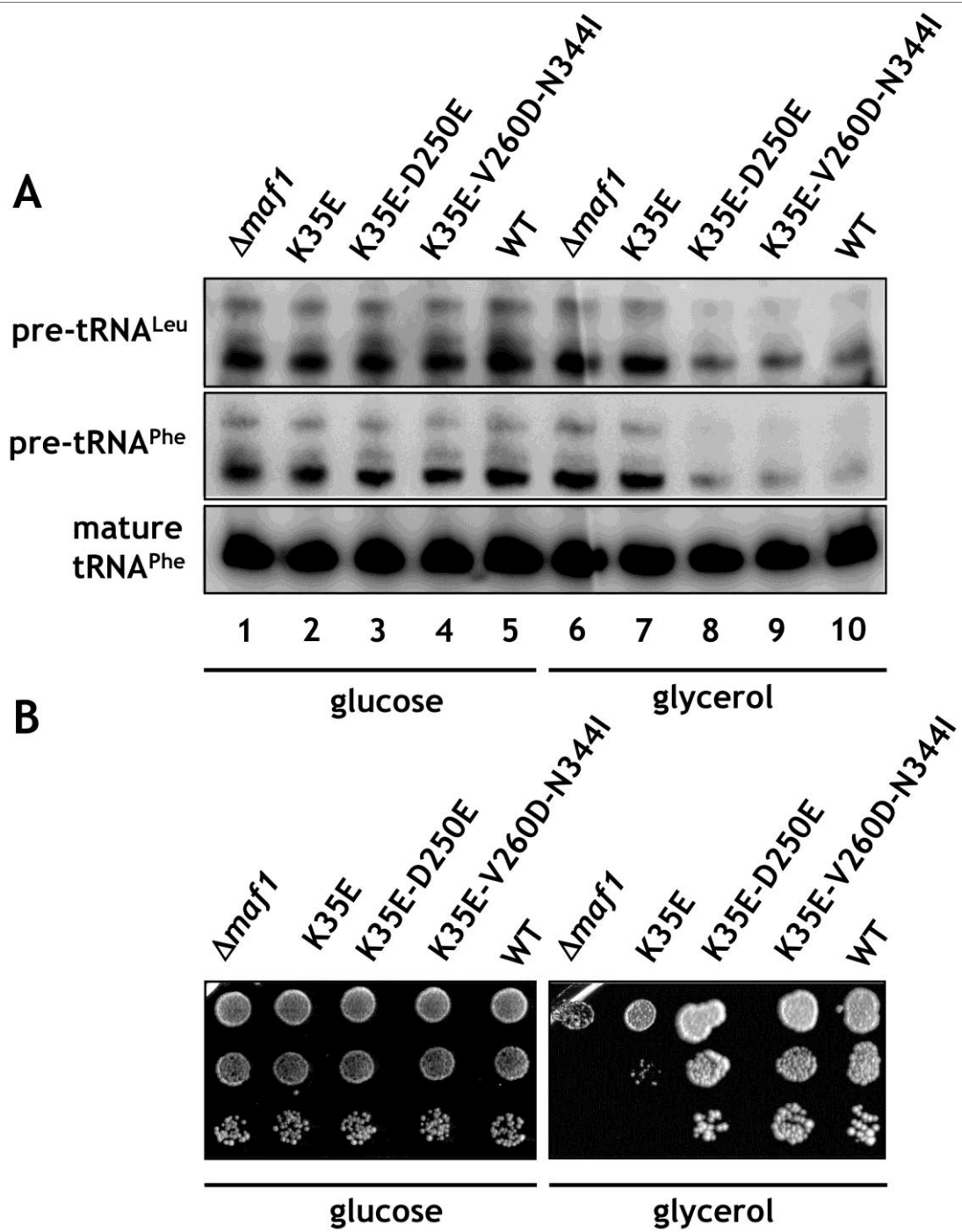
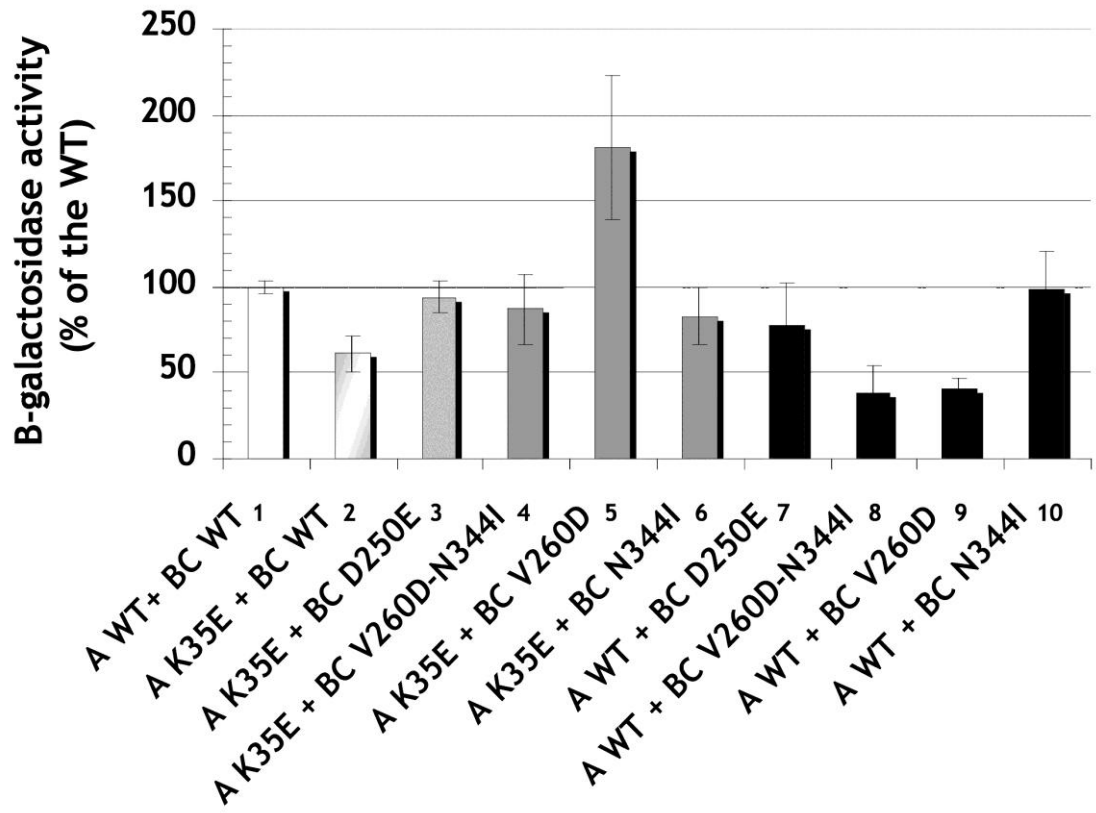


Fig. 5

A



B

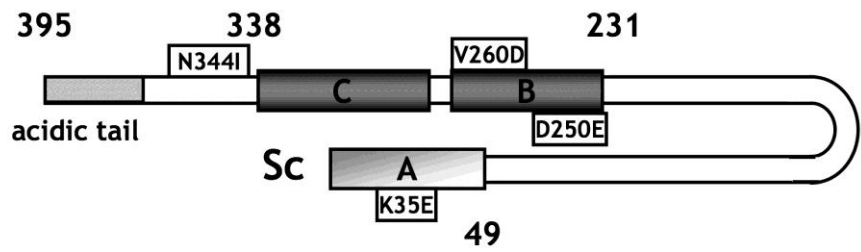


Fig. 6

