Two conserved modules of *Schizosaccharomyces pombe* Mediator regulate distinct cellular pathways

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ABSTRACT

Mediator is an evolutionary conserved coregulator complex required for transcription of almost all RNA polymerase II-dependent genes. The *Schizosaccharomyces pombe* Mediator consists of two disassociable components—a core complex organized into a head and middle domain as well as the Cdk8 regulatory subcomplex. In this work we describe a functional characterization of the *S. pombe* Mediator. We report the identification of the *S. pombe* Med20 head subunit and the isolation of ts alleles of the core head subunit encoding *med17*+.

Biochemical analysis of *med8*ts, *med17*ts, Δ*med18*, Δ*med20* and Δ*med27* alleles revealed a stepwise head domain molecular architecture. Phenotypical analysis of Cdk8 and head module alleles including expression profiling classified the Mediator mutant alleles into one of two groups. Cdk8 module mutants flocculate due to overexpression of adhesive cell-surface proteins. Head domain-associated mutants display a hyphal growth phenotype due to defective expression of factors required for cell separation regulated by transcription factor Ace2. Comparison with *Saccharomyces cerevisiae* Mediator expression data reveals that these functionally distinct modules are conserved between *S. pombe* and *S. cerevisiae*.

INTRODUCTION

The Mediator is a multiprotein coregulator complex that is required for the transcription of almost all RNA polymerase II (pol II)-dependent genes in fungi and metazoans (1). Mediator is thought to act as an interface between the general transcription machinery and sequence-specific transcriptional activators. A pure yeast *in vitro* transcription system consisting of pol II and all the general transcription factors (GTFs) cannot be stimulated by activators in the absence of Mediator (2,3).

Mediator was originally purified in *Saccharomyces cerevisiae* and has since then been isolated from several other eukaryotic species (1,4,5). These biochemical data revealed a core set of Mediator subunits that are conserved in most, if not all eukaryotes (6–8).

We have previously isolated and characterized the Mediator complex from *Schizosaccharomyces pombe* (6,9). The *S. pombe* Mediator exists in at least three states: a smaller core Mediator complex (S-Mediator) consisting of 15 subunits, a larger form (L-Mediator) consisting of core Mediator bound to a four-subunit module known as the Cdk8 module, and finally as a holoenzyme form with the core Mediator bound to pol II (9–11).

The Cdk8 module in both *S. cerevisiae* and *S. pombe* consists of four proteins: Med12 and Med13 as well as the cyclin-dependent kinase Cdk8 and its cyclin CycC (10,12). Both *S. cerevisiae* and *S. pombe* Cdk8 are able to phosphorylate the C-terminal domain of the largest subunit of pol II *in vitro*, which is thought to inhibit...
transcriptional initiation (10,13). Titration of the Cdk8 module-containing L-Mediator into an in vitro S. pombe transcription system has been shown to counteract the stimulatory effect of Mediator on basal transcription (14). Yet there is also evidence of a positive role for the Cdk8 stimulatory effect of Mediator on basal transcription (14).

The double null mutant module in activation (15,16). Yet there is also evidence of a positive role for the Cdk8

Isolation of med17*/mutants

A marker switch approach (27) was used for the construction of med17* mutants. The LEU2 gene was inserted in a PacI site at 159 bp 3' of med17* ORF in strain MP1 to generate TP384. The med17* gene from position 429 bp 5' to 212 bp 3' of the med17* ORF was cloned in pBluescript SK +/- and the 1.8-kb HindIII ura4* fragment of pREP42X (28,29) was inserted into the PacI site at 159 bp 3' of the med17* ORF to generate plasmid pTK1276. Mutagenesis of pTK1276 plasmid DNA was carried out with hydroxylamine (29) as follows: 10 µg of plasmid DNA was added to 500 µl ice-cold hydroxyamine solution (1 M H2NO-HCL, adjusted to pH 7 with NaOH) and incubated at 75°C for 90 min. Reactions were stopped by adding 200 mM NaCl, DNA was collected by EtOH precipitation and transformed in Escherichia coli strain DH5α. The med17*::ura4* fragment was exerted from a pool of mutagenized pTK1276 DNA with ApaLI and PacI and transformed into strain TP384. Ura+ transformants were selected on AA-Ura plates and replica-plated onto YES plates. After 3 days of incubation at 37°C, candidate colonies of temperature-sensitive (ts) mutants were isolated and tested for loss of the LEU2 gene on AA-Leu plates. Screening of 10000 yeast transformants for a ts phenotype at 37°C yielded four independent mutant strains with a ts phenotype at 37°C on YES plates.

Construction of the med17Δ50 allele

One hundred and fifty nucleotides from position 1601–1751 of the med17* ORF was deleted from plasmid pTK1276 introducing a Nhel site 3' of the stop codon in the process to generate plasmid pTK1439. The med17Δ50::ura4* sequence was exerted from pTK1439 with ApaLI and PacI and transformed into diploid strain TP384/MP2. Ura+ transformants were selected on AA-Ura plates and sporulated into AA-Ura spores were tested for a ts phenotype at 37°C and an ura4* leu2* med17Δ50 spore called TP390 isolated. The ura4* gene was removed from the med17Δ50 strain TP390 by transforming with an 812-bp fragment spanning the ura4* gene but not running into the med17* ORF followed by selection on 5-FOA plates giving strain TP392.

TAP-tagging of med7* in strain TP390 (med17Δ50) and strain MP10 (sep15-598/med8*)

The C-terminal part of the med7* gene fused to a TAP-tag was exerted from plasmid pFAd6a-kanMX6-CTAP2-spm7 (10) with AvrII and EcoRV and transformed into the med17Δ50 strain TP392 and sep15-598/med8* strain.

MATERIAL AND METHODS

Schizosaccharomyces pombe strains

All yeast strains used in this study are listed in Table 1. Schizosaccharomyces pombe cells were transformed by the lithium acetate procedure (26). Null mutants of med15*, med18* and med20* for expression profiling were generated using the kanMX selectable marker in the haploid h+ strain MP9 as described (6) using the primers listed in Supplementary Table S1. The double null mutant of med18* and med20* was constructed by crossing and tetrad analysis of the diploid TP396/TP235. Null mutants of med18*, med20* and med27* for protein purification were generated with the ura4* gene as a selectable marker in an Ura- background. A plasmid containing the wild-type ura4* gene was amplified and inserted into either side of the ura4* marker to generate the plasmids pURA4-spmmed20 and pURA4-spmmed27, respectively. The knockout fragments were released from the respective plasmids by digesting with PvuII and transformed into the diploid strain TP219/TP220 carrying a Tandem Affinity Purification (TAP) tag on both copies of the med7* gene. The obtained Ura+ diploids were sporulated and Ura+/G418-resistant spores were recovered, to generate strains TP416, TP417 and TP306, respectively.
MP10 giving strains TP315 and TP308, respectively. Correct integration of constructs in the genome was verified by PCR analysis. The MP10 strain was a kind gift from M. Sipiczki (University of Debrecen, Hungary). In all cases, tetrad analysis and/or Southern blot analysis were applied to demonstrate that only one copy of the construct had integrated in the genome.

**Saccharomyces cerevisiae strains**

All *S. cerevisiae* deletion strains were purchased from EUROSCARF. Deletion cassettes of the MED20 and MED18 genes were generated by amplifying the kanMX cassette from EUROSCARF deletion strains BY0661 (MED20) and BY04734 (MED18) with 500-bp flanking sequence on either side of the kanMX marker. Saccharomyces cerevisiae TAP-MED8 cells were transformed with the amplified deletion cassettes using the lithium acetate procedure. Correct genomic integration was assayed by PCR using primers positioned at the very 5’ end of either the upstream or coding region and conversely outside the 3’ flanking regions of the transformation constructs. The TAP-MED8 strain (12) was a kind gift from Roger Kornberg (Stanford University, CA, USA).
Protein purification

Cells were grown at 30°C unless otherwise specified. *Saccharomyces cerevisiae* cells were grown in YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) to OD _600_ 3.5–4.0, and *S. pombe* cells were cultured to OD _600_ 2.0–2.5 in yeast extract supplement (YES) medium (5 g/l yeast extract, 2 g/l casamino acids, 20 g/l glucose) supplemented with 0.2 g/l adenine. For preparation of whole-cell extract, we collected yeast cells from a 10 l culture by centrifugation (Beckman Instruments JLA-10 500 rotor, 9500 r.p.m., 10 min, 4°C), which were washed once with ice-cold water and suspended in 0.5 ml of 3× lysis buffer (200 mM HEPES-KOH, pH 7.8, 15 mM KCl, 1.5 mM MgCl _2_, 0.5 mM EDTA, 0.5% IGEPAL CA-630, 0.5 mM dithiothreitol and protease inhibitors) per gram of cell pellet. For all purifications, we used a 100× stock of protease inhibitors containing 100 mM phenylmethyl-sulfonyl fluoride, 200 mM pepstatin, 60 mM leupeptin and 500 μM dithiothreitol (Sigma). Antibodies specific for *S. cerevisiae* Mediator subunits Med1, Med2, Med8 and Med11 were kind gifts from Stefan Björklund (Umeå University, Sweden). Antibodies against *S. cerevisiae* Med18 and Med20 were kind gifts from R.A. Young (Whitehead Institute for Biomedical Research, Cambridge, MA, USA).

Affymetrix GeneChip probe array hybridization

*S. pombe* strains were grown in EMM medium at 30°C until 5×10^6 to 1×10^7 cells/ml. Heat-stressed wild-type and med17Δ50 cells were cultured in EMM at 30°C until 5×10^8 to 1×10^9 cells/ml, spun down briefly and then resuspended in media pre-warmed to 37°C. Cells were harvested after 2 h of incubation at 37°C. Total yeast RNA was isolated using a hot acid phenol extraction protocol (33). Labeling and array hybridization to Affymetrix Yeast Genome 2.0 arrays were performed at the Karolinska Institute Affymetrix core facility (Huddinge, Sweden). Labeling and hybridization protocols are described in the Affymetrix users’ manual (34). Washing and staining of arrays were performed using the GeneChip Fluidics Station 450. Arrays were scanned with the Affymetrix GeneArray scanner 3000 7G. Acquisition and quantification of array images as well as primary data analysis including normalization was performed using Affymetrix GeneChip Operating Software (GCOS). Data were processed with the MAS5 algorithm (scaling: all probe sets with target signal 100, normalization: user defined with normalization value 1). The expression profiling was done on two sets of samples. The first set contained transcript level data from three wild-type (MP9) cultures and three cultures of each of the *cdk8*Δ and *amed12, amed15, amed18* and *amed20* strains. The second set contained transcript level data from two *med17Δ50* mutant cultures and two corresponding wild-type (MP1) cultures. To rule out the possibility of global changes to the transcriptome in the Mediator mutants, all datasets were normalized to an internal housekeeping control mRNA. Through quantitative real-time PCR (see below) we established that the atp1 gene displayed <1.5-fold change in all mutants when normalized to 28 S rRNA levels and was therefore chosen as the internal mRNA control. Within each set of samples the transcript signal intensities was compared to all the corresponding wild-type baseline controls, generating a total of nine comparisons for each mutant in the first series. The Affymetrix change-call algorithm designated every transcript in each
RESULTS

Biochemical characterization of the *S. pombe* Mediator head domain

Mass spectrometric analysis of partially purified *S. pombe* Mediator identified the hypothetical protein SPAC17G8.05 (GenBank accession NP_593728) as a potential Mediator subunit with an apparent mass of around 20 kDa (data not shown). SPAC17G8.05 has previously been predicted to be the *S. pombe* homolog of *S. cerevisiae* and mammalian Med20 (7). To demonstrate its stable Mediator association, *S. pombe* Mediator was purified with TAP-tag on the Med8 subunit and resolved by Heparin–Sepharose chromatography. Western analysis showed co-elution of Med20 with Med17 and Med27 (Figure 1A). Thus, Med20 is a stable subunit of *S. pombe* Mediator.

In *S. cerevisiae*, Med20 and Med18 form a distinct submodule within the head domain (24,25,40). Med20 is completely lost from *S. cerevisiae* Mediator isolated from a *med18* strain (Figure 1B, right lane). However, we could still detect reduced amounts of Med18 associated with Mediator isolated from a *amed20* strain (Figure 1B, middle lane).

To establish whether the *S. pombe* head domain is organized as that of *S. cerevisiae* diploid strains heterozygous for either *amed18* or *amed20* were made. After tetrad analysis, viability segregated 4:0 showing that both med18+ and med20+ are nonessential genes in *S. pombe* like their *S. cerevisiae* homologs. Both *amed18* and *amed20* *S. pombe* cells have a slow growth phenotype and fail to sporulate (data not shown) (41). We purified Mediator from strains lacking either med18+ or med20+, respectively, utilizing a TAP-tag on Med7 (Figure 1C). In agreement with our observations in budding yeast, *amed20* *S. pombe* Mediator contained reduced amounts of the Med18 subunit, whereas mutant *amed18* Mediator completely lacked Med20 after purification over IgG–Sepharose. In addition, both the *amed18* and *amed20* mutant Mediator lacked the Med27 subunit. No homolog of Med27 has been identified in the *S. cerevisiae* Mediator. Cells lacking med27+ (systematic name SPAC17C9.05c) are viable without any apparent phenotype at 30°C (data not shown) (6). Mediator purified from *amed27* TAP-med7+ cells still contained both Med18 and Med20 (Figure 1D, middle lane) consistent with the wild-type growth phenotype at 30°C of *amed27* cells. This suggests that Med27 is located proximally to the Med18/Med20 dimer on the periphery of the head domain.

A ts allele of the essential head domain subunit Med8 called *sep15-598* (or *med8ts* for clarity) has been isolated (42–44). *Schizosaccharomyces pombe* cells carrying the *med8ts* allele display a similar growth phenotype as *amed18* cells suggesting that this allele could affect subunit interactions within the head domain (42). Western blot analysis revealed that Mediator purified from a *med8ts* TAP-med7+ strain had lost Med18, Med20 and Med27 (Figure 1D, right lane). Furthermore, Med8 interacts with Med20 in a yeast two-hybrid interaction analysis (data not shown). Thus, our observations suggest a step-wise structural organization of the nonessential *S. pombe* Mediator.
head domain subunits where Med27 is found on the very exterior connected to Med20, which in turn contacts Med18 that binds Med8 (Figure 1E). This model is in agreement with recent structural data of a Med8-Med18-Med20 trimer in *S. cerevisiae* (40).

Isolation and characterization of *S. pombe med17* ts alleles

The *S. cerevisiae* Med17 subunit is located in the head domain and essential for cell viability (20,24,45) reflecting its importance as a scaffold for the remaining head domain subunits (22,25). The ts allele of *S. cerevisiae* MED17 termed *srb4-138* was instrumental in demonstrating the universal requirement for Mediator in pol II-dependent transcription *in vivo* (45,46). The med17+ gene is also essential in *S. pombe* (6). We constructed a conditional med17 allele in *S. pombe* to understand Mediator function in fission yeast. We employed a marker switch approach (27) to establish a ts allele by targeted hydroxylamine mutagenesis at the med17+ locus and isolated four independent mutant strains with a ts phenotype at 37°C on YES plates. All four med17 mutants had acquired base changes in the C-terminal part of the med17+ coding sequence resulting in premature stop codons within the last 50 C-terminal amino acids of the Med17 protein. We considered the possibility that low levels of read-through of the premature stop codon could still produce full-length Med17 protein and that a de facto C-terminal truncation would be lethal. Thus, we constructed a strain lacking the last 50 C-terminal amino acids and termed this med17
allele med17Δ50 allele, which has a ts phenotype indistinguishable from the four previously isolated med17Δts alleles.

_Saccharomyces cerevisiae_ cells with the srb4-138 allele immediately stop growing after a shift to 37°C (45). _Schizosaccharomyces pombe_ med17Δ50 cells displayed a slightly more gradual response when heat stressed. After shifting a mid log culture of _S. pombe_ med17Δ50 cells growing at 30°C to 37°C an initial decrease of growth rate after 2.5 h was observed with growth ceasing altogether after 7.5 h (Figure 2A). The same pattern was obtained with the isolated med17Δts alleles (data not shown). As the _S. pombe_ med17Δ50 allele appeared subtly different from the _S. cerevisiae_ srb4-138 allele, we isolated total RNA from med17Δ50 cells and wild-type controls cultured at 30°C as well as 2 h after a shift to 37°C. In _S. cerevisiae_ srb4-138 cells, transcription ceases abruptly with most mRNA transcripts gone after 1 h (45). In contrast, northern blot analysis of heat-shocked _S. pombe_ med17Δ50 cells show no detectable decrease in mRNA levels even after 9 h at 37°C (Figure 2B).

Mediator was purified from _S. pombe_ med17Δ50 TAP-med7+ strain grown at 30°C in order to characterize its subunit composition. Western blotting of the TEV eluate revealed that the head module proteins Med17 and Med27 were completely lost while the amounts of Med18 and Med20 were severely reduced (Figure 2C). This drastic destabilization of _S. pombe_ med17Δ50 Mediator was reminiscent of _S. cerevisiae_ srb4-138 Mediator, which fractures at the head/middle domain boundary irrespective of growth temperature (19). We repeated the purification, culturing the med17Δ50 cells at 22°C and in this case Med17 was...
present in the med17Δ50 Mediator (Figure 2D). The head module subunit Med18 was still present whereas both Med20 and Med27 were lost from the mutant complex. This suggested that Med17 could be interacting with Med20 and Med27 independently of Med18. In support of this notion, yeast two-hybrid interaction analysis revealed that Med17 has physical contacts with Med20 and Med27 (data not shown). We have no two-hybrid data regarding interactions between Med17 and Med18. Direct interactions between Med17 and Med20 have also been reported previously in S. cerevisiae Mediator (22,24). Thus, we suggest the structural organization of the S. pombe head domain subunits depicted in Figure 1E with Med27 situated on the exterior connected to Med20, which contacts Med18 that ultimately binds Med8. Med17 could serve as a scaffold interacting with Med8, Med20 and Med27 could not serve as a scaffold interacting with Med8, Med20 and Med27.

Phenotypical classification of head and Cdk8 module Mediator mutant alleles

To discern functional subdivisions among Mediator subunits, we investigated the phenotypes of null alleles of med1+, med12+, med18+, med20+, med27+, med31+ as well as the SPBC146.01 gene encoding the proposed MED15 homolog. Deletion of med15+ in a diploid followed by tetrad analysis showed that med15+ is nonessential in S. pombe. We included the dead-kinase mutant allele of cdk8+ called cdk8mut (11) as well as med8ts (42) and med17Δ50. All mutants displayed one of two specific cellular morphologies—flocculation or hyphal growth (Figure 3A). Flocculation signifies the aggregation of yeast cells into larger clumps (47) and has previously been observed in Acd8, Amed12 and Amed13 cells (10,48). Hyphal growth occurs when cells are unable to cleave the primary septum that separates two daughter cells following medial septation at the end of mitosis (49). This phenotype had previously been observed in ts alleles of med8+, med18+ and med31+ (41,44). The cellular morphology of the head domain mutants med17Δ50, Amed18 and Amed20 corresponded to the hyphal growth seen for the med8+ ts allele sep1-598. The Amed27 mutant did not have a visible phenotype at 30°C, but showed some septation defects at 37°C (Figure 3A). A complete deletion of middle domain subunit med31+ also displayed a hyphal growth phenotype in agreement with previous report (41). Interestingly, the Amed15 mutant also showed this phenotype. We included the two null mutants of G2/M transcription factors Ace2 and Sep1 for comparison, both of which display a severe septation defect (50–52). The Cdk8 module mutants flocculated, but showed no septation defects. Mutants lacking the med1+ gene did not have a visible phenotype at 30°C, but flocculated at 37°C. In summary, we observed a clear distinction in cellular morphology between Cdk8 module mutants (and the associated middle domain subunit Med1) and those of the head domain as well as of middle domain subunit Med31 and the potential Mediator subunit Med15.

We observed varying degrees of severity in the hyphal growth phenotype in the head domain mutants, Amed15 and Amed31. A spot assay was carried out to further discern the magnitude of growth inhibition in the septating mutants (Figure 3B). We included Ace2 and Sep1 for comparison. Cells were challenged with elevated temperatures (37°C), high salt (750 mM KCl) and 1% formamide, respectively. The Amed27 mutant appeared nearly indistinguishable from wild-type cells at all conditions tested. The Amed20 mutant was slightly more sensitive than wild type at high salt, but was very sensitive to heat and formamide. The remaining head domain mutants med8ts, med17Δ50, Amed18 as well as a Amed18 Amed20 double mutant grow very poorly at all conditions tested. Thus, in these assays Amed18 is epistatic to Amed20. Equal degree of sensitivity was observed for the Amed31 and Amed15 mutants. The two mutants Ace2 and Sep1 were indistinguishable from wild type indicating that it was not the hyphal growth defect in itself that caused slow growth and stress sensitivity in the Mediator mutants. The Cdk8 module mutants were indistinguishable from wild type under all conditions tested (data not shown).

Expression profiling of Mediator mutants

We asked whether the distinct morphological phenotypes of Mediator mutants reflected distinct effects on global gene transcription. Hence, we performed expression profiling on six mutant alleles that we considered a representative cross section of the S. pombe Mediator. Global expression profiles of mutant alleles in conjunction with hierarchal clustering analysis can be used in an analogous way to phenotypes as a way to discern functional relationships (53).

We made two sets of comparisons. The first set of comparisons consisted of three independent cultures of each of the mutant strains cdk8mut (TP274), Amed12 (TP27), Amed15 (TP130), Amed18 (TP126) and Amed20 (TP234) as well as a wild-type (MP9) control. The second set of comparisons consisted of two independent med17Δ50 cultures (TP392) with two wild-type (MP1) controls. We employed the Affymetrix platform and the number of genes changing in each mutant by a defined ratio threshold is shown in Figure 4B.

We performed hierarchal clustering (54) on all genes changing 2-fold or more in the mutant datasets. The robustness of the clustering was tested by bootstrap analysis of 10,000 replicates with re-sampling of genes. The Cdk8 module mutants were clearly separated from the head domain subunits Med17, Med18 and Med20 (Figure 4A) as illustrated by the 100% bootstrap support of the Cdk8 module clade. The Amed15 mutant clustered within the head domain clade (Figure 4A), which is in agreement with previous results from S. cerevisiae Mediator (53). The genes that did change 2-fold or more belonged to diverse set of functional groups with no particular group of genes dominating any one expression profile—a reflection of the central role of Mediator in all pol II-dependent transcription. In addition, despite the significant correlation between expression profiles shown Figure 4A, each profile contained a set of genes particular
to each mutant that showed a 2-fold or more change in transcript levels—in total 531 genes.

**Mediator mutations elicit a partial stress response**

Expression profiling of *S. cerevisiae* Mediator mutants have reported the increase of stress response gene transcript levels (46,53,55). We investigated whether this is also the case for *S. pombe* Mediator mutants. The central environmental stress response (CESR) in *S. pombe* consists of a set of genes whose transcript levels consistently increase or decrease during various forms of stress (38). The Affymetrix array platform used in our study contained 134 of the induced CESR genes.

We studied the occurrence of CESR genes above a defined fold change threshold and then calculated *P*-values using the hypergeometric probability distribution. We applied a 2-fold ratio change threshold for the *cdk8mut*, *Δmed12*, *Δmed18* and *Δmed20* datasets, while the large number of genes changing in the *Δmed15* and *med17Δ50* datasets required us to apply a 3-fold threshold in order to allow computation. The induced set of CESR genes were significantly overrepresented in the genes increasing their transcript levels above the threshold in all six datasets (Table 2, *P* < 0.001). In some mutants we also observed a significant overlap between downregulated genes and the genes belonging to the repressed set of CESR genes (data not shown). We confirmed the elevated transcription levels...
Figure 4. Genome-wide expression analysis of nonlethal *S. pombe* Mediator alleles. (A) Heatmap cluster diagram of the 893 transcripts that change 2-fold or more in at least one of the mutants. Experiments and genes were hierarchically clustered according to average Pearson correlation linkage. The robustness of the experimental dataset clusters were analyzed by bootstrap analysis in MeV using 10,000 replicates. Numbers on branches indicate the range of bootstrap values expressed as percentages. (B) Bar diagram showing the number of genes changing 2-fold or more (yellow bars) and 10-fold or more (red bars) in each mutant strain. (C) Quantitative real-time PCR analysis of SPAC4H3.08 transcript levels of the mutant strains indicated as compared to wild-type cells. SPAC4H3.08 levels were normalized to 28S rRNA. Error bars indicate 1 SD. (D) Quantitative real-time PCR analysis of SPBC1289.14 levels as described in (C).
of the two induced CESR genes SPAC4H3.08 and SPBC1289.14 by quantitative real-time PCR analysis (Figure 4C and D). These genes are known to become induced 10-fold or more following heat shock and hydrogen peroxide treatment, respectively (38). Although all strains tested showed at least a 2-fold increase in transcript levels, a substantially higher degree of upregulation of these two genes were observed in the *Amed15* mutant closely followed by the *Amed18* mutant. These observations taken together suggest a central role for Mediator in the regulation of some CESR genes. The actual molecular mechanism of this regulation remains to be elucidated.

**Effects on genes involved in cell wall organization and metabolism**

We looked specifically at genes annotated in the *S. pombe* genome database as being involved in cell wall metabolism to explain the clear morphological differences between mutants of the Cdk8 module and those of the core Mediator. Specifically, we investigated genes known or proposed to be involved in the process of cell-to-cell adhesion as well as postmitotic septum dissolution. We could discern two major groups of genes with distinct expression patterns. The first group consisted of a family of genes that code for putative cell surface adhesins (56). These adhesins displayed increased transcript levels in the *cdk8*/*mut* and *Amed12* strains, but were largely unchanged in the head domain mutants (Figure 5A, orange bar). The increased transcript levels of these genes agree with the flocculation phenotype observed in *S. pombe* Cdk8 module mutants (Figure 3A) (10,48). The majority of the adhesin genes displayed only moderate increases in transcript levels (between 2- and 5-fold). One adhesin however, the DIPSY domain-containing SPAC186.01 gene, was upregulated nearly a 100-fold in the *cdk8*/*mut* strain as assayed by quantitative real-time PCR (Figure 5B). This corresponded well with the stronger flocculation phenotype in this strain as compared to the somewhat milder phenotype observed for the *Amed12* mutant (data not shown). Some of the other members of the *S. pombe* adhesin family that displayed more moderate increases in transcript levels are related to the *FLO1*, *FLO5* and *FLO9* genes of *S. cerevisiae* (56). The *FLO* genes are known to be upregulated in *S. cerevisiae* Cdk8 module mutants as well (46,53,55) and we conclude that the role of the Cdk8 module as a repressor of Flo-like adhesins appears conserved between *S. pombe* and *S. cerevisiae*.

The second group (Figure 5A, green bar) consisted of genes whose transcript levels were significantly decreased in the *Amed15* and head domain mutants, but with no significant change in the Cdk8 module mutants. This group included genes regulated by the transcription factor Ace2 as well as *ace2* itself (37,51,57). These genes are expressed late in the cell cycle and involved in the dissolution of the primary septum separating the newly formed daughter cells (51,57). Cells lacking these genes display septation defects. The Sep1 forkhead transcription factor regulates *ace2* expression (37,57). Sep1 precedes Ace2 in the regulatory pathway and the *sep1* gene was not affected by any of the mutants tested, suggesting that Sep1 requires the head domain subunits for *ace2* transcription.

We also compared our profiles with published datasets of ts alleles of *med8*/*+* and *med31*/*+* (36). In agreement with their hyphal phenotypes only the Ace2-regulated genes were affected in these two mutants. Likewise, *Ace2* and *Sep1* expression data (37) did not exhibit any significant changes in expression of surface adhesins indicating that the Cdk8 module does not play a role in the expression of Ace2-dependent genes. The *ace2*/*-regulated* *eng1* gene encodes an endoglucanase that is required for the dissolution of the primary division septum following mitosis, and its deletion leads to a septation phenotype similar to that of *Ace2* and *Sep1* cells (51). We performed quantitative real-time PCR analysis of *eng1* transcript levels in the Mediator mutants and the *Ace2* and *Sep1* strains to directly compare the magnitude of decrease in this particular gene. We observed that the decrease in the head domain mutants and *Amed15* was equivalent to that of the *Ace2* and *Sep1* mutants (Figure 5C). We therefore conclude that the *S. pombe* Mediator head domain and Med15 subunit play a crucial role in the transcription of *ace2*/*+/sep1*/*+*-dependent genes. We would like to point out that this analysis was done on asynchronous cells and therefore does not reveal any potential effect on the timing of cell cycle-dependent expression patterns.

Degradation of the primary septum in *S. cerevisiae* is controlled in part by the *ACE2* gene, the homolog of *S. pombe* *ace2*/*+* (58,59). However, primary septum dissolution and cell separation is fundamentally different and a distinct requirement of the *saccharomyces cerevisiae* *ACE2-AMN1CTS13, DSE2* and *DSE4/ENG1* (58,59) all displayed decreased transcript levels in head and tail domain mutants compared to wild-type cells when grown in rich medium (Figure 6). Analysis of previously published expression data for *S. cerevisiae* Mediator mutants grown in minimal media revealed that this effect can be observed in other *ACE2*-regulated genes as well (53). We therefore conclude that the requirement of the head and tail domain for Ace2-dependent expression appears conserved between *S. pombe* and *S. cerevisiae*.
DISCUSSION

In this study, we use a combined genetic and biochemical approach to perform a functional characterization of the *S. pombe* Mediator complex. Our data suggest a head module architecture with a stepwise structural organization: Med27 located on the very exterior connected to Med20, which in turn contacts Med18 which binds Med8 (Figure 1E). Biochemical characterization of the *med17Δ50* allele suggested that Med17 may act as scaffold for the head domain subunits and this function requires an intact C-terminus of the Med17 subunit (Figure 2C and D).

Removal of Med27 does not affect the association of Med18 or Med20 with Mediator to any detectable extent (Figure 1D). In addition, *Δmed27* cells do not display visible phenotypes unless heat stressed (Figure 3A) and appear only marginally less stress tolerant than wild-type cells (Figure 3B). Removal of Med20 also caused the

![Figure 5](https://academic.oup.com/nar/article-abstract/36/8/2489/2409930)

**Figure 5.** Mediator mutations affect genes involved in cell wall organization and metabolism. (A) Expression profiles of genes involved in cell wall metabolism, cell-to-cell adhesion and degradation of the post-mitotic primary septum. The first group (orange bar) includes cell surface proteins proposed to be involved in cell-to-cell adhesion during stress. The second group (green bar) includes genes previously known to cause hyphal growth when deleted. The corresponding datasets from previously described studies of other Mediator mutants (16) as well as null alleles of *ace2*+ and *sep1*+ (37) are included for comparison. (B) Quantitative real-time PCR analysis of SPAC186.01 transcript levels of the indicated mutant strains as compared to wild-type cells. SPAC186.01 levels were normalized to 28S rRNA. Error bars indicate 1 SD. (C) Quantitative real-time PCR analysis of *eng1*+ levels as described in (B).
also observed that transcription does not shut down globally at the nonpermissive temperature in *S. pombe me* 

\[ \text{med}17A50 \] cells (Figure 2B). In addition, global chromatin immunoprecipitation of \[ \text{med}17A50 \] Mediator showed a clear pattern of genomic reorganization after heat shock rather than a global reduction of Mediator binding (63).

In this work, we addressed the function of the potential *S. pombe* Mediator subunit Med15. Our data clearly demonstrate a functional link between Med15 and Mediator. Specifically, the phenotype and transcript profile of \[ \text{med}15 \] cells links Med15 to the head domain and to the middle domain subunit Med31 (Figures 3A and 4A). Presently, we do not know whether Med15 is able to stably associate with Mediator.

A relatively large number of core environmental stress-related (CESR) transcripts changed significantly in the expression profiles of all six Mediator mutants analyzed in this study. We interpret these data as the result of either one of two scenarios. First, the mutation might perturb cellular homeostasis to such a degree that the stress response is triggered as a secondary effect of the respective mutation. Alternatively, Mediator components could act as direct regulators of the stress response and so by removal of the regulator, the transcriptional program is triggered inadvertently. Further work will be required to shed light on this observation. However, Cdk8 in *S. cerevisiae* is known to specifically phosphorylate transcription factors directly involved in stress gene regulation (64), which therefore also would implicate Mediator as a direct regulator of the cellular stress response.

Expression profiles of genes known to be involved in cell wall metabolism correlated very well with the aberrant cellular morphologies observed in *S. pombe* Mediator mutants (Figures 3A and 5A–C). The flocculation phenotype seen in Cdk8 module mutants was likely the result of overproduction of surface adhesins (Figure 5A and B), a typical stress response in yeast and regulated by stress-related pathways such as MAP kinase cascades (47). Deletion of Cdk8 module genes in *S. cerevisiae* also leads to flocculation (46,65,66), suggesting that regulation of adhesins is conserved between *S. pombe* and *S. cerevisiae*. Indeed, transcript levels of the *S. cerevisiae* adhesin-coding genes *FLO1*, *FLO5* and *FLO9* are significantly elevated in Cdk8 module mutants (53,67).

Conversely, the decreased expression of genes involved in the degradation of primary septum would explain the hyphal growth in head domain mutants and the \[ \text{med}15 \] strain. Although transcription was not completely abolished the cumulative effect of several downregulated genes might give rise to the hyphal growth. The process of septum dissolution to achieve complete cell separation is fundamentally different between *S. pombe* and *S. cerevisiae* (49,60). In *S. pombe* the primary septum consists of 1,3-β-glucan which is primarily degraded by the Eng1 endoglucanase (51) while in *S. cerevisiae* the primary septum is made up of chitin and is subsequently degraded by the Cts1 endochitinase (51). The cell wall material surrounding the septum, the so-called septum edging, also differs in its composition between the two species. In *S. pombe* it is mainly composed of 1,3-x-glucan that is degraded by the Agn1 endoglucanase (68). In *S. cerevisiae*, the septum edging is 1,3-β-glucan and degraded by the complete loss of Med27 as well as an apparent decrease in the amount of Med18 (Figure 1C). A corresponding increase in severity is observed both in the cellular morphology of \[ \text{med}20 \] cells (Figure 3A) as well as their stress tolerance (Figure 3B). Similarly, the \[ \text{med}18 \] phenotype was more severe than that of \[ \text{med}20 \] cells. A \[ \text{med}18 \] \[ \text{med}20 \] double mutant appeared indistinguishable from a \[ \text{med}18 \] strain at 30°C demonstrating that \[ \text{med}18 \] is epistatic to \[ \text{med}20 \]. This is what we would expect as Med20 is unable to associate with \[ \text{med}18 \] Mediator (Figure 1C). Consolidation of the biochemical data with the observed phenotypes indicated that the loss of Med18, which occurred in \[ \text{med}18 \] (Figure 1C) and \[ \text{med}8ts \] cells (Figure 1D) as well as \[ \text{med}17A50 \] cells cultured above room temperature (Figure 2C), appeared sufficient to cause the septation defect and stress hypersensitivity. The somewhat milder phenotype in \[ \text{med}20 \] cell may well be caused by a decrease in the number of Med18 containing Mediator in the \[ \text{med}20 \] cells (Figure 1C) rather than the loss of the Med20 subunit in itself.

The Med17 subunit has played a key role in establishing the almost absolute requirement for Mediator in pol II-dependent transcription in *S. cerevisiae* (45,46). The conditional *S. pombe* \[ \text{med}17A50 \] allele isolated in this study is different to the *S. cerevisiae* \[ \text{sr}bA-138 \] allele in several respects. First, the *S. pombe* \[ \text{med}17A50 \] allele is a C-terminally truncated form while the \[ \text{sr}bA-138 \] allele has a number of single nucleotide substitutions along the entire coding region (62). Second, we do not see an equally rapid growth arrest at the nonpermissive temperature in *S. pombe* (Figure 2A) as reported in the *S. cerevisiae* mutant (45). The growth arrest in *S. cerevisiae* appears definitely linked to the immediate cessation of pol II-dependent transcription as the Mediator complex comes apart (19). The effect on Mediator stability in *S. pombe* \[ \text{med}17A50 \] is less clear-cut with an increase in severity at higher temperatures (Figure 2C and D). We also observed that transcription does not shut down

Figure 6. The requirement of the Mediator head and tail domain for Ace2-dependent transcription in *S. cerevisiae*. Quantitative real-time PCR analysis of transcript levels of the indicated mutant strains as compared to wild-type cells. Transcript levels for all genes were normalized to *TUB1* in accordance with previous studies of *S. cerevisiae* Mediator mutants (53).
Eng1 endo-glucanase (61, 69). The process is regulated by the conserved Ace2 transcription factor in both species but most of the target genes are not conserved between _S. pombe_ and _S. cerevisiae_. We were therefore quite struck by the fact that the Mediator head domain and tail domain (only the Med15 subunit in _S. pombe_) were required for proper expression of Ace2-dependent genes in both _S. pombe_ and _S. cerevisiae_ despite the many differences in the process (Figures 5A and 6) (53). Even though the tail domain is not conserved in _S. pombe_, the Med15 protein still has an essential role to play in the Ace2-regulated septation cascade.

In summary, the data presented in this article suggest a structural model for the _S. pombe_ head domain and clearly defines two distinct functional classes of subunits within the _S. pombe_ Mediator complex (Figure 7). The first class consists of all four components of the Cdk8 module as well as middle domain subunit Med1. If deleted, this group all flocculate due to de-repression of cell surface adhesions, some of which may be involved in mating, stress response or adaptation to starvation conditions. The second class is composed of the head domain as well as middle domain subunit Med31 and the as yet unassigned Med15 protein. When mutated these genes cause septation defects leading to hyphal growth due to impaired expression of the Ace2-dependent genes involved in cell separation. Our results and previously published expression data indicate that the head domain, Med31 and Med15 are required for the proper expression of the ace2+ gene, possibly by the Sep1 transcription factor. Thus, our work identifies two distinct functional submodules of the _S. pombe_ Mediator that have conserved roles in the regulation of specific cellular pathways.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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