PhD thesis
Michela Venturi

The *Schizosaccharomyces pombe* Mediator.
Roles in heterochromatinization and analysis of additional subunits Med9/Med11

This thesis has been submitted to the PhD School of The Faculty of Science, University of Copenhagen

Academic advisor: Dr. Steen Holmberg
Submitted: 28/03/13
Preface

This PhD thesis represents the results of my PhD project carried out at the Transcription, Chromatin and DNA Repair Laboratory at University of Copenhagen, Department of Biology under the supervision of Dr. Steen Holmberg and as result of a collaboration with Dr. Claes M. Gustafsson’s Laboratory in Stockholm and Gothenburg, Sweden.

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A paper written in collaboration with Michael Thorsen, Heidi Hansen, Steen Holmberg and Genevieve Thon is also included.
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<td>GTFs</td>
<td>General Transcription Factors</td>
</tr>
<tr>
<td>HATs</td>
<td>Histone Acetylases</td>
</tr>
<tr>
<td>HDACs</td>
<td>Histone De-Acetylases</td>
</tr>
<tr>
<td>CTD</td>
<td>Carboxy Terminal Domain</td>
</tr>
<tr>
<td>SAGA</td>
<td>Spt-Ada-Gnc5-Acetyltransferase</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-Initiation Complex</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding Protein</td>
</tr>
<tr>
<td>CDK8</td>
<td>Cyclin-dependent Kinase 8</td>
</tr>
<tr>
<td>RNA Pol II</td>
<td>RNA Polymerase II</td>
</tr>
<tr>
<td>IDRls</td>
<td>Intrinsically Disordered Regions</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatine Immuno-precipitation</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co Immuno-precipitation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-Assisted Laser Desorption/Ionization-Time Of Flight</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatin Proteins</td>
</tr>
<tr>
<td>PTM</td>
<td>Post Translational Modifications</td>
</tr>
<tr>
<td>Cnt</td>
<td>Centromere</td>
</tr>
<tr>
<td>Imr</td>
<td>Innermost regions</td>
</tr>
<tr>
<td>TGS</td>
<td>Transcriptional Gene Silencing</td>
</tr>
<tr>
<td>PTGS</td>
<td>Post Transcriptional Gene Silencing</td>
</tr>
<tr>
<td>ARC</td>
<td>Argonaute siRNA Chaperone</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-Induced Silencing Complex</td>
</tr>
<tr>
<td>RITS</td>
<td>RNA-Induced Transcriptional Silencing complex</td>
</tr>
<tr>
<td>SHREC</td>
<td>Snf2/HDAC-containing complex</td>
</tr>
<tr>
<td>RDRC</td>
<td>RNA-dependent RNA Polymerase Complex</td>
</tr>
<tr>
<td>CLRC</td>
<td>Clr4-Rik1-Cul4 complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble NSF Attachment Protein Receptor</td>
</tr>
<tr>
<td>GOI</td>
<td>Gene of Interest</td>
</tr>
<tr>
<td>DV</td>
<td>Destination Vector</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>PC</td>
<td>Positive Control</td>
</tr>
<tr>
<td>NC</td>
<td>Negative Control</td>
</tr>
<tr>
<td>HphR</td>
<td>Hygromycin Resistance</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase – Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem Affinity Purification</td>
</tr>
<tr>
<td>XLMR</td>
<td>X-linked Mental Retardation</td>
</tr>
<tr>
<td>REST</td>
<td>RE1 Silencing Transcription Factor</td>
</tr>
<tr>
<td>TLM</td>
<td>Telomere Length Maintenance</td>
</tr>
<tr>
<td>PEV</td>
<td>Position Effect Variegation</td>
</tr>
<tr>
<td>TERRA</td>
<td>Telomeric Repeat-containing RNA</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>AA</td>
<td>All Addition</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylen diammino tetracetic acid</td>
</tr>
<tr>
<td>YES</td>
<td>Yeast Extract Supplemented</td>
</tr>
<tr>
<td>EMM</td>
<td>Edinburgh Minimal Media</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylendiamine</td>
</tr>
<tr>
<td>TGM</td>
<td>Tris Glycine Methanol</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemi-Illuminescence</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>α-dCTP</td>
<td>DeoxyCytidine Triphosphate</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium Chloride Sodium Citrate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
</tr>
<tr>
<td>WCE</td>
<td>Whole Cell Extract</td>
</tr>
<tr>
<td>TBZ</td>
<td>Thiabendazole</td>
</tr>
<tr>
<td>6-AU</td>
<td>6-azauracil</td>
</tr>
<tr>
<td>TCA</td>
<td>TriChloroacetic acid</td>
</tr>
<tr>
<td>PVDF</td>
<td>PolyVynilidene Difluoride</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Ecth Virus</td>
</tr>
<tr>
<td>FT</td>
<td>Flow Through</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
</tbody>
</table>
English abstract

In the past several years great attention has been dedicated to the characterization of the Mediator complex in a different range of model organisms. Mediator is a conserved co-activator complex involved in transcriptional regulation and it conveys signals from regulatory transcription factors to the basal transcription machinery. Mediator was initially isolated from *Saccharomyces cerevisiae* based on its ability to render a RNA polymerase II *in vitro* transcription system responsive to activators. Additionally, structural studies have revealed striking structural similarities between *S. cerevisiae*, *Schizosaccharomyces pombe* and mammalian Mediator.

In our study, we have taken the *S. pombe* Mediator into consideration and characterized genetically and biochemically two subunits already known in *S. cerevisiae*, Med9 and Med11, but still not identified in the *S. pombe* Mediator. Genetic analysis has shown that med9+ is a non-essential gene, while deletion of med11+ resulted in unviable cells. These results are in line with those obtained in *S. cerevisiae*. Isolation of *S. pombe* Mediator by the tandem affinity purification method and Co-IP experiments lead to the conclusion that Med9 and Med11 might not belong to the Mediator complex, but our results did not exclude it completely either. Our attempts to demonstrate the presence of these two subunits in the Mediator complex remain inconclusive primarily due to the lack of proper expression of the tagged versions of the proteins. However, we have paved a way to further experiments that might shed light on the structure of the *S. pombe* Mediator.

*S. pombe* is a well known and used model organism for heterochromatin pathway studies. *S. pombe* has large and complex centromeres comprising a central core surrounded by inner and outer repetitive sequences. Silencing and heterochromatinization of the repeats depend on the RNA interference (RNAi) pathway. RNAi relies on transcription of the centromeric repeats by RNA Pol II. Centromeric transcripts are processed into siRNA by the RNAi machinery, leading to the recruitment and accumulation at centromeres of several interacting protein complexes and histone-modifying enzymes. Consistent with transcription being performed by RNA Pol II, centromeric transcripts are poly-adenylated and specific mutations in RNA Pol II subunits impair heterochromatin formation. The involvement of RNA Pol II in heterochromatin assembly indicates that the Mediator complex may also play a role in heterochromatin biology.

This feature allowed us to carry out several studies, taking three Mediator mutants into consideration: Δmed9, Δmed12, and Δmed18. We analyzed these Mediator deletion mutants in relation to the transcriptional regulation of centromeric repeats and thus in heterochromatin formation, centromere function and chromosome segregation. The mitotic loss of a non-essential mini-chromosome was affected in a Δmed18 strain. Also, our data demonstrated that Med18 regulates transcriptional repression of the centromeric repeats thereby affecting the functionality of
the centromeres. The same results were obtained by Thorsen et al. (*Epigenetics.Chromatin.*, 5, 19, 2012) using a different strain background. Moreover, we also observed that Med18 and Med9 were involved in telomere length maintenance. In summary, our data have demonstrated and confirmed the importance of Mediator function in the heterochromatic pathway, both at centromere and telomere levels.
**Danish abstract**


INTRODUCTION

Transcription of protein-coding genes in eukaryotes

Transcription of protein-coding genes is carried out in similar ways in all eukaryotes. Transcription can be divided into 5 phases: pre-initiation complex formation, initiation, promoter clearance, elongation and termination. In this study only the first three steps will be introduced in relation to the aim of this project, which focuses the events leading to pre-initiation complex assembly.

In addition to RNA polymerase II (RNA Pol II), General Transcription Factors (GTFs) are required for pre-initiation and initiation of transcription. Activators and co-activators are also required to recruit remodeling complexes and Histone Acetylases (HATs) to allow access of RNA Polymerase II to the promoter. The order of recruitment of GTFs changes according to the organism and type of cell (Eeckhoute et al. 2009), therefore in this section we will consider only the GTFs studied in Saccharomyces cerevisiae and Schizosaccharomyces pombe. Moreover, the Mediator complex, which will be at the core of this study, is considered as a GTF, being involved in recruitment of RNA Polymerase II. The function of individual GTFs is summarized in table 1.

Table 1. GTFs and their main functions (TAF: Transcription Activator Factors; TF: Transcription Factors)

<table>
<thead>
<tr>
<th>GTF</th>
<th>Main functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFIID:TBP+TAFs</td>
<td>Binds to the TATA-box; recognizes the promoter; recruits TFIIB</td>
</tr>
<tr>
<td>TFIIB</td>
<td>Recruits RNA Pol II and TFIIF</td>
</tr>
<tr>
<td>TFIIF</td>
<td>Binds to RNA Pol II, facilitating its recruitment to the Pre-Initiation Complex; recruits TFIIE/TFIIH.</td>
</tr>
<tr>
<td>TFIIE</td>
<td>Binds to the promoter at the transcription start site</td>
</tr>
<tr>
<td>TFIIH</td>
<td>Kinase and helicase activity</td>
</tr>
<tr>
<td>TFIIS</td>
<td>Transcription elongation factor with a role in initiation</td>
</tr>
<tr>
<td>Mediator</td>
<td>Binds to the RNA Pol II, conveying signals between regulatory TFs and Pol II.</td>
</tr>
</tbody>
</table>

The detailed study of transcription is of great potential interest. Studying transcription in cancer cells in particular, might allow the targeting of TFs to achieve proliferation or arrest of tumors. However, these studies are still at a pioneering stage.
**Activators and co-activators of transcription**

Activators and co-activators are essential for initiation of basal transcription due to their ability to bind to DNA and to recruit other general transcription factors. A multitude of activators is known from both budding and fission yeast. For example, Gal4 activates the metabolism of galactose and it also binds to DNA to support transcriptional activation. The GAL4 Upstream Activation Sequence (UAS) contains four sites where Gal4 binds to and this interaction leads to activation of transcription of the flanking genes Gal1 (Giniger et al. 1985) and Gal10 (Guarente et al. 1982). (Gill & Ptashne 1988) have shown that high concentration of Gal4 has an unexpected effect, therefore leads to inhibition of transcription in genes which do not bear the Gal4 binding site. For example, expression of the GAL1::lacZ fusion gene becomes very low when Gal4 is overproduced and its UAS binding site is replaced by other binding site of other activators like Gcn4. The same outcome has been observed for the HIS3 gene, where the transcription of the inducible start site is repressed by high levels of Gal4. Instead, transcription from the constitutive site of HIS3 does not incur in any change (Gill & Ptashne 1988). Therefore, over-expression of Gal4 leads to inhibition of transcription of other genes, a phenomenon often called squelching.

As already mentioned above, co-activators also help to recruit remodeling complexes to the DNA. One of the most studied co-activators in yeast is Gnc5, despite being initially found in *Tetrahymena thermophila*. Gnc5 was subsequently characterized in yeast as part of several high-molecular weight complexes like SAGA (see below) and Ada, both involved in acetylation of chromosomal histones (Grant et al. 1997). Gnc5 is also the catalytic subunit exhibiting HAT activity (Brownell et al. 1996). One of the aforementioned complexes containing Gnc5 is the SAGA complex, which is one of the keystones in transcriptional regulation.

The SAGA complex takes its name from the Spt-Ada-Gcn5-Acetyltransferase and it mainly acetylates lysine residues of H3 and H2B, at the N-terminal tail. It has a size of 1.8 MDa and it is formed by approximately 20 subunits, mainly from the Ada, Spt and TAF groups, as reviewed in (Baker & Grant 2007). The SAGA complex also includes the TAF5, 6, 9, 10 and 12 which, together with the TBP, form the TFIID (Grant et al. 1998).

**RNA polymerase II**

The RNA Pol II is formed by twelve subunits and I will focus on the description of Rpb1, Rpb4 and Rpb7 since they were shown to interact with the Head module of the Mediator complex which will constitute a central part of this study. Rpb1 is the largest subunit and it contains the Carboxy Terminal Domain (CTD) which consists of a sequence of heptapeptide repeats (Y_{1}S_{2}P_{3}T_{4}S_{5}P_{6}S_{7}). The number of these repeats changes according to the organism. In *Homo sapiens* there are 52, 25 in *Saccharomyces cerevisiae* (Corden 1990), while in *Schizosaccharomyces pombe* there are
29 repeats (Esnault et al. 2008; Schneider et al. 2010). The heptad repeats function as docking site for nuclear factors that regulate many aspects of transcription, from chromatin remodeling to initiation, elongation and termination of transcription (as reviewed in (Phatnani & Greenleaf 2006)). The scaffolding-like function of the CTD domain is regulated by the degree of phosphorylation (hyper- and hypophosphorylation) which occurs at the Ser2 and Ser5 residues (Dahmus 1995; Dahmus 1996). The RNA Pol II is recruited with an unphosphorylated CTD. From Pre-Initiation Complex (PIC) formation to initiation there is an increased phosphorylation at the Ser5 residue, due to two cyclin-dependent kinases Cdk7 (Kin28, TFIIH) and CDK8 (Mediator) as reviewed in (Hahn 2004).

Rpb4 and Rpb7 both have a role in transcription initiation. They can function as single proteins but also as a subcomplex of RNA Pol II. Rpb4 is important for the regulation of initiation but not elongation (Mitsuzawa et al. 2003). Rpb7 instead is a regulator of the polymerase synthesizing function and interacts with Rpb1 (Acker et al. 1997). Taken as a heterodimer, Rpb4-Rpb7 was shown to be the bridge between the Mediator Head module (which will be introduced below) and RNA Pol II. In fact, a physical interaction between the Mediator Head module and the Rpb4-Rpb7 dimer resulting in a change of conformation has been shown through a pull down assay with GST-tagged Head module for Rpb4-Rpb7, together with Electron Microscopy experiments carried out by (Cai et al. 2010).

**Pre-Initiation Complex formation**

The formation of the PIC is the first step toward transcription and it starts with the binding of the TBP (TATA-binding Protein, which is included in the TFIID) to the TATA-box promoter region. *In vitro*, an ordered assembly of GTFs at the promoter can be observed. First, TFIIA binds to the TBP stabilizing its binding to the DNA. Second, TFIIB joins the PIC in human and yeast (Maldonado et al. 1990) and this has been shown to be essential for RNA Pol II recruitment (Kuhlman et al. 1999). TFIIB binds to the TFIID followed by TFIIF in association with RNA Pol II. In fact, TFIIB bears two domains, one which interacts with TFIID and the other one with TFIIF and RNA Pol II (Ha et al. 1991). TFIIF has a “checkpoint” activity, helping reducing unspecific interactions between DNA and RNA Pol II (Flores et al. 1989). Once TFIE is recruited, the assembly of the PIC is almost completed. The addition of TFIIH causes phosphorylation of the CTD (Akoulitchev et al. 1995) and transcription will start when ATP and NTPs are provided. Once the transcription has started, the TF will be released, except for the TBP (Figure 1).
Among the TFs involved in transcription, TFIIS also deserves to be mentioned for its dual role in transcription. In fact, TFIIS was first discovered as an elongation factor (Wind & Reines 2000) but in vitro experiments have also shown its presence in complexes formed by RNA Pol II, TFIIB and TFIIE (Hirst et al. 1999), indicating that it is also part of the PIC. Moreover, in vivo deletion of TFIIS led to the loss of several Mediator subunits together with the Swi/Snf complex (Davie & Kane 2000). In vitro experiments have shown that the N-terminal domain of TFIIS interacts with the SAGA complex and Mediator, allowing PIC formation (Guglielmi et al. 2007). The Mediator, a conserved complex involved in transcription, can also be considered as a GTF. In fact, it has been shown in yeast that Mediator mutants (sbr4ts) fail to stimulate the basal RNA polymerase II transcription in vitro (Takagi & Kornberg 2006).

**Initiation and promoter clearance**

The assembly of the PIC is completed with the joining of TFIIH, and the transcription initiation complex is ready to synthesize the first RNAs. Small abortive RNAs of around 5-10 bp are first produced by RNA Pol II, followed by longer RNAs (Holstege et al. 1997). When the RNA reaches a length of about 30 bp, the RNA polymerase will dissociate from the promoter and the transcription initiation machinery, entering the stage of transcription elongation. TFIIE is known to be involved in recruitment of TFIIH, which will complete the PIC assembly, and also in the stabilization of the
transcription elongation complex, as well as in the promoter clearance (Maxon et al. 1994). In fact, the helicase activity of TFIIH will ensure promoter clearance through melting of the DNA at the promoter (Kim et al. 2000).

Mediator

Mediator discovery and structure

Mediator is a complex formed by approximately 20-30 subunits; it has several roles in transcription activation. It was discovered in 1991 in S. cerevisiae by (Flanagan et al. 1991) and since then its importance for RNA polymerase II transcription has been demonstrated. Mediator is composed of four modules: Head, Middle, Tail and CDK8 (Cyclin-dependent kinase 8) module (Figure 2) with some exceptions (e.g. S. pombe). Mediator can be identified in two forms: L- (large) and S-Mediator (small). The L-form includes the Head, Middle, Tail and the CDK8 module. The S-form lacks the CDK8 module (Elmlund et al. 2006). Recent studies in S. cerevisiae have shown that a free-from-RNA Pol II Mediator (the S-form) is the most common form present in the cell (Takagi et al. 2005). Each module is made of a different number of subunits, some of which are essential for cell viability (Table 2). Although the exact composition of Mediator differs from one organism to another (in S. pombe for example, the tail is missing), Table 2 shows that many subunits are highly conserved and that the overall structure is similar (Bourbon 2008; Rachez & Freedman 2001).

Despite Mediator being evolutionary conserved in all eukaryotes, there are additional subunits which are species specific (e.g. Med27 in S. pombe). For example, S. cerevisiae's Mediator consists of five more subunits than the one of S. pombe, but both Mediator complexes perform the same basal functions in transcription. The CDK8 module and the RNA Pol II mutually exclude each other during interaction with the Mediator complex (Elmlund et al. 2006).

In this work I will only focus on the yeast Mediator complexes, (S. cerevisiae and S. pombe).
### Table 2. Mediator subunits found in different organisms. For the *H. sapiens* Mediator, only the TRAP/SMCC domain is listed. The requirement for cell viability has been examined only for *S. cerevisiae* and *S. pombe*. (V) Stands for viable and (I) for inviable. Modified from (Bourbon et al. 2004).

<table>
<thead>
<tr>
<th>Mediator subunit</th>
<th><em>S. pombe</em></th>
<th><em>S. cerevisiae</em></th>
<th><em>C. elegans</em></th>
<th><em>D. melanogaster</em></th>
<th><em>H. sapiens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>MED1</td>
<td>Med1 (V)</td>
<td>Med1 (V)</td>
<td>MDT-1.1</td>
<td>Trap220</td>
<td>TRAP220</td>
</tr>
<tr>
<td>MED2</td>
<td>Med2 (V)</td>
<td></td>
<td>MDT-1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MED3</td>
<td>Med3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MED4</td>
<td>Med4 (I)</td>
<td>Med4 (I)</td>
<td>MDT-4</td>
<td>Trap36</td>
<td>TRAP36</td>
</tr>
<tr>
<td>MED5</td>
<td>Med5 (V)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>MED6</td>
<td>Med6 (I)</td>
<td>Med6(I)</td>
<td>MDT-6</td>
<td>Med6</td>
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<td>MED7</td>
<td>Med7(I)</td>
<td>Med7(I)</td>
<td>MDT-7</td>
<td>Med7</td>
<td>MED7</td>
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<td>MED8</td>
<td>Med8(I)</td>
<td>Med8(I)</td>
<td>MDT-8</td>
<td>Arc32</td>
<td></td>
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<td>Med9(V)</td>
<td></td>
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<td>CG5134</td>
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<td>MED10</td>
<td>Med10(I)</td>
<td>Med10 (I)</td>
<td>MDT-10</td>
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<td></td>
<td>MDT-11</td>
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<td>Med12 (V)</td>
<td>Med12 (V)</td>
<td>MDT-12</td>
<td>Kto</td>
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<td>Med13 (V)</td>
<td>Med13 (V)</td>
<td>MDT-13</td>
<td>TRAP240</td>
<td></td>
</tr>
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<td>CycC</td>
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*(V)* Stands for viable and (I) for inviable. Modified from (Bourbon et al. 2004)*
Figure 2. *S. cerevisiae* Mediator complex showing the four submodules Head, Middle, Tail and CDK8. Interaction with RNA Polymerase II is also shown. Modified from (Guglielmi et al. 2004).

### Mediator complex functions

One of the roles of the Mediator is to convey regulatory signals from the regulatory transcription factors to the RNA polymerase II and GTFs. Mediator also activates basal transcription and undergoes conformational changes between its modules and RNA polymerase II, respectively, during and after the formation of the Pre-Initiation Complex (Davis et al. 2002). The conformational changes within the Mediator complex are facilitated by the intrinsically disordered regions (IDRs) of the subunits. IDRs have been found in subunits situated in two out of four Mediator sub-modules: the Middle and Tail. This is especially true in *Homo sapiens* and *S. cerevisiae*, probably because Middle and Tail are the sub-modules which mostly interact with transcription repressors and activators. Only few Mediator subunits have highly conserved sequences, while some of them are species-specific (as already stated, Med27 in *S. pombe*). The disordered regions are considered to be the reason why Mediator is quite similar in subunit composition in many different organisms despite the limited homology between subunits from different organisms. The IDRs are probably involved in modulating the shape of Mediator. Being flexible, they can also be a liaison between domains, while allowing an easy coupling and uncoupling between sub-modules in response to...
regulatory signals. Moreover, the IDR\$s allow easy assembly between Mediator and the TF\$s during the PIC formation (Toth-Petroczy et al. 2008).

**Mediator sub-modules**

**Head module**


Experiments conducted with a *S. cerevisiae* temperature sensitive mutant for Med17 have shown that the Head module is important for RNA transcription *in vivo* (Holstege et al. 1998) and for stimulating the basal transcription *in vitro* (Takagi & Kornberg 2006). Five out of seven of the Head subunits are essential and when the head’s function is compromised, the mRNA synthesis is blocked at the promoters *in vivo* (Cai et al. 2010). Single particle Electron Microscopy of recombinant Mediator Head and Head sub-modules (Med6-Med8-Med11-Med17-Med22), has shown that the Head is important during promoter recognition and consequently in the formation of the PIC (Cai et al. 2010). Pull-down assay experiments, where TBP was mixed with Head, main core of the Head (Med17-Med6-Med8-Med20-Med11-Med22) and mini sub-complex (Med17-Med11-Med22) have shown that the TBP interacts with the Head module. Specifically, a strong physical connection occurs both between the TBP and the Head core and the full Head, respectively, while the mini sub-complex showed a weaker interaction with TBP (Cai et al. 2010). An *in vitro* reconstituted transcription assay conducted in order to measure the basal transcription according to the TBP level, has demonstrated that the Head influences the transcription level according to the concentration of TBP (Cai et al. 2010). These experiments have confirmed the absolute requirement for the Head module during transcription initiation.

**The Middle module**

In *S. cerevisiae*, the Middle module also consists of eight subunits (Figure 2), and acts as a bridge between the Head and the Tail modules. Deletion of Med19, a subunit of the Head module, causes a disconnection of the Middle module from the Mediator (Baidoobonso et al. 2007). The remaining Head and Tail module, however, stay intact, forming one stable complex. Therefore Med19 has a function in keeping the Middle module associated with the rest of the Mediator (Baidoobonso et al. 2007).
Moreover, it has been shown that Med21, a Middle module subunit, interacts with Med6, a Head module subunit, and this interaction is necessary for full function of transcriptional activators (Gromoller & Lehming 2000).

The CDK8 module

As already stated, Mediator can be identified in two forms: L-(large) and S-Mediator (small). The L-form includes the Head, Middle and Tail if present, plus the CDK8 module. The S-form instead, lacks the CDK8 module. The CDK8 module is formed by Med12, Med13, Cdk8 and CycC. The major function of CDK8 is to regulate basal transcription (Holstege et al. 1998). In S. pombe CDK8 has been isolated only in Mediators lacking RNA Polymerase II (Samuelsen 2003). In mammalian cells, a repressing activity of the CDK8 module for example, occurs when CDK8 phosphorylates TFIIH which is then unable to activate transcription (Akoulitchev et al. 2000). Another hypothesis suggests that CDK8 directly phosphorylates the CTD before the PIC formation, therefore blocking early transcription (Hengartner et al. 1998). 3D reconstruction analysis of S. pombe Mediator protein, has shown that the CDK8 module positions itself competes with RNA polymerase II for interaction with the S-Mediator (Head and Middle), thereby potentially inhibiting transcription (Elmlund et al. 2006). More recently, it has been shown that human CDK8 complex, which is often associated with Mediator, regulates transcription in different ways, acting like a switch. In reconstituted transcription experiments made using CDK8 kinase and kinase-dead CDK8, it has been shown that both types of kinases still work similarly during repression of activated transcription. Moreover, addition of core Mediator is able to re-establish activated transcription even when the CDK8 module is present. This leads to the conclusion that Mediator is probably not the only target of CDK8, even though is likely to be the main one. It has been proposed that after the PIC formation, Mediator can go through a structural change which allows interaction with the CDK8 sub-complex. At the same time though, Mediator is still able to RNA Pol II, therefore re-initiation can occur. When the CDK8 is absent, activated transcription can start over again, with the same RNA Pol II coming from the PIC. At the contrary, presence of CDK8 can block the re-initiation of the transcription during the formation of the scaffold and PIC (Knuesel et al. 2009).

The Tail module

In S. cerevisiae, the Tail module is composed of five subunits. It contains Med2, Med3, Med14, Med15 and Med16 which interact with various gene-specific activators, like Gal4 and Gcn4 as reviewed in (Bjorklund & Gustafsson 2005). Med2, Med3, Med15 and Med16 all together constitute a Gal11 sub-complex of the tail module. In S. cerevisiae, Med15 is known to interact with TFIIE and TFIIH and its function is coupled with TFIIE. In fact, deletion of MED2, MED3 or MED15 together with a mutation in TFIIE where the Gal11 binding region was deleted, leads to inviable cells. If MED15 or TFIIE are deleted, the transcription region will be affected by inefficient opening,
while no consequences in the occupancy of TBP at the TATA sequence can be detected (Bjorklund & Gustafsson 2005). This is likely due to the fact that Med15 and TFIIE functions are needed only after the PIC formation, when transcription levels are still quite low, as reviewed in (Bjorklund & Gustafsson 2005). In *S. pombe*, where the Mediator complex is known to lack the Tail module (Figure 3), a putative Med15 homologue has been found (Khorosjutina et al. 2010). Med15 is associated with Hrp1, a chromatin remodeling protein and they form a subcomplex of Mediator. Hrp1 is involved in regulation of nucleosome density and it forms interactions with other complexes beside Mediator (Walfredsson et al. 2007). The Med15-Hrp1 subcomplex is not part of the core Mediator complex but it interacts with the L-Mediator form. Genome-wide ChIP-on-chip analysis has shown an overlap of genomic target sites for Med15 and Hrp1. This could support the hypothesis that Hrp1 modulates transcription while Med15 could act as the main coordinator of interaction processes between CDK8, Hrp1 and Mediator (Khorosjutina et al. 2010).

**Novel Mediator subunits in *S. pombe***

In a previous work, the *S. pombe* RNA Pol II holoenzyme was purified and 15 polypeptides in the size range 20-200 kDa were described (Spahr et al. 2000). Six polypeptides corresponded to RNA Pol II subunits, whereas five were assigned as Mediator subunits. In another report, the remaining four polypeptides were also identified as Mediator subunits (Spahr et al. 2001). In addition, four bands in the molecular range below 21 kDa were isolated and identified as subunits of Mediator. Further characterization of the *S. pombe* L-Mediator revealed the presence of the CDK8 module. As already stated in the introduction, *S. pombe* Mediator is considerably smaller than its *S. cerevisiae* counterpart, lacking subunits Med2, Med3, Med9, Med11 and Med16. As the molecular range below 21 kDa was not analyzed with respect to the L-Mediator, we speculated that Med9 (16.5 kDa in *S. cerevisiae*) and Med11 (14.5 kDa in *S. cerevisiae*) might not have been identified.

Currently Mediator is known to consist of 20 to 30 subunits, depending on the organism. In this study of *S. pombe* Mediator, I will examine four subunits already studied in budding yeast: Med18 and Med20, Med9 and Med11.

**Med18 and Med20**

In *S. pombe*, *med18*, previously known as *pmc6* or *sep11*, belongs to the group of Sep genes implicated in the cell separation process and it was found in a screen for mutations that cause separation defects (Grallert et al. 1999). The majority of cloned Sep genes retain a high degree of similarity in many eukaryotes (Szilagyi et al. 2002) and three of these, *sep10* (*med31*), *sep11* (*med18*) and *sep15* (*med8*), are known to be Mediator subunits (Szilagyi et al. 2002; Zilahi et al. 2000). Med18 in *S. pombe* was shown to be Mediator subunit (Spahr et al. 2001). For this, purification of RNA polymerase II holoenzyme was followed by SDS-PAGE and MALDI-TOF analysis. The sequence obtained was then blasted and did not show sequence homology to any
other known sequence. Deletion of med18\(^{+}\) results in a temperature sensitive phenotype above 35°C, pseudo-hyphal growth and cells which tend to stick to each other (Szilagyi et al. 2002). As already discussed, a recent study carried out in budding yeast showed that Med18 physically interacts with Med20 and with another Head subunit, Med8 (Lariviere et al. 2008).

scMed20 is a non-essential Head subunit and it was first characterized in S. cerevisiae (Koleske et al. 1992). It was isolated as dominant suppressor of CTD truncation mutations, being involved in both basal and activated transcription. Moreover, the ability of Med20 to start and maintain transcription was shown through experiments in which med20 mutants were able to generate transcripts only when associated with a functional, restored MED20 or GAL4-VP16 promoter (Koleske et al. 1992). More recently, spMed20 has been identified in S. pombe as well, in a work in which Mediator was purified from a Med8-TAP tagged strain and chromatography analysis showed co-elution of Med20, Med17 and Med27 (Linder et al. 2008). Investigations of S. pombe med8ts TAP-Med7\(^{+}\) mutants have shown loss of Med20, together with Med18 and Med27 (Linder et al. 2008). These findings have thus again shown that Med18 and Med20, together with Med8, form a trimer in the Head module complex (Lariviere et al. 2008), not only in S. cerevisiae (Lariviere et al. 2008) (Shaikhibrahim et al. 2009) but also in S. pombe (Linder et al. 2008).

**S. pombe Mediator**

![Figure 3](image)

**Figure 3.** Hypothetical representation of S. pombe Mediator, indicating the putative position of Med9 and Med11 as suggested from studies in S. cerevisiae.
scCSE2/scMED9 and scMED11

In the early 90’s, a new group of genes was discovered in *S. cerevisiae*, genes which were involved in chromosome segregation: *CSE1* and *CSE2*. *CSE2*, afterwards identified as *MED9*, encodes a protein of 17 kDa. Deletion of *MED9* in combination with mutations in the centromere (19 or 45 bp deletion/mutation) leads to larger cells and defective meiosis (Xiao & Fitzgerald-Hayes 1995). Moreover, *med9* mutants in *S. cerevisiae* exhibit a slow growth phenotype and sensitivity to low temperature compared to wild-type (Xiao *et al.* 1993). Poor sporulation and aberrant ascus morphology have also been reported. Δ*med9* cells show a higher rate of nondisjunction of sister chromatids than the wild-type, especially when combined with mutations at the centromeres. In budding yeast, the role of Med9 might be to indirectly regulate the activity of RNA pol II (Gustafsson *et al.*, 1998). It has been speculated that *MED9* could also be involved in triggering cell cycle-dependent activation of several genes that are necessary for correct chromosome segregation. Moreover, the fact that *MED9* in *S. cerevisiae* can regulate different genes is probably due to its leucine zipper motif which can bind to DNA and the same effect has been noted in other Mediator subunits (Med7 and Med8) containing a leucine zipper motif (Gustafsson *et al.* 1998).

**Med11** is another Mediator subunit already characterized in budding yeast but still uncharacterized in *S. pombe*. In *S. cerevisiae*, Med11 was found during a co-immunoprecipitation experiment where Protein A sepharose beads were bound to anti-Cse2 antibody showing that Med10, Med9, and Med11 were all part of the Mediator complex (Gustafsson *et al.* 1998). Purified Mediator was analysed through SDS-PAGE showing several bands belonging to different subunits and, among them, a band of 14 kDa in size. Mass spectroscopy of the band resulted in a sequence which did not have any similarity to any known ORF and thereafter it was named Med11. Another work has confirmed the existence of Med11 in *S. cerevisiae* and that it is part of Mediator complex (Han *et al.* 1999). Mutants with deletion of *med11* are not viable (Han *et al.* 1999).

Using the yeast two-hybrid system, it was shown that MED11 interacts with Rad3, a helicase present in TFIIH (Esnault *et al.* 2008). *med11* temperature sensitive and truncation mutants showed a decreased interaction with Rad3, Med17 and Med22 in the two-hybrid assay. This reduced interaction did not affect the association of Mediator with the RNA Pol II. When the effect of Med11 mutants on TFIIH occupancy at the promoter was analyzed, mutants in which the association of Med11 with the Rpb1 was decreased did not show any sign of affected promoter occupancy by TFIIH. This result led to the conclusion that Mediator interacts with TFIIH (through Rad3) independently of RNA polymerase II (Esnault *et al.* 2008).
Chromatin

In this study we will also take into consideration the effect and possible roles of several Mediator mutants in the heterochromatin pathway, at centromere and telomere levels. In a recent work, it has been proposed that in *S. cerevisiae* mutated Med4 and Med7 have synthetic interactions with proteins involved in remodeling and modification of chromatin in the elongation step (Zhu *et al.* 2011). Moreover, it has been shown that post-translational modifications of histones have a role in Mediator occupancy. In fact, the occupancy of Mediator increased in conjunction with low levels of acetylation of H4K16 (Zhu *et al.* 2011).

Chromatin is a nucleoprotein structure formed by the core nucleosome and various non-histone proteins (e.g., scaffold proteins and HP1 heterochromatin proteins) and DNA. The basic structure of the nucleosomal unit is an octamer of histone proteins consisting of a histone H3-H4 tetramer flanked by two histone H2A-H2B dimers. One hundred and forty-seven base pairs of DNA are wrapped around each octamer, forming the nucleosome. The histone H1 serves to stabilize the DNA strand to make two turns around the nucleosome core. Each nucleosome core particle is separated from the next one through a region of linker DNA of varying length (Figure 4).

The histones are posttranslationally modified by acetylation, methylation, phosphorylation, ubiquitination and sumoylation which are the most studied (Kouzarides 2007). The post translational modifications (PTMs) of the histones are what generally characterize the chromatic state of the genome.

Chromatin can be divided into euchromatin and heterochromatin. In yeast, the majority of the genome is composed of euchromatin. Euchromatin contains regions of active transcription, where the access to DNA is regulated by chromatin remodeling factors such as SWI/SNF and also posttranslational modifications (PTM) like acetylation. One of the PTMs of histones that contribute

![Figure 4](Image.png)

*Figure 4.* Octamer of histones around which DNA is wrapped, forming a nucleosome. From *Life, the Science of Biology*, by Purves, Orians, & Heller, 5th ed., 1997
to the relaxed chromatin structure is the acetylation of histone 3 at lysine 9 (H3K9) and lysine 14 (H3K14) and of histone H4 at K5, K8, K12 and K16. This creates an open chromatin structure which is easily accessible by transcription factors. Moreover, methylation of histone 3 at lysine 4 (H3K4) is another conserved mark of euchromatin and it is found at the promoters of active genes (Bernstein et al. 2002). Also, it is characterized by poor staining by G-Banding through Giemsa and low density.

In contrast, heterochromatin in S. pombe and higher eukaryotes is characterized by high compactness, containing few genes which are mostly transcriptionally repressed. The heterochromatin is not easily accessible, thereby preventing transcription, and its PTMs are characterized by hypoacetylation and di- or trimethylation of histone H3K9. Moreover, a characteristic mark for heterochromatin is the presence of heterochromatin proteins (HP1) which are associated with gene repression. The HP1 proteins, also called chromdomain (Chromatin organization modifier) proteins, encompass Chp1, Chp2 and Swi6. All three bind to methylated H3K9 through their chromdomain (Bannister et al. 2001). It is worth to mention that S. cerevisiae does not follow the same pattern as S. pombe in heterochromatin formation and maintenance. In fact, all the RNAi components found in S. pombe are missing in budding yeast which, instead, relies both on ncRNA as alternative gene regulation process (Harrison et al. 2009) and Sir2-dependent deacetylation of histones (Bryk et al. 2002). However, a recent work has shown that different yeast species as S. castelli use a different type of Dcr1 and Ago1 which, when expressed in S. cerevisiae, would restore the RNAi pathway (Drinnenberg et al. 2009). Heterochromatin is also characterized by deep staining by G-Banding through Giemsa.

**Location of heterochromatin in S. pombe**

Heterochromatin is mainly located at three distinct chromosome regions: the mating-type locus, the telomeres and the centromeres. The repetitive elements which share a high degree of homology in all three regions are the target of heterochromatin formation. The homology is found at two repeated regions, dh and dg (Figure 5). In S. pombe, three genes are present at the mating-type locus: mat1+, mat2+ and mat3+. Mat2P+ (plus) and mat3M+ (minus) loci both contain the mating-type information. However, they are silenced by the 20 kb heterochromatin domain present at the mating-type region (Noma et al. 2004; Thon & Klar 1993). Expression of mat2P+ and mat3M+ occurs after translocation to the mat1+ locus. The mat1+, mat2+ and mat3+ loci are located at approximately 15 kb intervals (Thon et al. 1994). In fact, only mat2+ and mat3+ are located at heterochromatic domain, together with the cenH element which is entirely homologous to the dh and dg repeats of the centromere and partially to the RecQ helicase gene at the telomeres (Figure 5).
Another region with condensed chromatin is the telomere, where the subtelomeres, containing also the \( dh \) and \( dg \) repeats are involved in the heterochromatin formation process (Kanoh et al. 2005). One of the functions of heterochromatin at telomeres is to prevent degradation of the ends of the chromosome and thereby shortening of the chromosomes (Grewal & Jia 2007).

The centromere structure has been extensively studied in \( S. \) pombe because of its repetitive pattern. The three chromosomes in fission yeast display a different positioning of \( dh \) and \( dg \) repeats (Figure 6) which delimit the central core (\( cnt \)) and the innermost regions (\( imr \)). At centromeres, the integrity of repressive chromatin is important for proper segregation of sister chromatides during cell division. In fact, heterochromatic regions are essential for cohesion between sister centromeres, with the help of the HP1 protein Swi6. Swi6 is a conserved protein which has a multitude of roles and effects on the cells: for example, it recruits chromatin-modifying complexes (Grewal & Elgin 2002; Hall et al. 2002); it recruits cohesin required for sister centromere cohesion (Bernard et al. 2001); it binds to the methylated H3 at lysine 9 through its chromodomain, strengthening the Clr4-H3K9Me bond (Zhang et al. 2008). Further, it was shown that recruitment of cohesin at centromeres depends on Swi6 binding to H3K9 which will allow correct chromosome segregation (Bernard et al. 2001). In \( S. \) pombe, the three centromeres display significant differences in the length and number of repeats. In chromosome I, the \( dg \) repeats are inverted compared to the chromosome II. In chromosome III, instead, both the \( dg \) and \( dh \) repeats are present in several copies (Figure 6).
Figure 6. *S* *pombe* centromeres display different organization of dh/dg repeats and their orientation. The *cen3* contains a higher number of repeats compared to the other two.

Heterochromatin assembly: initiation, spreading and maintenance

The two main model organisms used for heterochromatin studies are *S. pombe* and *D. melanogaster*. The RNA interference (RNAi) pathway, which is involved in heterochromatin establishment, has been extensively studied in *D. melanogaster*, and *S. pombe*, but was first discovered in *C. elegans*. Even though they have similar mechanisms, small differences can be found due to the proteins involved in the heterochromatin assembly process as reviewed in (Grewal 2010; Montgomery 2004). The heterochromatic repeated regions are the target of protein complexes involved in heterochromatin establishment. Genes present in these regions are silenced through two different mechanisms: Transcriptional Gene Silencing (TGS) and Post-Transcriptional Gene Silencing (PTGS). TGS occurs at heterochromatic regions, through prevention of transcriptional machinery binding to the DNA. PTGS instead is induced by dsRNA through the RNAi pathway.

As already stated, the transcribed *dg* and *dh* repeats present at the mating-type locus, telomeres and centromeres are the preferential target of heterochromatin formation through the RNAi machinery (Volpe *et al.* 2002). The RNAi machinery is composed by the following proteins and complexes which, in turn, will affect the heterochromatin formation steps: Dicer (Dcr1), Argonaute (Ago1), RNA-dependent RNA polymerase complex (RDRC, which includes Rdp1, Cid12 and Hrr1), RNA-Induced Transcriptional Silencing complex (RITS, which contains Chp1, Ago1 and Tas3) and RNA-Induced Silencing Complex (RISC) (Pratt & MacRae 2009). Dcr1 has a slicing activity towards dsRNA which is processed into small interfering RNAs (siRNAs) (Hannon 2002). Ago1 contains two conserved domains PAZ and PIWI. PAZ helps tethering of siRNA to the RISC complex (Yan *et al.* 2003), while the PIWI domain is required for slicing activity towards target RNA (Song *et al.* 2003). The RDRC complex has polymerase activity which is required for binding of siRNA by the RITS complex (Motamedi *et al.* 2004). The RITS complex utilizes the siRNA produced by Dcr1 to target heterochromatic regions through a base pairing interaction system (Motamedi *et al.* 2004).
Heterochromatin initiation

Two different mechanisms for heterochromatin assembly have been recently proposed and both have shed a different light on the mechanism of heterochromatin assembly (Halic & Moazed 2010; Shanker et al. 2010).

However, according to (Halic & Moazed 2010), both pathways can be found in the cells. In fact, different classes of small RNAs, called pre-siRNA are produced by bidirectional transcription of \( dh \) and \( dg \) repeats. Afterwards, they are degraded into priRNA and loaded onto Ago1. At this point, heterochromatin formation can proceed by either the first pathway, which leads to propagation of heterochromatin through Swi6 targeting, or the second one through binding of nascent transcripts to CRLC complex (Clr4-Rik1-Cul4), leading to a decreased H3K9 methylation and therefore, inefficient silencing, due to the fact that the RITS and RDRC are not recruited.

More specifically, in the first pathway (Figure 7), after the priRNAs (also identified as siRNA molecules) are loaded onto Ago1, they are used as complementary strand for targeting nascent transcripts. Once the targeting has happened, it will trigger the binding of the Tas3 subunits to the RITS complex, which is necessary for heterochromatin-independent siRNA amplification. As soon as the \( dg \) siRNA is accumulated at the centromere, it is loaded into the Argonaute siRNA chaperone (ARC) (Iida et al. 2008). Ago1 also has slicing activity, which is however restrained by Arb1, another protein which, together with Arb2 is found in the ARC (Buker et al. 2007). The loading of the siRNA onto the ARC will trigger the RITS complex targeting of centromeric transcripts which, in turn, will trigger the methylation of H3K9 and also the amplification of siRNA at the \( dg \) repeats. Afterward, the Clr4-Rik1-Cul4 (CLRC) is recruited, giving rise to further H3K9 methylation. Clr4 is a conserved methyltransferase which is the fission yeast homolog of SU(VAR) protein family both in \( D. \ melanogaster \) and mammalian cells (Nakayama et al. 2001; Rea et al. 2000). The methylation started by CLRC will then set off the recruitment of Swi6 and Chp1, two HP1 proteins which will ensure spreading of heterochromatin (Figure 7) (Halic & Moazed 2010).
In the second pathway, always according to (Halic & Moazed 2010), the heterochromatin assembly starts once again with the slicing activity of Ago1. priRNAs are loaded into Ago1 and they will target nascent centromeric transcripts. This event occurs only at the pericentromeric regions because the centromeric repeats are transcribed in a bidirectional fashion, giving rise to sense and antisense transcript. As soon as the priRNAs-Ago1 complexes are formed, they will target the centromeric transcripts, the CLRC complex will be recruited, leading low levels of H3K9 methylation. This pathway is not as efficient in heterochromatin spreading as the first one and produces low levels of methylation because Tas3 and Chp1 of the RITS complex do not intervene in this process, leading to a diminished H3K9 methylation (Figure 8).
Concurrently, (Shanker et al. 2010) have also tried to identify a more specific role for the CLRC complex in different mutants and to test the CLRC or RNAi components chronological interactions in the heterochromatin assembly. In order to do so, they constructed a background strain, \textit{tas3\textsubscript{WG}} (where an alanine substitution at W265 and G266 residues makes the Chp1-Tas3 unable to associate with Ago1) to which a TAP-tag was then attached. In their work, they considered the effects of \textit{rik1\textsuperscript{+}, pcu4\textsuperscript{+}}, CLRC complex, \textit{dcr1\textsuperscript{+}, ago1\textsuperscript{+}} and RDRC components in their transient deletion.

**Effect of \textit{rik1\textsuperscript{+}, raf1\textsuperscript{+}, raf2\textsuperscript{+}, pcu4\textsuperscript{+}} and CLRC components transient deletion on heterochromatin establishment**

Rik1 belongs to the CLRC complex and its main function is to bind CLRC to chromatin. When \textit{tas3\textsubscript{WG}} cells were depleted of \textit{rik1\textsuperscript{+}, raf1\textsuperscript{+} and raf2\textsuperscript{+}}, \textit{dg} and \textit{dh} centromeric repeats were highly transcribed. Reintroduction of \textit{rik1\textsuperscript{+}, raf1\textsuperscript{+} and raf2\textsuperscript{+}}, did not restore the normal levels of \textit{dg} and \textit{dh} transcript repeats. \textit{pcu4\textsuperscript{+}} mutants followed the same pattern as \textit{Δrik1} cells, even when reconstituted, leading to the conclusion that CLRC components are strictly required for heterochromatin initiation in cells which bear a defective RITS complex. Taken together, these results showed once again that reintroduction of CLRC components is not enough to lead to H3K9Me2 on centromeric repeats.
Effect of $dcr1^+$ and RDRC components transient deletion on centromeric heterochromatin and H3K9Me2

In $\Delta dcr1$, $\Delta rdp1$, $\Delta hrr1$ and $\Delta cid12$ cells, high level of centromere transcripts could be detected, and then reduced by reintegration of the deleted gene. The H3K9Me2 levels at centromeres were still low in all mutants (Shanker et al. 2010) but normal levels were reached again after reintroduction of all the genes. These results supported the fact that $dcr1^+$ and siRNA are not essential for CLRC activity, but they are required for proper H3K9Me2 during late heterochromatin assembly. Similar effects were obtained with $ago1^+$ null mutants, both for the centromeric transcript level and H3K9Me2 levels.

The whole set of data demonstrated that $\Delta ago1$ cells and RNAi defective cells both have a reduced amount of H3K9Me2 which, in $\Delta ago1$ cells is however enough for allowing heterochromatin assembly (Figure 9). This supports the idea that the heterochromatin assembly is triggered by the levels of H3K9Me2 at the centromere and not by the siRNA, therefore putting CLRC as first complex for heterochromatin assembly. Therefore, RNAi independent factors and RNAi pathway are both required for full heterochromatin assembly.

The results shown by (Halic & Moazed 2010) and (Shanker et al. 2010) are clearly conflicting regarding the factors that initiate heterochromatin assembly. According to (Halic & Moazed 2010), Ago1 is the first factor involved in heterochromatin assembly, while (Shanker et al. 2010) postulate a CLRC recruitment totally independent of RNAi. However, a common clear point is given by the fact that minimal levels of H3K9Me2 must be present at the centromere in order to start the heterochromatin assembly.

The reason why the results obtained by the two studies are different, considering the fact that they are both based on H3K9me level in $\Delta ago1$ cells, could be due to the fact that both studies have
been carried out using different strategies and also different strains. (Halic & Moazed 2010) have used high-throughput sequencing of small RNAs for single mutants and for double mutants in which genes were rendered inactive (e.g. rdp1+ or ago1*). (Shanker et al. 2010) instead, used tas3+ mutants as a background in combinations with other deletion mutants.

**Heterochromatin spreading and maintenance**

The H3K9me is the heterochromatic mark which spreads beyond nucleation centers, through a cooperation between histone modifying enzymes and other proteins which bind to the modified histones (Grewal & Elgin 2007). H3K9me sites will then be the target of a group of heterochromatin proteins (HP1) (Fischer et al. 2009). The HP1 proteins (Chp1, Chp2 and Swi6) and Clr4 form the chromodomain group and, with Clr3, they cooperate in the silencing process (Kim & Workman 2010). As shown by (Hall et al. 2002), HP1 proteins, in turn, serve as a platform for recruiting chromatin remodeling complexes which ensure preservation and spreading of heterochromatin. Clr4 is the principal factor for spreading of heterochromatin because it both methylates H3K9 and binds to the methylated site (Zhang et al. 2008). Moreover, Clr4 bound to H3K9me causes the modification of surrounding nucleosomes (Zhang et al. 2008). The process of heterochromatin spreading and maintenance is shown in figure 10.

Swi6 in cooperation with Chp2 recruits the Snf2/Hdac-containing complex (SHREC), which contains Clr1, Clr2, Clr3 and Mit1. The main function of SHREC is to decrease occupancy of Pol II at heterochromatic regions assembling high order chromatin structures and also through HDAC Clr3 action (Sugiyama et al. 2007). Moreover, boundary elements prevent the spreading of heterochromatin beyond heterochromatic regions (Zhang et al. 2008).

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**Figure 10.** Nucleation and spreading of heterochromatin through interactions between CLRC and HP1 proteins. The methylated sites (red dots) will attract the CLRC complex, contributing to create more docking site for other CLRC and HP1 proteins after nucleosome modifications. The SHREC complex will also be recruited by HP1 proteins and it will decrease the occupancy of RNA Pol II, while creating higher order chromatin structure (Zhang et al. 2008).
Aims of the project

In the past years great attention has been dedicated to the understanding of the transcription process, leading to an unrivaled amount of data from several model organisms. Two of the most exploited ones are *S. cerevisiae* and *S. pombe*. Several works have shown the complexity of the transcription process and all the players which are involved in it. The Mediator complex is one of them and it deserves a special attention due to its conserved structure through evolution. Mediator has been extensively studied in *S. cerevisiae*, while there are more unanswered questions regarding the *S. pombe* Mediator. Therefore, we decided to focus our attention on two Mediator subunits which have been already discovered and described in *S. cerevisiae* but not in *S. pombe*, MED9 and MED11. Protein alignments will help us to identify the plausible ORF. As we would like to characterize Med9 and Med11, we first need to assess the viability of deletion mutants. After that, a tagging process will take place, allowing us to pull down the protein of interest and thus assessing the possible presence of Med9 and Med11 in the Mediator complex.

Moreover, it was shown that Mediator has also a role in heterochromatin maintenance in *S. pombe* (Linder et al. 2008). To follow up on these studies, mutant strains for med12+, med18+ and med9+ will be constructed and tested for chromosome segregation defects, silencing at the centromere and telomere length. The chromosome segregation defects will be tested with mutants constructed in the Ch16 mini-chromosome bearing strain, while silencing at the centromere will be quantified through analysis of RT-PCR for the aforementioned mutants. Telomere length will also be measured through Southern Blot.
RESULTS

Identification of S. pombe Med11 (SPAC23A1.15c)

Since Mediator is involved in the regulation of transcription, identification and characterization of all its subunits would lead to a better understanding of the complex and its different roles in transcription.

A work from (Boube et al. 2002) has reported sequence similarity among S. pombe, S. cerevisiae, A. thaliana, D. melanogaster and H. sapiens for several Mediator subunits through sequence blasting. The Med11/TRAP250 sequences showed a similarity with SPAC23A1.15c from S. pombe (Figure 11).

Genetic analysis of SPAC23A1.15c

In order to assess if SPAC23A1.15c (hereafter called med11a+ in figures and figure texts) is the MED11 counterpart in S. pombe, a series of experiments were conducted.

First, as the MED11 gene in S. cerevisiae is known to be essential (Boube et al. 2002), it was investigated if med11a+ in S. pombe is essential. To this end, S. pombe diploid strains heterozygous for Δmed11a were constructed, employing the long flanking region PCR strategy (see Materials and Methods), marking the deletion with the hygromycin cassette.
Two sets of primers (YecE and YecF; YecG and YecH, see Primer table 7), which included a recognition site for a restriction enzyme also present on the hygromycin containing pTK1435 vector polylinker, were designed and used for PCR amplification of med11a+ flanking regions using WT (MP9) DNA as a template.

First, the amplified fragments were sequenced and cloned into p-Gem T-Easy Vector. Second, the 446 bp 5' flanking fragment and the 414 bp 3' flanking fragment were sub-cloned into pTK1435, thereby marking the knock out fragment with hygromycin resistance, giving plasmid pTK1599. Third, the 2051 bp knock out fragment was released from pTK1599 by digestion with BamHI and SpeI (Figure 12). The 2501 bp fragment was used to transform diploid strains homozygous for TAP-med7+ (TP219XTP220) or TAP-TRAP240+ (TP66XTP67) respectively, for subsequent use in Tandem Affinity Purification (TAP) of Mediator. The Med7 subunit is located in the Middle module of the Mediator complex, while TRAP240 belongs to the CDK8 module. Correct insertion of the hygromycin cassette into med11a+ was verified with PCR analysis.

Once correct yeast transformants were obtained, genetic analysis through dissection of tetrads from a med11a+/med11a+:Hph/med7+::TAP-KanMX/med7+::TAP-KanMX (or med11a+/med11a+:Hph/TRAP240+::TAP-KanMX/TRAP240+::TAP-KanMX) strain was performed. Two diploid strains for each combination, were dissected, and viability segregated 2:0 in 28 tetrads and 32 tetrads, respectively, showing that SPCC23A1.15c is essential. Also, all living spores were hygromycin sensitive. Representative tetrads are depicted in Figure 13.

![Figure 12. Map of plasmid pTK1599 containing the med11a+ knock-out fragment. Relevant restriction enzyme sites are depicted. White arrows indicate the position of primers used to amplify the 5'- and 3' region of med11a+, respectively.](image-url)
Tagging of Med11a

In order to biochemically establish whether Med11a is a stable Mediator subunit, it was C-terminally HA-tagged for affinity purification.

The same strategy as constructing the deletion strain was used, but in this case the long flanking regions were cloned into pFA6A-3HA-HphMX. The resulting plasmid (pTK1601) is shown in Figure 14. Again, the targeting construct was transformed into both TP66XTP67 (TAP-TRAP240') and TP219XTP220 (TAP-med7'), leading to a triple HA-tagged Med11a allele marked with HphR. Strains bearing a TAP on TRAP240' or med7' were chosen in order to allow for subsequent Tandem Affinity Purification (TAP) which would pull down the TAP-tagged proteins and interaction partners. After transformation, cells were first plated on YES plates followed by replication to YES+Hph plates where only resistant colonies would grow.

Genetic analysis of med11a'-3HA

To confirm the viability of the strains harboring Med11a-3HA as the sole copy of med11a', two transformants for each strain (TP528, TP529, TP530 and TP532) were dissected on YES plates. Twenty-nine four-spore tetrads out of a total of 36, and 31 four-spore tetrads out of a total of 36 tetrads were obtained, respectively. In all four spore tetrads, 2:2 segregation of the hygromycin resistance confirmed the presence of one copy of the hygromycin cassette in the diploids (Figure 15) and also the lack of interference of the tag with viability.
Western blot analysis of Med11a-HA

Once it was confirmed that the strain was correct through Southern Blot (see Materials and Methods), a Western Blot was performed to assess the expression of the Med11a-3HA fusion protein. Strain TP530 was used for Western blot analysis. The strain was grown in liquid YES and a whole cell extract (WCE) was prepared as described in Materials and Methods. SDS-PAGE was run and then transferred to a membrane which was then incubated with anti-HA antibody. The Western blot displayed in figure 16 shows that the TP530 cell extract shows a band of the expected size of 31 kDa, demonstrating that Med11a-3HA has successfully been tagged.

In order to further assess the functionality of the 3HA tag of the Med11a-3HA fusion protein, immunoprecipitation analysis of Med11a-3HA was performed. However, strain TP530 was backcrossed to parental strain MP13 in order to remove the TAP-TRAP240 TAP tagged C-term to avoid capture of TAP-TRAP240 with the Protein A beads used in the IP. The resulting strain was called TP531. Whole cell extract was obtained from strain TP531 and then subdivided in 3 aliquots in order to

Figure 15. med11a'-3HA (SPAC23A1.15c-3HA ade6- M216 TRAP240 TAP tagged C-term) does not interfere with cell viability. Spores from Med11a-3HA strain TP530 were dissected on a YES plate and then replicated on a YES+Hygromycin plate.

Figure 16. Western blot analysis in order to visualize the endogenously expressed med11a'-3HA. ScRpb3 serves as a loading control. No tag is strain MP9. 400 µg of WCE were separated on a SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with anti-HA antibody.
treat them differently to discriminate among different outcomes and possible cross reaction to the anti-HA antibody. The aliquots were processed as follows: the WCE from TP531 and TP61 was divided into two aliquots (see flow chart 1).

Flow chart 1. WCE treatments for TP531 and TP61 (in red) samples, related to figure 17. The WCE was divided in two aliquots. One was incubated overnight with anti-HA antibody, while the other was not. Beads were added to both aliquots and incubated for one hour at 4°C. After this step, samples were spun down and supernatant and pellets from the beads were both collected and used in the Western blot analysis shown in figure 17. The same procedure was followed for the aliquots not containing antibody. The numbers in brackets refer to the lane in which the sample is displayed in figure 17. Red font refers to the TP61 strain.

One aliquot was incubated overnight with anti-HA antibody, while the other aliquot was kept free from antibody. Thereafter, Protein A beads were added to both aliquots and incubated for one hour at 4°C. After incubation, all samples were spun down and supernatant and pellets were collected and analyzed in a Western blot.
Figure 17. Western blot analysis in order to visualize the endogenously expressed Med11a-3HA. Immunoprecipitation of Med11a-3HA. The sample TP531 is the Med11a-3HA tagged strain. TP61 is the positive control with Med7-3HA tagged (48 kDa) and WT is MP9, used as negative control. The WCE was incubated without or with Protein A beads (with or without anybody). Lane 1 contains the whole cell extract; lane 2 and 4 Protein A beads without and with antibody, respectively. Lane 3 and 5 the supernatant derived from the bead samples, without and with antibody, respectively. Lane 6 contains the partially purified Mediator sample used as negative control. Lane 7 displays the WCE from the positive control TP61, which bears a 3HA tag on Med7. Lanes 8 and 10 show the TP61 strain incubated with beads, without and with antibody, respectively. Lanes 9 and 11 contained the supernatant from the beads for the TP61, without and with antibody, respectively. Lanes 12 and 13 display the WT, incubated with beads and without or with antibody, respectively.

TP531 is the Med11a-3HA tagged strain, TP61 is a positive control with Med7-3HA tag and the WT (MP9) and the partially purified Mediator extract (kind gift of Claes Gustafsson) were the negative control. The membrane was incubated with anti-HA antibody. The samples which were immunoprecipitated with Protein A beads show the light and heavy chain from the beads IgG at 25 kDa and 51 kDa, respectively (Figure 17, lanes 2, 4, 8 and 10). Protein extract, supernatant with or without antibody and Protein A beads with or without antibody show a band with the correct size of 31 kDa (Lanes 1 to 5). Also the positive control TP61, showed a band of the correct of 46 kDa from Med7-3HA (Lanes 7 to 11). The final outcome showed that Med11a was successfully tagged, therefore Co-IP experiments followed in order to seek for the presence of Med11a in the Mediator complex.

In order to test if Med11a belongs to the Mediator complex, aliquots from samples 2, 4 and 5 from the Western blot shown in figure 17, together with the Mediator extract, were also probed with an antibody against the Med17 Mediator subunit (Figure 18). Med17 is an already characterized Mediator subunit (Holstege et al. 1998; Spahr et al. 2000). Aliquots from sample TP531 incubated with Protein A beads and anti-HA antibody, from which both pellet and supernatant were collected, were probed with anti-Med17 antibody to seek for precipitation of Med17. Also, an aliquot from the
TP531 sample incubated with beads but not containing antibodies, and a Mediator extract, were included in the analysis. Since Med17 is part of the Mediator complex, we expected Med11a to be present in the Co-Immuno precipitation. In figure 18, the Mediator extract sample shows a band of 64 kDa, which is the expected size for the Med17 subunit, while Med11a-3HA samples (both with Protein A beads, with or without antibody, respectively) lack the 64 kDa band, indicating that Med11a does not belong to the Mediator complex. However, the specificity of the Med17 antibody is probably not good enough to show the Med17 band in the supernatant, leaving a question mark about the presence of Med17 in the WCE.

Figure 18. Western blot of Co-Immuno precipitation of Med11a and Med17. IP was performed with anti-HA antibody to test the co-precipitation of Med11a with Med17. Lane 1 shows sample TP531 incubated with Protein A beads, without antibody (see flowchart 1, sample 2). Lane 2 also shows the beads from sample TP531 incubated with anti-HA antibody (see flowchart 1, sample 4). Lane 3 displays Med17 from the partially purified Mediator extract (Kind gift of Claes Gustafsson). Lane 4 shows the supernatant from sample 5 shown in flowchart 1. The membrane was incubated with anti-Med17 antibody.

After completion of the work described above, it has been proposed that S. pombe med11a⁺, could be the S. pombe sec20⁺ essential homologue of Candida albicans and other yeasts. The GeneDB database from the Sanger Institute has indicated homology between Med11a sequence and the Sec20 sequence of several model organisms (Figure 19).
Figure 19. Alignment of \textit{SPAC23A1.15c/sec20*} to six different model organisms according to GeneDB database. Drome= \textit{Drosophila melanogaster}; Yeast= \textit{Saccharomyces cerevisiae}; Schpo= \textit{Schizosaccharomyces pombe}; Human= \textit{Homo sapiens}; Arath= \textit{Arabidopsis thaliana}; Canal= \textit{Candida albicans}. Colour code as follows: Red: Ser; Dark red; Dark pink: Glu; Yellow: Ala; Bright yellow: Cys; Orange: Thr; Asp; Blue: Arg; Dark yellow: Gly; Green: Ile, Leu, Met; Light green: Val; Ochre: Pro; Turquoise: Trp; Teal: Phe; Tyr; Light blue: His; Dark blue: Lys; Purple: Asn; Light purple: Gln.

The amino acids are grouped by similar colours according to R substituent. Red and dark pink shades: Polar, uncharged R groups. Green, ochre and yellow shades: nonpolar, aliphatic R groups. Turquoise and teal shades: aromatic R groups. Blue shades: positively charged R groups. Purple shades: polar uncharged R groups.

In \textit{Candida albicans}, Sec20 belongs to the SNARE complex (Soluble NSF Attachment Protein Receptor) (Weber \textit{et al.} 2002), which is involved in membrane transport. Moreover, it has a role in posttranslational glycosylation and transport through the Golgi (Schleip \textit{et al.} 2001). From our results it seems that Med11a does not belong to the Mediator complex, but no further analysis was conducted to confirm its belonging to the SNARE complex. However, we cannot be completely sure about Sec20. In fact, it could be still associated to Mediator through a weak or transient interaction or maybe a different choice of control strain would have helped to obtain a more clear answer. Our experimental conditions though, did not allow us to prove it.

Identification of \textit{S. pombe} Med9 SPCC645.12c

In \textit{S. cerevisiae}, Med9 is not essential for cell survival and has been thoroughly characterized. Its characterization was missing in \textit{S. pombe} because previous studies about Mediator subunits involved only proteins with a large size (Linder \textit{et al.} 2008), while the Med9 ORF is predicted to encode a protein of only 21.7 kDa. Interestingly, (Boube \textit{et al.} 2002) indicated that the already characterized Med9 in \textit{S. cerevisiae} has homology to SPCC645.12c in \textit{S. pombe} (Figure 20A). This homology convinced us to undertake genetic and phenotypic analysis of the hypothetical Med9.
Figure 20A. Protein alignment for Med9 in five different organisms. A blast search was carried out using *S. cerevisiae* as starting model organism in order to compare the evolutionary conserved motifs between different organisms in the Mediator complex. The search was done using Blast, PSI-Blast, DbClustal. Flanking numbers indicate the position in the complete peptide sequence, while numbers in parentheses show the intercalary regions. The asterisks indicate a homology or similarity in all five sequences, while the plus sign shows that at least four out of five organisms share a high similarity. Sc= *Saccharomyces cerevisiae*; Sp= *Schizosaccharomyces pombe*; Ce= *Caenorhabditis elegans*; Dm= *Drosophila melanogaster*; Hs= *Homo sapiens*. H= predicted helical.

Subsequently, during our analysis, a new protein alignment comparing SPCC645.12C sequence to *S. cerevisiae* Med9 sequence with 11 different model organisms was released from GeneDb, revealing increasing similarity (Figure 20B) compared to the ones shown in figure 20A. In *S. pombe*, gaps between position 33 and 74 were filled, as well as the gaps from position 86 to 94.

**Figure 20B.** Up to date protein alignment for MED9 in 11 different organisms. The alignment is based on a blast search among several organisms, according to the GeneDB database. The search was performed comparing 11 organisms for the putative MED9 protein sequence. Vanpo: *Vand erwaltizyma polyspora*; Klula: *Klu veromyces lactis*; Ashgo: *Ashbya gossypii*; Canga: *Candida glabrata*; Yeast: *Saccharomyces cerevisiae*; Aedae: *Aedes aegypti*; Schpo: *Schizosaccharomyces pombe*; Mouse: *Mus musculus*; Xenla: *Xenopus laevis*; Drops: *Drosophila pseudoobscura* pseudobscura; Tetng: *Tetraodon nigroviridis*.

Colour code as follows: Red: Ser; Dark red: Dark pink: Glu; Yellow: Ala; Bright yellow: Cys; Orange: Thr; Asp: Blue: Arg: Dark yellow: Gly; Green: Ile, Leu, Met; Light green: Val; Ochre: Pro; Turquoise: Trp; Teal: Phe; Tyr; Light blue: His; Dark blue: Lys; Purple: Asn; Light purple: Gln; The amino acids are grouped by similar colours according to R substituent. Red and dark pink shades: Polar, uncharged R groups. Green, ochre and yellow shades: nonpolar, aliphatic R groups. Turquoise and teal shades: aromatic R groups. Blue shades: positively charged R groups. Purple shades: polar uncharged R groups.
Genetic analysis of \textit{med9}’ (\textit{SPAC645.12c})

Characterization of the putative Med9 in \textit{S. pombe} started with the ascertainment of its essentiality for cell survival. To this end, \textit{S. pombe} diploid strains were constructed heterozygous for Δ\textit{SPCC645.12c} (hereafter called \textit{med9}’ in figures and figure texts), employing the long flanking region PCR strategy marking the deletion with the hygromycin cassette. Two sets of primers (YecA and YecB; YecC and YecD, see appendix) (Figure 21) which included a restriction site, were made. The yeast DNA template came from strain MP9 (WT). The two fragments made using YecA-YecB and YecC-YecD sets, amplified the 5’- and 3’-flanking region of \textit{med9}’, respectively. After cloning in pGem-T Easy (Promega), the fragments were inserted into the hygromycin carrying plasmid (pTK1435), giving pTK1573. All cloned fragments were verified by sequencing after each cloning step. The final targeting fragment was released from pTK1573 using BsiWI and Clal (Figure 21). The fragment was then transformed into two diploid strains homozygous for TAP-\textit{med7}’ (TP219XTP220) or TAP-\textit{TRAP240}’ (TP66XTP67). The TAP tag present in the diploids strain was strategically used for subsequent purification of the L- or S- Mediator form.

Transformants with correct insertion of the hygromycin cassette into \textit{med9}’ were verified by PCR analysis and subsequent Southern blot analysis (data shown in Materials and Methods).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig21.png}
\caption{Map of plasmid pTK1573 containing the \textit{med9}’ knock-out fragment. Relevant restriction enzyme sites are depicted. White arrows indicate the position of primers used to amplify the 5’- and 3’ region of \textit{med9}’, respectively.}
\end{figure}

Once correct colonies in both diploid backgrounds, where \textit{med9}’ was exchanged with the hygromycin cassette, were identified, genetic analysis through dissection of tetrads from \textit{SPCC645.12c/SPCC645.12c::hph/TAP-TRAP240’/TAP-TRAP240 }’ was performed. In the correct strain, now called TP448 (background strain with \textit{TAP-TRAP240}’) twenty four-spore asci out of 32 total asci showed a segregation pattern of 4:0 for viability (Figure 22), demonstrating the viability of Δ\textit{med9} cells. After replication to YES+Hph plates, the segregation pattern was 2:2, confirming the presence of one hygromycin cassette. Similar results were obtained for both diploids (TAP-\textit{med7}’ and TAP-\textit{TRAP240}).
Resistance of Δmed9 strains to stress

Studies of several Mediator mutants (e.g. med15) have shown that Mediator recruitment to promoters under osmotic stress is reduced (Fan et al. 2006). More recently (Linder et al. 2008) have shown that in Δmed18 and Δmed31 cells, growth is impaired by osmotic stress and heat shock. To test for possible phenotypes associated with the lack of med9+, a spotting assay was carried out with Δmed9 cells (Figure 23). Cells were challenged with elevated temperature (37°C), high salt (750 mM KCl) and 1% or 2% formamide.

![Figure 22](image)

**Figure 22.** med9+ is a non-essential gene. Spores from strain TP448 were dissected on a YES plates. The master plate was then replicated to YES+Hph, in order to score for hygromycin resistant spores.

![Figure 23](image)

**Figure 23.** Growth test of Δmed9. Drop assay of Δmed9 (spores a and b) and WT (MP9, single colonies a and b) on AA, AA+KCl, AA+ formamide plates. The first dot spot concentration is $10^7$ cells/ml, followed by 10 fold serial dilutions.

The result showed that the growth of Δmed9 mutant cells was indistinguishable from WT cells under all the conditions tested. Similarly, experiments conducted in order to test the growth rate of Δmed9 cells showed a doubling time comparable to the WT (Table 3). Specifically, growth in liquid media (AA) and at different temperatures (30°C and 37°C) was tested for some of the Δmed9 and WT strains (Table 3). Cultures of the different strains were grown overnight in AA at 30°C and the next morning diluted to OD$_{600}$ 0.01. The growth was followed through the day by spectrophotometer reading and the growth rate was then calculated after 8 hours. The liquid culture assay did not show any significant impairment of growth rate for any of the tested strains, both at 30°C and 37°C. Taken together, these results have shown that absence of med9+ has no effect on resistance to stress and growth rate.
Table 3. Doubling time comparison among deletion mutants for Δmed9, med9+ -13Myc tag and WT, at 30°C and 37°C. The growth rate was calculated sampling the cultures every 2.5 hours by O.D. measurement. The final O.D. measurement was taken after 8 hours.

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Doubling time/h</th>
<th>Doubling time/h</th>
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<td>med9’::HphR TAP-TRAP240*</td>
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<td>med9’::HphR TAP-TRAP240*</td>
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</table>

Affinity tagging of Med9

In order to establish if Med9 has a stable association with Mediator, a C-terminal 13Myc tag was attached to Med9. The same cloning strategy as used for Med11a was applied. Two new sets of primers were designed, Myc1-Myc2 and Myc3-Myc4 (see appendix for sequence) and the regions flanking Med9 were cloned into pFA6A-13Myc-HphMX (pTK1496). The final fragment was released from the vector using Bsu36I and XmnI restriction endonucleases and then transformed into strains TP66 and TP219, selecting for hygromycin resistance. Twenty single colonies from each transformation were picked and checked by colony PCR and among those, four of each group were taken and further analyzed by Southern blot.

Moreover, in order to confirm that the tag does not exert any dominant negative effects on cell growth, the tagged strains were tested by osmotic stress and poisoning by formamide (Figure 24) together with determination of growth rate (shown in Table 3), because Mediator defects are known to interfere with the ability of cells to respond to osmotic stress and poisoning by formamide (Linder et al. 2008). The Δmed9 strain was included along with WT strains as reference. The osmotic stress and poisoning tests indicated that the 13Myc tag on Med9 did not interfere with the cell viability (Figure 24). However, a light sensitivity to 750mM KCl was noticeable. On the other hand, as the med9’ deletion strain did not show any phenotype, we might not expect any phenotype for the tagged strains.
Figure 24. Drop test sensitivity assay for Δmed9 spore a, TP476 (med9^-13Myc in TP219), TP474 (med9^-13Myc in TP66) and WT strains. Five different 10-fold serial dilutions (starting at 10^7 cells/ml) of cells were plated on AA, AA+750 mM KCl and AA+formamide (1%) supplemented plates. No significant growth impairment was detected for any of the strains.

**Western blot analysis of 13Myc-tagged Med9**

After obtaining and checking the constructed Med9-13Myc strain, a Western blot was performed in order to examine expression of Med9-13Myc. Three strains (TP474, TP475, and TP476) were grown to OD_{600} 0.5 in liquid YES media, and whole cell extracts prepared, followed by TCA precipitation (see Materials and Methods). The proteins were separated on an SDS-PAGE and transferred to a membrane which was then incubated with anti-Myc antibody. The Western blot in figure 25 shows a band of the expected size of 44 kDa, thereby demonstrating that Med9 was correctly tagged.

Figure 25. Western blot in order to visualize endogenously expressed Med9, both for TCA precipitate and lysate, incubated with α-Myc antibody. ScMrn1-Myc serves as a positive control, with an expected size of 72 kDa. WT is the strain TP66. 400µg of WCE or TCA precipitate, were separated on a SDS-PAGE and transferred to PVDF membrane. The two bands show Med9-13Myc and protein degradation, respectively.
Tandem Affinity Purification (TAP) of Mediator

After the Med9 tagged strains were tested both by Western and Southern blot (see Materials and Methods), they were then used for purification of Mediator using the TAP-med7+ strain TP476 (see flowchart 2). These experiments were performed at Claes Gustaffson’s Lab at Karolinska Institut in Stockholm. Around 12 liters of cultured cells were used to obtain a partially purified Mediator from strain TP476 (see Materials and Methods). TAP-med7+ purification of Mediator was performed with IgG Beads and Mediator was then eluted from the beads by TEV cleavage, followed by Western blot analysis. The membrane was incubated with anti-Myc antibody in order to visualize the Med9-13Myc in the Mediator extract. Two different extractions for strain TP476 were performed and the results of one are showed in figure 26. In the elution, no band of the expected size of 44 kDa for 13Myc tagged Med9, was visible. IgG beads, flow through and input samples all showed high degradation patterns for unknown reasons.

Flowchart 2. TAP purification of Med9-13Myc with TAP-Med7. Cells from 12 liters of yeast culture were collected, washed and frozen in liquid nitrogen. Cells were subsequently broken in a freezer mill and then re-suspended in TAP buffer. Supernatant was collected and then ultra-centrifugated. The resulting supernatant was then incubated with IgG beads and spun down. The beads were then collected and a purification column was then used for the next two washes. The first one was performed with IgG buffer, producing the FT. In the second wash, TEV cleavage buffer was used and Mediator was eluted. Aliquots were collected at every step and then loaded on a SDS-PAGE gel, which was then transferred to a nitrocellulose membrane and incubated with anti-Myc antibody (Figure 26). The numbers in brackets refer to the lane in which the sample is displayed in figure 26.
The elution sample from Mediator did not show any band, while the FT and the input both showed a degradation pattern. The beads lane also showed few unspecific bands.

Another Western Blot analysis was performed using the same elution samples in order to test the Mediator TAP purification. The membranes were probed with anti-Med7 antibody and anti-Med27 antibody, respectively, which are both characterized Mediator subunits in *S. pombe* (Figure 27).

**Figure 26**: Western blot analysis of TAP purifications of Med9-13Myc with TAP-Med7 in order to visualize the endogenously expressed protein of interest. Twenty µl of each sample were loaded on a SDS-PAGE gel and transferred to a nitrocellulose membrane. Lane 1, 2 and 3 show the input, FT and wash, respectively. The lane 4, which contained the elution of Mediator from the IgG beads, did not show any band. Lane 5 shows the IgG beads. The membrane was incubated with anti-Myc antibody.

**Figure 27**: Western blot analysis of TAP purified Mediator with anti-Med7 and anti-Med27 antibody, respectively. A 31 kDa band was expected for Med27, while a 75 kDa band was expected for Med7. The elution loaded on the SDS-PAGE gel is the same as used in the experiment shown in Figure 26. This Western blot was performed in order to prove the correct Mediator purification, which was confirmed by the two positive outcomes.
The presence of Med7 and Med27 in the elution products is indicative of a correct Mediator purification (Figure 27). This final outcome from our experiments led us to the conclusion that Med9 does not belong to the Mediator complex. However, we cannot completely exclude that Med9 belongs to the Mediator complex due to the fact that different approaches might have led to different results. This result led to a series of questions regarding the possibility that med9* is poorly expressed or that the 13Myc-tag would interfere with the association of Med9 with Mediator. In order to investigate these possibilities, several immuno-precipitations with anti-Myc antibody were performed. This procedure requires a strain devoid of the Med7*-TAP tag and new strains were created through backcrossing to the parental strains and subsequent tetrad analysis in order to remove the TAP-tag in the Med9-13Myc tagged strains. One of these new strains, TP600, was then analyzed at Claes Gustaffson’s lab at Gotenburg University. Whole cell extract was extracted from 100 ml of cell culture as explained in Materials and Methods and used first for a Western Blot assay and second for an anti-Myc IP experiment.

The final outcome (not shown) could not provide a clear proof that the 13Myc tag on Med9 was functional for pull down by the anti-Myc antibody. The fact that this might have been due to low transcription of Med9 suggested that a bigger volume of cells was needed for a correct visualization of the bands. Therefore, whole cell extracts of strain TP600 (Med9-13Myc, expected size 44 kDa), TP52 strains (Med4-13Myc, with an expected size of 61 kDa) and WT were obtained from one liter culture and processed as indicated in flow chart 3. In order to immunoprecipitate Med9-13Myc, one aliquot from sample TP600 was incubated with IgG beads only, another aliquot with IgG beads together with a mono- anti-Myc and a third one with polyclonal anti-Myc antibody, respectively. From the washing step of all the samples containing beads, the respective supernatants were obtained. The positive control was TP52 which underwent the same procedure. The WT cells were also incubated with beads (see flowchart 3).
*Flow chart 3.* WCE treatments for TP600, related to *figure 28*. WCE was incubated with monoclonal or polyclonal antibody. Beads were added and after one hour incubation, the samples were spun down. Pellet and supernatant were collected from all the samples and loaded on a SDS-PAGE gel. Gel was transferred to a membrane and then incubated with anti-Myc antibody. The same procedure was followed for TP52 samples. Numbers in brackets refer to the lane in which the sample is displayed in *figure 28*.

After blotting of the gel to a nitrocellulose membrane, it was incubated with anti-Myc antibody. The result is shown in *figure 28*. All the samples from TP600, which were immuno-precipitated with anti-Myc Protein A beads, show the light and heavy chains from the beads IgG at 25 kDa and 51 kDa, respectively (*Figure 28*). The supernatants from the beads for TP600 (with or without antibody) all showed a band of approximate size of 44 kDa. The same band was not visible in the precipitated samples containing the pellet from the beads, where the displayed heavy chain from the antibody might have covered the 44 kDa band, due to its thickness. A different outcome was visible between samples treated with monoclonal or polyclonal antibody. The polyclonal antibody gave a stronger band which made it difficult to discriminate band size. From the No-Tag sample, it is also clear that the beads themselves produce a quite strong signal. Taken together, all these data led to the conclusion that the polyclonal antibody pulls down Med9-13Myc and Med4-13Myc; however, not all Med4-13Myc is bound by the beads.
Figure 28. Immunoprecipitation of Med9-13Myc and Med4-13Myc. Western blot of proteins extracted from one liter culture TP600 (Med9-13Myc), TP52 (Med4-13Myc) and WT. Lane 1 show the No tag control incubated with beads only (TP66). Lane 2 shows the supernatant remains from TP52 after pull down. Lane 3 displays the beads pellet from TP52 extract incubated with polyclonal antibody. Lane 4 contained a supernatant from TP52 incubated with beads but without any antibody. Lane 5 contained the WCE from TP600. Lanes 6 and 7 display the supernatant and the beads pellet from TP600 after incubation with monoclonal antibody, respectively. Lanes 8 and 9 show supernatant and beads pellet of TP600 after incubation with polyclonal antibody, respectively. Lane 10 shows TP600 supernatant from the beads without any kind of antibody. Lane 11 displays the pellet from the beads for TP600, without any antibody. The membrane was probed with anti-Myc antibody.

Five of the samples precipitated with the different anti-Myc antibodies were also used for Western blot analysis with anti-Med7 antibody (Figure 29). The samples shown in lanes 5, 6, 8, 9 and 11 from figure 28, were analyzed in the SDS-PAGE and Western blot analysis with anti-Med7 antibody and a purified Mediator as a control (from Claes Gustafsson Lab). Extracts from strain TP600 Med9-13Myc never showed any band corresponding to the expected size for Med7 (64 kDa, Med7, which was clearly visible in the purified Mediator sample (Figure 29), while the band was absent in the TP600 samples incubated with anti-Myc antibody). The fact that Med9 did not precipitate Med7 led to the conclusion that Med9 possibly does not belong to the Mediator complex. However, it is possible that the affinity of the anti-Med7 antibody is not sufficient to show the corresponding band in the input.
Since the 13Myc tag could interfere with the association of Med9 with Mediator, a new approach was used to further analyze whether Med9 is a part of the Mediator complex or not.

**Tagging of Med9 with a FLAG epitope using Gateway® Recombination Cloning Technology**

The failure of Med9-13Myc to immunoprecipitate Med7 could be due to the Myc-Tag interfering with the interaction of Med9 with the Mediator complex. Therefore, the Gateway® technology was used to attach a different tag to Med9. I chose the FLAG-His₆ tag because of its small size. This versatile and efficient system involves the use of a library of “Entry clones” which contain the sequenced verified gene of interest (GOI), together with a “Destination vector” (DV) containing an epitope, which will be fused to the gene of interest. These vectors and Entry clones contain specific att sequences that are used for the proper insertion of the GOI. The destination vectors have been constructed with the possibility to modulate the protein expression through the *S. pombe nmt1* promoter, with three different levels of expression available. The DVs, which are called pDUAL, bear different kinds of tags, including, FLAG-Tag, His₆, GFP, to be fused either C- or N- terminally.

In our experiments, an intermediate expression level was chosen for both N- and C-terminally located 2xFLAG-His₆ tags. After the construction of the expression clones (see Materials and

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**Figure 29.** Med9 does not precipitate Med7. IP of one liter culture Med9-13Myc probed with α-Med7 antibody. Lanes 1 and 4 show the sample TP600 incubated with beads with no antibody and polyclonal antibody respectively. Lanes 2 and 5 display the supernatant coming from the samples in lane 1 and 4. Lane 3 shows Mediator extract and lane 6 the input.
Methods for details), we had the possibility to transform the whole plasmid bearing Med9-FLAG-His\textsubscript{6} (either C- or N-terminally) into a yeast strain in order to keep it as a self replicating plasmid at a high copy number to increase expression. With respect to the \textit{med9}+ gene two destination vectors were used, the HFF41c vector with a FLAG-His\textsubscript{6} tag N-terminally located with respect to the GOI, and the FFH41c vector with the same tag C-terminally located. The following expression vectors were made as described in Materials and Methods and checked through digestion with EcoRI and Bsu36I.

- pTK1661: Med9-2xFLAG-His\textsubscript{6} (N-terminal) (Figure 30A)
- pTK1659: Med9-2xFLAG-His\textsubscript{6} (C-terminal) (Figure 30B)

**Figure 30A and 30B.** Map of plasmid pTK1661 and pTK1659 containing the Med9-2xFLAG-His\textsubscript{6} construct with N- and C- terminal tags, respectively. Relevant NotI restriction sites for integration in \textit{leu1-32} strain are depicted.

Once the validity of the newly made plasmids was confirmed, a yeast transformation was set up. The yeast strain used for these experiments was MP46 which is \textit{Δura4} to allow selection of transformants. The \textit{ura4}+ gene on the plasmid will allow growth of the yeast cells in AA–Ura medium. Four \textit{ura4}+ colonies from each transformation (for C-terminal and N-terminal tag vector) were picked and checked by Western blot analysis for expression of the Med9-2xFLAG-His\textsubscript{6} fusion protein and its functionality for IP. Whole cell extract was prepared after growth in Edinburgh Minimal Media (EMM) and incubated overnight with monoclonal anti-FLAG M2 antibody (raised in mouse) (see Materials and Methods for details). Samples were then incubated with Protein G Dynabeads and afterwards the beads were washed and the proteins eluted from the beads were loaded on a SDS-PAGE gel. In order to avoid the signal of the light and heavy chain from the anti-FLAG antibody, as the expected Med9-FLAG-His\textsubscript{6} band size would have been 25 kDa, the Western Blot membrane was then incubated overnight with rabbit anti-FLAG (TM) polyclonal primary antibody. The day after, the secondary antibody (\textalpha rabbit) was added and the membrane was then developed and exposed to an X-ray film. The resulting Western blot did not give any conclusive result regarding the functional tagging because of the beads interference (data not
shown). Therefore, as we were not able to identify the correct bands, we decided to try another strategy.

The Gateway® system can also be used together with the pDUAL vectors which have been created by (Matsuyama et al. 2004). These vectors allow the expression of multiple copies of genes with episomal features or a single copy through homologous recombination after digestion of the desired fragment. In order to integrate our gene of interest with the tags in the yeast genome, a fragment from the plasmid containing the Med9 fused to 2xFLAG-His₆ was isolated. Digestion of the Med9-2xFLAG-His₆ carrying plasmid with restriction enzyme NotI provided a fragment containing the fusion that would integrate at the leu1 locus in a leu1-32 strain and thereby restore leucine prototrophy. Thus, a leu1-32 strain (EG1190) (kind gift of Olaf Nielsen) was transformed with the cut plasmid. The cells were plated on AA-leu plates and two colonies of each of the following transformants were picked:

- TP509 and TP510: SPCC645.12c-2xFLAG-His₆
- TP511 and TP512: 2xFLAG-His₆-SPCC645.12c

Three different PCRs were performed, to verify the correct integration of the 2xFLAG-His₆ tag (primers were HFFM911-5’ - HFFM11-3’ for TP511 and TP512; FFHM11-5’- FFHM911-3’ for TP509 and TP510; see Materials and Methods for primers sequence). The results obtained confirmed the correct integration of the fragment. Immunoprecipitation and Western Blot were again performed, using Protein A Dynabeads (Invitrogen) and antibodies as described above. No band of the expected size of 25 kDa for Med9-2xFLAG-His₆ was obtained in any of the different single colonies. A strong protein degradation pattern was also visible and therefore we could conclude that the IP did not work as expected (data not shown).

Another Western blot analysis, shown in figure 31 was carried out, where beads only were also loaded on the gel in order to see if they would give a pattern and their result compared to the Med9 samples. It was clear that the beads themselves already produced a strong pattern. However, no band of 25 kDa belonging to the Med9-2xFLAG-His₆ strains was detected (lanes 6, 7, 8 and 9 in Figure 31), showing that the immunoprecipitation of Med9 did not work.
Figure 31. Western Blot of different immune-precipitated Med9-2xFLAG-His6, in order to verify any possible interference given by the Protein A beads. Lane 1 shows the bands given by the beads alone. Lane 2 and 3 display the positive control WCE and TP509, both incubated with beads but without antibody. Lane 4 shows WCE form TP509 incubated with beads, without antibody. Lanes 5 (EG2537, Cia1-2xFLAG-His6) and 6 (MP46) display positive and negative control, respectively. Lanes 7, 8 and 9 were all loaded with WCE from the indicated strains. The membrane was incubated with anti-FLAG antibody.

In conclusions, we were unsuccessful in the Co-Immunoprecipitation of Mediator with Med9. All the attempts made to show the belonging of Med9 to the Mediator complex have been unsuccessful, with our choice of tags. At this point and with the collected data, we cannot confirm that Med9 does belong to the Mediator complex.
Identification of med11b\(^+\) (SPAC644.10) in \textit{S. pombe} 

Just like SPCC645.12c, which has been previously described in this project, Med11b falls in the same category of small size peptides belonging to the \textit{S. cerevisiae} Mediator complex which were not previously identified in \textit{S. pombe} Mediator. In \textit{S. cerevisiae}, biochemical characterization of the gene confirmed that it belonged to the Mediator complex and its requirement for transcriptional activation (Gustafsson \textit{et al.} 1998).

Moreover, another scientific work indicated the Mediator head (Srb4 sub-complex) as the position for Med11, which was then assigned the role of head module assembler and keeper, together with Med22 (see \textbf{figure 2}). Loss of Med11 in fact, led to absence of the Srb4 sub-complex (Takagi \textit{et al.} 2006).

Several protein alignments have shown a similarity between the \textit{S. cerevisiae}'s ORFs YMR112c/MED11 and \textit{S. pombe} SPAC644.10. An alignment among 27 different organisms was carried out using the GeneDB database as reference (\textbf{Figure 32}), leading to the conclusion that \textit{S. cerevisiae} ORF YMR112C could correspond to \textit{SPAC644.10} in \textit{S. pombe}.

We therefore decided to undertake the genetic and biochemical characterization of the putative SPAC644.10/Med11b (hereafter called Med11b in figures and figure text).
**Figure 32.** Alignment originated by blast search according to the GeneDB database. The search was performed comparing 27 different organisms for MED11b protein sequence.

Vanpo: Vanderwaltozyma polyspora; Klula: Kluyveromyces lactis; Danre: Danio rerio; Picst: Pichia stipitis; Debha: Debaryomyces hansenii; Botfb: Botryotinia fuckeliana; Denve: Demansia vestigiata; Neuer: Neurospora crassa; Arath: Arabidopsis thaliana; Piegu: Meyerozyma guilliermondii; Human: Homo sapiens; Chagb: Chaetomium globosum; Lodel: Lodderomyces elongisporus; Ashgo: Ashbya gossypii; Canga: Candida glabrata; Xentr: Xenopus tropicalis; Drome: Drosophila melanogaster; Neofi: Neosartorya fischeri; Cael: Caenorabditis elegans; Maggr: Magnaporthe grisea; Orysj: Oryza sativa; Yeast: Saccharomyces cerevisiae; Ajecn: Ajellomyces capsulate; Aedae: Aedes aegypti; Schpo: Schizosaccharomyces pombe.

Colour code as follows: Red: Ser; Dark red; Dark pink: Glu; Yellow: Ala; Bright yellow: Cys; Orange: Thr; Asp; Blue: Arg; Dark yellow: Gly; Green: Ile, Leu, Met; Light green: Val; Ochre: Pro; Turquoise: Trp; Teal: Phe; Tyr; Light blue: His; Dark blue: Lys; Purple: Asn; Light purple: Gin; White: gaps

The amino acids are grouped by similar colours according to R substituent. Red and dark pink shades: Polar, uncharged R groups. Green, ochre and yellow shades: nonpolar, aliphatic R groups. Turquoise and teal shades: aromatic R groups. Blue shades: positively charged R groups. Purple shades: polar uncharged R groups. Alignments between 40 and 50 and 120 and 150 have been cropped for figure fitting.
Genetic analysis of \textit{med11}^	extsuperscript{b}^	extsuperscript{+}

In order to assess if SPAC644.10/\textit{med11}^	extsuperscript{b}^	extsuperscript{+} was the \textit{S.cerevisiae} MED11 counterpart in \textit{S. pombe}, the following experiments were performed. As Med11 in \textit{S. cerevisiae} is known to be essential for cell viability (Han \textit{et al}. 1999), it was investigated whether \textit{med11}^	extsuperscript{b}^	extsuperscript{+} was also essential in \textit{S. pombe}.

The procedures used were similar to the ones employed for \textit{med9}^	extsuperscript{a} and \textit{med11a}^	extsuperscript{+}, with different primer sets (YecL1-YecM1, YecN-YecO (see appendix for sequences)). A deletion fragment carrying an HphR cassette was obtained by cutting pTK1645 with restriction enzymes SspI and Pmel (Figure 33). The correctness of the fragment was verified by sequencing, and it was then transformed in a diploid strain (TP219XTP220) homozygous for TAP-\textit{med7}^	extsuperscript{a} (or TAP-TRAP240, TP66XTP67).

![Figure 33. Map of plasmid pTK1645 containing the \textit{med11}^	extsuperscript{b} knock-out fragment. Relevant restriction enzyme sites are depicted. White arrows indicate the position of primers used to amplify the 5'- and 3' region of \textit{med11}^	extsuperscript{b}](image)

Four transformants growing on the YES+Hph plates were picked and checked by colony PCR, followed by tetrad analysis (Figure 34). Two diploid strains for each combination of crosses (\textit{med7}^	extsuperscript{a} and TRAP240) were dissected. In 44 4-spored tetrads out of a total of 72 dissected, the segregation was 2:0 for viability, showing that \textit{med11}^	extsuperscript{a} is essential for cell viability. As expected, the viable spores were sensitive to hygromycin.

![Figure 34. \textit{med11}^	extsuperscript{b} is an essential gene. Spores A-D from strain TP529 were dissected on a YES plate and then replicated on a YES+Hygromycin plate.](image)
Affinity tagging of Med11b

In order to identify Med11b within the Mediator complex, the protein was C- or N-terminally tagged with 2xFLAG-His$_6$ tag, using the Gateway strategy. The whole procedure was similar to the one adopted for Med9 (see page 47). The destination vectors were the same as used for Med9 (see page 47).

At first, the circular plasmid bearing *med11b*-2xFLAG-His$_6$ (Figure 35A and 35B) was transformed into a yeast strain (MP46; *h* ade6-M210 *leu1-32* *ura4-D18*) to keep it as a self replicating plasmid at high copy number. This feature should increase the expression of the tagged protein. In order to assess the functionality of the tag, several Western Blots were performed in the same way as for Med9. Unfortunately, a correct sized band was never obtained (results not shown). We therefore decided to integrate the plasmid following the same procedure as adopted for Med9. The plasmid was cut with the restriction endonuclease NotI and it was transformed into a *leu1-32* strain (EG1190) in order to establish leucine prototrophy. Cells were plated on AA-Leu plates and two colonies of each of the following strains were picked:

- TP513-TP514: *Med11b*-2xFLAG-His$_6$-Med11b (C-terminal)
- TP515-TP516: Med11b-2xFLAG-His$_6$ (N-terminal)

These strains proved to be correct by PCR (results not shown) using the following primer combinations: HFFM911-5’ - HFFM11-3’ for TP515 and TP516; FFHM11-5’ - FFHM911-3’ for TP513 and TP514 (see Materials and Methods for primers sequence). In order to check the expression of the tag, several Western Blot analyses of the strains with integrated 2xFLAG-His$_6$
were performed. An immuno-precipitation of Med11b was carried out using Protein A Dynabeads (Invitrogen) and anti-FLAG antibodies as already described for SPCC645.12c (Figure 36 and flow chart 4). The expected size for Med11b-FLAG-His$_6$ tag is 16 kDa but no bands below 25 kDa were visualized for both samples. Strain MP46, which is the No tag control, also showed some bands and a strong background as well as the positive control strain EG2537, expressing Cia1. Those bands are probably derived from the Dynabeads and produce a background which makes it difficult to identify specific bands. A similar pattern was also obtained from the Med9 IP.

Flow chart 4. WCE treatments for TP515, TP515 and EG2537, related to figure 36. WCE was incubated with anti-FLAG antibody. Protein A Beads were added and after one hour incubation, the samples were spun down. Pellet and supernatant were collected from all the samples and loaded on an SDS-PAGE gel. The gel was transferred to a membrane and then incubated with anti-FLAG antibody. Numbers in brackets refer to the lane in which the sample is displayed in figure 36.

Figure 36. Western blot analysis of several immunoprecipitated Med11b-2xFLAG-His$_6$ after incubation with mouse α-FLAG antibody and Protein A dynabeads in order to pull down the endogenously expressed protein. The membrane was incubated with rabbit anti-FLAG primary antibody. Lanes 1 and 2 show the N-terminal tagged Med11b. Lanes 3 shows the No Tag, which is the negative control; while lanes 4 and 5 show Cia1 (EG2537), which is the positive control (kind gift of Christian Holmberg), with an expected size of 37 kDa. Cia1 was incubated both with and without antibodies.
In order to test if med11b\(^{\prime}\)-2xFLAG-His\(_6\) would give an mRNA transcript, total RNA was isolated and an RT-PCR was performed (see Materials and Methods for details) with a positive outcome (Figure 37A). The primers used in the experiments are; FFHM11-5’-FFHM911-3’ for TP513 and TP514; HFFM911-5’-HFFM11-3’ for TP515 and TP516. The RT-PCR result showed the expression of the Med11b-2xFLAG-His\(_6\) mRNA in the cells.

![Figure 37A](image1)

**Figure 37A and 37B.** A: med11b\(^{\prime}\)-2xFLAG-His\(_6\) expression. RT-PCR for med11b\(^{\prime}\)-2xFLAG-His\(_6\) strains in order to visualize the mRNA transcripts. Lanes 1 and 2 show the C-terminal Tagged Med11b, with expected size of 410 nt. Lanes 4 and 5 show N-Terminal Tagged Med11b, with expected size of 368 bp. Lanes 3 and 6 show the Negative controls (-RT). B: Primer map is also shown for both primers combination, with the respective expected sizes.

However, the discouraging results obtained with Med11b Western Blot analysis of Immuno-precipitations were not due to the lack of expression of the mRNA.
Contribution of Mediator to heterochromatin formation and maintenance

The Mediator complex was first identified by Kornberg’s group in a study about RNA Pol II transcriptional activation in vitro (Flanagan et al. 1991). Further work has then shown Mediator’s ability to both increase activated transcription and to repress it (Kornberg 2005), depending on the interaction with the CDK8 module (Elmlund et al. 2006).

Different studies have shown that the function of the CDK8 module (also known as Med12 module) is to compete with RNA Pol II for interaction with the S-form of Mediator. As reviewed by (Bjorklund & Gustafsson 2005), orthologue Mediator modules of both budding and fission yeast are involved in repression of transcription. Moreover, a work carried out in mammalian cells, where transcription factor C/EBPβ and retinoic acid receptor are able to recruit Med12 in order to repress the transcription (Mo et al. 2004), supported this hypothesis.

In Metazoans, Med12 has a recognized role in cell fate and nervous system development. In higher organism like humans, a role has been attributed to Med12 in nervous system diseases, like psychosis and schizophrenia. Recent works have shown an involvement of the Med12 subunit in gene expression with X-linked mental retardation (XLMR). Med12 has been identified in a network of proteins which are associated with XLMR. This network comprises, besides Med12, also a G9 histone methyltransferase and the REST (RE1 silencing transcription factor). Med12 deletion has shown increased transcription of genes which are predominantly REST targets, confirming the participation of Med12 in extra neuronal gene silencing (Ding et al. 2008).

These results suggested that other Mediator subunits would potentially be involved in transcription silencing, despite not being part of the CDK8 module. An epistasis map of genetic interactions constructed in S. pombe by (Roguev et al. 2008) revealed a positive interaction between Mediator subunits like Med1, Med6, Med19 and Med20 and the RNAi machinery. A transcriptome analysis of 893 non-lethal S. pombe alleles showed that Δmed12, Δmed15, Δmed18 and Δmed20 mutants upregulated several telomere positioned genes including pot1+, rif1+ and rap1+. The same upregulation was observed in Δclr4 mutants (Linder et al. 2008). Therefore, we decided to undertake a project in order to assess if SPCC645.12c (which we consider as homologue of S. cerevisiae Med9), Med12 and Med18 Mediator subunits could be involved in the silencing process in S. pombe.

Chromosome loss assay

Heterochromatin has been widely studied in several organisms, including S. pombe. The centromeric heterochromatin is known to have an effect in the chromosome segregation process. A way to check if genes are involved in chromosome segregation is to estimate the loss rate of a
non essential mini-chromosome (Osami Niwa et al. 1986). In a previous work, other genes involved in heterochromatin formation, \( clr7^+ \) and \( clr8^+ \) (Thon et al. 2005) and \( clr3^+ \) and \( clr4^+ \) (Hansen et al. 2005), have been tested for chromosome loss using the strains developed by (Osami Niwa et al. 1986). Deletion of \( clr7^+ \) and \( clr8^+ \) showed an increase in mini-chromosome loss (Thon et al. 2005). Mutations in \( clr4^+ \) cells also led to an increased mini-chromosome loss, while mutations in \( clr3^+ \) showed a phenotype similar to wild type (Allshire et al. 1995).

The mini-chromosome strain is derived from the pericentric part of \( S.\ pombe \) chromosome III and it is called Ch16m23::ura4-Tel. Strains carrying the mini-chromosome are stable and partially aneuploid. In fact, they contain an \( ade6\text{-}M216 \) allele on the Ch16, and an \( ade6\text{-}M210 \) allele on the regular chromosome 3. The intra-allelic complementation between the two alleles makes this type of strain Ade+. These strains are used for chromosome loss assay since loss of Ch16 results in red or sectored colonies, which are scored on media containing a low concentration of adenine. Low levels of adenine are necessary to keep the cells which have lost the mini-chromosome alive and to allow development of red color. The cells which do not lose the mini-chromosome appear white on this media. The sectoring indicates the division phase during which the mini-chromosome is lost (Figure 38). If lost at the first mitotic division after plating, a precise half sectored colony is formed; if lost during subsequent divisions, the red sector would decrease in size. Only half sectored colonies are counted and divided by the total number of white and half sectored colonies. This is a powerful tool to investigate which mutants are potentially causing chromosome segregation defects and therefore conceivably in silencing at the centromere region.

![Figure 38](image.png)

**Figure 38.** Typical phenotype of cells which have lost the mini-chromosome, with half-sectored pattern or fully red (not shown). The white colonies still bear the mini-chromosome.

\( med12^+ \) (Srb8) (Samuelsen 2003) and \( med18^+ \) (Szilagyi et al. 2002) have been characterized in \( S.\ pombe \). Being non-essential genes, they allow further investigation of deletion mutants, which have been previously described in \( S.\ cerevisiae \) (Xiao & Fitzgerald-Hayes 1995). Therefore, deletion strains of \( med12^+ \) and \( med18^+ \) were constructed in strain MP55 carrying the mini-chromosome Ch16 in order to assess any involvement in chromosome segregation of the Med12 and Med18 Mediator subunits.
**Mini-chromosome loss assay in Δmed12 mutants**

Construction of a Δmed12 strain was performed (see Material and Methods for details) and verified by Southern Blot. Three independent colonies were grown in AA-Ade liquid medium and plated onto YES-Ade and incubated at 30°C for a few days. YES-Ade plates contain a very low concentration of adenine from the Yeast Extract. As explained before, this quantity is sufficient to reveal chromosome loss. The Δclr4 is used as control for its involvement in centromere assembly (Ekwall et al. 1996). Half-sectored colonies were counted, and the chromosome loss rate calculated (Table 4).

Table 4. Chromosome loss rate

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sectored colonies</th>
<th>Total colonies</th>
<th>Chromosome loss rate</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>72</td>
<td>14100</td>
<td>0.0050</td>
<td></td>
</tr>
<tr>
<td>Δmed12</td>
<td>48</td>
<td>8500</td>
<td>0.0056</td>
<td>5.9·10⁻¹</td>
</tr>
<tr>
<td>Δmed18</td>
<td>105</td>
<td>10400</td>
<td>0.0100</td>
<td>6.1·10⁻⁵</td>
</tr>
<tr>
<td>Δclr4</td>
<td>124</td>
<td>5570</td>
<td>0.0200</td>
<td>10⁻¹¹</td>
</tr>
</tbody>
</table>

The p-value is obtained comparing to the WT.

The Δmed12 mutation exhibits a chromosome loss rate comparable to the wild type.

**Mini-chromosome loss assay in Δmed18 mutants**

Transcription of centromeric repeats is de-regulated in a Δmed18 mutant (Heidi Hansen 2010). In addition, Δmed18 cells typically show a hyphal phenotype associated with cell aggregation and separation defects (Szilagyi et al. 2002). Approximately 95% of Δmed18 cells showed hyphal growth with obvious separation defects. These phenotypes could affect the chromosome loss assay to some extent. In order to assess the effect of the deletion of med18⁺ in the mini-chromosome strain, mutants in the Ch16 background were constructed and verified by PCR and Southern blot. It was clear that Δmed18 cells encountered slight stress when plated or grown in liquid medium. The chromosome loss assay was performed as described above for Δmed12 mutants and results are shown in table 6. The mini-chromosome loss rate for the Δmed18 mutant is significantly higher than the one obtained from the wild type.
**Sensitivity of Δmed12 and Δmed18 mutants to Thiabendazole (TBZ)**

TBZ is a spindle body poisoning compound which gives rise to multipolar and asymmetric metaphase spindles. It is extensively used in assays for mutants who are defective in centromeric chromatin structure. Δclr4 mutants are known to be sensitive to TBZ, consistent with the increased chromosome loss rate shown in **table 6**. Therefore these mutants were used as positive control. In our assay we wanted to ascertain if any other single mutant would resemble the Δclr4 phenotype. Cells were plated on two different TBZ concentrations and grown for 3 days at 30°C (**Figure 39B**).

No significant difference in growth was noticeable among the two different concentrations of TBZ (**Figure 39B**) compared to the control plate (shown in **figure 39A**) for Δmed12 and the WT, while Δmed18 showed growth impairment, indicating that med18+ has a cell growth defect similar to the phenotype of the Δclr4 mutant.

**Figure 39.** Dot spot analysis for Δmed12, Δmed18, Δclr4 mutants and WT on different plates. A: Ten-fold serial dilutions of cells were plated on two different YES plates. The first dilution was adjusted to OD 0.5. Plates were incubated for 4 days at 30°C or 37°C to score for resistance to non-permissive temperature. All strains except Δmed18 grew on both plates. B: YES+TBZ plates. Different concentrations of cells were plated on two YES plates with different TBZ concentration (12 mg/ml and 14 mg/ml). The first dilution was adjusted to OD 0.5 and followed by four 10-fold serial dilutions. Plates were incubated for 4 days at 30°C to score for mutants with weak centromeric chromatin structure. Δmed18 and Δclr4 were the most affected strains. C: Dot spot analysis for Δmed12, Δmed18, Δclr4 mutants and WT. Cells were plated on YES plates containing 1% or 2% formamide. The first dilution was adjusted to OD 0.5 and followed by four 10-fold serial dilutions. Plates were incubated for 4 days at 30°C. All strains except Δmed18, whose growth was greatly impaired, grew on both plates. D: Dot spot analysis for Δmed12, Δmed18, Δclr4 mutants and WT. Different concentrations of cells were plated on YES+6-azauracil (300 mg/ml) and YES+750 mM KCl plates. The first dilution was adjusted to OD 0.5 and followed by four 10-fold serial dilutions. Plates containing 6-azauracil were incubated for 4 days at 30°C. Plates containing 750 mM KCl were also incubated for 4 days at 30°C.
Sensitivity assay of Δmed12 and Δmed18 mutants to stress

To test whether the Δmed12 and Δmed18 single mutants were sensitive to different kind of drugs and stressful conditions (Linder et al. 2008), we set up a dot spot assay on YES plates and YES plates containing 6-azauracil (6-AU), formamide (1% and 2 %) and KCl (750 mM) at 30°C, challenging for transcription elongation defect, formamide poisoning and osmotic stress, respectively. Δclr4 and WT were also used in the screening as controls. Moreover, the same colonies were tested for high temperature sensitivity on YES plates at 37°C (Figure 39A). Three single colonies for each strain were spotted and they all responded in the same way, therefore only one representative colony is shown for each genotype. All the strains grew as well as WT except Δmed18. This is consistent with the already observed growth defect of the strain. Moreover, Δmed18 is dramatically sensitive to high temperature. Similarly, the Δmed18 mutant also showed sensitivity to formamide poisoning at 1% or 2% concentration (Figure 39C).

6-AU is a drug which inhibits the synthesis of purines and pyrimidines leading to elongation defects. For example, in S. pombe, it has been shown that mutants in the elongation factor Spt5, associated with other mutations in the rpb1" gene, are sensitive to 6-AU (Schneider et al. 2010). We wanted to test if our single mutants are sensitive to 6-AU and therefore potentially involved in transcription elongation. The mutants were spotted onto YES plates supplemented with 300 mg/ml of 6-AU and incubated for 3 days at 30°C. All the strains except Med18 mutants show the same degree of growth impairment on 6-AU relative to the no drug control, indicating that Med18 is likely to be involved in transcription elongation (Figure 39D).

Moreover, osmotic stress response was tested on YES plates containing KCl (750 mM), which were incubated at 30°C for 3 days (Figure 39D). Once again, when compared to growth at 30°C on YES plate, none of the strains exhibited sensitivity to KCl.

In conclusion, the drug sensitivity assay shows that Δmed18 is sensitive to TBZ, high temperature and formamide, while resistant to 6-AU and KCl, pointing to a role of Med18 in centromeric transcription. In contrast, Δmed12 did not show any sensitivity to any of the conditions tested.

Centromere transcript quantification for the Δmed18 mutant

The RNAi pathway involves many different known proteins and likely even more factors might have a direct or indirect role and effect into it. As Mediator is actively involved in transcription, a role for it in heterochromatin maintenance is conceivable. Several subunits have been shown to be involved in silencing at the centromeres, specifically at the dh repeats. In fact, deletion of the subunits Med1, Med6, Med19 (Roguev et al., 2008), Med18 and Med20 (Heidi Hansen 2010) lead to a moderate increase of dh transcription. This result has sparked the idea that more Mediator subunits could be involved in silencing. Moreover, RT-PCR of deletion mutants of med18" and
med20+ cause a small increase of transcription at the dh repeats during S-phase (Heidi Hansen 2010). RT-PCR was initially performed for three independent single colonies of WT and Δmed18 strains (Figure 40A). Our Δmed18 mutants on mini-chromosome strain background were tested to seek for confirmation of the transcription level increment. Pictures from the gels were then analyzed with ImageJ software and the quantitated band intensities plotted in figure 40B. The final outcome was given by the average of the three single colonies. The dh repeat accumulation was normalized to the act1+ transcription level. The p-value was 2.55·10⁻⁴, which confirmed that dh transcription in the Δmed18 mini-chromosome background is significantly elevated compared to the wild-type. As the colonies came from the same strain, they should be genetically identical. However, the results obtained for both WT and Δmed18 showed different amounts of transcripts for individual colonies coming from a single strain. This might be due to an epigenetic effect. As no differences should be present in the DNA sequence, regulation of histone modifications and heterochromatin might have caused this effect. This variability was obtained also in other Mediator mutants tested, but not shown in this study.

Figure 40A: RT-PCR amplifying dh repeats transcripts in the WT and Δmed18. Three different single colonies for each strain were analyzed by RT-PCR in order to check the dh repeat expression levels. WT single colonies all show a slightly different level of transcript, while the Δmed18 colonies all show remarkable different degrees of transcription. RT-PCR amplification of the euchromatic transcript serves as a reference. Figure 40B Transcript level of dh repeats. The bands obtained with the RT-PCR were quantitated with ImageJ software and plotted in order to see accumulation of transcripts at the centromere in WT and the single mutants of Δmed18. Error bars represent the standard deviation for three samples.

The results obtained so far lend further support for a function of med18+ in transcription at the centromere, and a role in chromosome segregation.
Telomere length in Mediator mutants

Telomeres are complexes between DNA and proteins and are found at the very end of the eukaryotic chromosomes. Their functions are to protect and stabilize chromosomes, preventing fusion between chromosomes and degradation of the chromosome ends. Telomeres tend to become shorter, due to the end replication problem (Greider 1996; Rieko Ohki et al. 2013). This occurs at every generation, but it is compensated by telomerase (Savitsky et al. 2006).

Telomere composition is different between S. cerevisiae and S. pombe but both have been widely studied in the past years. Several genome-wide screens in S. cerevisiae have shown the implication of different genes in telomere length maintenance (TLM) (Askree et al. 2004; Gatbonton et al. 2006). Many of the genes are also involved in chromatin modifications. Moreover, 10 among all the TLM genes include subunits of the Mediator complex, which have shown an indirect effect on telomere shortening (Gatbonton et al. 2006).

Many works have shown the serious consequences of telomere shortening for cell fate. In S. pombe, it has been shown that several genes like pot1+ and ccq1+ are involved in telomere maintenance (Jain et al. 2010). Moreover, heterochromatin proteins have also shown an effect on telomere maintenance and silencing. Some of these proteins are Taz1, Rap1 (Cooper et al. 1997), Swi6 (Kanoh et al. 2005), Chp2 (Thon & Verhein-Hansen 2000) and Clr4. (Allshire et al. 1995; Bisht et al. 2008; Cooper et al. 1997) have shown that deletion or mutation of Taz1 leads to abnormally long telomeres. Also, mutations in Rap1 result in extremely long telomeres, accompanied by increased transcription of the flanking region of the telomeres. Another work from (Kanoh et al. 2005) has shed light on the effect of Swi6 on telomeres. Swi6 is also a known homologue of HP1 (Heterochromatin Protein 1) and it binds to the heterochromatic regions along the chromosome. To do so, it strongly cooperates with Taz1. Moreover, Swi6 binds to the subtelomeric regions which contain sequences similar to the centromere dh repeat. When Taz1 is deleted, alone or in conjunction with RNAi-RITS, Swi6 becomes heavily destabilized (Kanoh et al. 2005). On the other hand, Chp2 function partially overlaps with Swi6. When Chp2 is deleted, it alleviates the telomere positioning effect in S. pombe (Thon & Verhein-Hansen 2000). It has been shown that Δclr4 mutants are prone to develop a high level of recombination in the sub-telomeric regions (Bisht et al. 2008).

Interestingly, other genes which are involved in chromatin formation and maintenance have been linked to telomere shortening. In fact, it was shown that dcr1+, ago1+ and rdp1+ play a major role in heterochromatin formation and maintenance at the centromere (Volpe et al. 2002), through the RNAi pathway. Dcr1 is an RNase-III like ribonuclease which cuts dsRNA into 22-23 nt small RNAs (Bernstein et al. 2001). Ago1 belongs to the RITS (RNA-induced Transcriptional Silencing) complex (Verdel et al. 2004), while Rdp1 is involved in the RDRC (RNA-directed RNA polymerase complex) (Motamedi et al. 2004).
From our experience, we knew that also Mediator subunits could play a role in telomere shortening. In fact, data obtained in collaboration with Heidi Hansen (Heidi Hansen 2010) have shown that deletions of dcr1+ , rdp1+ , ago1+ , all genes involved in the silencing pathway, lead to telomere shortening when compared to the WT (Figure 41; lane 1, 3 and 6, respectively). ∆dcr1 and ∆ago1 were the two mutated strains which displayed a more dramatic effect compared to the WT, while a milder consequence can be seen with the ∆rdp1 strain. However, this effect dramatically increased when the deletion of any of the above mentioned heterochromatin genes was combined with the deletion of med18+ and med20+ , especially with the latter. A Southern blot analysis of strains deleted for dcr1+ , rpd1+ or ago1+ , alone or in conjunction with med18+ or med20+ is shown in figure 41. Consistently, a single deletion for med18+ or med20+ also displayed a small effect on telomere length compared to the WT strain (Figure 41, lane 11). We can therefore conclude that med18+ and med20+ both have an effect on telomere length, which is exacerbated when their deletion is associated with the deletion of dcr1+ , rdp1+ or ago1+. Taken together, these data confirm the role for Mediator in telomere length control, probably via heterochromatin maintenance pathway.

![Figure 41](image.png)

**Figure 41.** Telomere shortening in heterochromatin and Mediator mutants. Samples were digested with EcoRI and probed with 32P-labeled pNSU70 DNA (Kind gift of Genevieve Thon). Lanes 1, 3, 6 display single mutants for the heterochromatin proteins, while lanes 9 and 10 show single mutants of Mediator, respectively. Lanes 2, 4, 5, 7 and 8 show the double mutants. All the mutations have been performed in the FY498 strain background (shown on lane 11). A part from ∆rdp1 mutant, all the telomeres display shortening. The depicted line is used to compare the length of the telomeres, using the WT as a reference.

The analysis of the double deletion mutants of Mediator and the RNAi pathway provided an interesting hint regarding a possible involvement of Mediator subunits into telomere length control. Therefore, we decided to test whether ∆med9 (in a TP66XTP67 background), ∆med18 and ∆med12 (both in the Ch16 background) mutants constructed during this project had a significant difference in telomere length when compared to the WT.
Δmed9, Δmed12 and Δmed18 were analyzed by Southern blot analysis, together with WT strain. The Δmed9 mutant exhibit decreased telomere length (Figure 42). In contrast, the Δmed12 and Δmed18 strains showed no significant difference between each other, but when compared to the WT their telomeres were a bit longer than the WT (data not shown). The same data for Δmed12 have been reported for S. cerevisiae (Peng & Zhou 2012). A Southern blot analysis with strains digested with Apal was also performed in order to confirm the data obtained after digestion with EcoRI. Apal cuts the telomeric repeats in smaller fragments, and the analysis performed with Apal validated the EcoRI digestion results (data not shown).

In summary, our data have shown a very likely involvement of Mediator in heterochromatin formation, both at the centromere and the telomere. Moreover, the degree of influence varies between subunits. Δmed18 mutants showed a high rate of chromosome segregation defects and also shorter telomeres. Δmed9 mutants have also displayed significantly shorter telomeres compared to WT. It is also worth to mention that the lack of med20+ in double mutants for proteins involved in RNA silencing like dcr1+, ago1+ and rdp1+, led to dramatically shorter telomeres compared to single mutants. This result supports the hypothesis that med20+ can have a strong effect on telomere control via the establishment and/or maintenance of heterochromatin at telomeres. Taken together, these results led to the conclusion that med9+, med18+ and med20+ might have a role in telomeres length regulation.

Figure 42. Telomere shortening in Δmed9 mutant. Samples were digested with EcoRI. Lane 1 shows Δmed9 (TP448) mutant; lane 2 shows TP66, which is the background strain of TP448. The yellow line is used as a reference, marking the middle of the WT band. All samples were digested with EcoRI and probed with 32P-labeled pNSU70.
Mediator regulates non-coding RNA transcription at fission yeast centromeres

Michael Thorsen, Heidi Hansen, Michela Venturi, Steen Holmberg and Genevieve Thon
Mediator regulates non-coding RNA transcription at fission yeast centromeres

Michael Thorsen, Heidi Hansen, Michela Venturi, Steen Holmberg* and Genevieve Thon*

Abstract

Background: In fission yeast, centromeric heterochromatin is necessary for the fidelity of chromosome segregation. Propagation of heterochromatin in dividing cells requires RNA interference (RNAi) and transcription of centromeric repeats by RNA polymerase II during the S phase of the cell cycle.

Results: We found that the Med8-Med18-Med20 submodule of the Mediator complex is required for the transcriptional regulation of native centromeric dh and dg repeats and for the silencing of reporter genes inserted in centromeric heterochromatin. Mutations in the Med8-Med18-Med20 submodule did not alter Mediator occupancy at centromeres; however, they led to an increased recruitment of RNA polymerase II to centromeres and reduced levels of centromeric H3K9 methylation accounting for the centromeric desilencing. Further, we observed that Med18 and Med20 were required for efficient processing of dh transcripts into siRNA. Consistent with defects in centromeric heterochromatin, cells lacking Med18 or Med20 displayed elevated rates of mitotic chromosome loss.

Conclusions: Our data demonstrate a role for the Med8-Med18-Med20 Mediator submodule in the regulation of non-coding RNA transcription at Schizosaccharomyces pombe centromeres. In wild-type cells this submodule limits RNA polymerase II access to the heterochromatic DNA of the centromeres. Additionally, the submodule may act as an assembly platform for the RNAi machinery or regulate the activity of the RNAi pathway. Consequently, Med8-Med18-Med20 is required for silencing of centromeres and proper mitotic chromosome segregation.

Keywords: S. pombe, Chromatin, RNA Pol II, Mediator, Centromere, Chromosome segregation

Background

Mediator is a large (approximately 1 MDa) protein complex that conveys regulatory signals to RNA polymerase II (Pol II). The Saccharomyces cerevisiae Mediator was the first to be characterized but Mediators have since then been described in many other species. A comparative genomics approach of approximately 70 eukaryotic genomes shows that although its exact subunit composition varies, Mediator is conserved across the eukaryotic kingdom [1]. The Schizosaccharomyces pombe Mediator consists of at least 20 subunits, all of which appear to have orthologues in Drosophila melanogaster, Caenorhabditis elegans and Homo sapiens [2].

Three distinct domains (head, middle and tail) have been identified by electron microscopy on single Mediator particles from S. cerevisiae [3]. Electron microscopy on the S. pombe Mediator also shows a head and a middle domain, but no tail domain consistent with the lack of S. pombe orthologues of the S. cerevisiae tail components [4]. The head domain can structurally be further divided (for example, a head domain submodule consisting of Med8-Med18-Med20 is found in both S. pombe and S. cerevisiae) [5,6]. In S. pombe, Med27 may also be part of this submodule [7]. A specific role for the Med8-Med18-Med20 submodule has hitherto not been described, although it is known from work in S. cerevisiae that Med18-Med20 interacts directly with the RNA Pol II subunits Rpb4 and Rpb7 [8].

Like metazoans, S. pombe has large and complex centromeres. S. pombe centromeres comprise a central core surrounded by inner and outer repetitive sequences, imr and otr respectively. The otr repeats consist of alternating dh and dg repeats (Figure 1A). Both imr and otr are heterochromatic, and reporter genes inserted into the repeats are silenced [9]. Silencing and heterochromatinization of...
complex RDRP [13], the Clr4 histone 3-lysine 9 (H3K9) methyltransferase complex CLRC [14-18] and the trimethyl H3K4 demethylase Lid2 [19]. These protein complexes are capable of interacting with modified nucleosomes and, possibly, non-coding centromeric RNAs and both types of interactions are believed to be required for proper heterochromatin formation and chromosome segregation [20,21].

In spite of the central role played by non-coding RNAs at S. pombe centromeres, little is known regarding the regulation of transcription in pericentromeric repeats. Transcription of the dg and dh repeats peaks during the S-phase of the cell cycle in a window where histone modifications change as a consequence of other cell-cycle regulated events [22-24]. Presently, only one promoter controlling transcription of a centromeric repeat has been described [25]. Consistent with transcription being performed by RNA Pol II, centromeric transcripts are poly-adenylated [26] and specific mutations in RNA Pol II subunits impair heterochromatin formation [25,27,28]. The involvement of RNA Pol II in heterochromatin assembly indicates that the Mediator complex [25,27,28]. The involvement of RNA Pol II in heterochromatin formation and chromosome segregation [20,21].

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heterochromatin, and we identify roles played by the Med8-Med18-Med20 submodule in the transcriptional regulation of centromeric repeats and thus in heterochromatin formation, centromere function and chromosome segregation.

Results and discussion

A subset of Mediator subunits are required for silencing of a centromeric ura4+ reporter gene

Genes encoding non-essential subunits of Mediator were individually deleted in FY498, a strain with the S. pombe ura4+ gene ectopically inserted in the centromere of chromosome 1, at imr1R(NcoI) [33]. In addition, a med8ts allele [34] was crossed into FY498. We found that silencing of ura4+ at imr1R(NcoI) depends on all three components of the Med8-Med18-Med20 Mediator submodule, whereas the other four Mediator subunits tested (Med1, Med12, Med27, and Med31) were dispensable for silencing ura4+ at this location (Figure 1B-D). A variegated phenotype was observed for both med18Δ and med20Δ as some clones showed a robust silencing of ura4+ whereas others showed only weak silencing. Likewise, deletion of med1+ did occasionally show derepression of centromeric ura4+; however, this was a modest phenotype compared to the phenotype of med18Δ and med20Δ. Quantification of ura4+ transcript by RT-qPCR confirmed derepression of imr1R(NcoI)::ura4+ in strains with a compromised Med8-Med18-Med20 submodule (Figure 1E).

dh and dg transcripts accumulate in the absence of Med18 or Med20

To test whether the derepression observed with the ura4+ reporter extends to the native centromeric repeats, RT-PCRs and qPCRs were performed to compare the abundance of centromeric transcripts in the wild type and the med18 and med20 deletion strains. We found that dh and dg transcripts accumulated following deletion of med20+ or med18+ (Figure 2A, B, and data not shown). The changes in transcript levels did not appear to be strand specific (Figure 2C). The size of the transcripts from the dh and dg repeats estimated by Northern blotting for the med18Δ and med20Δ mutants were similar to wild type (Figure 2D). Combined, these data indicate that the Med8-Med18-Med20 submodule is not involved in choosing promoters or transcription termination sites but that it more likely influences transcription rate or efficiency of transcript processing.

The steady-state level of centromeric siRNA depends on Med18 and Med20

The increased abundance of dh and dg transcripts in med18Δ and med20Δ mutants could be explained by either elevated transcription or reduced processing of the

Figure 2 Mutations in the Med8-Med18-Med20 submodule cause an accumulation of centromeric transcripts. (A) The steady-state level of centromeric non-coding RNA was estimated by RT-PCR in the indicated mutants. The actin transcript was used as reference. (B) RT-qPCR shows that the dh transcript accumulates in med18Δ and med20Δ strains. (C) Strand specific RT-PCR shows that med18Δ and med20Δ strains have wild-type ratios of forward to reverse transcripts. (D) Northern blot analysis shows that the length of major centromeric transcripts is unchanged in the mutants. The strains for this figure were: WT (FY498), med18Δ (MT42), med20Δ (MT26), med8ts (MT31), and dcr1Δ (TP480).
transcripts. To estimate whether \( dh \) transcripts were processed into siRNA, we performed Northern blot analyses on total RNA. A random-primed probe was generated from a PCR fragment corresponding to a region of the \( dh \) repeats known to yield high levels of siRNA [35]. Using this probe clearly showed that the processing of centromeric transcripts was not abolished when \( med18^+ \) or \( med20^+ \) was deleted as siRNA remained easily detectable in the mutants. However, the deletion strains contained approximately 20 to 30% less siRNA than the wild-type control indicative of a partial impairment of siRNA biogenesis in the two mutants. A strain without \( dcr1^+ \) did not show any detectable siRNA in this assay (Figure 3A, B). Thus, the increase in non-coding RNA levels did not result in higher, but lower siRNA production indicating that wild-type regulation of \( dh \) transcription is required for effective \( dh \) siRNA formation.

**Lack of Med18 or Med20 does not influence Mediator recruitment to centromeres**

The modest decrease in siRNA levels observed in the \( med18 \Delta \) and \( med20 \Delta \) mutants suggested that reduced processing of centromeric transcripts might not on its own account for the elevated levels of \( dh \) and \( dg \) transcripts in these mutants. Elevated transcript levels could also be a consequence of the Med18-Med20-Med8 submodule functioning as a negative regulator of transcription from the \( dh \) and \( dg \) repeats in wild-type cells. A single pericentromeric promoter driving expression of \( dg \) and \( dh \) repeats has been described in the literature [25]. We estimated Mediator occupancy at this promoter and at the \( dg \) repeat regulated by the promoter by chromatin immunoprecipitation (ChIP). The Mediator subunit Med7 was pulled down followed by qPCRs for promoter and \( dg \) sequences, respectively. The assay showed that Mediator is associated with the centromeric regions tested and that its association is not affected by deletion of \( med18 \Delta \) or \( med20 \Delta \) (Figure 4). These observations are consistent with a direct role of Mediator at centromeres and suggest that the Med8-Med18-Med20 submodule negatively regulates transcription downstream of Mediator association with centromeres.

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**Figure 3** siRNA levels in \( med18^+ \) and \( med20^+ \) deletion strains.
(A) Representative Northern blot of siRNA in wild type and indicated mutants. Total RNA was run on a 17.5% polyacrylamide/7M urea gel, blotted and hybridized as described in Materials and Methods. Ethidium-bromide staining of the same RNA preparations was used as loading control. (B) Quantification of the blots (n=4) *P <0.05; **P =5.2e to −12. The strains for this figure were: WT (FY498), \( med18 \Delta \) (MT42), \( med20 \Delta \) (MT26), and \( dcr1 \Delta \) (TP480).

**Figure 4** Mediator occupancy (Med7-TAP) at the centromeric \( dg \) promoter and \( dg \) repeat in \( med18^+ \) and \( med20^+ \) deletion strains. ChIP analyses show that the relative Mediator occupancy at (A) the centromeric promoter as well as at (B) the \( dg \) repeat is unchanged in \( med18 \Delta \) and \( med20 \Delta \) mutant strains. The strains for this figure were: WT (FY498), \( med18 \Delta \) (MT42), and \( med20 \Delta \) (MT26).
Strains lacking Med18 or Med20 display increased RNA Pol II occupancy on the dg promoter and on the dg repeat itself

One well-documented function of the Mediator complex is to regulate RNA Pol II activity [36]. We therefore assayed RNA Pol II occupancy in pericentromeric repeats by ChIP in wild-type, med18Δ, med20Δ, and clr4Δ cells. ChIP-qPCR performed both on the putative dg promoter and on the dg repeat showed an RNA Pol II enrichment of two and five fold in med20Δ and med18Δ, respectively, compared to wild-type. The enrichment of RNA Pol II in med18Δ is similar to the enrichment seen in a clr4Δ strain in a parallel experiment (Figure 5). The fact that Clr4 limits RNA Pol II occupancy at centromeres was previously reported [24] but the precise mechanism through which exclusion occurs is unknown. Our results strengthen the view that the Med8-Med18-Med20 submodule negatively regulates non-coding RNA transcription at centromeres by reducing the ability of Mediator to recruit RNA Pol II. This process might be part of the mechanism through which the Clr4 H3K9 methyltransferase excludes RNA Pol II from centromeres.

Desilencing of centromeric heterochromatin in med18 and med20 mutants correlates with decreased H3K9 methylation

The increased abundance of non-coding centromeric transcripts in strains deleted for med18+ or med20+ prompted us to investigate the methylation levels of histone H3K9. Figure 6 shows that dimethylation of H3K9 was reduced on the putative dg promoter in the med18Δ and med20Δ mutants. H3K9 methylation at the dg repeat next to the promoter was also reduced, but less significantly (data not shown). This observation is consistent with the Med8-Med18-Med20 submodule acting upstream ofClr4 to facilitate H3K9 methylation. The Med8-Med18-Med20 submodule might recruit Clr4, which would in turn inhibit RNA Pol II through H3K9 methylation. Because RNAi-directed heterochromatin formation forms a self-enforcing loop, indirect effects could also account for reduced H3K9me in Mediator mutants as depicted in the model we present in a later section.

Mutations in the Med8-Med18-Med20 submodule and deletion of clr4Δ lead to similar changes in transcription profile

More generally, we noticed that the genome-wide expression profiles of clr4 and Mediator mutants display striking similarities indicating the Med8-Med18-Med20 submodule and H3K9me act in concert at many locations other than centromeres. A total of 42/110 genes upregulated more than 1.5x in clr4-481 [26] are upregulated more than 2x in the med8Δ mutant ([37]; 164 genes are upregulated more than 2x in the med8Δ mutant). A total of 24/58 genes upregulated more than 1.5x in clr4Δ are upregulated more than 2x in the med8Δ mutant. These genes are enriched in large subtelomeric regions extending approximately 100 kb into chromosomes 1 and 2; 39/164 genes upregulated more than 2x in the med8Δ mutant are subtelomeric. These regions share properties with centromeric heterochromatin [26,38,39] The same subtelomeric gene clusters are controlled by Spt6 [40] suggesting Spt6, Clr4, and the Med8-Med18-Med20 Mediator submodule cooperate in heterochromatic gene silencing both at centromeres and at other chromosomal locations.
Chromosome segregation is affected in med18Δ and med20Δ strains
Defects in heterochromatin impair the association of cohesins with centromeric regions and increase mitotic and meiotic chromosome loss [33,41,42]. To further investigate whether mutations in the Mediator complex affect the functionality of centromeres, we measured the rate of mitotic loss of a non-essential mini-chromosome, Ch16m23::ura4+Tel[72] [43], in med18Δ, med20Δ and wild-type strains. For comparison we included a clr4Δ strain in the analysis. Chromosome segregation was affected in med18Δ and med20Δ mutants corroborating the alleviated-silencing phenotype of these mutants. These strains lost their mini-chromosome in approximately 0.3 to 0.8% of cell divisions compared to approximately 4% in a clr4Δ background and more than 0.025% in wild-type cells (Figure 6A and Table 1). These changes correspond to a 32- and 12-fold increase in mini-chromosome loss rates in med18Δ and med20Δ, respectively, compared to wild-type. In addition, strains without Med18 or Med20 were sensitive to the microtubule destabilizing agent thiobendazole (Figure 7B), further implicating Med18 and Med20 as crucial factors for maintaining centromere function.

Conclusions
The central observations presented here, that long centromeric non-coding RNAs accumulate in mutants compromised in the Med8-Med18-Med20 submodule of Mediator, that centromeric H3K9me is reduced in these mutants, and that the levels of siRNAs are not dramatically altered but, if anything, slightly reduced in the mutants can be understood as depicted in Figure 8. The model in Figure 8 proposes that one role of the Med8-Med18-Med20 Mediator submodule is to prevent the recruitment of RNA Pol II to centromeric heterochromatin. By analogy with S. cerevisiae where the Med8-Med18-Med20 submodule was reported to interact with the Rpb4/Rpb7 RNA polymerase II subunit complex [8], we propose that S. pombe Med8-Med18-Med20 also interacts with Rpb4/Rpb7. The structural studies monitoring Med18-Med20 interaction with Rpb4/Rpb7 in S. cerevisiae reveal that Med18-Med20 modulates the conformation of RNA Pol II, regulating its

Table 1 Mini-chromosome loss rate is higher in strains deleted for med18* or med20*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Half sectored</th>
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<th>Loss Rate</th>
</tr>
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<tr>
<td>WT</td>
<td>1</td>
<td>4012</td>
<td>0.025%</td>
</tr>
<tr>
<td>clr4Δ</td>
<td>85</td>
<td>2181</td>
<td>3.9%</td>
</tr>
<tr>
<td>med18Δ</td>
<td>26</td>
<td>3195</td>
<td>0.8%</td>
</tr>
<tr>
<td>med20Δ</td>
<td>7</td>
<td>2339</td>
<td>0.3%</td>
</tr>
</tbody>
</table>

Figure 6 Mutations in the Med8-Med18-Med20 submodule compromise H3K9 methylation at the centromeric dg promoter. ChIP analyses show that the level of H3K9 dimethylation at the centromeric dg promoter is reduced in med18Δ and med20Δ mutants relative to wild-type. A clr4Δ strain was processed in parallel for comparison. *P <0.003. The strains for this figure were: WT (FY498), med18Δ (MT42), med20Δ (MT26), and clr4Δ (PG3423).

Figure 7 Deletion of med18* or med20* impairs centromere function. (A) A non-essential mini-chromosome, Ch16m23::ura4+-Tel[72], is frequently lost in strains deleted for med18*, med20* or clr4*. Cells containing the mini-chromosome form white colonies on medium with low concentration of adenine while cells lacking the mini-chromosome form red colonies. Loss of the mini-chromosome in the first cell division after plating results in a half-sectored colony. (B) Deletion of med18* or med20* renders the cells sensitive to the microtubule destabilizing agent thiobendazole (12 μg/ml). The strains for this figure were: WT (FY520), med18Δ (TP527), med20Δ (TP527), and clr4Δ (PG3420).
interaction with DNA. Thus, a mutation in the Med8-Med18-Med20 submodule is likely to affect RNA Pol II function. In *S. pombe*, the Rpb7 subunit of RNA Pol II is required for initiation of transcription of centromeric non-coding RNAs. In the *rpb7-G150D* mutant reduced transcription initiation at centromeres leads to compromised heterochromatin which allows for more spurious transcription and accumulation of non-coding transcripts [25]. We propose that the Med8-Med18-Med20 submodule limits centromeric transcription in wild-type cells by inhibiting transcription initiation through Rbp4/Rpb7.

Mechanistically, the interaction between the Med18-Med20 sub-complex and the Rpb4/Rpb7 sub-complex of Pol II has been proposed to alter the conformation of the Pol II clamp domain to facilitate opening of its active-site cleft and thereby the access of promoter DNA to the Pol II cleft [44]. This interaction would facilitate pre-initiation-complex (PIC) formation. We suggest that in heterochromatin specific interactions of other components with Mediator and/or Pol II might prevent clamp movement and thereby the productive interaction of Pol II with DNA.

Since the above proposed function of Med8-Med18-Med20 might not account for the decrease in siRNA or H3K9me in the mutants, we suggest that the Med8-Med18-Med20 submodule also facilitates the processing of long non-coding RNAs into siRNA. This second function might be carried out together with the two largest *S. pombe* RNA Pol II subunits, Rpb1 [28] and Rpb2 [27]. A mutation in Rpb2, *rpb2-m203*, increases the steady-state levels of centromeric transcripts and reduces siRNA to undetectable levels [27]. The *rpb2-m203* phenotype has been taken to suggest that Rpb2 provides an interaction interface with RNAi complexes and/or a means of distinguishing non-coding centromeric transcripts from mRNA, triggering processing of the former into siRNA [27]. This presumed function of RNA Pol II, which would be compromised by the *rpb2-m203* mutation, may also be affected by mutation in the Med8-Med18-Med20 submodule. A non-mutually exclusive possibility is that Med8-Med18-Med20 facilitates processing of centromeric non-coding RNA into siRNA together with Rpb1 [28]. The *S. pombe* C-terminal domain of Rpb1 contains 28 conserved YSPTSPS repeats acting as an assembly platform for various mRNA processing factors, thus coupling transcription to pre-mRNA processing and export. A mutant form of Rpb1 (rpb1-11) retaining 16 of the 28 hepta-repeats apparently does not affect transcription of the pericentromeric repeats, but nevertheless compromises downstream RNAi function [28]. As for Rpb2, given the ubiquitous interactions between the Mediator complex and active RNA Pol II, it seems plausible that a mutation in Med8-Med18-Med20 might disturb the Rpb1-dependent RNAi machinery assembly function. Alternatively, the Med8-Med18-Med20 submodule might itself be a site where pre-siRNA processing is regulated.
Consistent with our conclusions, a very recent study by Zhu and colleagues [45], published during the writing of this article, reports an accumulation of centromeric non-coding RNA and reduced processing of the dh repeat transcript into siRNA in a med20A strain. In addition, an independent large-scale epistasis map revealed genetic interactions between subunits of the Mediator and RNAi and heterochromatin components [29]. Neither med8 nor med18 mutants were included in this screen but probing the bioGRID [46] with Osprey [47] lists 101 gen
nor med18 and heterochromatin components [29]. Neither med8 interactions between subunits of the Mediator and RNAi independent large-scale epistasis map revealed genetic transcript into siRNA in a this article, reports an accumulation of centromeric non-

cen-h+ ura4-DS/E ade6-210 imr1R(NcoI)::ura4+ori1 FY498

Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>FY498</td>
<td>h+ ura4-DS/E ade6-210 imr1R(NcoI)::ura4+ori1</td>
<td>[9]</td>
</tr>
<tr>
<td>MT6</td>
<td>h+ ura4-DS/E ade6-210 imr1R(NcoI)::ura4+ori1</td>
<td>This study med20A::KanMX</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>MT14</td>
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<td>This study med20A::KanMX</td>
</tr>
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<td>This study med20A::KanMX</td>
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<tr>
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<tr>
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<td>h+ ura4-DS/E ade6-210/216 Ch16m23::ura4*-Tel[72]</td>
<td>[43]</td>
</tr>
<tr>
<td>TP528</td>
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</tr>
<tr>
<td>PG3420</td>
<td>h+ Ch16m23::ura4*-Tel[72] leu1-32 ura4-DS/E ade6-210/216 cl4Δ∪LEU2</td>
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<tr>
<td>PG3423</td>
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<td>[17]</td>
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Methods

Strains and primers

The *S. pombe* strains used in this study are listed in Table 2 and the primers are listed in Table 3.

Table 3 Oligonucleotides used in the study

<table>
<thead>
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<th>Name</th>
<th>Sequence</th>
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<td>dhH-siRNA</td>
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</tr>
<tr>
<td>Cen-dh-FOR2</td>
<td>CGCAGAATCTCAGTGTGACAGTCC</td>
</tr>
<tr>
<td>GTO265</td>
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</tr>
<tr>
<td>GTO266</td>
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</tr>
<tr>
<td>GTO223</td>
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</tr>
<tr>
<td>GTO226</td>
<td>CTGGTGTCTTTGCTTGAGTAGG</td>
</tr>
<tr>
<td>OKR70</td>
<td>GTAGTCGCTTGAAGAGGTTAGG</td>
</tr>
<tr>
<td>OKR71</td>
<td>TGGAGACTGAGTCGTTGTGAGTCA</td>
</tr>
<tr>
<td>Act1 q-PCR FW</td>
<td>CTGTGTTCGCTTTGTTATGC</td>
</tr>
<tr>
<td>Act1 q-PCR RV</td>
<td>TAAAGTAGTCGTCGACGTCATCA</td>
</tr>
<tr>
<td>dhA q-PCR FW</td>
<td>GCAAAAGACCCCTCACATACAG</td>
</tr>
<tr>
<td>dhA q-PCR RV</td>
<td>CAAGGACTAAGGCAAGAGAC</td>
</tr>
<tr>
<td>ura4 q-PCR FW</td>
<td>CTTTGGTCTTTGCTTGAGTAGG</td>
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<tr>
<td>ura4 q-PCR RV</td>
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</tr>
<tr>
<td>p33F</td>
<td>TCGAAGTGGAAGTGGGCTTCA</td>
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<td>p33R</td>
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</tr>
<tr>
<td>p30F</td>
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<tr>
<td>omIT142</td>
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</tr>
<tr>
<td>omIT143</td>
<td>ACATCCGCAAGGTCTAGTACAG</td>
</tr>
</tbody>
</table>

RT-PCR/qPCR

RNA extraction and RT-PCR were as in [48] except for the final step where quantification was performed by ethidium-bromide staining using a Bio-Rad Laboratories imaging station and the Quantity One image analysis software (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences are listed in Table 3. For RT-PCR, the oligonucleotides GTO-265 and GTO-266 were used to amplify *ura4* and *ura4-DS/E*; GTO-223 and GTO-226 were used to amplify RNA originating from centromeric repeats or mating-type region; OKR70 and OKR71 were used to amplify actin mRNA. Strand-specific RT-PCR was achieved by using GTO-226 to prime reverse transcription on centromeric forward transcripts or GTO-223 on centromeric reverse transcripts prior PCR amplification.

RNA used in RT-qPCR was isolated using an RNeasy™ mini kit (Qiagen, Hilden, Germany) and an RNase-Free DNase set (Qiagen, Hilden, Germany). Reverse transcription of the purified RNA was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) and random hexamer primers. qPCR was performed on a CFX96 real time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) supplied with SYBR Green.
Reference Dye. Three technical replicates were performed for each of the biological triplicates. Technical replicates with standard deviations above 10% were repeated or excluded from the experiment. Primers used to amplify act1\textsuperscript{+} and the dh repeat are shown in Table 3.

**Chromatin immunoprecipitations**

ChIP was performed according to standard procedures. Antibodies used to immunoprecipitate RNA Pol II and H3K9me2 were ChIPAb RNA Pol II (Merck Millipore, Billerica, MA, USA) and histone H3 (dimethyl K9) antibody ChIP Grade ab1220 (Abcam, Cambridge, MA, USA), respectively. Protein G Dynabeads were used to pull down the antibody captured proteins. Rabbit Anti-Mouse Immunoglobulins (Dako, Glostrup, Denmark) were covalently coupled to the surface of Dynabeads with the Dynabeads Antibody Coupling Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) and these beads were used to pull down the Mediator complex through a TAP-tagged Med7. Presence of RNA Pol II, Mediator or dimethyl H3K9 was detected by qPCR using the primers dhA q-PCR FW and dhA q-PCR RV for the dh repeat, oMiT142 and oMiT143 for the dg repeat, or oMiT127 and oMiT128 for the putative promoter.

**Abbreviations**

ChIP: Chromatin immunoprecipitation; RNA Pol II: RNA polymerase II; RT-PCR: Reverse transcription PCR; RT-qPCR: Quantitative reverse transcription PCR.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

MT, HH and MV carried out the research. MT, GT and SH wrote the manuscript. GT and SH provided guidance in experimental design and interpretation of data. All authors read and approved the final manuscript.

**Acknowledgements**

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**References**


DISCUSSION

Mediator subunits Med9, Med11a and Med11b and their role in heterochromatin

In this project, great attention has been dedicated to the possible roles that Med9, Med11a and Med11b, subunits of the Mediator complex, can have both in transcription and in heterochromatin maintenance. As already explained in the chapter regarding med9+ (SPCC645.12c), med11a+ (SPAC23A1.15c) and med11b+ (SPCC644.10), our attempts to demonstrate the presence of these two subunits in the Mediator complex remain inconclusive.

A TAP purification of Med9-13Myc did not show the presence of the expected 44 kDa band. The fact that no Med9-13Myc signal was obtained in the elution fraction shown in figure 26 could be interpreted as the fact that Med9 is not part of the Mediator complex but it is known from literature (Khorosjutina et al. 2010) that Gal11/Med15 as well was at first disregarded as Mediator subunit and then reinstated because its roles in the complex were identified. Also, the pull down experiments performed with the strains constructed with Med9, subsequently bearing a 2xFLAG-His6 tag were inconclusive. However, immunoprecipitation of Med9-13Myc in figure 28, has shown that the polyclonal α-Myc antibody was able to pull down Med9, but without co-immunoprecipitation of Mediator subunits. Taken together, these results show that we did not succeed in the co-immunoprecipitation of Mediator with Med9.

Nevertheless, med9+ being not essential (this work), it was possible to carry out different kinds of experiments on deletion mutants for this gene, trying to find a connection between SPCC645.12c and heterochromatin function.

Moreover, two already characterized Mediator subunits, Med12 and Med18, have been included in this project, in order to see if they would also have a possible role in heterochromatin function at the centromere and telomere. Three different assays for centromere function were applied in this study: 1) chromosome loss; 2) TBZ sensitivity; 3) quantification of dh repeat transcript.

Chromosome loss in Mediator mutants

In this project we have analyzed three Mediator subunits for their possible involvement into the heterochromatin pathway. med12+ and med18+ were deleted from a S. pombe strain bearing a non essential mini-chromosome. med9+ was deleted in the wild type strain (TP66 and TP219).

Our experiments carried out in strains mutated for med12+ and med18+ in the mini-chromosome strain aimed to show involvement of Mediator in heterochromatin function at the centromere. The results obtained in this study for Δmed12 and Δmed18, have shown that Δmed12 mutants exhibit a chromosome loss rate comparable to the wild type, while Δmed18 mutants show an increased chromosome loss. Similar results were obtained by (Thorsen et al. 2012) for Δmed18 mutants.
Both results led to the conclusion of a very likely involvement of med18’ heterochromatin function at the centromere and in chromosome segregation.

**Drug sensitivity assay of Mediator mutants**

Other works have shown that strains with mutations in genes encoding for proteins involved in chromatin functions, like Pst1, which belongs to the SIN3-HDAC family (Silverstein et al. 2003) or Crlr4 and Swi6 (Ekwall et al. 1996) are sensitive to the Spindle pole body poisoning agent (TBZ). When Δmed18 mutants were tested for sensitivity to TBZ, both at 12 mg/ml and 14 mg/ml, they showed a slight growth impairment compared to the WT and the Δmed12 mutants, indicating the involvement of med18’ in chromosome segregation. (Thorsen et al. 2012) did obtain similar results with Δmed18 mutants. This is consistent with the results achieved in the chromosome loss assay, where we observed an increased chromosome missegregation for the Δmed18 mutants, but not for Δmed12 cells. Moreover, the results obtained in the 6-AU and KCl stress assay showed that Δmed12 and Δmed18 strains are probably not involved in the transcription elongation process. In conclusion, the results point to an involvement of med18’ in centromeric transcription, while med12’ has not shown any role in transcription in the assays we used.

**Silencing of the centromeric regions in Mediator mutants**

As indicated previously by the Master thesis project of Heidi Hansen (Heidi Hansen 2010), med18’ appears to play a role in transcriptional silencing at centromeres and telomeres. Accordingly, we confirm here that accumulation of the dh repeat transcripts at the centromere was significantly higher in Δmed18 compared to the WT (Figure 40A). RT-PCR for quantification of the level of dh transcripts was performed for Δmed18 in the Ch16 background, and gave similar results to those obtained by Heidi Hansen (Heidi Hansen 2010) with Δmed18 and Δmed20 mutants in the FY498 background, as well as in (Thorsen et al. 2012). The three individual clones tested in the RT-PCR assay gave a variable transcript amount at the dh repeat (Figure 40A). Similar variability was observed in the chromosome loss and TBZ sensitivity assays.

The amount of transcript at the dh repeat varies significantly from one colony to another, as shown in figure 40A, not giving a clear and reproducible result. Therefore, a possible explanation is given by an epigenetic effect affecting the colonies which we were not able to control. The Position Effect Variegation (PEV) is one example (Allshire et al. 1994). In fact, several factors could interfere with the dh transcript level at the centromere. Nucleosome remodeling and post-translational modifications like histone modifications are some of the possible causes of the epigenetic effect. The fluctuation, however, is seen in both WT and in the Δmed18 mutant. Taken together, these results lead to the conclusion that Mediator is involved in the regulation of transcription at different levels. In fact, the chromosome segregation defects observed in Δmed18 together with the TBZ sensitivity and centromere silencing defects prove that Mediator is required for proper
heterochromatin maintenance at the centromere, but still holding a major role in transcription promotion at the euchromatic sites too.

**Mediator and telomere length**

Interesting results have also been obtained by our group regarding Mediator function and telomere length. $\Delta med18$ and $\Delta med20$ strains were shown to have shortened telomeres compared to WT. However, the length difference increased when $\Delta med20$ mutation was combined with mutations in proteins involved in RNA silencing such as Dcr1, Ago1 and Rd1 (Heidi Hansen 2010).

In order to confirm the previous results, we decided to test if also the $\Delta med12$ and $\Delta med18$ mutants we have constructed for the heterochromatin project would have any influence on telomere length. The results obtained have corroborated the role for $med12^+$ and $med18^+$ in telomere length regulation. Moreover, the telomere length of a $\Delta med9$ (TP448) mutant strain was also assessed.

(Gatbonton et al. 2006) have shown, during a genome-wide screen in *S. cerevisiae*, the involvement of Mediator subunits in telomere length regulation. In their study, they have found that Med1, Med3, Med12, Med13 (Gatbonton et al. 2006) Med15, Med18 and Med20 subunits (Gatbonton et al. 2006; Peng & Zhou 2012) and the CDK8 complex all have an effect on telomere length (Askree et al. 2004). According to (Peng & Zhou 2012), both $\Delta med9$ and $\Delta med12$ mutants in *S. cerevisiae* have longer telomeres than WT, while $\Delta med18$ mutants have shortened telomeres.

These results do partially match with our results where $\Delta med9$ and $\Delta med18$ mutants in *S. pombe* all displayed shorter telomeres while $\Delta med12$ mutants showed longer telomeres (data not shown). The different result for our strains could be due to a different model organism (*S. pombe* versus *S. cerevisiae*). In *S. cerevisiae*, in fact, the heterochromatin formation follows a different pathway compared to *S. pombe*. Therefore, our data suggest that only some of the *S. pombe* Mediator subunits are involved in maintaining telomere length and in our case $\Delta med9$ was the one that showed the most remarkable effect.

An important factor recently discovered in heterochromatin maintenance at the telomeric level in animals and fungi is called TERRA (Telomeric Repeat-containing RNA). It is formed by a large non-coding RNA, containing both subtelomeric and telomeric sequences (Azzalin et al. 2007). Despite being a conserved structure, TERRA does not seem to be essential for maintenance of telomeric chromatin but it likely has a structural function during telomere assembly, as reviewed by (Luke & Lingner 2009).

A recent work from (Bah et al. 2012) have shed light on TERRA in *S. pombe*, through the discovery of telomeric G-rich TERRA, a complementary C-rich sequence called ARIA, ARRET sequences (subtelomeric RNA transcribed in the opposite direction of TERRA) and αARRET,
(which are complementary to ARRET). In their work, they have also shown the direct involvement of rap1*, cid12* and cid14*. rap1* is involved in telomere length maintenance and also has a role in PVE (Kanoh & Ishikawa 2001), while cid12* has a role in RNAi pathway as part of the RDRC complex (Motamedi et al. 2004). cid14* is a poly (A) polymerase (Win et al. 2006). They have also shown by RNA-FISH that ARIA and TERRA localize in the nucleus and, while ARIA is mainly found in the nucleoplasm, TERRA seems to be recruited to chromatin domains probably not fully depending on rap1*. Moreover, they have described the involvement of the rbp7* RNA Pol II subunit into ARRET and αARRET transcription, showing the requirement of rbp7* to keep ARRET and αARRET cellular levels. Since Mediator is involved in transcription, there could be a relationship between ARRET, αARRET and Mediator subunits. In fact, Mediator mutants might negatively interfere with the ARRET and αARRET transcription, therefore leading to shortened telomeres. Taken together, these results have shown that dsRNA and siRNAs can be formed by the two complementary RNAs ARRET and αARRET, in vivo. It is still not clear, though, if the subtelomeric siRNAs which have been found in S. pombe derives from ARRET and αARRET.

Final conclusions and perspectives

Our results have shown that Mediator is quite likely involved in heterochromatin assembly and maintenance. The two different Mediator subunits, Med9 and Med18 have a function in heterochromatin, while Med12, which belongs to the repressive CDK8 complex, is the only one that did not show any strong affiliation with the heterochromatic pathways. However, Med12 is known to be involved in several pathways leading to brain formation in Zebrafish (Hong & Dawid 2011). It is also related to X-linked disorders with autistic features (Kaya et al. 2012) and mental retardation (Ding et al. 2008). These facts could possibly open to new prospects about Med12 in a wide variety of roles and perhaps, a role into heterochromatin will soon be discovered. Regarding med18*, our results demonstrate involvement in the heterochromatin maintenance at the centromere. A work from (Thorsen et al. 2012) has also confirmed this conclusion. Moreover, the chromosome loss assay showed and confirmed that med18* is required for chromosome stabilization during mitotic chromosome segregation in WT cells, also confirmed by (Thorsen et al. 2012). Although these results are quite comforting, the role of med18* in the telomere length regulation is not so striking. We can therefore speculate that med18* has an important role in heterochromatin maintenance at the centromere but quite reduced at the telomere.

med9* mutants, instead, have given quite interesting results on telomere length. A substantial difference in length between Δmed9 cells and its isogenic WT strain was observed. At the present time no data is available from other published works, so all our conclusions about med9* are novel.

Our experiments corroborate the proposed role of Mediator in transcription. Many works have shown the interaction between Med18 and Med20 subunits (Cai et al. 2010; Carlsten et al. 2012),
which, as heterodimer, are known to interact with TBP, leading to structural changes in Mediator complex. This relation could control the interaction with RNA Pol II, which directly interact with the RNA Pol II through Rpb4 and Rpb7 subunits (Cai et al. 2010).

The fact that med18+ deletion leads to increased transcription at the centromere, confirms a direct effect of Mediator on transcription, also supported by the ChIP experiment carried out by (Thorsen et al. 2012) for the Med8-Med18-Med20 submodule. Mediator seems to be always associated with the centromeric regions and deletion of med18+, did not change any occupancy level of Mediator at the centromere (Thorsen et al. 2012). Moreover, the same data have been confirmed by experiments on the subcomplex Med8-Med18-Med20 from (Carlsten et al. 2012), in which Δmed18, Δmed12 and Δmed20 strains, have shown a similar behavior to our mutants when tested for TBZ sensitivity.

**Perspectives**

In our study about the effect of Mediator mutants in heterochromatin function using the mini-chromosome strain as background, we have considered only single mutants, which provided a lot of useful and novel information. The next step would be to construct a Δmed9 strain in the mini-chromosome background as well, as we obtained quite interesting results in the telomere length assay with Δmed9 mutants. Moreover, it would probably be interesting to construct double mutants with Δmed18 and Δmed9 deletions. In fact, the double mutation may alleviate the corresponding phenotypes, especially regarding the hyphal growth in Δmed18 single mutants.

Also, as shown in the Results chapter, we tested Δmed9 mutants on TP66xTP67 background.

The deletion mutant for med9+ (TP448) was the only one which showed shortened telomeres when compared to its background strain (TP66) ([Figure 45](#)). In order to confirm this result, it would probably be advisable to repeat the same Southern Blot analysis for TP448 and TP449 (another Δmed9 mutant isogenic to TP66) and also for other Δmed9 strains isogenic to TP219 and TP220. In case the result would be confirmed, this would lead to the hypothesis that Med9/SPCC645.12c might have a significant role in telomere maintenance.

With this work, we have laid the foundations about Med9 and Med11. Being results novel, we have shed new light on the future possible studies about Med9 and Med11. Our experiments were based on previously characterized Mediator subunits but it is understandable that different approaches are needed in order to achieve the complete characterization of Med9 and Med11.

Even though we cannot totally exempt them from being Mediator subunits, we could still study Med9 and Med11 from a different point of view or pursue with our initial plan, but with different scientific approaches.
MATERIALS AND METHODS

Construction of strains

ΔSPCC645.12c, ΔSPAC23A1.15c and Δmed11 mutants were constructed and all the detailed protocols are shown below.

Long flanking region PCR strategy

Our strategy was based on the method of constructing a fragment to be transformed in yeast using a vector containing a Hygromycin resistance cassette as a “frame”. The newly made primer sets (Yec sets, see appendix) included a recognition site for a restriction enzyme also present on the hygromycin vector polylinker pTK1435 (that was used as an attachment site) plus around 25 bp of a yeast genome sequence homologous to the 5’ end or 3’ end region both preceding and following the target gene ORF. Once cloned and then transformed into E. coli strains bearing a hygromycin resistance cassette, restriction enzymes will allow the release of a fragment containing the hygromycin resistance cassette together with a fragment of around 50 bp which has homology with regions beside the gene of interest. This homology leads to the exchange of the gene with the cassette marker through homologous recombination.

Two primer sets were made and a general illustration of primer positions for all three genes is shown in figure 43.

![Figure 43](image)

**Figure 43.** General schematic for primers position. The schematic is valid for all three genes analyzed. YecX and Yec Y correspond to the 5’ primer set, while the YecW and YecZ correspond to the 3’ primer set.

The yeast DNA template came from the WT strain MP9.

After PCR amplification with the Yec primers, A-tailing of the amplified fragment and ligation of the same were performed according to the Promega protocol using pGEM T-Easy vector for cloning. 1 μl of YecX-YecY standard reaction was transformed in SURE2 competent cells from Stratagene and then plated on LB + ampicillin plates. Colony PCR was performed for 10 selected colonies to prove their correctness.
Further digestions were carried out to confirm that the recognition site for the chosen restriction nuclease was present in the cloned PCR product. Once that the presence of the restriction site was assessed, the sequencing of the chosen samples was always carried out after any transformation step, in order to make sure that a correct integration had occurred. Midi preps of correct bacterial clones were prepared, followed by a double digestion in order to transfer the proper fragment to the hygromycin vector (TK1435, pFA6a-HphMX6), which was previously cut with the respective restriction endonucleases and gel purified. Ligation was performed according to the manufacturer protocol (T4 DNA Ligase, NEB, cat no. M0202S).

Afterwards, transformation was performed with SURE2® High competent cells (Stratagene) and DNA was extracted from the obtained colonies. A restriction digest was carried out in order to confirm the correct assembly of the new vectors (see individual vectors for restriction enzyme choice).

Colony PCRs were performed on E. coli transformants and some of the colonies which gave the expected size (details not shown) were grown in order to extract DNA. Midi preps were prepared and the knock out fragment was cut out with proper restriction enzyme and was then transformed in yeast diploids (TP66xTP67 and TP219xTP220) after sequencing.

After transformation and plating on YES plates, cells were replicated to YES+Hph plates and the surviving colonies were those who became hygromycin resistant. Eight of them were picked and checked by PCR.

**Extraction of genomic DNA from S. pombe**

15 ml of S. pombe cell were grown overnight in YES medium at 30°C then collected through centrifugation for 5 minutes at 3000rpm at 4°C. They were then re-suspended in 1.8 ml SP1 buffer (1.2 M Sorbitol 50 mM Sodium Citrate, 50 mM Sodium Phosphate, 40 mM EDTA, pH 5.6). 10 mg glucanex dissolved in 0.2 ml SP1 buffer were used for degrading cell walls and incubation at 37°C for 20 minutes followed. Spheroplasts were collected by centrifugation at 2000 rpm for 2 minutes at 4°C, re-suspended in 675 μl of 5X TE (50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0) and broken by addition of 100 μl 10% SDS and incubation at 65°C for 15 minutes. Cell content was precipitated adding 225 μl 5M KAc and incubation on ice for 20 minutes, followed by centrifugation at 20000 rpm for 10 minutes at 4°C in order to clear the supernatant that was then transferred to a clean tube. One volume of -18°C isopropanol was used to precipitate nucleic acids and followed by centrifugation at 15000 for 5 minutes at 4°C. The pellet was then washed with -18°C 70% ethanol, re-suspended in 500 μl 5X TE and 8 μl of 5 mg/ml RNase and incubated at 37°C for 20 minutes. 1.3 μl 15 mg/ml Proteinase K was added to the suspension and incubated at 55°C for 1 hour. Phenol extraction was performed and the final upper layer was transferred to a new tube. Precipitation of DNA was carried out with 50 μl 3 M NaAc and 1250 μl 96% -18°C ethanol and then
collected through centrifugation for 20 minutes at 20000 rpm at 4 °C. The pellet was then washed with -18°C 70% Ethanol and dissolved in 30 μl TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0).

**Mini/MiniPrep of Escherichia coli**

5 ml/100 ml of *E. coli* were grown overnight at 37°C and collected by centrifugation at 13000 for 10 minutes. Extraction of DNA was performed using the Qiagen Plasmid Midi Kit (cat. No. 12125) or the GeneJET™ Plasmid Miniprep Kit (cat no. K0502, Fermentas), according to the manufacturer's instructions.

**Cloning of PCR products**

Cloning of PCR product was carried out using the pGEM®-T Easy Vector System from Promega (cat. No. A1380) following the manufacturer's instructions. A PCR product was obtained using Pfu polymerase and running a program different according to the length of the desired fragment then A-tailed.

**Extraction and purification of DNA from agarose gel**

The agarose gel containing the appropriate DNA was run until ready. Extraction of DNA was performed using QIAquick Gel Extraction Kit from Qiagen (cat. No. 28706) or the GeneJET™ Gel extraction KIT (cat.no. K0691, Fermentas) according to the manufacturer's instructions.

**PCR product purification**

PCR product was purified using the QIAquick PCR purification Kit from Qiagen (cat. No. 28106), according to the manufacturer's instructions.

**Yeast transformation**

100 ml of *S. pombe* cells were grown on YES 10⁷ cell/ml then collected by centrifugation for 3 minutes at 3000 rpm. Cells were washed once with water, re-suspended in 1 ml of water, spun down to pellet and washed once with 1 ml LiAcTE. Cells were collected by centrifugation at 1000 rpm and re-suspended in LiAc/TE in order to obtain 2x10⁹ cells/ml. 100 μl of cells were mixed with 2 μl of carrier DNA and 10 μl of transforming DNA then incubated for 10 minutes at room temperature. 260 μl of 40% PEG/LiAc/TE was added, and then incubated at 30°C for 45 minutes.

43 μl of DMSO were added and cells were heat-shocked for 5 minutes at 42°C. 500 μl of water were added, mixed and cells were pelleted at 1000 rpm for 2 minutes. Cells were re-suspended in 500 μl of water and plated.
Tagging of SPCC645.12c, SPAC23A1.15c and Med11

The proteins of choice had to be tagged in order to perform TAP-tag purification with the aim to confirm their belonging to the fission yeast Mediator complex.

The tagged strain construction was performed by the same principle as used for making of the deletion strains, using different primers (see appendix) and a different plasmid vector which was carrying either a 3HA or 13Myc tag together with a hygromycin resistance cassette. Correct integrations were confirmed through PCR and Southern blot (Figure 44 for SPCC645.12c and figure 45 for SPAC23A1.15c).

**Figure 44.** Southern blot of SPCC645.12c-13Myc cells. In order to prove a correct insertion of the 13Myc tag, Aliquots of SPCC645.12c-13Myc strain were digested with different restriction enzyme, which would cut both inside and/or on the side of the hygromycin resistance cassette. Lane 1, 2 (strains carrying also a TAP tag on TRAP240’) and 3 (TAP tag on med7’) show the outcome after cutting with BamHI (expected size 3160). Lanes 4, 5 (TAP-tag on TRAP240”) and 6 (TAP-tag on med7”) show the expected bands after cutting with BanI and BclI (1222bp and 2219bp). Lanes 7, 8 and 9 show the WT digested with three different restriction enzymes. The bands in the WT and all the other visible extra bands in all the lanes are due to the kanamycin band present in the background strain where the SPCC645.12c-13Myc fragment was transformed in. The red stars indicate the correct bands for the hygromycin cassette.
Figure 45. Southern blot of SPAC23A1.12c-3HA cells. In order to prove a correct insertion of the 3HA- tag, Aliquots of SPAC23A1.12c-3HA were digested with different restriction enzyme, which would cut both inside and/or on the side of the hygromycin resistance cassette. Lane 2, 3 (strains carrying also a TAP tag on TRAP240), 1 and 4 (TAP tag on med7') show the outcome after cutting with BsrGI and HpaI (expected size 3244). Lanes 6, 7 (TAP-tag on TRAP240') and 5, 8 (TAP-tag on med7') show the expected bands after cutting with BanI and BsrDI (1553bp and 2171bp). All the other visible extra bands in all the lanes are due to the kanamycin band present in the background strain where the SPAC23A1.12c-3HA fragment was transformed in. The red stars indicate the correct bands for the hygromycin cassette.

Whole cell extract preparation for Western blot

100 ml of yeast culture was grown at 32°C overnight to OD_{600} 0.5. Cells were collected through centrifugation at 3000 rpm for 3 minutes then washed once with water. Pellet was re-suspended in 400 μl 3X TAP buffer (100 mM KOH-Hepes, 100 mM KCl, 1mM MgCl2, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT and PI (Roche Complete Protease Inhibitor Cocktail Tablets), rinsed once and then transferred to a screw cap tube with around 600 μl of 425-600 μm glass beads. Cells were broken with FastPrep machine for 5 times at setting 6.5 with intervals of 2 minutes between each breaking. Tube bottom was pinched with a needle and centrifuged briefly to get the extract that was then centrifuged at 15000 rpm for 15 minutes. Both supernatant and pellet were kept. An SDS-PAGE gel was made at 11% concentration of Bis-acrylamide (1.84 ml 37.5% polyacrylamide, 1.25 ml 4x separating buffer (181.7g/l Tris, 3 g/l EDTA, 4 g/l SDS, pH 8.8), 1.90 ml H_{2}O, 10 μl TEMED and 10 μl 10% APS) using the BioRad equipment. The separating gel was cast between two sealed glass plates and then H_{2}O was added on top. In the meantime, the 11%
stacking gel was prepared with 600 µl 37.5% polyacrylamide, 1 ml stacking buffer (60.6 g/l Tris, 3 g/l EDTA, 4 g/l SDS, pH 6.8), 2.35 ml H₂O, 25 µl TEMED and 25 µl 10% APS. After the polymerization for the separating gel was concluded, the water on top was discarded and the stacking gel was added on top. To 10 µl aliquots of each sample (supernatant), 10 µl of Sample Buffer (1.6 ml 1M Tris-HCl (pH 6.8), 4 ml 10% SDS, 2 ml Glycerol, 1 ml β-mercaptoethanol, 4 mg Bromophenol blue; 1.4 ml H₂O) were added and then denaturation was carried out at 95°C for 5 minutes. Samples were loaded on the gel and run with 1X Running buffer (10X Running buffer SDS: 144 g/l Glycin, 30.3 g/l Tris-Base, 10 g/l SDS) for around 1 hour at 120V. When finished, the gel was transferred to a nitrocellulose membrane (MFS, Membrane Filter Mixed cellulose Ester, Advantec) through wet transfer system using the BioRAD (Mini Protean II) and the TGM buffer (10X TGM: 12.1 g Tris-Base; 72.3 g Glycine). Transfer was carried out at 200 mV for 90 minutes at 4°C with constant stirring. Membrane was blocked in 10 ml of 1X TBS + 5% milk for 1 hour at 4°C. The membrane was then incubated overnight at 4°C with primary antibody (according to the experiment) on a shaker, then rinsed 3 times for 10 minutes with TBS + 0.5% Tween 20. Choice of secondary antibody was then added according to the experiment and incubated at room temperature for 1.5 hours on shaker. Membrane was rinsed 5 times for five minutes with 1X TBS + 0.5 Tween 20 and last rinse was made with 1XTBS. The development solution was then prepared with Amersham™ ECL Plus Western Blotting Detection System (cat. No. RPN2132) from GE Healthcare on Amersham Hyperfilm™ ECL High performance chemiluminescence film (cat. No. 92667).

**TCA precipitation**

100%TCA was added to samples in order to have a final 20% TCA concentration. After 20 minutes on ice, WCE was spun down for 15 minutes. After removing all TCA, the pellet was re-suspended in 2X sample buffer and then loaded on the SDS-PAGE.

**Protein purification of TAP-tagged proteins**

15 liters of *S. pombe* cells were grown to OD 3.0- 4.5 (600 nm) in YES medium supplemented with 0.2 g/liter adenine. Cells were collected by centrifugation. After supernatant was cleared by centrifugation, at 9,000 rpm, for 15 min, at +4°C, 1/9 vol. of 2 M KCl was added and stirred for 15 minutes, washed once with ice-cold water, and frozen in the liquid nitrogen. Cells were broken in a Freezer/Mill, using the following program: 10 minutes pre-cooling, 5 cycles with 2 minutes beating at stringency 14 and 2 minutes rest. Broken cells were suspended in 0.5 ml of buffer TAP buffer (200 mM KOH–Hepes, pH 7.8/15 mM KCl/1.5 mM MgCl₂/0.5 mM EDTA/15% glycerol) per g of cell pellet 0.5 mM DTT, and protease inhibitors (Roche Complete Protease Inhibitor Cocktail Tablets) in the total volume. After supernatant was cleared by centrifugation (JA-10, at 9,000 rpm, for 15 minutes, at +4°C), 1/9 vol. of 2 M KCl was added and stirred for 15 minutes. After
ultracentrifugation (Ti45, Beckman Coulter, at 42,000 rpm, for 30 minutes, at +4°C) supernatant can be frozen, or processed further.

300 µl of IgG beads (slurry) (Amersham Biosciences) was added per 30-45 ml of extract and incubated for 1 h at +4°C. IgG beads were collected by centrifugation at 1,000 rpm, for 2 minutes, at +4°C, all beads were combined into one column and washed with 30 ml of IgG buffer (10 mM Tris-HCl, 150 mM KOAc/or 150 mM NaCl, pH 8.0). After washing with 20 ml of tobacco etch virus (TEV) protease cleavage buffer (10 mM Tris-Hcl pH7.6, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 0.05% NP-40, pH 8.0), Mediator was eluted by incubation for 1.5-2 h at +16°C with 200 units of TEV protease in 1.5-2 ml of TEV buffer (for cells from 15 l of yeast). Aliquots of IgG beads, elution and input were all loaded on a SDS-PAGE gel and run for approximately one hour at 100V. The gel was then transferred to a nitrocellulose membrane and incubated with Amersham™ ECL Plus Western Blotting Detection System (cat. No. RPN2132) and developed with Amersham Hyperfilm™ ECL High performance chemilluminescence film (cat. No. 92667).

Gateway® Recombination Cloning Technology

The Gateway® Recombination Cloning Technology from Invitrogen was used to attach a 2xFLAG tag and His6 tag to our proteins of interest. 2xFLAG Tag and His6 tag were attached to both SPCC645.12c and Med11 genes. All the strains were kindly provided by Christian Holmberg. The Gateway® system (Invitrogen) is based on a site-specific recombination system of the bacteriophage lambda from E. coli leading to integration of the former’s DNA into the latter’s chromosome. Different kinds of clones are used in the gateway system. In our case we used the following Entry clones:

- 4FI (ORF 645.12C)- SPCC645.12c (Figure 46A)
- 3BI (ORF 644.10) — med11+ (Figure 46B)

![Figure 46A](image1.png) **Figure 46A.** Entry clone for SPCC645.12c, to be used in conjugation with a destination vector.

![Figure 46B](image2.png) **Figure 46B.** Entry clone for med11b+, to be used in conjugation with a destination vector.
These entry clones were then inserted on specific Destination Clones bearing a 2xFLAG-His<sub>6</sub> tag. The destination vectors were:

- FFH41C (tag on C-terminal)
- HFF41C (tag on N-terminal)

**Figure 47A.** Final outcome of the destination vector after insertion of the entry clone for the gene of interest. The 2xFlag and His<sub>6</sub> tag are in N-terminal position. GOI= Gene of Interest

**Figure 47B.** Final outcome of the destination vector after insertion of the entry clone for the gene of interest. The 2xFlag and His<sub>6</sub> tag are in C-terminal position. GOI= Gene of Interest

Mini preps of both Entry clones and Destination clones were prepared, followed by a LR recombination reaction (Figure 47A and 47B) carried out according to the manufacturer's instruction (LR Clonase® II Enzyme mix, Invitrogen). The LR reaction occurs between the att sites (attachment sites) that are slightly similar between each other, but not enough to recombine with each other. Expression clones were then transformed into E. coli competent cells (DH5α) and DNA was extracted from the resulting clones. In order to check the correctness of the recombinants, digestion reactions were performed using NotI. This restriction nuclease is supposed to cut three times if the vectors made are correct and this can be proved according to the bands visible on the electrophoresis gel and showed in the Results session.

**Southern blot**

~25 μg of digested DNA were loaded into 0.7% agarose TBE gel and run overnight at 50 V, together with λ DNA marker. DNA was depurinated by soaking the gel in 0.25 M HCl twice for 20 minutes, denatured by soaking the gel in 0.5 M NaOH, 1.5M NaCl twice for 20 minutes. Neutralization followed in 1.5 M NaCl, 1 M Tris-HCl (pH 7.4) twice for 30 minutes. DNA was then blotted onto a nylon membrane (Hybond-XL, Amersham) in 10X SSC (1.5 M sodium chloride, 150 mM sodium citrate; pH 7.0) overnight. The day after the membrane was rinsed in 1XSSC and then cross linked by exposure to ~120mJ/m<sup>2</sup> of UV.
Hybridization

The membrane was incubated with 10 ml of pre-heated ULTRAhyb™ (Ambion) for prehybridization at 42°C for 2-4 hours.

Probes preparation

Hybridization probes were obtained through digestion of pTK1435 with restriction enzymes BglII and SacI for the hygromycin resistance cassette, while the plasmid pTK1049 was digested with the same restriction enzymes in order to obtain the KanMX6 cassette. Antibiotic resistance cassettes were gel purified and diluted to 12.5 ng/µl. Probes were labeled with α^{32}P-dCTP isotope and Stratagene Prime IT® Random Primer labeling kit (cat. No.300385) according to the manufacturer protocol.

The λ DNA marker was also used as a probe and prepared in the same way as the antibiotic resistance cassettes.

The probes were purified with Amersham G-50 columns following the manufacturer instructions. After purification, 6 µl of the ladder-probe were added to the other probes (resistance cassettes) and then denature 95°C for 5 minutes, ice for 3 minutes. All the ~56 µl of each cassette resistance probe were pipetted in the glass tube containing the hybridization buffer and the respective membrane and left overnight at 42°C turning constantly. The day after, membranes were washed twice for 5 minutes in 2x SSC and 0.1% SDS at 42°C. The third rinsing was done twice with 0.1X SSC and 0.1% SDS at 42°C for 15 minutes. The last rinse was done with 1X SSC. After rinsing process, the membranes were wrapped on cling film and then the screen was set, using the KODAK BioMax MS Film (cat. No. 8222648) and put at -80°C for at least 16 hours. The film was developed using the KODAK X-omat 1000 machine. The same procedure was followed for telomere length Southern blot, where a pNSU70 probe was used. The pNSU70 contains S. pombe telomeric repeats sequences which will hybridize to the endogenous S. pombe telomeres. Digestion of pNSU70 with Apal and EcoRI gives short sequences which allowed hybridization to all 6 telomeres, because of the small telomeric repeats found at the end of all S. pombe chromosomes.

Chromosome loss assay

Cells were grown in AA-Ade liquid medium overnight and diluted the day after in order to have around 500 cells per plate. After plating on 10 YES-Ade plates, incubation at 30°C for few days and at 4°C for two to enhance the red colour was followed by cell counting. The number of half sectored cells was divided by the total number of white plus half-sectored colonies, giving the chromosome loss ratio.
Appendix 1

Proteins and Bioinformatics

Proteins consist of a sequence of amino acids referred to as their primary structure. The 3D protein structure instead, is given by the secondary, tertiary and quaternary structures. The primary structure of two proteins can be completely different, but if their higher order structures are similar or almost identical, it is likely that they will have similar functions.

Prediction models

The three dimensional structure of proteins is predicted through the amino acids sequence using computer algorithms. The proteins structures are predicted based on structure of already known proteins which can be used as a template. When no template is available, a correct prediction becomes difficult to obtain. For this reason, several new generations of software are now available to collect and analyze all the possible data in order to give a likely protein structure.

Scores

Since all our attempts to insert a functional tag have proven to be difficult, we then thought it was due to the protein structure. In our study, we relied on Protein Structure Prediction to assign a hypothetical structure to the two proteins we tried to identify as Mediator subunits. Therefore we have used I-Tasser features to predict the structure of SPCC645.12c (Med9) and SPAC644.10 (Med11) in S. pombe, as well as MED9 and MED11 in S. cerevisiae. The I-Tasser server builds 3D models using multiple threading alignments taking advantage of other alignment tools like LOMET or TM-Align. Three different tools are used for interpretation of the accuracy of the models: C-score, TM-Align and COFACTOR. Each prediction model has a C-score which is based on the consensus significance score of multiple threading templates. It serves to estimate the accuracy of the I-TASSER predictions, whose value range from -5 to +2, where -5 indicates the less likely protein structure, while values toward +2 would indicate a high likelihood of similarity with the real protein structure (Zhang 2008). When a structure is assigned a low C-score, for example close to -5, it means that the proteins probably belong to a family of still unknown proteins so it cannot be identified correctly. TM-Align compares protein structure prediction with known structures in the Protein Data Bank (PDB), giving several outcomes with different structures and functions and the best hits are the most probable ones. On the other hand, COFACTOR compares the proteins based on their biological function.

Hypothetical structures for Med9 (at the 22nd of August 2012)

Five different hypothetical structures for SPCC645.12c (Med9) were obtained and only the first three with the highest C-score were taken into consideration in the top ten structures with similar
protein sequence. The highest C-score was -2.02. The first 3 hits for C-score, TM-Align and COFACTOR are all summarized in Table 5. Predicted models for *S. pombe* and *S. cerevisiae* Med9/MED9 are shown in Figures 48A and 48B. The color code proceeds according to the amino acid sequence position from blue to red, for N- to C- terminal, respectively.

**Table 5.** First, second and third hits for C-score, TM-Align and COFACTOR for Med9 in both *S. pombe* and *S. cerevisiae*

<table>
<thead>
<tr>
<th>Med9</th>
<th><em>S. pombe</em></th>
<th><em>S. cerevisiae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C-score</strong> 1st hit</td>
<td>C-terminal tropomyosin fragment with N- and C-terminal extensions of the leucine zipper from <em>S. cerevisiae</em> (C-score -2.02)</td>
<td>Structure of Human Prolylcarboxypeptidase (C-score -2.16)</td>
</tr>
<tr>
<td>2nd hit</td>
<td>Dimerization domain of LAP2ALPHA in <em>Mus musculus</em></td>
<td>Crystal structure of syntaxin 10 from <em>Homo sapiens</em></td>
</tr>
<tr>
<td>3rd hit</td>
<td>Crystal Structure of Repeats 8 and 9 of Human Erythroid Spectrin</td>
<td>Crystal structure of PCRH in complex with the chaperone binding region of POPD in <em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td><strong>TM-align</strong> 1st hit</td>
<td>Crystal Structure of Repeats 8 and 9 of Human Erythroid Spectrin</td>
<td>NMR structure of two different apolipophorin-III from <em>Locusta migratoria</em></td>
</tr>
<tr>
<td>2nd hit</td>
<td>Crystal structure of repeats 15, 16 and 17 of chicken brain alpha spectrin</td>
<td>As above</td>
</tr>
<tr>
<td>3rd hit</td>
<td>Crystal structure of repeats 16 and 17 of chicken brain alpha spectrin</td>
<td>Crystal structure of the pathogenicity island 1 effector protein from <em>Chromobacterium violaceum</em></td>
</tr>
<tr>
<td><strong>COFACTOR</strong> 1st hit</td>
<td>Crystal structure of the <em>E. coli</em> histidine kinase sensor TorS sensor domain</td>
<td>Crystal structure of pyruvate dehydrogenase kinase isoform 4 in complex with ADP in <em>Homo sapiens</em></td>
</tr>
<tr>
<td>2nd hit</td>
<td>Structure of the full-length <em>E. coli</em> ParC subunit</td>
<td>Crystal structure of the PDK3-L2 complex in <em>Homo sapiens</em></td>
</tr>
<tr>
<td>3rd hit</td>
<td>Crystal structure of the full-length autotransporter EstA from <em>Pseudomonas aeruginosa</em>.</td>
<td>Branched chain alpha-ketoacid dehydrogenase kinase complexed with ADP in <em>Rattus norvegicus</em></td>
</tr>
</tbody>
</table>

According to the results obtained, no structures similar to any other Mediator subunits were identified. Also, the C-scores for both Med9 in *S. pombe* and MED9 in *S. cerevisiae* showed a low value. Despite the fact that MED9 has been described in *S. cerevisiae*, the low C-score and the results for the hits shown in Table 5 demonstrate that it might also belong to a family of unknown
proteins. This analysis has helped us to understand why our tagging process probably didn’t work. It is possible that, due to its different nature, special procedures (for example, a different choice of tag and maybe also different protein extraction protocols) were necessary in order to effectively tag *S. pombe* Med9.

**Hypothetical structure for Med11b/SPAC644.10**

Med11 in *S. pombe* hasn’t been characterized yet, but protein alignment among 27 different organisms done by GeneDB has shown similarity between scMed11 and SPCC644.10 in *S. pombe*. The I-Tasser results for *S. cerevisiae* Med11 and *S. pombe* Med11 are shown in figures 49A and 49B. The red and blue ribbons show the C- and N- terminal residues respectively. The first and second hits for *S. pombe* Med11 both showed similarity to Mediator head subcomplex Med11/22 in *S. cerevisiae*, with a C-score of -2.20 and -3.35 respectively. The third hit referred to the architecture of the Mediator Head module, still in budding yeast. The PDB hit confirmed that this is the genuine Structure of the Mediator head subcomplex Med11/Med22. The first 3 hits for C-score, TM-Align and COFACTOR are all summarized in table 6.

![Figure 49A](image1.png) **Figure 49A**: Med11 prediction structure in *S. pombe*, according to the I-Tasser software. The C-score for this prediction is -1.64.

![Figure 49B](image2.png) **Figure 49B**: MED11 prediction structure in *S. cerevisiae*, according to the I-Tasser software. The C-score for this prediction is -1.87.
In order to check for structure similarity to other Mediator subunits, the protein sequence for *S. cerevisiae* MED11 was also run into i-Tasser. The first and third hits were both the structure of the Mediator head subcomplex MED11/MED22, with C-score of -1.87 and -2.26 respectively (Figure 49B, first hit). The second hit was the structure of the Mediator Head module, with a C-score of -1.98. The first 3 hits for C-score, TM-Align and COFACTOR are all summarized in Table 6. The three positive hits obtained with the prediction model tool have shed a light on the possibility that the protein we considered to be Med11 in *S. pombe* might be a bona fide homologue of scMED11.

<table>
<thead>
<tr>
<th>Med11</th>
<th>S. pombe</th>
<th>S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C-score 1st hit</strong></td>
<td>Mediator head subcomplex Med11/22 in <em>S. cerevisiae</em> (C-score -2.20)</td>
<td>Mediator head subcomplex MED11/22 (C-score -1.87)</td>
</tr>
<tr>
<td><strong>2nd hit</strong></td>
<td>As above (C-score -3.35)</td>
<td>Architecture of the Mediator Head module (C-score)</td>
</tr>
<tr>
<td><strong>3rd hit</strong></td>
<td>Structure of the Mediator Head module in <em>S. cerevisiae</em></td>
<td>Mediator head subcomplex Med11/22 (C-score -2.26)</td>
</tr>
<tr>
<td><strong>TM-align 1st hit</strong></td>
<td>Crystal structure of insecticidal delta-endotoxin in <em>Bacillus thuringiensis</em></td>
<td>Crystal structure of the neuronal T-SNARE syntaxin-1A in <em>Rattus norvegicus</em></td>
</tr>
<tr>
<td><strong>2nd hit</strong></td>
<td>As above</td>
<td>N-terminal NTS-DBL1-alpha and CIDR-gamma double domain of the PIEMP1 protein from <em>Plasmodium falciparum</em></td>
</tr>
<tr>
<td><strong>3rd hit</strong></td>
<td>Insecticidal crystal protein CRY2AA, in <em>Bacillus thuringiensis</em></td>
<td>Crystal structure of the CorA Mg2+ transporter in <em>Thermotoga maritima</em></td>
</tr>
<tr>
<td><strong>COFACTOR 1st hit</strong></td>
<td>Crystal structure of the R2 subunit of ribonucleotide reductase from <em>Chlamydia trachomatis</em></td>
<td>Anisotropically refined structure of FTCD in <em>Rattus norvegicus</em></td>
</tr>
<tr>
<td><strong>2nd hit</strong></td>
<td>Cholesterol bound form of human beta2 adrenergic receptor</td>
<td>Crystal structure of the R2 subunit of ribonucleotide reductase from <em>Chlamydia trachomatis</em></td>
</tr>
<tr>
<td><strong>3rd hit</strong></td>
<td>Crystal structure of a replication fork DNA polymerase editing complex in <em>Enterobacteria phage</em></td>
<td>R2-like ligand binding Mn/Fe oxidase from <em>Mycobacterium tuberculosis</em></td>
</tr>
</tbody>
</table>

**Table 6.** First, second and third hits for C-score, TM-Align and COFACTOR for Med11/MED11 in both *S. pombe* and *S. cerevisiae*
Final conclusion on Bioinformatics

Generally speaking, the problems we have encountered during the tagging process might have been due to the choice of a tag incompatible with the structure of the proteins we took into consideration in this study. The 13Myc tag for Med9 or the 3HA tag for Med11a/Sec20 tag were tested first. The 13Myc tag never showed a clear signal, with the exact size. Simple Western blot and Co-IPs were always dubious due to the presence of extra bands, protein degradation or beads interference. On the other hand, the 3HA tag attached to Med11a allowed us to conclude that Med11a did not belong to the Mediator complex.

The second choice of tag, inserted through the Gateway system to both Med9 and Med11b, also failed to show a correct size band due to the beads interference or high degradation patterns. We could then speculate that the 2xFLAG-His tag was probably the wrong choice of a tag. Possibly, a tag with a substantial size like GFP would have allowed us to obtain a better signal, as shown by (Matsuyama et al. 2006). However, our primary aim was not to do microscopy assays on our proteins, so the GFP or YFP were not considered.
### Table 7. List of primers used for ORF deletions and tagging of the proteins of interest

<table>
<thead>
<tr>
<th><strong>med9</strong> deletion primers</th>
<th>Colour code: restriction enzyme; hygromycin cassette</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-MED9 (XhoI)</td>
<td>5'-GGCTCGAGAACTAAGGATACATGCTTTTTATAGTTTCGAATGCAATATTTTTAGATTAATGATATTGTTTGAGAAAGAGCTTCAAAATCGGATCCCCGGTTAAATTA-3'</td>
</tr>
<tr>
<td>3'-MED9 (XhoI)</td>
<td>5'-GGCTCGAGAAGGAAGAAATATGTAATAAAACAGTTACTTTTTTAGTTGGTGCTATTTTTTTTTTGATTGTTACAGGTGAATATTGTTAGAAAAGGAGCTTCAAAAATCGGATCCCCGGTTAAATTA-3'</td>
</tr>
<tr>
<td>YecA-5' (BsiWI)</td>
<td>5'-GGCGGTACGAGCAAAGTTACCTCTTTCGGAATTTAC-3'</td>
</tr>
<tr>
<td>YecB-3' (BglII)</td>
<td>5'-GAAGATCTGCGATGTCTCTTTTCGACG-3'</td>
</tr>
<tr>
<td>YecC-5' (Pmel)</td>
<td>5'-GGGGTTAAAACCGGAAGAATGTGAAATTATTTTGTTTGG-3'</td>
</tr>
<tr>
<td>YecD-3' (ClaI)</td>
<td>5'-GGGACTGCAATGCATATGTAAATGCCTAGTTTTCGACG-3'</td>
</tr>
<tr>
<td>Sp1 (deletion check)</td>
<td>5'-GCCGTCCTCTCTAGTTT-3'</td>
</tr>
<tr>
<td>Sp2</td>
<td>5'-GCATAGGGCCAAAGAAATGAGCCC-3'</td>
</tr>
<tr>
<td>Sp3</td>
<td>5'-GTTGAGGTTAATGCGAAGTGGC-3'</td>
</tr>
<tr>
<td>Sp4</td>
<td>5'-GGGACTGCAATGGAATATGTAATAAACAGTATTCTTTTAGTTGGTGCTATTTTTTTTTTGATTGTTACAGGTGAATATTGTTAGAAAAGGAGCTTCAAAAATCGGATCCCCGGTTAAATTA-3'</td>
</tr>
<tr>
<td>Delta med9 5'</td>
<td>5'-GGGTTAAAACCGGAAGAATGTGAAATTATTTTGTTTGG-3'</td>
</tr>
<tr>
<td>Delta med9 3'</td>
<td>5'-GGGACTGCAATGGAATATGTAATAAACAGTATTCTTTTAGTTGGTGCTATTTTTTTTTTGATTGTTACAGGTGAATATTGTTAGAAAAGGAGCTTCAAAAATCGGATCCCCGGTTAAATTA-3'</td>
</tr>
<tr>
<td>KanMX5'</td>
<td>5'-GGAGGAGGCAAGCTAAACAGATC-3'</td>
</tr>
<tr>
<td>KanMX3'</td>
<td>5'-GGAGGAGGCAAGCTAAACAGATC-3'</td>
</tr>
<tr>
<td>KanMX5' rev</td>
<td>5'-GGAGGAGGCAAGCTAAACAGATC-3'</td>
</tr>
<tr>
<td>Primers for med9** tag</td>
<td>5'-GCCGTCCTCTCTAGTTT-3'</td>
</tr>
<tr>
<td>Myc1-5'</td>
<td>5'-CCCAGATCCATCTCGTAGCTGGCAGTA-3'</td>
</tr>
<tr>
<td>Myc2-3'</td>
<td>5'-GGGACTCACAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>Myc3-5'</td>
<td>5'-GGGACTCACAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>Myc4-3'</td>
<td>5'-GGGACTCACAAGCTTTTCGACAGC-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>med11a</strong> deletion primers (Sec20)</th>
<th>Colour code: restriction enzyme; hygromycin cassette</th>
</tr>
</thead>
<tbody>
<tr>
<td>YecE-5' (BamHI)</td>
<td>5'-GGGACTCCGGCAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>YecF-3' (BglII)</td>
<td>5'-GGGACTCCGGCAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>YecG-5' (Pmel)</td>
<td>5'-GGGACTCCGGCAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>YecH-3' (ClaI)</td>
<td>5'-GGGACTCCGGCAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>Delta med11a 5' (deletion check)</td>
<td>5'-GGAGGAGGCAAGCTAAACAGATC-3'</td>
</tr>
<tr>
<td>Delta med11a 3' (deletion check)</td>
<td>5'-GGAGGAGGCAAGCTAAACAGATC-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primers for med11a** tag</th>
<th>Colour code: restriction enzyme; hygromycin cassette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc5-5'</td>
<td>5'-GGGACTCCGGCAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>Myc6-3'</td>
<td>5'-GGGACTCCGGCAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>Myc7-5'</td>
<td>5'-GGGACTCCGGCAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>Myc8-3'</td>
<td>5'-GGGACTCCGGCAAGCTTTTCGACAGC-3'</td>
</tr>
<tr>
<td>Tag</td>
<td>Sequence</td>
</tr>
<tr>
<td>-----</td>
<td>----------</td>
</tr>
<tr>
<td>Sec20 tag-5'</td>
<td>5'-GCCTCGACATCATCTGCCCAGATGC-3'</td>
</tr>
<tr>
<td>Sec20 tag-3'</td>
<td>5'-CGGCGGGGAACAAGGCAAGCTAAAC-3'</td>
</tr>
</tbody>
</table>

**med11b** deletion primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>YecL1-5' (SalI)</td>
<td>5'-GGGTGTGACCATATGCCATATTTGTAATATTTTG-3'</td>
</tr>
<tr>
<td>YecM1-3' (XmaI)</td>
<td>5'-CCCCCGGGCCGTCCGTCACTATTTGATCATCG-3'</td>
</tr>
<tr>
<td>YecN-5' (BsmI)</td>
<td>5'-GGGAAATTGCACTACCTGAGCGGTTCAGCCTTTTC-3'</td>
</tr>
<tr>
<td>YecO-3' (PmeI)</td>
<td>5'-GGGTATTAACCATTTGCAAGGCTTGACGTACGATTG-3'</td>
</tr>
</tbody>
</table>

**Primers for med11b** tag

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc9-5' (SalI)</td>
<td>5'-GCCTCGACCAACCCAGATTTCCAGGTA-3'</td>
</tr>
<tr>
<td>Myc10-3' (XmaI)</td>
<td>5'-CCCCCGGGTGCAGCAATCTTTAGGACTGT-3'</td>
</tr>
<tr>
<td>Myc11-5' (BsmI)</td>
<td>5'-GGGAATTGCTTTGACATGCAACTTTTGAGCAC-3'</td>
</tr>
<tr>
<td>Myc12-3' (PmeI)</td>
<td>5'-CGGTATTAACGGTGCTATATAGCATTTGCTCTTG-3'</td>
</tr>
<tr>
<td>Med11-Tag 5'</td>
<td>5'-CATCTGCCAGATGCGAAGTTAA-3'</td>
</tr>
<tr>
<td>Med11-Tag 3'</td>
<td>5'-CGGGGACAAGGAAGCTAAACAGATC-3'</td>
</tr>
</tbody>
</table>

**Primers for Gateway®**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH- TermF</td>
<td>5'-CTCTTTAGTGCACACACTCTTACC-3'</td>
</tr>
<tr>
<td>Leu1-R</td>
<td>5'-GGTCATAAAGTTGACCGATGRTG-3'</td>
</tr>
<tr>
<td>ffh M11 5'</td>
<td>5'-CCTCGAGACTTTGACGCAAGATCC-3'</td>
</tr>
<tr>
<td>ffh M119 3'</td>
<td>5'-CTTGTAGTCCATGCGGTATTATCGTC-3'</td>
</tr>
<tr>
<td>ffh M9 5'</td>
<td>5'-GTGAGCTGGCAGTAAGTTAATATG-3'</td>
</tr>
<tr>
<td>hff m911 5'</td>
<td>5'-GGATGATGCAGTAAGTTAATATG-3'</td>
</tr>
<tr>
<td>hff M9 3'</td>
<td>5'-GGCCATAAATGTTGCTTGAGGAG-3'</td>
</tr>
<tr>
<td>hff m11 3</td>
<td>5'-GAGTAGACGCATAACGTTCTTAG-3'</td>
</tr>
</tbody>
</table>
List of strains

Table 8. List of yeast strains used in this project

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP9</td>
<td>h' wildtype</td>
</tr>
<tr>
<td>MP13</td>
<td>h' ade6-M216</td>
</tr>
<tr>
<td>MP46</td>
<td>h' ade6-M210 leu1-32 ura4-D18</td>
</tr>
<tr>
<td>MP54</td>
<td>h? Ch16m23::ura4*-TEL[72] leu1-32 ura4-DS/E ade6-M210 (Ch16 ade6-M216) clr4Δ::LEU2</td>
</tr>
<tr>
<td>MP55</td>
<td>h' leu-32, ade6-210, ura4-DS/E Ch16 ade6-216 Ch16 m23::ura4-TEL[72]</td>
</tr>
<tr>
<td>TP28</td>
<td>h' srb8&quot;::KanMX6</td>
</tr>
<tr>
<td>TP52</td>
<td>h' ade6-M216 med4*-13Myc</td>
</tr>
<tr>
<td>TP66</td>
<td>h' ade6-M216 TRAP240* -TAP-KanMX6</td>
</tr>
<tr>
<td>TP67</td>
<td>h' ade6-M210 -TRAP240* -TA-KanMX6</td>
</tr>
<tr>
<td>TP219</td>
<td>h' ade6-M216 ura4-D18 med7*-TAP-KanMX6</td>
</tr>
<tr>
<td>TP220</td>
<td>h' ade6-M210 ura4-D18 med7*-TAP-KanMX6</td>
</tr>
<tr>
<td>TP421</td>
<td>h' med12&quot;::KanMX6 ura4-DS/E, leu1-32, ade6-m210/Chr 16 m 23 (ade6-m216) ura-tel</td>
</tr>
<tr>
<td>TP422</td>
<td>h' med18&quot;::KanMX6 ura4-DS/E, leu1-32, ade6-m210/Chr 16 m 23 (ade6-m216) ura-tel</td>
</tr>
<tr>
<td>TP448*</td>
<td>SPCC645.12c/SPCC645.12c::HphMX6 /TAP-TRAP240* / TAP-TRAP240*</td>
</tr>
<tr>
<td>TP474*</td>
<td>SPCC645.12c-13Myc::HphMX6 ade6-M216 TRAP240* -TAP-KanMX6</td>
</tr>
<tr>
<td>TP475*</td>
<td>SPCC645.12c-13Myc::HphMX6 ade6-M216 TRAP240* -TAP-KanMX6</td>
</tr>
<tr>
<td>TP476</td>
<td>h' SPCC645.12c-13Myc::HphMX6 ade6-M216 med7*-TAP-KanMX6</td>
</tr>
<tr>
<td>TP509*</td>
<td>SPCC645.12c-2xFLAG-His6</td>
</tr>
<tr>
<td>TP510*</td>
<td>SPCC645.12c-2xFLAG-His6</td>
</tr>
<tr>
<td>TP511*</td>
<td>2xFLAG-His6-SPCC645.12c</td>
</tr>
<tr>
<td>TP512*</td>
<td>2xFLAG-His6- SPCC645.12c</td>
</tr>
<tr>
<td>TP513*</td>
<td>med11* -2xFLAG-His6</td>
</tr>
<tr>
<td>TP514*</td>
<td>med11* -2xFLAG-His6</td>
</tr>
<tr>
<td>TP515*</td>
<td>2xFLAG-His6-med11*</td>
</tr>
<tr>
<td>TP516*</td>
<td>2xFLAG-His6-med11*</td>
</tr>
<tr>
<td>TP531*</td>
<td>SPAC23A1.15c-3HA::HphMX6 ade6-M216</td>
</tr>
<tr>
<td>TP530</td>
<td>h' SPAC23A1.15c-3HA::HphMX6 ade6-M210 TRAP240* TAP-KanMX6</td>
</tr>
<tr>
<td>TP600*</td>
<td>SPCC645.12c-13Myc::HphMX6</td>
</tr>
<tr>
<td>FY498</td>
<td>h' leu-32, ade6-210 ura4-DS/ E imlrR(NcoI)::ura4 oril</td>
</tr>
<tr>
<td>EG1190</td>
<td>leu1-32</td>
</tr>
<tr>
<td>EG2537</td>
<td>cia1*-2xFLAG-His6</td>
</tr>
<tr>
<td>*</td>
<td>The mating type was not determined</td>
</tr>
</tbody>
</table>

Table 9: List of plasmids used in this project

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTK1435</td>
<td>DH5α pFA6a-HphMX6</td>
</tr>
<tr>
<td>pTK1573</td>
<td>DH5α pFA6a-HphMX6 SPCC645.12c::HphMX6</td>
</tr>
<tr>
<td>pTK1599</td>
<td>DH5α pFA6a-HphMX6 SPAC23A1.15c ::HphMX6</td>
</tr>
<tr>
<td>pTK1601</td>
<td>DH5α pFA6a-HphMX6 SPAC23A1.15c -3HA</td>
</tr>
<tr>
<td>pTK1645</td>
<td>med11*::HphMX6</td>
</tr>
<tr>
<td>pTK1655</td>
<td>med11* -2xFLAG-His6</td>
</tr>
<tr>
<td>pTK1657</td>
<td>2xFLAG-His6-med11*</td>
</tr>
<tr>
<td>pTK1659</td>
<td>SPCC645.12c-2xFLAG-His6</td>
</tr>
<tr>
<td>pTK1661</td>
<td>2xFLAG-His6- SPCC645.12c</td>
</tr>
</tbody>
</table>
Acknowledgments

For such a long time I waited for this moment...the acknowledgments!!

Of course, the first one I would like to thank is my supervisor Steen Holmberg, who with I spent about 5 years of my life....time spent with him trying to teach me, discussing about weird or positive results, chatting about my private life....and rarely about his! He has been a great guide, with up and downs in our relation, with tears (mine), laughs (ours) and some grumpiness (mine mostly). I really loved the fact that I could always tell him what I thought, good or bad, and especially I will be eternally grateful to him for the humanity he showed towards me during several difficult times. During the thesis writing up he suggested many times I should have avoided to show my feeling in the thesis results....I tried to and hopefully I succeeded but now, in the acknowledgment it would be feelings galore!

My biggest thank goes to my parents who supported me psychologically and especially financially during these years and they have always encouraged me not to give up, despite dark moments. I would have never made it without you both!!

I would also like to thank Chris Conners for being beside me for four years during the PhD and for having supported me, visited me from Cardiff and showed me love and affection. Sadly we are not a couple anymore but his support was so important to me that he really deserves the third position in the acknowledgments.

I would also like to thank Claes Gustafsson for his hospitality at both Labs at Karolinska Institut in Stockholm and Gotenborg, as well as Olga Khorosjutina and Zsolt Szilagy for their great help.

And now.... people from the lab, begin to tremble!

The one and only Michael Lisby, with his precious and countless advices and who has taken care of me and my needs so many times: under that armor of shyness hides a huge heart. Thank you ever so much, I’ll never forget it!

All my girls from the office: Susi, Irene, Sonia, Heidi and Nadine who I spent hours talking with, gossiping, watching YouTube videos and eating (Sonia is my partner in “food crime”).

A heartfelt thank you goes also to Heidi, who was also another “partner in crime” in the lab, where we often spent evening with our experiments gladdened by lots of cheesy songs from the 80’s! I
also enjoyed our rehearsal in the microscope room when I practiced “Video Video”...it was a great
fun!! Thank you so much for that!

A special thank goes to my dear friend Susi, who has been always with me in the late evenings in
the lab, trying to help me and cheer me up. Also outside the lab she was the friend that anyone
would wish for. And despite being now busy with her little toddler she always finds time for me and
I am so utterly grateful for that!

I would also like to thank all the other members of the lab (too many to mention all of them) and
especially I would like to apologize for having forced you to listen to me while singing during tetrads
dissection…I know your ears must have bled!! And also for all my constant talking…I am sure the
lab is a better and silent place now without me around!

A part from the lab rats, I also would love to thank all the Italian friends I hooked up in these years:
Alessia, Christian, Irene (both in and outside the lab), and Valeria: you have been my biggest
company and support and I hold you all very dear...you are my family in Copenhagen.

And Christian, the brother I have never had and the man I adore deserves an extra score for
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suggestions.

Thank you for everything!


