Genome-wide analysis of the Mediator transcription complex

Genoom-brede analyse van het Mediator transcriptie complex

(met een samenvatting in het Nederlands)

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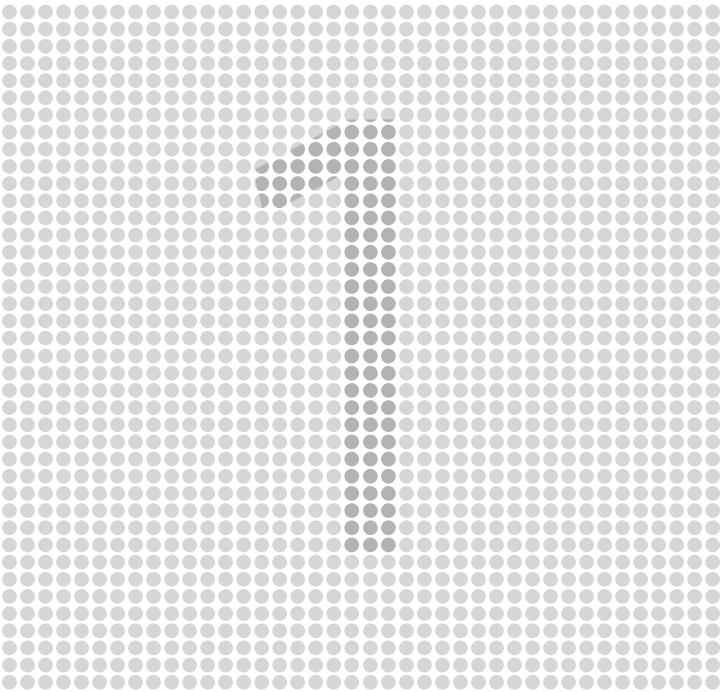


General Introduction

In 1958, Francis Crick described the central dogma of molecular biology that deals with the detailed residue-by-residue transfer of sequential information within living organisms (Crick, 1958). The genetic information pathway can be summarized in a short and simplified way as "DNA makes RNA makes Proteins, which in turn facilitates the previous two steps as well as the replication of DNA". Furthermore, it states that such information can't be transferred from proteins to either proteins or nucleic acids. With some exceptions (Brosius, 2003; Bussard, 2005; Hunter, 1999; Mendes Soares and Valcarcel, 2006) this dogma still holds and can be broken down into four biological processes: transcription, splicing, translation and replication.

Eukaryotic cells possess a highly complex mechanism to accurately transcribe genes and can react rapidly to extra-cellular signals, environmental changes or during development. Rapid response is necessary to survive and requires both accuracy and regulation. Transcription, the first step in the central dogma, involves reading the genetic code (DNA), the production of intermediates (RNA) and finally the proteins that are necessary for living organisms to survive. Depending on the state or fate of the cell, different genes need to be transcribed to different degrees.

In the bakers yeast, *Saccharomyces cerevisiae*, more than 320 different proteins (http://mips.gsf.de/, http://www.yeastgenome.org/) (Cherry et al., 1998; Guldener et al., 2005) are directly involved in efficient transcription of genes. These can be subdivided into basal transcription machinery, co-regulatory complexes, gene-specific transcription factors, chromatin regulating complexes as well as many other proteins. All factors interact and interplay in different combinations for proper expression of all genes.

This introduction describes the process of transcription regulation of protein coding genes, concentrating on *S. cerevisiae*. A short overview of the basal transcription machinery present in eukaryotic cells and in particular in yeast is introduced. In depth information about the discovery, function and mechanism of the multi-protein Mediator complex, which functions as a co-regulator, will be discussed. Current research suggests that Mediator is more than a multi-protein molecular bridge between gene-specific transcription factors and the basal transcription machinery (Bjorklund and Gustafsson, 2005; Kim and Lis, 2005; Kornberg, 2005; Myers and Kornberg, 2000; Taatjes et al., 2004a). New insights indicate novel roles for Mediator functioning as a target of signal-transduction pathways and affecting downstream processes. This opens new complexities in the already multifaceted regulation of transcription.

Eukaryotic transcription and the basal transcription machinery

Eukaryotic transcription is more complex than transcription in eubacteria due to the diversity of chromatin structure and the greater complexity of gene regulatory pathways (Levine and Tjian, 2003). Eukaryotic cells package their DNA, using nucleosomes, forming chromatin to accommodate the small space available in the nucleus (Mellor, 2005). Nucleosomes consist of 147 basepairs (bp) of DNA wrapped around octamers of four histone subunits. The octamers of histone subunits are essential and among the best-conserved proteins among eukaryotes.

In yeast, the structure of DNA coding for genes can be separated in 3 functional elements: UAS/URS (Upstream Activating Sequence/Upstream Regulating Sequence), core-promoter and the coding region/ORF (Open Reading Frame) (**Figure 1.1**) (Smale and Kadonaga, 2003). The UAS/URS is a binding platform for gene-specific transcription factors to regulate the locus. Activators and repressors are recruited at these sites and subsequently recruit additionally regulatory complexes, such as chromatin modifying complexes, to control different stages of transcription. Chromatin-modifying complexes modify the tails of histones necessary to open the chromatin structure and make it accessible for the general transcription machinery. There are different types of chromatin regulating complexes, those that covalently modify nucleosomes by acetyl-transferases (e.g. SAGA) and those that remodel chromatin (e.g. Swi/Snf) in the presence of ATP (Perez-Martin, 1999).

The core-promoter, including TATA-box and transcription start site, is sufficient for

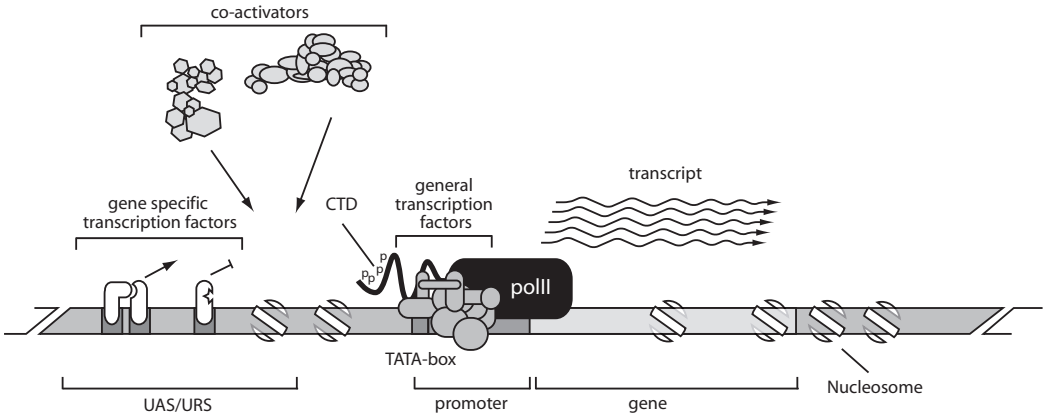


Figure 1.1: Yeast transcription regulation
Gene specific activators and repressors bind to regulatory elements (UAS/URS) upstream of the gene and recruit various co-activator complexes necessary to modify or remodel chromatin structure and to activate/repress transcription. Subsequently, the general transcription machinery is recruited to the promoter. After initiation of transcription, RNA Pol II elongates and mRNA is synthesised.

directing transcription initiation by the basal transcription machinery (TFIIB, TFIID, TFIIE, TFIIF, TFIIH) (Hampsey, 1998). In higher eukaryotes the TATA-box is located 25-30 bp upstream of the transcription start site, whereas in *S. cerevisiae* it is located 40-120 bp upstream of the transcription start site and is the main binding site for TFIID.

RNA Polymerase is the central enzyme transcribing genes resulting in RNA and is recruited after the general transcription machinery. Three different RNA polymerase complexes are present in eukaryotic cells. RNA Pol I is involved in the transcription of rRNA, RNA Pol III is required for the transcription of tRNA, and RNA Pol II transcribes protein-coding genes and small non-coding RNAs. RNA Pol II consists of 12 subunits and is well conserved between eukaryotes. The carboxy terminal domain (CTD) of the largest subunit (Rpb1) of RNA Pol II is essential for its function. In yeast, the CTD contains 26 repeats of a hepta-peptide sequence (Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7) whereas in higher eukaryotes the CTD contains more than 50 copies of this repeat. The CTD is involved and required in diverse transcriptional processes such as: transcription initiation, elongation, capping, splicing, cleavage and poly-adenylation of mRNAs (Conaway et al., 2000; Corden and Patturajan, 1997; Proudfoot et al., 2002; Riedl and Egly, 2000). Most of these processes are dependent on the phosphorylation events occurring on the CTD. Ser2 and Ser5 of the CTD consensus repeat are the main residues subjected to phosphorylation. In general, Ser5 phosphorylation occurs in promoter proximal regions indicating the start of transcription whereas Ser2 phosphorylation occurs more distal from the promoter and indicates elongating transcription. Several kinases such as Cdk7, Cdk8, Ctk1, and Bur1 were shown to phosphorylate the CTD *in vitro* and/or *in vivo* (Kobor and Greenblatt, 2002; Meinhart et al., 2005). In addition, regulation of CTD phosphorylation by phosphatases such as Fcp1, Scp1 and Ssu72 has a crucial function during the transcription cycle and recycling of RNA Polymerase II (Meinhart et al., 2005). Therefore different combinations of phosphorylated residues can occur on one heptapeptide repeat of the CTD, currently leading to speculations about the existence of a "CTD-code", that is a specific phosphorylation pattern at specific points during the transcription cycle (Buratowski, 2003).

Identification and purification of yeast Mediator

Several co-activators/repressors have been identified in the past. These are involved in the regulation of transcription either by forming a molecular bridge between gene specific transcription factors and the basal transcription machinery or are recruited to modify the

chromatin structure at the promoter (Naar et al., 2001). Characterisations of these multi-protein complexes have identified new aspects and additional complexities in the regulation of transcription. The Mediator complex is one of these complexes and conserved from yeast to higher eukaryotes (Boube et al., 2002).

In the late 80's beginning 90's, several proteins were discovered which could suppress phenotypes caused by defects in proteins involved in transcription (Carlson, 1997). Initially, these were distinct proteins that were not known to belong to the same identical complex. In a genetic screen, Nonet *et al.* (Nonet and Young, 1989) selected suppressors that restore growth of the temperature sensitive mutation caused by a CTD truncation of Rpb1. After the identification of *MED20* (*SRB2)*, further analysis resulted in an additional eight genes; *MED17 (SRB4), MED18 (SRB5), MED22 (SRB6), MED21 (SRB7), MED12 (SRB8), MED13 (SRB9), CDK8 (SRB10)* and *CycC (SRB11)* (Liao et al., 1995; Thompson et al., 1993), able to suppress the cold-sensitive mutation. In 1994, biochemical fractionation of yeast extracts identified a stable multi-protein complex (~1MDa) containing all SRB (Suppressor of RNA Polymerase B) proteins, co-purifying with RNA Pol II and able to bind a CTD affinity column (Thompson et al., 1993). In addition, Med18 and Med20 were required for efficient basal and activated transcription *in vitro*. Kornberg and colleagues isolated a similar complex containing Med9 (Rox3), Med14 (Rgr1), Med15 (Gal11), Med16 (Sin4) and seven additional novel polypetides (Med1, Med4, Med7, Med8, Med9, Med10, Med11) and showed that this complex was required for the response to the activators Gal4-VP16 and Gcn4 *in vitro* (Kim et al., 1994). Moreover, this complex stimulated CTD phosphorylation by TFIIH. In parallel, Srb/Mediator genes (*MED9, MED12, MED13, MED14, MED15, MED16, CDK8* and *CYCC*) were identified in various genetic screens for negative regulators (Balciunas and Ronne, 1995; Chang et al., 2001; Song et al., 1996; Strich et al., 1989; Tabtiang and Herskowitz, 1998). Six Srb/Mediator genes were identified as negative regulators of *SUC2* gene expression (Song et al., 1996). Mutations were selected as suppressors of the *snf1* (*ssn*; suppressor of *snf1*) mutant defect in *SUC2* de-repression. Mutations in *MED16* and a c-terminal deletion of *med14* were recovered as suppressors that allowed transcription of *HO-LacZ* in the absence of the Swi5 activator (Stillman et al., 1994). In addition, mutations in *MED12, MED13, CDK8* and *CYCC* were identified to de-repress early meiotic genes (*ume*; unscheduled meiotic gene expression). Phenotypes of these mutations suggested that their wild-type alleles encode negative regulators of genes (*SPO11*, *SPO13*, *SPO16*) expressed early in meiotic development (Strich et al., 1989).

Srb/Mediator genes were mainly found in genetic screens searching for negative regulators because this is more easily detected than (partial) loss of activation (Carlson, 1997). However, Med15 was identified as a positive regulator. A mutation of *MED15* was recovered which decreased expression up to 30% of wild type levels of the enzymes (*GAL1*, *GAL7*, *GAL10*) involved in galactose catabolism under induced conditions (Suzuki et al., 1988). The decrease of expression was not due to decreased levels of the transcription factor Gal4, suggesting that Med15 is necessary for the response to galactose. Moreover, a different *MED15* mutation (*GAL11P*) could recover transcriptional defects of Gal4 mutations (Barberis et al., 1995).

Taken together, biochemical experiments and genetic screens indicate that Mediator enhances basal transcription and mediates the response to activators. Furthermore, Mediator is associated with RNA Pol II via the CTD and possesses negative as well as positive regulatory functions. The diversity of genetic screens showed moreover that Mediator functions in the regulation of a wide variety of genes and therefore in diverse physiological processes. In addition, microarray experiments performed with *med17ts* (Holstege et al., 1998) cells indicate that Mediator is necessary for the transcription of virtually all protein coding genes in *S. cerevisiae*.

Purification of Mammalian Mediator

In the late 90's, the first biochemical purifications of a co-activator complex similar to yeast Mediator were reported in higher eukaryotes using different strategies (Ito and Roeder, 2001). Jiang *et al.* isolated a multi-protein complex (mMED) (Jiang et

al., 1998) from a mouse hybridoma cell-line. The complex contained the murine homologs of yeast Med6, Med7, Med14 and Med21 (Srb7). As in yeast, the purified complex was able to bind the CTD of RNA Pol II and stimulate CTD phosphorylation by TFIIH. A similar complex was isolated from HeLa cell nuclear extracts by Sun *et al.* named NAT (Negative regulator of Activated Transcription) (Sun et al., 1998). Using an affinity purification with antibodies directed at Cdk8, ~20 polypeptides including Med6, Med14, Med21, Cdk8 and CycC were isolated and the purified complex was able to repress activated transcription *in vitro*. TRAP (Thyroid Receptor Activating Protein) (Fondell et al., 1996; Fondell et al., 1999; Ito et al., 1999) was purified using a epitope tagged ligand binding domain of the thyroid receptor transcription factor. The purified multi-protein complex was associated with thyroid receptor dependent on the ligand T3. One of the best-characterized metazoan Srb/Mediator-like complexes is SMCC (Srb and Med Containing Cofactor) (Gu et al., 1999; Ito et al., 1999), which was purified from human cell lines with epitope tagged Med21, Cdk8 or CycC on the basis of its ability to mediate activation by Gal4 derivatives and through direct interactions with activators. SMCC contains approximately 25 proteins, including orthologues of yeast Mediator and did not contain RNA Polymerase II. Because SMCC and TRAP were essentially identical, this complex was named TRAP/SMCC (Ito and Roeder, 2001; Ito et al., 1999). Using an epitope tagged ligand binding domain of the vitamin D receptor, DRIP (vitamin D Receptor Interacting Protein complex) (Rachez et al., 1999; Rachez et al., 1998) was isolated. DRIP is associated with the VDR (Vitamin D Receptor), TR (Thyroid Receptor) and PPARγ (Peroxisomal Proliferating Activated Receptor gamma), all dependent on the presence of its ligand. DRIP association is dependent on the AF-2 domain of VDR and contains HAT (Histone Acetyl Transferase) activity (Rachez et al., 1998). In addition, other Mediator-like complexes were reported and named: CRSP (co-factor required for SP1) (Ryu and Tjian, 1999; Ryu et al., 1999), ARC (Activator Recruited Cofactor) (Naar et al., 1999) and PC2 (Positive Co-factor 2) (Malik et al., 2000).

The composition of the isolated complexes differed slightly (Sato et al., 2004). The Kinase module was present in the larger complexes TRAP/SMCC/NAT/ARC/DRIP, but absent in the purification of CRSP. CRSP contained an additional subunit, CRSP70 (Med26), which is similar to TFIIS, involved in transcription elongation and was absent in other complexes with the exception of DRIP. To verify whether previous differences in subunit compositions are due to insufficient sensitive analytical methods and loss during different purification strategies, Sato *et al.* recently systematically investigated the subunit composition of mammalian Mediator (Sato et al., 2004) using MudPIT (multidimensional Protein Identification Technology) (Washburn et al., 2001; Wolters et al., 2001). They identified 29 of 30 Mediator proteins present in previously purified complexes, including a novel homologue of Med13 and an additional kinase, Cdk11. In addition, they found that the Med26 purified fraction was more enriched for RNA Pol II and sub-stochiometric for Cdk8/CycC.

Isolations of mammalian Mediator illustrate that it interacts with gene-specific transcription factors and is ligand dependent for several nuclear hormone receptors. Furthermore, Mediator can interact with RNA Pol II and the majority of the subunits of yeast Mediator are conserved in higher eukaryotes (**Table1.1**).

Modular structure of Mediator

Structural characterization of the yeast and mammalian Mediator using single particle electron microscopy (EM) shows a discrete complex with a well-defined structure (Asturias et al., 1999; Chadick and Asturias, 2005). Alignment and averaging of individual particle images resulted in a low-resolution structure of Mediator. The structure with a resolution of ~40 Å, revealed that Mediator has an elongated, roughly conical shape and is ~400 Å in length. Upon incubation with RNA Pol II, Mediator particles changed from a compact to an extended conformation (Asturias et al., 1999; Davis et al., 2002) forming a Holoenzyme (**Figure 1.2a**). Furthermore, no Holoenzyme complexes were observed when incubated with RNA Pol II lacking the CTD and no structural changes were observed when the purified complex was incubated with

recombinant CTD alone. This suggests that the CTD and subunits of RNA Pol II are both necessary to induce a conformational change of Mediator. Determination of the precise interaction surface between RNA Pol II and Mediator suggests an interaction with the Rpb3 and Rpb11 subunits (Davis et al., 2002). The extended structure adopted by Mediator upon interaction with RNA Pol II revealed three distinct structural modules called: Head, Middle and Tail module, together forming core Mediator (**Figure 1.2b,c**). The Head (Med6, Med8, Med11, Med17, Med18, Med19, Med20, Med22) and Middle (Med1, Med4, Med5, Med7, Med9, Med10, Med21, Med31) module associates most closely to RNA Pol II (Davis et al., 2002; Dotson et al., 2000). Whereas the subunits from the Tail module (Med2, Med3, Med14, Med15, Med16), which are less conserved between yeast and mammalian Mediator, do not interact with RNA Pol II. Moreover, proteins within this module are tightly associated and dependent on each other. For example, biochemical purifications from cells lacking Med2 results in the loss of other Tail subunits such as Med3 and Med15 (Lee et al., 1999; Myers et al., 1999). The modular structure of Mediator was also observed in dissociation studies of immobilized purified Mediator complex (Lee and Kim, 1998), *in vitro* reconstitution experiments performed with recombinant proteins (Kang et al., 2001) and yeast 2 hybrid analyses (Guglielmi et al., 2004). EM studies with mammalian Mediator demonstrated that different activators can also trigger different specific conformational changes of Mediator upon binding and can target different patches on the surface of Mediator (Taatjes et al., 2002; Taatjes et al., 2004b). This may explain the diversity of transcription factors able to interact with Mediator (Blazek et al., 2005; Taatjes et al., 2004a). The Kinase module (Med12, Med13, Cdk8, CycC), absent in the structural and biochemical

Table 1.1 Mediator nomenclature
Nomenclature of Mediator subunits according to Bourbon et al., 2004.

Name	*S. cerevisiae* synonym	Module	Essential	Mammalian orthologue
Med1	Med1	Middle	N	TRAP220
Med2	Med2	Tail	N	-
Med3	Pgd1, Hrs1	Tail	N	-
Med4	Med4	Middle	Y	TRAP36, DRIP36
Med5	Nut1	Middle	N	-
Med6	Med6	Head	Y	hMed6, DRIP33
Med7	Med7	Middle	Y	hMed7, DRIP34, CRSP33
Med8	Med8	Head	Y	ARC32
Med9	Cse2	Middle	N	Med25
Med10	Nut2	Middle	Y	hNut2, hMed10
Med11	Med11	Head	Y	HSPC296
Med12	Srb8, Ssn5, Nut6	Kinase	N	TRAP230, DRIP240
Med13	Srb9, Ssn2, Nut8, Ume2, Rye3	Kinase	N	TRAP240, DRIP250
Med14	Rgr1	Tail	Y	TRAP170, DRIP150, CRSP150
Med15	Gal11, Rar3, Sds4, Spt13, Abe1	Tail	N	ARC105
Med16	Sin4, Ssn4, Gal22, Rye1	Tail	N	TRAP95, DRIP92
Med17	Srb4	Head	Y	TRAP80, DRIP77, CRSP77
Med18	Srb5	Head	N	p28b
Med19	Rox3, Ssn7, Nut3	Head	Y	LCMR1
Med20	Srb2, Hrs2	Head	N	hTRFP, p28a
Med21	Srb7	Middle	Y	hSrb7, p21
Med22	Srb6	Head	Y	Surf5
Med23	-	-	-	TRAP150b, DRIP130, CRSP130, hSur2
Med24	-	-	-	TRAP100, DRIP100, CRSP100
Med25	-	-	-	ARC92
Med26	-	-	-	ARC70, CRSP70
Med27	-	-	-	TRAP37, CRSP34
Med28	-	-	-	Fksg20
Med29	-	-	-	Hintersex
Med30	-	-	-	TRAP25
Med31	Soh1	Middle	N	hSoh1
Cdk8	Srb10, Ssn3, Nut7, Ume5, Rye5	Kinase	N	hSrb10, Cdk8
CycC	Srb11, Ssn8, Nut9, Ume3, Rye2	Kinase	N	hSrb11, Cycc

studies, is the fourth module of Mediator.

Mediator recruitment

So far, genetic and biochemical experiments have revealed that Mediator can affect transcription both positively and negatively and is essential for the transcription of virtual all protein coding genes in yeast. In addition, Mediator is associated with RNA Pol II via the Middle and Head Module whereas the Tail module is proposed to interact with gene-specific transcription factors.

Holoenzyme preparations devoid of a functional Tail module, i.e. prepared from cells lacking Med15, was defective for activated transcription *in vitro* due to the crippled physical interaction between transcription activators and the Tail module (Lee et al., 1999). Similar results were obtained *in vivo* with the artificial activator LexA-VP16 and the yeast gene-specific transcription factors Gal4 and Gcn4 (Park et al., 2000). Upon deletion of subunits from the Tail, cells were not able to respond properly anymore. Furthermore, each activator could interact with different domains of the Tail subunits. This in agreement with EM studies with mammalian Mediator described before.

These results indicate that the Tail module is essential for proper recruitment of Mediator to the promoter by gene-specific transcription factors. But how are Mediator and the general transcription machinery recruited upon activation? Initial experiments suggested that gene-specific transcription factors bind the response element at the UAS, which subsequently recruit the RNA Pol II-holoenzyme resulting in transcriptional activation.

Investigations using ChIP (Chromatin Immuno-Precipitations) with Mediator at several model genes provided more detailed information of how Mediator is recruited and functions *in vivo*. Mediator is necessary for proper activation and expression of galactose metabolising genes (Balciunas et al., 1999) and several Mediator mutants are unable to grow on non-fermentable carbon sources (http://mips.gsf.de/, http://www.yeastgenome.org/). Kuras *et al*. (Kuras et al., 2003) showed that Mediator is recruited to the *GAL* UASs independent of the GTFs (General Transcription Factors, in particularly TBP, TFIIE and TFIIH), TATA box and RNA Pol II. Furthermore, Bryant *et al*. (Bryant and Ptashne, 2003) investigated the ordered recruitment of Mediator, SAGA, GTFs and RNA Pol II at the same model genes and found that upon activation, the transcription factor Gal4 recruits first SAGA followed by Mediator and subsequently the general transcription factors and RNA Pol II. Recruitment of Mediator is unaffected

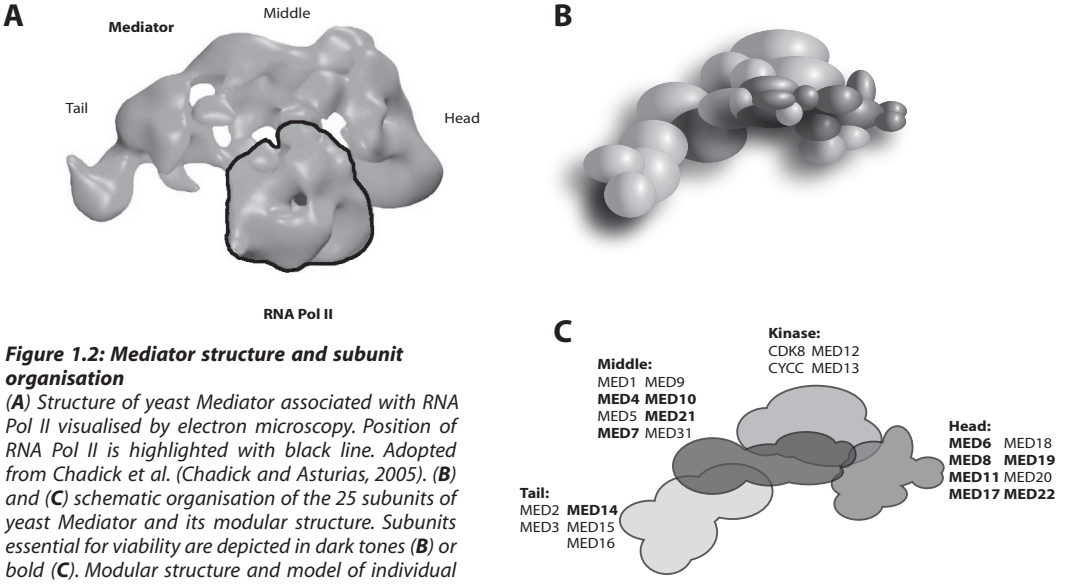


Figure 1.2: Mediator structure and subunit organisation

*(**A**) Structure of yeast Mediator associated with RNA Pol II visualised by electron microscopy. Position of RNA Pol II is highlighted with black line. Adopted from Chadick et al. (Chadick and Asturias, 2005). (**B**) and (**C**) schematic organisation of the 25 subunits of yeast Mediator and its modular structure. Subunits essential for viability are depicted in dark tones (**B**) or bold (**C**). Modular structure and model of individual subunit organisation were taken from Guglielmi et al. (Guglielmi et al., 2004).*

in the absence of SAGA and vice versa. However, recruitment of RNA Pol II at the *GAL* promoter is dependent on SAGA but not Mediator. Furthermore, recruitment of Mediator is necessary for proper recruitment of the chromatin remodelling complex SWI/SNF (Lemieux and Gaudreau, 2004). This suggests that Mediator is recruited independent of RNA Pol II, and besides Mediator, other co-activator complexes are necessary for proper *GAL* gene activation. Separate recruitment of Mediator and the general transcription machinery has also been demonstrated at the HO promoter (Cosma et al., 2001). Early biochemical experiments in the past suggested that Mediator is recruited together with RNA Pol II as a RNA Pol II-Holoenzyme (Kim et al., 1994; Thompson et al., 1993; Thompson and Young, 1995). However the separate recruitment as observed in ChIP experiments suggest that Mediator recruitment is initiated by gene specific transcription factors whereas the RNA Pol II-Mediator interaction occurs only transiently at the promoter during transcription initiation/ activation.

The presences of subunits containing negative regulatory effects, such as the Kinase module, also suggest a role for Mediator in transcriptional repression. Investigations on the general repressor Ssn6/Tup1 revealed that Ssn6/Tup1 recruits Mediator by direct interaction with Cdk8 (Zaman et al., 2001). In addition, genes encoding components of the Kinase module have all been identified in genetic screens for loss of Tup1-mediated repression (Lee et al., 2000). This suggests that Mediator, including the repressive Kinase module, is recruited to the promoter by the repressor Tup1. Experiments in mammalian cells support this idea. The human transcription factor C/EBPβ (CCAAT/ enhancer binding protein beta) regulates genes involved in a wide variety of biological processes including energy homeostasis (Tanaka et al., 1997), immune functions (Tanaka et al., 1995) and tumorigenesis (Zahnow et al., 1997). It is converted from a repressive into an active transcription factor by extra-cellular signals. *Mo et al.* (Mo et al., 2004) showed that structural alterations of C/EBPβ induced by Ras, lead to interaction and recruitment of different Mediator compositions. During repressive conditions C/EBPβ interacts with Mediator containing the Kinase module via Med23 (CRSP130/Sur2), whereas upon activation of the RAS pathway, C/EBPβ interacts with Mediator via Med23 lacking the Kinase module but containing CRSP70, a subunit of TFIIS. Furthermore, expression profiles of cells lacking any protein of this Kinase module result in mainly up-regulated genes in the mutant cells compared to wildtype (Holstege et al., 1998). Initial biochemical purification were absent for proteins from this Kinase module because purified complexes were specifically selected on its ability to activate transcription *in vitro* (Kim et al., 1994; Thompson et al., 1993). Moreover, purified Mediator was isolated from cells in post exponential growth in which Cdk8 is absent (Holstege et al., 1998). Nevertheless, later a stable sub-complex (Borggrefe et al., 2002) or Mediator with the Kinase module (Gavin et al., 2002) was purified and able to phosphorylate CTD of RNA Pol II (Hengartner et al., 1998). Altogether this illustrates that the Kinase module is an important factor in the repressive function of Mediator. Beside the negative effects described for the Kinase module, some studies have described that the Kinase module can also have positive effects on transcription. For example, phosphorylation of the transcription factor Gal4 by Cdk8 is required for proper activation of galactose inducible genes (Hirst et al., 1999; Rohde et al., 2000).

Several studies have investigated the recruitment of different transcription factors or additional complexes including Mediator at model genes. Such studies can result in the understanding and function of Mediator at a specific promoter/gene during activation or repression. While previous data suggest differential function, mechanisms and recruitment dependent on activator/repressor and associated complexes, recent genome-wide localization studies of Mediator in *S. cerevisiae* (Andrau et al., 2006) and *S. pombe* (Zhu et al., 2006) indicate that core-Mediator and the Kinase module display very similar binding patterns during exponential growth. Furthermore, Mediator binds downstream of active as well inactive genes. This suggests, that Mediator is not only recruited during activation but is also already present at some genes to facilitate an efficient and quick transcriptional response.

The correlation between RNA Pol II binding during yeast stationary phase (Radonjic et al., 2005) and Mediator binding during exponential growth (Andrau et al., 2006), suggests that Mediator can act as a binding platform for RNA Pol II for quick activation of inactive genes. Finally, Mediator also binds coding regions, suggesting novel roles in elongation and might explain the presence of Med26 (CRSP70) in purified mammalian Mediator (Ryu et al., 1999; Sato et al., 2004).

Mediator as a target of signal transduction pathways

As described before, Mediator is recruited by gene-specific transcription factors and is essential for proper activation of several model genes in yeast. However, genome-wide recruitment of Mediator indicated that its presence is not immediately associated with transcription activation. Therefore the questions remain: how is the specific activity of Mediator regulated for different transcription factors and what is the regulatory role of the Kinase module? The existence of signal transduction pathways targeting Mediator could explain specificity and activity of Mediator. Recent progress suggests the existence of such pathways and indicates that Mediator is more than a multi-protein complex connecting gene-specific transcription factors and RNA Pol II.

One of the first indications that Mediator could function as a target for signal transduction pathways were found by Jiang *et al.* (Jiang et al., 1998). In their purifications of mammalian Mediator, a Ring3-like protein was present which later was identified as Brd4 and contained 2 bromodomains (Houzelstein et al., 2002). Brd4 is involved in leukaemia and is activated by phosphorylation (Denis and Green, 1996). The yeast homolog Bdf1 interacts with TFIID (Matangkasombut et al., 2000), binds to acetylated histone H3/H4 and limits spreading of heterochromatin into euchromatin (Ladurner et al., 2003). Furthermore, others found that Bdf1 and Taf2 can be phosphorylated by Cdk8 (Liu et al., 2004). Until now, it is not clear whether Cdk8 is the only kinase targeting Bdf1, what the functions of this phosphorylation event are in yeast, and whether interaction with Mediator occurs. Other Mediator-related complexes purified and characterized until now do not contain any Bdf-family member as a constitutive unit (Gavin et al., 2006; Krogan et al., 2006).

More direct evidence that Mediator can function as a target of signal transduction pathways is illustrated by research from Chang *et al.* (Chang et al., 2004) and Pandey *et al.* (Pandey et al., 2005). Chang *et al.* found that increased activity of the Ras/PKA signalling pathway suppress transcriptional defects associated with Mediator subunits in yeast. Genetic data identified two residues (Ser608 and Ser1236) of Med13 that were functionally redundant and only Ser608 was identified as a substrate of PKA *in vitro* and *in vivo*. This result suggested that signal transduction pathways could directly influence the activity of Mediator. Pandey *et al.* illustrated that Mediator is a target of MAPK signalling in mammalian cells. Human Med1 (Trap220) is embryonic lethal and interacts with a wide variety of nuclear receptors and other transcription factors. Phosphorylation of 2 threonine residues of Med1 by ERK1-MAPK resulted in stabilization of Med1.

Modifications of Med4 and Med8 in yeast were previously described, based on the existence of different mobility forms (Balciunas et al., 2003). Whereas it is not clear what modification is responsible for the different migrating forms of Med8, Med4 is phosphorylated at T237 by Kin28 (Cdk7), a kinase of the CDK-family and a member of TFIIH (Guidi et al., 2004). It remains unclear whether Kin28 is the only kinase responsible for Med4 phosphorylation. Experiments with *kin28-ts3,* still shows residual phosphorylation at the non-permissive temperature, whether this is due to reduced phosphatase activity or redundant kinases is unknown. Nevertheless, phosphorylation is not required for its essential function *in vivo*. In a different approach using an analog-sensitive (as) kinase mutant, Liu *et al.* identified two proteins (Med4 and Med14) in a purified PIC (Pre-Initiation Complex) that were phosphorylated by Kin28 (Liu et al., 2004). In this approach, the ATP-binding pocket of the kinase is mutated, resulting in an enlarged pocket not present in other kinases. The resulting kinase will accept [γ-^{32}P] N^6-benzyl-ATP much more efficiently than endogenous kinases

(Shah and Shokat, 2003; Witucki et al., 2002).

Altogether this indicates that different signal transduction pathways can target Mediator and are essential for proper activation/repression of transcription. In addition, Mediator contains several subunits that have a negative effect on transcription. The presence of subunits containing enzymatic activity such as a cyclin dependent kinase and histone acetyl transferase additionally indicates that the post-translational modifications initiated by Mediator can have an important role in the regulation of transcription. This illustrates that Mediator is not only a co-activator creating a molecular bridge between gene-specific transcription factors and the general transcription machinery but might be regulated by signal transduction pathways and modify downstream processes.

Downstream modifications by Mediator

Different types of posttranslational modification on both mammalian and yeast Mediator subunits have been described and suggest that several different regulatory pathways can influence abundance, recruitment or activity at specific promoters in combination with different transcription factors. Besides functioning as a target, several proteins within Mediator contain enzymatic activity and may therefore themselves regulate other proteins.

The catalytic activity of Cdk8/CycC is the most well known and best characterized (**Table 1.2**). The CTD of RNA Pol II was the first identified substrate of Cdk8 and suggested a role for Mediator and the Kinase module in transcription initiation (Hengartner et al., 1998). Cdk8/CycC phosphorylation occurs mainly at Ser5 of the CTD, although Ser2 phosphorylation has been reported too (Borggrefe et al., 2002; Hengartner et al., 1998; Rickert et al., 1999). In addition, Cdk8 can phosphorylate mammalian CycH of the general transcription factor TFIIH thereby repressing its CTD kinase activity and its ability to activate transcription (Akoulitchev et al., 2000).

Besides the phosphorylation of components of the general transcription machinery, several transcription factors were found as a target of Cdk8/CycC. The transcription factor Gal4 was the first gene-specific transcription factor found to be phosphorylated by Cdk8/CycC (Hirst et al., 1999). Interestingly, phosphorylation of Gal4 is required for proper activation of galactose metabolising genes illustrating the positive effect of Cdk8/CycC. Similar effects were observed upon phosphorylation of the gene-specific transcription factor Sip4 (Vincent et al., 2001). Sip4 binds to the carbon source response elements of gluconeogenic genes. Cdk8 kinase activity is required for phosphorylation of Sip4 during growth on non-fermentable carbon sources and

Table 1.2 Putative targets of Cdk8/CycC

Target	Remarks	Reference
Rpb1	Largest subunit of RNA PolII, Phosphorylation on Ser2 and Ser5 of the CTD.	(Borggrefe et al., 2002; Hengartner et al., 1998; Rickert et al., 1999)
Gal4	Transcription factor, S699 phosphorylation is required for efficient Gal4 activation.	(Hirst et al., 1999)
Sip4	Transcription factor, necessary for growth on non-fermentable carbon sources.	(Vincent et al., 2001)
Gcn4	Transcription factor, phosphorylation occurs on 5 residues (S17, T61, T105, T165 and S218), results in degradation.	(Chi et al., 2001)
Msn2	Transcription factor, results in translocation to cytoplasm.	(Chi et al., 2001)
Ste12	Transcription factor, S261 and S451 phosphorylation inhibits filamentous growth by destabilization of Ste12.	(Nelson et al., 2003)
Ecm22	Transcription factor, regulates transcription of the sterol biosynthetic genes	(Ptacek et al., 2005)
Med2	Subunit of Mediator, S208 phosphorylation up-regulates 2micron coded genes and repress expression of Aft1/Rcs1 targets. Residual phosphorylation occurs in cells lacking Cdk8 or CycC.	(Hallberg et al., 2004; van de Peppel et al., 2005)
Bdf1	Subunit of TFIID, C-terminal region of mammalian TAF1.	(Liu et al., 2004)
Taf2	Subunit of TFIID.	(Liu et al., 2004)
CycH	Component of TFIIH, S5 and S304 phosphorylation represses both the ability of TFIIH to activate transcription and its CTD kinase activity.	(Akoulitchev et al., 2000)
Sgf73	Subunit of SAGA, co-purifies with Med1.	(Gavin et al., 2006; Ptacek et al., 2005)
Ubp10	Ubiquitin-specific protease, has telomeric and gene-silencing functions.	(Ptacek et al., 2005)
Notch ICD	Intracellular domain of Notch receptor, phosphorylated within the TAD and PEST domains.	(Fryer et al., 2004)

its activity stimulated Sip4 to activate transcription of a reporter gene. Moreover, Sip4 was found to interact with Cdk8 but it remains unclear whether Cdk8/CycC directly phosphorylates Sip4 or regulates the activity of other kinases.

Phosphorylation of the gene–specific transcription factors Gcn4 and Msn2 by Cdk8/CycC resulted in negative regulation by either degradation of the transcription factor or translocation to the cytoplasm (Chi et al., 2001). Gcn4 was partially stabilized in the absence of Cdk8. Upon mutation of five Cdk8 dependent phosphorylated residues or removal of both Cdk8 and Pho85, Gcn4 was almost completely stabilized suggesting that both CDKs are essential for Gcn4 turnover. In addition, phosphorylation of Ste12 inhibits filamentous growth by decreasing the stability of Ste12 (Nelson et al., 2003). Upon nitrogen-limitation, loss of Cdk8 increases Ste12 stability and activation of filamentous growth.

Besides the kinase activity, other Mediator components exhibit enzymatic activity. In *S. cerevisiae*, Med5, was found to contain HAT activity (Lorch et al., 2000), which is not found in other eukaryotic Mediator complexes except DRIP. Microarray analysis of cells lacking Med5 are until now inconclusive whether this HAT activity is functional *in vivo* (Beve et al., 2005; van de Peppel et al., 2005). Furthermore mammalian MED8 can assemble with Elongins B and C, Cul2, and Rbx1 to reconstitute a ubiquitin ligase (Brower et al., 2002). However, the targets of ubiquitylation have not been described yet.

Scope and aim of the work described in this thesis

Prior to the work described in this thesis, Mediator was established to work as a molecular bridge between gene-specific activators and the basal transcription machinery, mediating activation of transcription by facilitating activator-dependent recruitment of RNA polymerase II. The presence of Mediator components that act negatively on transcription, as well as the presence of subunits with enzymatic activities do not fit into this model, giving rise to the otherwise unsubstantiated proposal that Mediator may function as a signal transducer, capable of integrating multiple cellular signals. Although some post-translational modification of Mediator components had been described, their direct effect on transcription had not been determined. The goal of the work described in this thesis was to investigate the possibility that Mediator may process multiple signals by systematically analysing the effects of deleting different components. The underlying idea was that if Mediator is capable of integrating various signals, it may do so through different subunits and that deletion of different subunits would result in different effects on transcription of identical genes.

DNA microarray mRNA expression profiling was therefore used to carry out a systematic analysis of the effects of deleting different components of Mediator. Because some Mediator components had previously been shown to be required for expression of virtually all protein-coding genes, it was necessary to first develop a microarray data normalisation strategy that could properly cope with large, unbalanced changes in possibly the entire mRNA population. **Chapter 2** describes such a normalisation strategy that is based on using spiked-in external RNA controls. Besides being useful for normalisation of experiments investigating mutations in components of the generally required transcription machinery, this work also demonstrates that unbalanced changes in large populations of mRNAs occurs more frequently than is currently assumed. The vast majority of microarray studies miss finding such global changes because they lack the proper controls.

Chapter 3 presents a systematic DNA microarray expression-profiling analysis of the effects of individually losing 15 non-essential Mediator subunits. Besides providing evidence that Mediator can indeed function to integrate more than just activator interactions, the work shows four new applications of DNA microarray expression-profiling: structure-function analysis; discovery of signal-transduction pathway components; determination of epistatic relationships; pin-pointing the precise effects of regulatory protein modification on transcription. The results also lead to the surprising finding that modification of a single amino acid within a component of the generally required transcription machinery can have highly specific effects on transcription.

Whereas **chapter 3** shows that the Cdk8 subunit of Mediator regulates the activity of the Med2 component, **chapter 4** investigates Cdk8 phosphorylation of the Med15 subunit. **Chapter 5** presents a model for the novel regulatory mechanisms discovered here, placing this in the context of current models of transcription regulation, Mediator and how specificity in transcription regulation is achieved. The implications of the new DNA microarray expression-profiling applications are also discussed, especially in light of so-called systems approaches to investigating cellular processes such as transcription.

REFERENCES

Akoulitchev, S., Chuikov, S., and Reinberg, D. (2000). TFIIH is negatively regulated by cdk8-containing mediator complexes. Nature *407*, 102-106.

Andrau, J. C., van de Pasch, L., Lijnzaad, P., Bijma, T., Koerkamp, M. G., van de Peppel, J., Werner, M., and Holstege, F. C. (2006). Genome-Wide Location of the Coactivator Mediator: Binding without Activation and Transient Cdk8 Interaction on DNA. Mol Cell *22*, 179-192.

Asturias, F. J., Jiang, Y. W., Myers, L. C., Gustafsson, C. M., and Kornberg, R. D. (1999). Conserved structures of mediator and RNA polymerase II holoenzyme. Science *283*, 985-987.

Balciunas, D., Galman, C., Ronne, H., and Bjorklund, S. (1999). The Med1 subunit of the yeast mediator complex is involved in both transcriptional activation and repression. Proc Natl Acad Sci U S A *96*, 376-381.

Balciunas, D., Hallberg, M., Bjorklund, S., and Ronne, H. (2003). Functional interactions within yeast mediator and evidence of differential subunit modifications. J Biol Chem *278*, 3831-3839.

Balciunas, D., and Ronne, H. (1995). Three subunits of the RNA polymerase II mediator complex are involved in glucose repression. Nucleic Acids Res *23*, 4421-4425.

Barberis, A., Pearlberg, J., Simkovich, N., Farrell, S., Reinagel, P., Bamdad, C., Sigal, G., and Ptashne, M. (1995). Contact with a component of the polymerase II holoenzyme suffices for gene activation. Cell *81*, 359-368.

Beve, J., Hu, G. Z., Myers, L. C., Balciunas, D., Werngren, O., Hultenby, K., Wibom, R., Ronne, H., and Gustafsson, C. M. (2005). The structural and functional role of Med5 in the yeast Mediator tail module. J Biol Chem *280*, 41366-41372.

Bjorklund, S., and Gustafsson, C. M. (2005). The yeast Mediator complex and its regulation. Trends Biochem Sci *30*, 240-244.

Blazek, E., Mittler, G., and Meisterernst, M. (2005). The mediator of RNA polymerase II. Chromosoma *113*, 399-408.

Borggrefe, T., Davis, R., Erdjument-Bromage, H., Tempst, P., and Kornberg, R. D. (2002). A complex of the Srb8, -9, -10, and -11 transcriptional regulatory proteins from yeast. J Biol Chem *277*, 44202-44207.

Boube, M., Joulia, L., Cribbs, D. L., and Bourbon, H. M. (2002). Evidence for a mediator of RNA polymerase II transcriptional regulation conserved from yeast to man. Cell *110*, 143-151.

Bourbon, H. M., Aguilera, A., Ansari, A. Z., Asturias, F. J., Berk, A. J., Bjorklund, S., Blackwell, T. K., Borggrefe, T., Carey, M., Carlson, M., *et al.* (2004). A unified nomenclature for protein subunits of mediator complexes linking transcriptional regulators to RNA polymerase II. Mol Cell *14*, 553-557.

Brosius, J. (2003). The contribution of RNAs and retroposition to evolutionary novelties. Genetica *118*, 99-116.

Brower, C. S., Sato, S., Tomomori-Sato, C., Kamura, T., Pause, A., Stearman, R., Klausner, R. D., Malik, S., Lane, W. S., Sorokina, I., *et al.* (2002). Mammalian mediator subunit mMED8 is an Elongin BC-interacting protein that can assemble with Cul2 and Rbx1 to reconstitute a ubiquitin ligase. Proc Natl Acad Sci U S A *99*, 10353-10358.

Bryant, G. O., and Ptashne, M. (2003). Independent recruitment in vivo by Gal4 of two complexes required for transcription. Mol Cell *11*, 1301-1309.

Buratowski, S. (2003). The CTD code. Nat Struct Biol *10*, 679-680.

Bussard, A. E. (2005). A scientific revolution? The prion anomaly may challenge the central dogma of molecular biology. EMBO Rep *6*, 691-694.

Carlson, M. (1997). Genetics of transcriptional regulation in yeast: connections to the RNA polymerase II CTD. Annu Rev Cell Dev Biol *13*, 1-23.

Chadick, J. Z., and Asturias, F. J. (2005). Structure of eukaryotic Mediator complexes. Trends Biochem Sci *30*, 264-271.

Chang, Y. W., Howard, S. C., Budovskaya, Y. V., Rine, J., and Herman, P. K. (2001). The rye mutants identify a role for Ssn/Srb proteins of the RNA polymerase II holoenzyme during stationary phase entry in Saccharomyces cerevisiae. Genetics *157*, 17-26.

Chang, Y. W., Howard, S. C., and Herman, P. K. (2004). The Ras/PKA signaling pathway directly targets the Srb9 protein, a component of the general RNA polymerase II transcription apparatus. Mol Cell *15*, 107-116.

Cherry, J. M., Adler, C., Ball, C., Chervitz, S. A., Dwight, S. S., Hester, E. T., Jia, Y., Juvik, G., Roe, T., Schroeder, M., *et al.* (1998). SGD: Saccharomyces Genome Database. Nucleic Acids Res *26*, 73-79.

Chi, Y., Huddleston, M. J., Zhang, X., Young, R. A., Annan, R. S., Carr, S. A., and Deshaies, R. J. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. Genes Dev *15*, 1078-1092.

Conaway, J. W., Shilatifard, A., Dvir, A., and Conaway, R. C. (2000). Control of elongation by RNA polymerase II. Trends Biochem Sci *25*, 375-380.

Corden, J. L., and Patturajan, M. (1997). A CTD function linking transcription to splicing. Trends Biochem Sci *22*, 413-416.

Cosma, M. P., Panizza, S., and Nasmyth, K. (2001). Cdk1 triggers association of RNA polymerase to cell cycle promoters only after recruitment of the mediator by SBF. Mol Cell *7*, 1213-1220.

Crick, F. H. (1958). On protein synthesis. Symp Soc Exp Biol *12*, 138-163.

Davis, J. A., Takagi, Y., Kornberg, R. D., and Asturias, F. A. (2002). Structure of the yeast RNA polymerase II holoenzyme: Mediator conformation and polymerase interaction. Mol Cell *10*, 409-415.

Denis, G. V., and Green, M. R. (1996). A novel, mitogen-activated nuclear kinase is related to a Drosophila

developmental regulator. Genes Dev *10*, 261-271.

Dotson, M. R., Yuan, C. X., Roeder, R. G., Myers, L. C., Gustafsson, C. M., Jiang, Y. W., Li, Y., Kornberg, R. D., and Asturias, F. J. (2000). Structural organization of yeast and mammalian mediator complexes. Proc Natl Acad Sci U S A *97*, 14307-14310.

Fondell, J. D., Ge, H., and Roeder, R. G. (1996). Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc Natl Acad Sci U S A *93*, 8329-8333.

Fondell, J. D., Guermah, M., Malik, S., and Roeder, R. G. (1999). Thyroid hormone receptor-associated proteins and general positive cofactors mediate thyroid hormone receptor function in the absence of the TATA box-binding protein-associated factors of TFIID. Proc Natl Acad Sci U S A *96*, 1959-1964.

Fryer, C. J., White, J. B., and Jones, K. A. (2004). Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol Cell *16*, 509-520.

Gavin, A. C., Aloy, P., Grandi, P., Krause, R., Boesche, M., Marzioch, M., Rau, C., Jensen, L. J., Bastuck, S., Dumpelfeld, B., *et al.* (2006). Proteome survey reveals modularity of the yeast cell machinery. Nature *440*, 631-636.

Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., *et al.* (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature *415*, 141-147.

Gu, W., Malik, S., Ito, M., Yuan, C. X., Fondell, J. D., Zhang, X., Martinez, E., Qin, J., and Roeder, R. G. (1999). A novel human SRB/MED-containing cofactor complex, SMCC, involved in transcription regulation. Mol Cell *3*, 97-108.

Guglielmi, B., van Berkum, N. L., Klapholz, B., Bijma, T., Boube, M., Boschiero, C., Bourbon, H. M., Holstege, F. C., and Werner, M. (2004). A high resolution protein interaction map of the yeast Mediator complex. Nucleic Acids Res *32*, 5379-5391.

Guidi, B. W., Bjornsdottir, G., Hopkins, D. C., Lacomis, L., Erdjument-Bromage, H., Tempst, P., and Myers, L. C. (2004). Mutual targeting of mediator and the TFIIH kinase Kin28. J Biol Chem *279*, 29114-29120.

Guldener, U., Munsterkotter, M., Kastenmuller, G., Strack, N., van Helden, J., Lemer, C., Richelles, J., Wodak, S. J., Garcia-Martinez, J., Perez-Ortin, J. E., *et al.* (2005). CYGD: the Comprehensive Yeast Genome Database. Nucleic Acids Res *33*, D364-368.

Hallberg, M., Polozkov, G. V., Hu, G. Z., Beve, J., Gustafsson, C. M., Ronne, H., and Bjorklund, S. (2004). Site-specific Srb10-dependent phosphorylation of the yeast Mediator subunit Med2 regulates gene expression from the 2-microm plasmid. Proc Natl Acad Sci U S A *101*, 3370-3375.

Hampsey, M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. Microbiol Mol Biol Rev *62*, 465-503.

Hengartner, C. J., Myer, V. E., Liao, S. M., Wilson, C. J., Koh, S. S., and Young, R. A. (1998). Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Mol Cell *2*, 43-53.

Hirst, M., Kobor, M. S., Kuriakose, N., Greenblatt, J., and Sadowski, I. (1999). GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase SRB10/CDK8. Mol Cell *3*, 673-678.

Holstege, F. C., Jennings, E. G., Wyrick, J. J., Lee, T. I., Hengartner, C. J., Green, M. R., Golub, T. R., Lander, E. S., and Young, R. A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell *95*, 717-728.

Houzelstein, D., Bullock, S. L., Lynch, D. E., Grigorieva, E. F., Wilson, V. A., and Beddington, R. S. (2002). Growth and early postimplantation defects in mice deficient for the bromodomain-containing protein Brd4. Mol Cell Biol *22*, 3794-3802.

Hunter, N. (1999). Prion diseases and the central dogma of molecular biology. Trends Microbiol *7*, 265-266.

Ito, M., and Roeder, R. G. (2001). The TRAP/SMCC/Mediator complex and thyroid hormone receptor function. Trends Endocrinol Metab *12*, 127-134.

Ito, M., Yuan, C. X., Malik, S., Gu, W., Fondell, J. D., Yamamura, S., Fu, Z. Y., Zhang, X., Qin, J., and Roeder, R. G. (1999). Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. Mol Cell *3*, 361-370.

Jiang, Y. W., Veschambre, P., Erdjument-Bromage, H., Tempst, P., Conaway, J. W., Conaway, R. C., and Kornberg, R. D. (1998). Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways. Proc Natl Acad Sci U S A *95*, 8538-8543.

Kang, J. S., Kim, S. H., Hwang, M. S., Han, S. J., Lee, Y. C., and Kim, Y. J. (2001). The structural and functional organization of the yeast mediator complex. J Biol Chem *276*, 42003-42010.

Kim, Y. J., Bjorklund, S., Li, Y., Sayre, M. H., and Kornberg, R. D. (1994). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell *77*, 599-608.

Kim, Y. J., and Lis, J. T. (2005). Interactions between subunits of Drosophila Mediator and activator proteins. Trends Biochem Sci *30*, 245-249.

Kobor, M. S., and Greenblatt, J. (2002). Regulation of transcription elongation by phosphorylation. Biochim Biophys Acta *1577*, 261-275.

Kornberg, R. D. (2005). Mediator and the mechanism of transcriptional activation. Trends Biochem Sci *30*, 235-239.

Krogan, N. J., Cagney, G., Yu, H., Zhong, G., Guo, X., Ignatchenko, A., Li, J., Pu, S., Datta, N., Tikuisis, A. P., *et al.* (2006). Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. Nature *440*, 637-643.

Kuras, L., Borggrefe, T., and Kornberg, R. D. (2003).

Association of the Mediator complex with enhancers of active genes. Proc Natl Acad Sci U S A *100*, 13887-13891.

Ladurner, A. G., Inouye, C., Jain, R., and Tjian, R. (2003). Bromodomains mediate an acetyl-histone encoded antisilencing function at heterochromatin boundaries. Mol Cell *11*, 365-376.

Lee, M., Chatterjee, S., and Struhl, K. (2000). Genetic analysis of the role of Pol II holoenzyme components in repression by the Cyc8-Tup1 corepressor in yeast. Genetics *155*, 1535-1542.

Lee, Y. C., and Kim, Y. J. (1998). Requirement for a functional interaction between mediator components Med6 and Srb4 in RNA polymerase II transcription. Mol Cell Biol *18*, 5364-5370.

Lee, Y. C., Park, J. M., Min, S., Han, S. J., and Kim, Y. J. (1999). An activator binding module of yeast RNA polymerase II holoenzyme. Mol Cell Biol *19*, 2967-2976.

Lemieux, K., and Gaudreau, L. (2004). Targeting of Swi/Snf to the yeast GAL1 UAS G requires the Mediator, TAF IIs, and RNA polymerase II. Embo J *23*, 4040-4050.

Levine, M., and Tjian, R. (2003). Transcription regulation and animal diversity. Nature *424*, 147-151.

Liao, S. M., Zhang, J., Jeffery, D. A., Koleske, A. J., Thompson, C. M., Chao, D. M., Viljoen, M., van Vuuren, H. J., and Young, R. A. (1995). A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature *374*, 193-196.

Liu, Y., Kung, C., Fishburn, J., Ansari, A. Z., Shokat, K. M., and Hahn, S. (2004). Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. Mol Cell Biol *24*, 1721-1735.

Lorch, Y., Beve, J., Gustafsson, C. M., Myers, L. C., and Kornberg, R. D. (2000). Mediator-nucleosome interaction. Mol Cell *6*, 197-201.

Malik, S., Gu, W., Wu, W., Qin, J., and Roeder, R. G. (2000). The USA-derived transcriptional coactivator PC2 is a submodule of TRAP/SMCC and acts synergistically with other PCs. Mol Cell *5*, 753-760.

Matangkasombut, O., Buratowski, R. M., Swilling, N. W., and Buratowski, S. (2000). Bromodomain factor 1 corresponds to a missing piece of yeast TFIID. Genes Dev *14*, 951-962.

Meinhart, A., Kamenski, T., Hoeppner, S., Baumli, S., and Cramer, P. (2005). A structural perspective of CTD function. Genes Dev *19*, 1401-1415.

Mellor, J. (2005). The dynamics of chromatin remodeling at promoters. Mol Cell *19*, 147-157.

Mendes Soares, L. M., and Valcarcel, J. (2006). The expanding transcriptome: the genome as the 'Book of Sand'. Embo J *25*, 923-931.

Mo, X., Kowenz-Leutz, E., Xu, H., and Leutz, A. (2004). Ras induces mediator complex exchange on C/EBP beta. Mol Cell *13*, 241-250.

Myers, L. C., Gustafsson, C. M., Hayashibara, K. C., Brown, P. O., and Kornberg, R. D. (1999). Mediator protein mutations that selectively abolish activated transcription. Proc Natl Acad Sci U S A *96*, 67-72.

Myers, L. C., and Kornberg, R. D. (2000). Mediator of transcriptional regulation. Annu Rev Biochem *69*, 729-749.

Naar, A. M., Beaurang, P. A., Zhou, S., Abraham, S., Solomon, W., and Tjian, R. (1999). Composite co-activator ARC mediates chromatin-directed transcriptional activation. Nature *398*, 828-832.

Naar, A. M., Lemon, B. D., and Tjian, R. (2001). Transcriptional coactivator complexes. Annu Rev Biochem *70*, 475-501.

Nelson, C., Goto, S., Lund, K., Hung, W., and Sadowski, I. (2003). Srb10/Cdk8 regulates yeast filamentous growth by phosphorylating the transcription factor Ste12. Nature *421*, 187-190.

Nonet, M. L., and Young, R. A. (1989). Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of Saccharomyces cerevisiae RNA polymerase II. Genetics *123*, 715-724.

Pandey, P. K., Udayakumar, T. S., Lin, X., Sharma, D., Shapiro, P. S., and Fondell, J. D. (2005). Activation of TRAP/mediator subunit TRAP220/Med1 is regulated by mitogen-activated protein kinase-dependent phosphorylation. Mol Cell Biol *25*, 10695-10710.

Park, J. M., Kim, H. S., Han, S. J., Hwang, M. S., Lee, Y. C., and Kim, Y. J. (2000). In vivo requirement of activator-specific binding targets of mediator. Mol Cell Biol *20*, 8709-8719.

Perez-Martin, J. (1999). Chromatin and transcription in Saccharomyces cerevisiae. FEMS Microbiol Rev *23*, 503-523.

Proudfoot, N. J., Furger, A., and Dye, M. J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501-512.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., *et al.* (2005). Global analysis of protein phosphorylation in yeast. Nature *438*, 679-684.

Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A. M., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999). Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. Nature *398*, 824-828.

Rachez, C., Suldan, Z., Ward, J., Chang, C. P., Burakov, D., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1998). A novel protein complex that interacts with the vitamin D3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system. Genes Dev *12*, 1787-1800.

Radonjic, M., Andrau, J. C., Lijnzaad, P., Kemmeren, P., Kockelkorn, T. T., van Leenen, D., van Berkum, N. L., and Holstege, F. C. (2005). Genome-wide analyses reveal RNA polymerase II located upstream of genes poised for rapid response upon S. cerevisiae stationary phase exit. Mol Cell *18*, 171-183.

Rickert, P., Corden, J. L., and Lees, E. (1999). Cyclin C/CDK8 and cyclin H/CDK7/p36 are biochemically distinct CTD kinases. Oncogene *18*, 1093-1102.

Riedl, T., and Egly, J. M. (2000). Phosphorylation in transcription: the CTD and more. Gene Expr *9*, 3-13.

Rohde, J. R., Trinh, J., and Sadowski, I. (2000). Multiple signals regulate GAL transcription in yeast. Mol Cell Biol *20*, 3880-3886.

Ryu, S., and Tjian, R. (1999). Purification of transcription cofactor complex CRSP. Proc Natl Acad Sci U S A *96*, 7137-7142.

Ryu, S., Zhou, S., Ladurner, A. G., and Tjian, R. (1999). The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1. Nature *397*, 446-450.

Sato, S., Tomomori-Sato, C., Parmely, T. J., Florens, L., Zybailov, B., Swanson, S. K., Banks, C. A., Jin, J., Cai, Y., Washburn, M. P., *et al.* (2004). A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. Mol Cell *14*, 685-691.

Shah, K., and Shokat, K. M. (2003). A chemical genetic approach for the identification of direct substrates of protein kinases. Methods Mol Biol *233*, 253-271.

Smale, S. T., and Kadonaga, J. T. (2003). The RNA polymerase II core promoter. Annu Rev Biochem *72*, 449-479.

Song, W., Treich, I., Qian, N., Kuchin, S., and Carlson, M. (1996). SSN genes that affect transcriptional repression in Saccharomyces cerevisiae encode SIN4, ROX3, and SRB proteins associated with RNA polymerase II. Mol Cell Biol *16*, 115-120.

Stillman, D. J., Dorland, S., and Yu, Y. (1994). Epistasis analysis of suppressor mutations that allow HO expression in the absence of the yeast SW15 transcriptional activator. Genetics *136*, 781-788.

Strich, R., Slater, M. R., and Esposito, R. E. (1989). Identification of negative regulatory genes that govern the expression of early meiotic genes in yeast. Proc Natl Acad Sci U S A *86*, 10018-10022.

Sun, X., Zhang, Y., Cho, H., Rickert, P., Lees, E., Lane, W., and Reinberg, D. (1998). NAT, a human complex containing Srb polypeptides that functions as a negative regulator of activated transcription. Mol Cell *2*, 213-222.

Suzuki, Y., Nogi, Y., Abe, A., and Fukasawa, T. (1988). GAL11 protein, an auxiliary transcription activator for genes encoding galactose-metabolizing enzymes in Saccharomyces cerevisiae. Mol Cell Biol *8*, 4991-4999.

Taatjes, D. J., Marr, M. T., and Tjian, R. (2004a). Regulatory diversity among metazoan co-activator complexes. Nat Rev Mol Cell Biol *5*, 403-410.

Taatjes, D. J., Naar, A. M., Andel, F., 3rd, Nogales, E., and Tjian, R. (2002). Structure, function, and activator-induced conformations of the CRSP coactivator. Science *295*, 1058-1062.

Taatjes, D. J., Schneider-Poetsch, T., and Tjian, R. (2004b). Distinct conformational states of nuclear receptor-bound CRSP-Med complexes. Nat Struct Mol Biol *11*, 664-671.

Tabtiang, R. K., and Herskowitz, I. (1998). Nuclear proteins Nut1p and Nut2p cooperate to negatively regulate a Swi4p-dependent lacZ reporter gene in Saccharomyces cerevisiae. Mol Cell Biol *18*, 4707-4718.

Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N., and Kishimoto, T. (1995). Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell *80*, 353-361.

Tanaka, T., Yoshida, N., Kishimoto, T., and Akira, S. (1997). Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. Embo J *16*, 7432-7443.

Thompson, C. M., Koleske, A. J., Chao, D. M., and Young, R. A. (1993). A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell *73*, 1361-1375.

Thompson, C. M., and Young, R. A. (1995). General requirement for RNA polymerase II holoenzymes in vivo. Proc Natl Acad Sci U S A *92*, 4587-4590.

van de Peppel, J., Kettelarij, N., van Bakel, H., Kockelkorn, T. T., van Leenen, D., and Holstege, F. C. (2005). Mediator expression profiling epistasis reveals a signal transduction pathway with antagonistic submodules and highly specific downstream targets. Mol Cell *19*, 511-522.

Vincent, O., Kuchin, S., Hong, S. P., Townley, R., Vyas, V. K., and Carlson, M. (2001). Interaction of the Srb10 kinase with Sip4, a transcriptional activator of gluconeogenic genes in Saccharomyces cerevisiae. Mol Cell Biol *21*, 5790-5796.

Washburn, M. P., Wolters, D., and Yates, J. R., 3rd (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol *19*, 242-247.

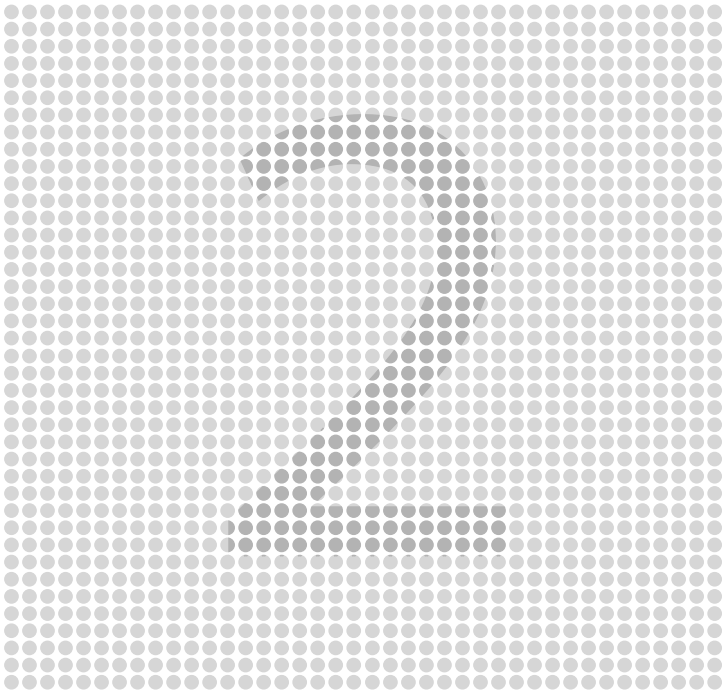
Witucki, L. A., Huang, X., Shah, K., Liu, Y., Kyin, S., Eck, M. J., and Shokat, K. M. (2002). Mutant tyrosine kinases with unnatural nucleotide specificity retain the structure and phospho-acceptor specificity of the wild-type enzyme. Chem Biol *9*, 25-33.

Wolters, D. A., Washburn, M. P., and Yates, J. R., 3rd (2001). An automated multidimensional protein identification technology for shotgun proteomics. Anal Chem *73*, 5683-5690.

Zahnow, C. A., Younes, P., Laucirica, R., and Rosen, J. M. (1997). Overexpression of C/EBPbeta-LIP, a naturally occurring, dominant-negative transcription factor, in human breast cancer. J Natl Cancer Inst *89*, 1887-1891.

Zaman, Z., Ansari, A. Z., Koh, S. S., Young, R., and Ptashne, M. (2001). Interaction of a transcriptional repressor with the RNA polymerase II holoenzyme plays a crucial role in repression. Proc Natl Acad Sci U S A *98*, 2550-2554.

Zhu, X., Wiren, M., Sinha, I., Rasmussen, N. N., Linder, T., Holmberg, S., Ekwall, K., and Gustafsson, C. M. (2006). Genome-wide occupancy profile of mediator and the srb8-11 module reveals interactions with coding regions. Mol Cell *22*, 169-178.



Monitoring global messenger RNA changes in externally controlled microarray experiments

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ABSTRACT

Expression profiling is a universal tool, with a range of applications that benefit from the accurate determination of differential gene expression. To allow normalization using endogenous transcript levels, current microarray analyses assume that relatively few transcripts vary, or that any changes that occur are balanced. When normalization using endogenous genes is carried out, changes in expression levels are calculated relative to the behavior of most of the transcripts. This does not reflect absolute changes if global shifts in messenger RNA populations occur. Using external RNA controls, we have set up microarray experiments to monitor global changes.

The levels of most mRNAs were found to change during yeast stationary phase and human heat shock when external controls were included. Even small global changes had a significant effect on the number of genes reported as being differentially expressed. This suggests that global mRNA changes occur more frequently than is assumed at present, and shows that monitoring such effects may be important for the accurate determination of changes in gene expression.

INTRODUCTION

Microarray expression profiling is a universal tool, with a range of applications that benefit from the accurate determination of differential gene expression (Brown and Botstein, 1999; Young, 2000). The detection of changes in messenger RNA expression requires normalization between samples. This counters non-biological variation, such as differences in labeled material, local array differences, dye-specific biases and so on (Quackenbush, 2001; Tseng et al., 2001; Yang et al., 2002).

Normalization methods consist of an algorithm and the features on the array to which the algorithm is applied. Ideally, such features should have identical signals in all the samples under investigation. Most researchers carrying out microarray analyses have dismissed the idea of using invariant house-keeping genes that are stably expressed across a wide range of experimental conditions (Lee et al., 2002). With a few exceptions (Talaat et al., 2002; Yang et al., 2002), most microarray experiments make use of the expression levels of all genes as normalization features. The assumption underlying this 'all-genes' approach is that relatively few transcript levels vary between samples, or that any changes that occur are balanced. Normalization using the expression levels of endogenous genes means that changes are calculated relative to the majority of transcripts. These relative changes do not reflect the absolute changes at the cellular level that occur if global shifts in mRNA populations take place.

The aim of this study was to set up microarray experiments, incorporating external controls, to monitor the effects of inactivating components of the generally required transcription machinery, such as RNA polymerase II (Holstege et al., 1998; Wang et al., 2002). The purpose of external normalization controls is to derive a set of signals for which the final outcome is known to be

equal among samples. This can be achieved by the addition of equal amounts of control RNA molecules to samples before processing. Here, we present results from experiments in which we observed global changes occurring under conditions that, in the past, have been studied without the use of external controls. As well as showing how global effects can be monitored, the results suggest that global mRNA changes occur more frequently than is presently assumed by researchers carrying out microarray analyses. This has important implications for the interpretation of such experiments.

RESULTS

External normalization controls

A generalized scheme for carrying out microarray analyses using external controls for normalization is shown in **Figure 2.1a**. In this study, total RNA was spiked with a mixture of nine control RNAs. The concentrations of the control RNAs were varied over three orders of magnitude to cover a range of mRNA expression levels (**Figure 2.1b**). Oligonucleotide probes (of 70 nucleotides in length) representing each control were spotted at least twice onto the microarray subgrids to generate sufficient data points (960 in total; 20 per subgrid). This allows local, expression-dependent or intensity-dependent normalization (Yang et al., 2002). The microarrays also incorporated other controls in addition to the gene probes (6,371 for *Saccharomyces cerevisiae*, each spotted twice, and 16,735 for the human arrays).

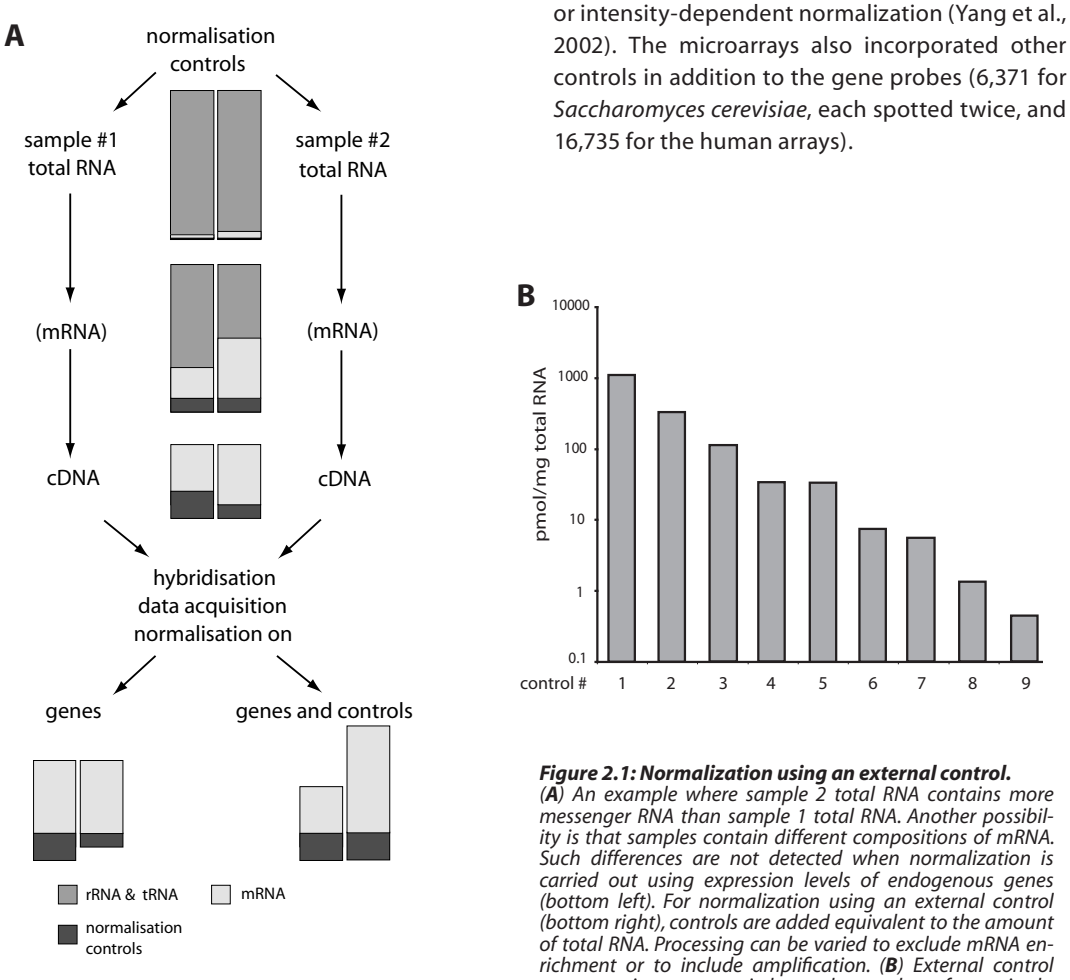

Figure 2.1: Normalization using an external control. *(**A**) An example where sample 2 total RNA contains more messenger RNA than sample 1 total RNA. Another possibility is that samples contain different compositions of mRNA. Such differences are not detected when normalization is carried out using expression levels of endogenous genes (bottom left). For normalization using an external control (bottom right), controls are added equivalent to the amount of total RNA. Processing can be varied to exclude mRNA enrichment or to include amplification. (**B**) External control concentrations were varied over three orders of magnitude. The amounts of each in vitro transcribed control RNA added as a single mix to total RNA are shown. rRNA, ribosomal RNA; tRNA, transfer RNA.*

Global changes during mammalian heat shock

One study that has shown global changes in the mRNA population is that of the heat-shock response of primary human umbilical vein endothelial cells (HUVECs). This is similar to many previous microarray studies that have examined cellular responses, and was investigated as part of our program to understand transcriptional regulation. When external controls were used, a change in global mRNA levels during heat shock was shown, as indicated by the gradual separation of the green gene spots away from the blue control spots (**Figure 2.2a-d**). This culminated in an almost twofold median drop in mRNA levels on average in the dye-swap experiments (**Figure 2.2d**). Typically, these responses have previously been normalized using expression levels of all genes. Approximately equal numbers of genes were interpreted as being up- or downregulated after normalization of the experiment in this way (**Figure 2.2e**, left column), which is a consequence of making the assumption that there is no overall change. Such changes in individual transcript levels are relative to the behavior of most of the transcripts. If a global change occurs, as is the case here (**Figure 2.2d**), normalization using genes does not correlate with changes at the cellular level. The interpretation of these experiments differs markedly when external controls are used (**Figure 2.2e**, middle column). The number of genes reported as being downregulated increased from 506 to 6,872 when an arbitrary twofold threshold was used. These changes are relative to equivalent amounts of total RNA because spiking of the controls was carried out at the total RNA stage. Counting cells before harvesting allows differences in RNA content in the two

states to be taken into account (**Figure 2.2e**, right column).

Results of small global changes during serum starvation

A second example shows the results of subjecting human mammary gland adenocarcinoma (MCF7) cells to serum deprivation for 30 h (**Figure 2.3**). Although this resulted in only a small change in global mRNA levels (1.3-fold median drop; **Figure 2.3a**), it had a significant effect on the outcome. More than twice as many genes were found to be differentially expressed as when normalization using the all-genes approach was carried out (**Figure 2.3b**).

Yeast stationary phase

To rule out the possibility that global changes are restricted to mammalian cell cultures, we also studied stationary phase in *S. cerevisiae*. This showed a 1.8-fold median drop in mRNA levels when normalized using external controls (**Figure 2.4a**). Significantly, there is a group of more than 1,000 genes that appear to be upregulated when normalized using the all-genes method, although their mRNA levels had actually decreased. This was only revealed after normalization using external controls was carried out (**Figure 2.4c**, left and middle columns). The apparent upregulation when the experiment was normalized using the all-genes approach was seen because the amount of downregulation for these genes is less than that of most transcripts. When cellular RNA content was also taken into account, the changes were found to be more extreme (**Figure 2.4c**, right column).

Changes dependent on expression levels

An important issue highlighted by the stationary phase experiment is that of an inconsistent distribution of changes across the range of expression levels: that is, along the x axis in **Figure 2.4a,b**. This results in the apparently aberrant behavior of the controls seen when normalization using the all-genes approach was carried out (**Figure 2.4b**). Highly expressed genes (on the right halves of the graphs in **Figure 2.4**) show far more downregulation. One group of highly expressed mRNAs are the ribosomal protein genes (Holstege et al., 1998).The high degree of downregulation of these genes is consistent with the significant decrease in translation that occurs during stationary phase (Dickson and Brown, 1998). Unlike approaches using endogenous transcripts, normalization using external controls is not confounded by an uneven distribution of changes, and also contributes to a more accurate determination of mRNA changes in this respect.

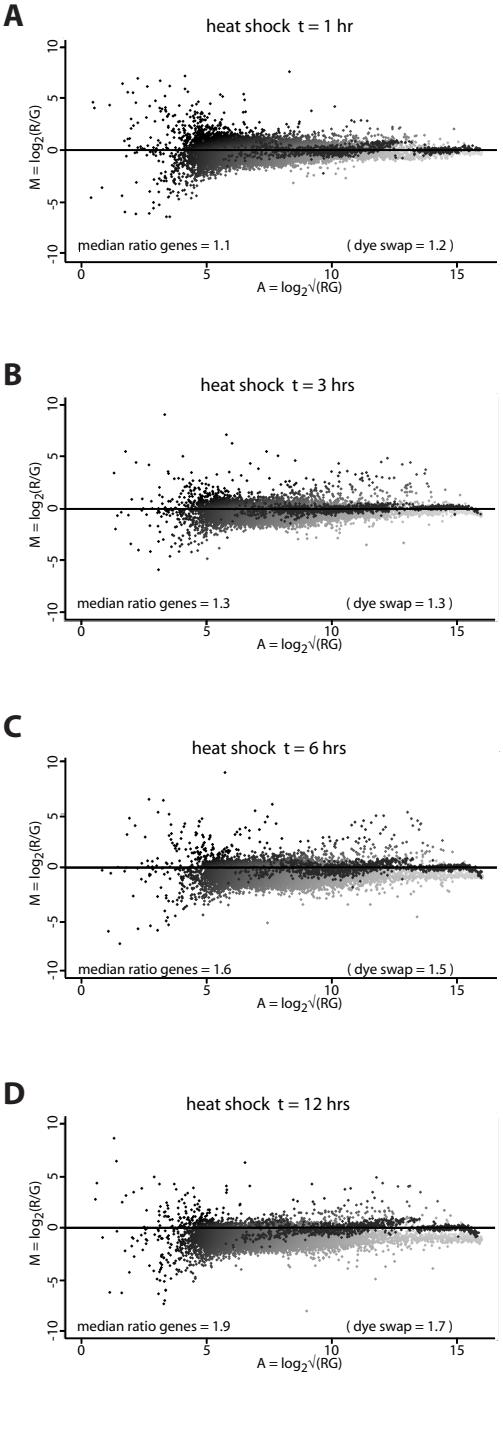

Figure 2.2: Global messenger RNA changes during mammalian heat shock.
*Human umbilical vein endothelial cells were heat-shocked and RNA was isolated at the timepoints shown (**A–D**). The result of hybridization of each sample (R) against that of the non-heatshocked reference cells (G) is shown. Each graph is an MA scatterplot (where M= log2(R/G) and A = log2√(RG); Yang et al., 2002). The y axis shows the log2 ratio (mean spot intensity minus mean local area background) after normalization using genes and controls (see Methods). The values plotted on the x axes are derived from the intensities of both channels. The median change for all genes is indicated, as well as the result of a corresponding dye-swap experiment. (**E**) Comparison of different normalization strategies. From left to right are the results of three strategies applied to the 12-h timepoint. Each column is made up of 16,735 coloured lines, stacked vertically. Each line represents the change for a single gene. Upregulated genes are shown in red and downregulated genes are shown in green. Numbers below the bars indicate the median change of all genes after normalization (as a ratio) and how many genes are reported as being upregulated or downregulated if an arbitrary twofold cut-off is applied. The first column shows the result of Lowess normalization per subgrid using endogenous genes (see Methods). The second column shows the result of incorporating external controls into the normalization strategy by carrying out normalization to equivalent amounts of total RNA (normalization using genes and controls; see Methods). This leads to a markedly altered perception of the changes that have taken place, with many more transcripts reported as being downregulated. The third column shows the result when the slight drop in the total RNA content of the cells is taken into account.*

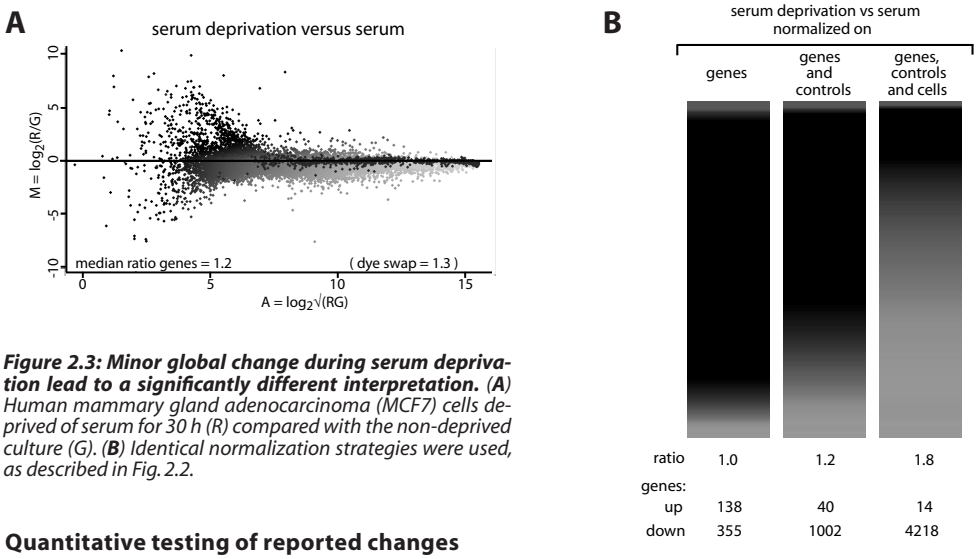

Figure 2.3: Minor global change during serum deprivation lead to a significantly different interpretation. *(**A**) Human mammary gland adenocarcinoma (MCF7) cells deprived of serum for 30 h (R) compared with the non-deprived culture (G). (**B**) Identical normalization strategies were used, as described in Fig. 2.2.*

Quantitative testing of reported changes

How accurate are the changes observed when normalization is carried out using external controls? Carrying out RT–PCR (reverse transcription followed by PCR) on a selected set of mRNAs leads to the question of what reference transcript should be chosen for the normalization of such experiments. The accuracy of the reported global mRNA changes was therefore tested by spiking the control mixtures in different ratios in identical pairs of RNA samples. The ratios chosen were 1:1, 1:2 and 1:10, thereby simulating 1-, 2- and 10-fold changes in the mRNA population relative to the controls. Normalization using external controls allowed the accurate determination of changes in mRNA levels relative to the controls. The gene spots drifted away from the control spots as the spiking ratio was increased to 1:10 (**Figure 2.5a-c**). The median amount of change observed in the expression of the genes after normalization was almost identical to the spiking ratios, with values of 1.0-, 1.9- and 9.3-fold, respectively, for the average of each dye swap. This shows that the global changes reported here (up to twofold median changes; **Figure 2.2-4**) are likely to be correct.

DISCUSSION

This study focuses on the features used for single-slide normalization and shows how the incorporation of external controls can markedly alter the interpretation of microarray experiments.

External controls have been used previously for normalization in studies examining the artificial inactivation of RNA polymerase II (Holstege et al., 1998; Wang et al., 2002). In these cases, global changes were expected. The experiments presented here show that global changes can also occur under more conventional experimental conditions. We have also shown how these controls can be applied to cope with local, intensity-dependent systematic variation (Yang et al., 2002) by representation in sufficient numbers on each microarray subgrid, and by spiking over a range of levels. As well as being useful for normalization, controls such as these are useful for monitoring sample labeling, optimization of all microarray protocols, and as external controls for the reported ratios.

Recent papers have discussed the importance of experimental design and normalization algorithm choice (Kerr and Churchill, 2001; Kroll and Wolfl, 2002; Quackenbush, 2001; Tseng et al., 2001; Yang et al., 2002; Yang and Speed, 2002). An important improvement has been the adoption of normalization algorithms that take into account local, intensity-dependent systematic variation (Yang et al., 2002). Regardless of the algorithm

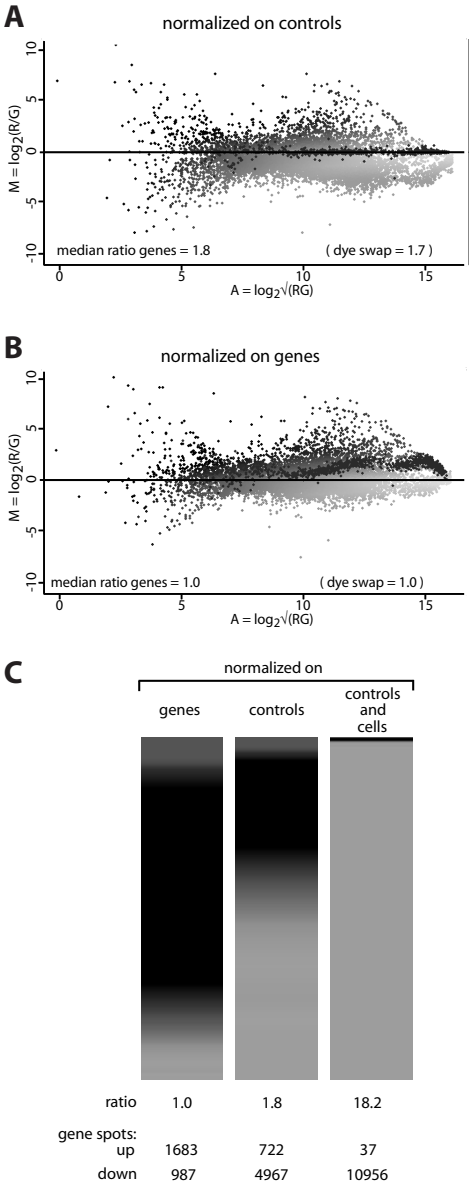

Figure 2.4: Yeast stationary phase culture compared with mid-log phase culture.
*(**A**) MA scatterplot (where M = log2(R/G) and A = log2√(R/G)) after Lowess normalization for each subgrid using controls (see Methods). (**B**) Lowess normalization for each subgrid using genes (see Methods). The aberrant pattern of external control spots (blue) occurred because messenger RNA levels had not changed uniformly across the entire range of expression levels (along the x axis). RNA levels for genes with higher expression levels had dropped to a greater degree than those of genes expressed at lower levels. Due to saturation of the signals in the scanned images, this effect seemingly decreases for a small group of genes expressed at the highest levels, resulting in the curve of the control spots to the far right of the graph. (**C**) Comparison of different normalization strategies. The left and middle columns correspond to the graphs shown in (**B**) and (**A**), respectively. The drop in total RNA yields per cell (see Methods) can also be taken into account (right column). Because each probe was spotted twice on the yeast arrays, the numbers reflect how many spots have changed. R, stationary phase culture; G, mid-log phase culture.*

applied, most current analyses rely on assumptions of evenly distributed changes and/or on the absence of global shifts. As well as the wide use of the level of expression of all genes as an invariant feature, alternative normalization features that have been proposed include housekeeping genes, spotted microarray sample pools or spots containing genomic DNA (for overviews, see (Kroll and Wolfl, 2002; Yang et al., 2002)). Normalization using such features does not reveal global, unbalanced changes. The use of a common reference sample, made up of a collection of all the DNAs represented on arrays, has also been proposed (Dudley et al., 2002; Sterrenburg et al., 2002). This allows better comparisons between slides and also overcomes the problem of obtaining negligible signals for underrepresented mRNAs. However, such approaches do not address the possibility of global, unbalanced changes. It is likely that the use of a combination of external controls and a common pooled, spotted reference sample may be the best way of overcoming several problems simultaneously.

Monitoring global effects is not required, or feasible, for every microarray experiment. The advantage of the use of external controls is that it allows the possibility of detecting such changes in an experimental set-up that is otherwise unchanged. The disadvantage is the requirement for the robust preparation of RNA samples and the reliable quantitation of yields. When RNA yields are too low to be monitored reliably, or when RNA preparations vary qualitatively, the use of external controls for normalization will not be reliable.

The importance of monitoring global and/or unbalanced mRNA changes is dictated by the goals of the experiment. For example, in disease classification studies, determination of the most extreme (relative) markers of a particular state without consideration of the actual changes does not require external control normalization.

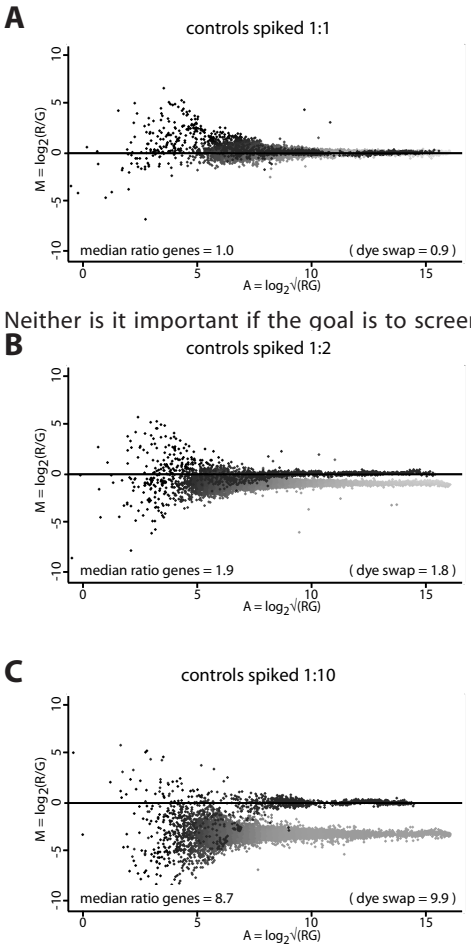

Figure 2.5: External controls can accurately detect global transcript changes.
*The external control mix was added at a ratio of (**A**) 1:1, (**B**) 1:2 and (**C**) 1:10 to paired aliquots of a single yeast total RNA preparation.*

only the most responsive genes in particular experimental conditions. However, as experimental biology becomes more comprehensive and quantitative due to the availability of whole-genome sequences and to the increased focus on systems biology (Ideker et al., 2001), it will become more of a necessity to monitor with greater precision the actual, rather than the relative, changes in different cellular states. Methods that take into account the possibility of global changes will contribute towards such goals. Examples of studies that will benefit from the use of external controls include comprehensive studies of gene regulation and analyses of drug side-effects, as well as microarray studies incorporating only limited sets of genes.

METHODS

Accession numbers and protocols

For MIAME (minimum information about a microarray experiment)-compliant (Brazma et al., 2001) protocols, data sets in Microarray Gene Expression Markup Language (MAGEML) (Spellman et al., 2002) and normalization scripts, see our website (http://www.genomics.med.uu.nl/pub/jvp/ext_controls) or the public microarray database ArrayExpress (http://www.ebi.ac.uk/microarray/ArrayExpress/arrayexpress.html) (**Table 2.1**).

Table 2.1 Arrayexpress accession numbers

Accession number	Description
A-UMCU-1	UMC Utrecht *Saccharomyces cerevisiae* 16-k array, version 1.1
A-UMCU-2	UMC Utrecht *Homo sapiens* 19-k array, version 1.0
P-UMCU- 1	*S. cerevisiae* culture
P-UMCU-2	Human umbilical vein endothelial cell culture
P-UMCU-3	MCF7 cell culture
P-UMCU-4	Total RNA isolation (*S. cerevisiae)*
P-UMCU-5	Total RNA isolation (mammalian cell culture)
P-UMCU-6	Messenger RNA enrichment (*S. cerevisiae*)
P-UMCU-7	Amino-allyl labelling
P-UMCU-8	Microarray production
P-UMCU-9	Hybridization
P-UMCU-10	Scanning protocol
P-UMCU-11	Image analysis
E-UMCU-1	Yeast spiked controls
E-UMCU-2	Human umbilical vein endothelial cell culture heat shock
E-UMCU-3	MCF7 serum deprivation
E-UMCU-4	Yeast stationary phase

MCF7, human mammary gland adenocarcinoma cell line; UMCU, University Medical Centre Utrecht.

External controls

Constructs containing *Bacillus subtilis* genes (*ycxA*, *yceG*, *ybdO*, *ybbR*, *ybaS*, *ybaF*, *ybaC*, *yacK* and *yabQ*) cloned between the *Xba*I and *Bam*HI sites in pT7T3 (Amersham Pharmacia Biotech) were made, with an additional 30-nucleotide poly(A) sequence between the gene and the *Xho*I site. For making RNA, plasmids were digested with *Xho*I for use in *in vitro* transcription reactions using MEGAscript-T7 (Ambion).

Cell culture

HUVECs were isolated as described in Jaffe et al. (Jaffe et al., 1973). Cells were cultured in endothelial

growth medium (EGM-2) at 37°C in the presence of 5% CO2. Before heat shock, the medium was removed, preheated EGM-2 was added and cells were incubated at 42.5°C in EGM-2.

MCF7 cells were cultured in DMEM/Ham's F12 medium (1:1) containing 5% fetal calf serum, glutamine (300 mg/ml), penicillin (100 inhibitory units/ml) and streptomycin (100 mg/ml). Cells at 70% confluence were grown for 30 h in phenol-red-free, serumfree medium with 0.2% BSA, transferrin (10 mg/ml) and 30 nM sodium selenite.

S. cerevisiae S288c (*MATa*; *met15*; *ura3*; *his3*Δ1; *leu2*) (Research Genetics) was grown in YEP medium (containing yeast extract and peptone) supplemented with 2% glucose. Cultures for the spiking experiments (Figure 5) were grown to mid-log phase (OD_{600} = 0.5), and for the stationary phase experiment were grown to mid-log phase or to stationary phase (OD_{600} = 10.0; 10-day culture).

RNA isolation and labeling

For mammalian cells, total RNA was prepared using Trizol (Gibco BRL) in accordance with the manufacturer's instructions. External controls were added in an appropriately diluted 5-µl mixture to 10 µg of total RNA. Yeast total RNA was prepared using hot phenol. External controls were added, as an appropriately diluted 5-µl mixture, to 500 µg of total RNA, and mRNA was isolated using Oligotex (Qiagen). Complementary DNA synthesis was carried out using 10 µg of mammalian total RNA, or 3 µg of yeast mRNA, in the presence of 2-aminoallyl-dUTP. Samples were purified using Microcon-30 (Millipore) columns and were coupled to Cy3 and Cy5 fluorophores. Before hybridization, free dyes were removed using Chromaspin-30 (Clontech) columns, and the efficiency of cDNA synthesis and dye incorporation was measured using a spectrophotometer (UV1240mini, Shimadzu).

Microarray hybridization

From each sample, 300 ng cDNA (with a specific activity of 2–4% dye-labeled nucleosides) was hybridized for 16–20 h at 42°C. Slides were scanned in a Scanarray 4000 XL (Perkin Elmer Biosystems). Image analysis was carried out using Imagene 4.0 (Biodiscovery).

Microarray production

C6-amino-linked oligonucleotides (70 nucleotides in length), the Yeast Genome ArrayReady and the Human Genome ArrayReady Oligo set (version 1.1) were purchased from Qiagen, and were printed on Corning UltraGAPS slides with a MicroGrid II (Apogent Discoveries) using 48-quill pins

(Microspot2500; Apogent Discoveries) in 3 x SSC at 50% humidity and at 18°C, and were processed by ultraviolet crosslinking (2,400 millijoules, 10 min) with a Stratalinker2400 (Stratagene).

Normalization

Algorithms were based on Lowess print-tip normalization (Yang et al., 2002), applied in the statistical package R (Ihaka and Gentleman, 1996), using the existing packages SMA (http://www.stat.berkeley.edu/users/terry/zarray/Software/smacode.html) and com.braju.sma (http://www.maths.lth.se/help/R/com.braju.sma). Alterations were made for the import of Imagene 4.0 files, flagging of control spots, Lowess line calculation on subsets of spots (either controls or genes) and extrapolation to all spots in the subgrid. This has been incorporated in an R package called genomics.sma.

The first method, normalization using the expression levels of endogenous genes, uses gene spots to calculate the Lowess line for each subgrid and then applies these lines to all spots. The second method, normalization using the expression levels of all genes, and external controls, also shifts all spots linearly according to the median ratio of the control spots. The third method, normalization using external controls, uses the controls to calculate the Lowess line for each subgrid and then applies these lines to all spots for normalization.

The second and third methods gave almost identical results for the experiments shown in **Figures 2.2, 2.3 and 2.5**. Due to the non-uniform distribution of changes in mRNA levels in the stationary phase experiment, only the third method (normalization using external controls) gave accurate results overall. This would be the method of choice for all experiments. However, there was a significant variation in the amount of the individual oligonucleotides supplied in the collections compared with the control oligonucleotides. This resulted in suboptimal extrapolation of the Lowess line to those gene spots that have extremely low intensity values, as a result of both low amounts of oligonucleotides and low or non-existent levels of mRNA. The variation in the amount of oligonucleotides supplied has been rectified in later versions of the collections.

To take into account differences in RNA yield per cell, cells were counted, and a linear shift was applied to the normalized data using the ratio derived from the total RNA yield per cell from each pair of samples.

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REFERENCES

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C., *et al.* (2001). Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet *29*, 365-371.

Brown, P. O., and Botstein, D. (1999). Exploring the new world of the genome with DNA microarrays. Nat Genet *21*, 33-37.

Dickson, L. M., and Brown, A. J. (1998). mRNA translation in yeast during entry into stationary phase. Mol Gen Genet *259*, 282-293.

Dudley, A. M., Aach, J., Steffen, M. A., and Church, G. M. (2002). Measuring absolute expression with microarrays with a calibrated reference sample and an extended signal intensity range. Proc Natl Acad Sci U S A *99*, 7554-7559.

Holstege, F. C., Jennings, E. G., Wyrick, J. J., Lee, T. I., Hengartner, C. J., Green, M. R., Golub, T. R., Lander, E. S., and Young, R. A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell *95*, 717-728.

Ideker, T., Galitski, T., and Hood, L. (2001). A new approach to decoding life: systems biology. Annu Rev Genomics Hum Genet *2*, 343-372.

Ihaka, R., and Gentleman, R. (1996). R: a language for data analysis and graphics. J Comp Graph Statist *5*, 299-314.

Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest *52*, 2745-2756.

Kerr, M. K., and Churchill, G. A. (2001). Statistical design and the analysis of gene expression microarray data. Genet Res *77*, 123-128.

Kroll, T. C., and Wolfl, S. (2002). Ranking: a closer look on globalisation methods for normalisation of gene expression arrays. Nucleic Acids Res *30*, e50.

Lee, P. D., Sladek, R., Greenwood, C. M., and Hudson, T. J. (2002). Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res *12*, 292-297.

Quackenbush, J. (2001). Computational analysis of microarray data. Nat Rev Genet *2*, 418-427.

Spellman, P. T., Miller, M., Stewart, J., Troup, C., Sarkans, U., Chervitz, S., Bernhart, D., Sherlock, G., Ball, C., Lepage, M., *et al.* (2002). Design and implementation of microarray gene expression markup language (MAGE-ML). Genome Biol *3*, RESEARCH0046.

Sterrenburg, E., Turk, R., Boer, J. M., van Ommen, G. B., and den Dunnen, J. T. (2002). A common reference for cDNA microarray hybridizations. Nucleic Acids Res *30*, e116.

Talaat, A. M., Howard, S. T., Hale, W. t., Lyons, R., Garner, H., and Johnston, S. A. (2002). Genomic DNA standards for gene expression profiling in Mycobacterium tuberculosis. Nucleic Acids Res *30*, e104.

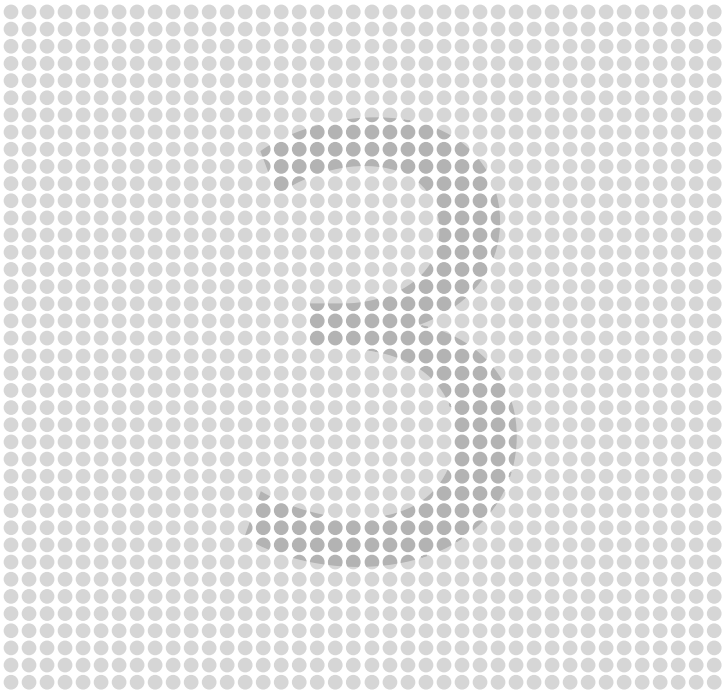
Tseng, G. C., Oh, M. K., Rohlin, L., Liao, J. C., and Wong, W. H. (2001). Issues in cDNA microarray analysis: quality filtering, channel normalization, models of variations and assessment of gene effects. Nucleic Acids Res *29*, 2549-2557.

Wang, Y., Liu, C. L., Storey, J. D., Tibshirani, R. J., Herschlag, D., and Brown, P. O. (2002). Precision and functional specificity in mRNA decay. Proc Natl Acad Sci U S A *99*, 5860-5865.

Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., and Speed, T. P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res *30*, e15.

Yang, Y. H., and Speed, T. (2002). Design issues for cDNA microarray experiments. Nat Rev Genet *3*, 579-588.

Young, R. A. (2000). Biomedical discovery with DNA arrays. Cell *102*, 9-15.



Mediator Expression-Profiling Epistasis Reveals a Signal Transduction Pathway with Antagonistic Submodules and Highly Specific Downstream Targets

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SUMMARY

Mediator is an evolutionarily conserved coregulator of RNA polymerase II transcription. Microarray structure-function analysis of *S.cerevisiae* Mediator reveals functional antagonism between the cyclin dependent kinase (Cdk) submodule and components from the Tail (Med15, Med2, Med3), Head (Med20, Med18) and Middle (Med31). Certain genes exhibit increased or decreased expression, depending on which subunit is deleted. Epistasis analysis with expression-profile phenotypes, shows that *MED2* and *MED18* are downstream of *CDK8*. Strikingly, Cdk8-mediated modification of a single amino acid within Mediator represses the regulon of a single transcription factor, Rcs1/Aft1. Highly specific gene regulation is thought to be determined by activators and combinatorial use of cofactors. Here, subtle modification of the general transcription machinery through one of its own components is shown to determine highly specific expression patterns. Expression-profiling can therefore precisely map regulatory cascades and our findings support a role for Mediator as a direct processor of signaling pathways for determining specificity.

INTRODUCTION

In eukaryotes, transcription regulation of protein-coding genes depends on a complex interplay between signal-transduction pathways, gene-specific regulators, coregulatory protein complexes including those that modify chromatin, general transcription factors and RNA polymerase II (Hochheimer and Tjian, 2003; Lee and Young, 2000; Roeder et al., 2005; Sims et al., 2004). An important multi-subunit complex that functions as a cofactor for transcription regulation is the Mediator complex.

A well-established role of Mediator is to mediate the response to transcription activators (Blazek et al., 2005; Conaway et al., 2005; Lewis and Reinberg, 2003; Myers and Kornberg, 2000). Roles in activator binding have been allocated to several Mediator subunits in diverse organisms. This has lead to a model whereby Mediator acts positively in transcription through activator-mediated recruitment and interaction with RNA polymerase II. Alternative Mediator structures are induced by binding of different activators and through interaction with RNA polymerase II itself (Davis et al., 2002; Naar et al., 2002; Taatjes et al., 2002). Structural modification may therefore also play an important role in transmitting activator signals. Mediator can function as a scaffold for repeated rounds of reinitiation by RNA polymerase II (Yudkovsky et al., 2000) and a post-initiation role has also recently been proposed (Wang et al., 2005). The importance of Mediator is exemplified by the immediate drop in virtually all transcripts upon inactivation of the Med17 subunit in the yeast *Saccharomyces cerevisiae* (Holstege et al., 1998).

Although Mediator is generally thought to play a positive role, several subunits have been implicated in negative regulation (Hampsey, 1998; Myers and Kornberg, 2000). Mediator from several organisms exists in at least two forms that differ mainly by the presence or absence of a negative

regulatory submodule (Liu et al., 2001; Sato et al., 2004; Spahr et al., 2003). This negative regulatory submodule consists of a cyclin-dependent kinase (Cdk8), its cyclin partner (CycC) as well as two additional subunits, Med12 and Med13, all of which are well-conserved (Bourbon et al., 2004). Interestingly, it has recently been shown that some mammalian Mediator complexes alternatively harbor Cdk11 (Sato et al., 2004). The negative role of Cdk8 is exemplified by upregulation of a significant subset of genes in yeast cells bearing kinase defective Cdk8 (Holstege et al., 1998).

Cdk8 is the most frequently studied Mediator subunit and several different models have been proposed for the mechanism of Cdk8 repression (Akoulitchev et al., 2000; Chi et al., 2001; Fryer et al., 2004; Hengartner et al., 1998; Nelson et al., 2003). Studies of mammalian transcription show that for some genes, Cdk8 is associated with inactive transcription complexes (Pavri et al., 2005). In other cases Cdk8 is located within the initiation complexes of active genes (Wang et al., 2005). Examples of a positive role for Cdk8 have also been put forward (Liu et al., 2004; Vincent et al., 2001). The nature of Cdk8 activity, its submodule partners and the functional interplay with other submodules remains elusive.

Early after its initial identification, it was suggested that Mediator may form a direct end-point of signal-transduction pathways. This notion was based on the presence of a protein with homology to a RING-3 kinase (Jiang et al., 1998). Except for the recent demonstration that Mediator activity is modulated by the Ras/PKA pathway (Chang et al., 2004), such a direct signaling processor role for Mediator has not been substantiated.

In studies of transcription regulation, microarrays are most frequently applied to determine putative target genes. Here, we describe a structure-function analysis of each non-essential Mediator subunit in *S. cerevisiae* using expression-profiles as a molecular phenotype. This reveals new submodule relationships, including the presence of several mutually antagonistic components within Mediator that form part of a signal-transduction pathway. The functional consequences of one of the antagonistic couples are mapped further. This shows that with appropriate controls, gene expression-profiling can accurately uncover signal-transduction pathways, determine epistatic relationships and pin-point the gene-specific targets of protein phosphorylation .

It is currently thought that high specificity in transcription regulation is achieved by transcriptional activators in combination with differential use of multiple regulatory cofactors (Hochheimer and Tjian, 2003). Intriguingly, our results demonstrate that internal modification of a single amino acid within Mediator results in activation of a single transcription factor regulon. Subtle modifications in the general transcription machinery can therefore also be pivotal for achieving highly specific regulation. Together, the analyses support a direct signal-transduction processing model for Mediator function and indicate its internal mechanism.

RESULTS

To investigate the role of individual components of Mediator, *S.cerevisiae* strains bearing deletions of all 15 non-essential Mediator genes were obtained or generated (**Figure 3.1a**, Experimental Procedures). Two independent cultures of each deletion strain were grown to early mid-log phase in synthetic complete (SC) medium supplemented with glucose. Dye-swap mRNA expression profiles were generated using microarrays with 70-mer oligonucleotides representing each gene in duplicate (Experimental Procedures). This results in four measurements of transcript level changes for each mutant strain compared to a common reference laboratory wild type (wt). Several strains obtained from the yeast gene deletion consortium (Giaever et al., 2002) exhibited aneuploidy (Hughes et al., 2000b) or aberrant phenotypes (Experimental Procedures). These strains were regenerated in an identical genetic background and reanalyzed. The experimental design included obtaining expression-profiles for nine wt cultures, grown in parallel to the deletion strains (**Figure S1**). cDNA from the additional wt cultures were also hybridized versus reference wt to determine variance. This is essential to prevent genes with

variable transcript levels from artificially increasing overlaps between expression-profiles (Hughes et al., 2000a). The collection of expression profiles was examined using analysis of variance (ANOVA), thereby excluding genes with high variance, dye bias or array batch effects. **Figure 3.1b,c** shows an example of a deletion strain and control analysis. Genes with changes in expression levels specific to one or more deletion strains are depicted in **Figure 3.2**. Tight co-clustering is observed for replicate dye-swap profiles derived from independent cultures. This is required if accurate comparisons of different deletion strains are to be made.

Mediator structure-function expression-profiling

Hierarchical clustering of the expression profiles (**Figure 3.2**) reveals three major groups within the non-essential Mediator subunits; 1) positive subunits whereby deletion results mainly in decreased transcript levels (Tail components Med15/Gal11, Med3, Med2, Head components Med20/Srb2, Med18/Srb5 and the recently found Middle component Med31/Soh1 (Guglielmi et al., 2004; Linder and Gustafsson, 2004); 2) negative subunits exhibiting mainly increased transcript levels (Cdk submodule components Cdk8/Srb10, CycC/Srb11, Med12/Srb8, Med13/Srb9 and partial deletion of Head component Med19/Rox3); 3) subunits whereby deletion does not show many significant changes under these growth conditions (Middle submodule components Med5/Nut1, Med1, Med9 and Tail component Med16/Sin4).

The cluster diagram partially reflects previously known physical interactions within Mediator. **Figure 3.1a** depicts a model of Mediator architecture (Guglielmi et al., 2004). Some of the similarities between expression profiles can be interpreted based on this structural information. For example, the *med20*Δ and *med18*Δ profiles are nearly identical (**Figure 3.2**). This likely reflects the fact that deletion of either gene results in loss of the other subunit from the complex (Lee et al., 1999). Similarly, incorporation of Med15 and Med3 into Mediator requires the presence of Med2 (Myers et al., 1999). This explains why all *med3*Δ or *med15*Δ effects are observed within the profile of *med2*Δ. Throughout this study the

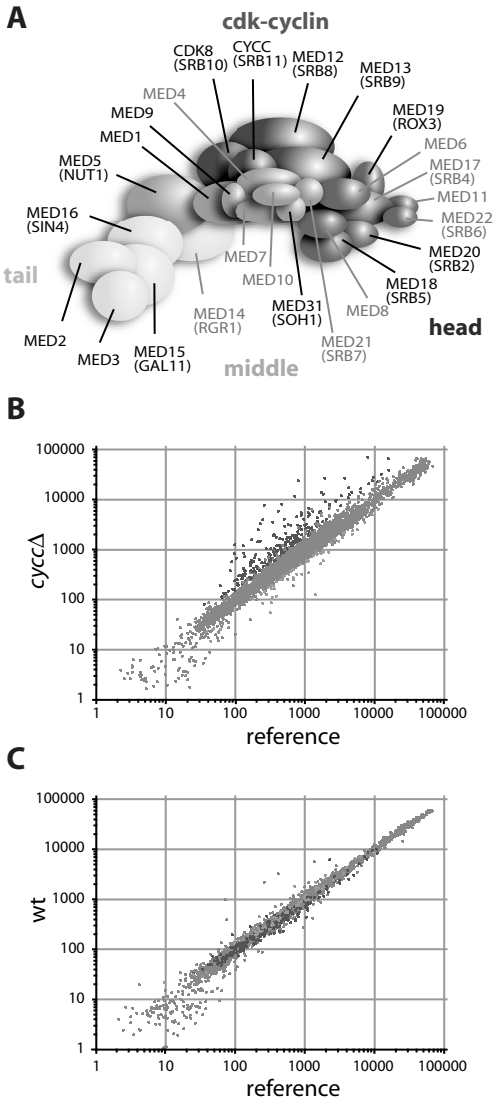

Figure 3.1: Mediator structure-function by expression-profiling.
*(**A**) Mediator consists of 25 subunits which can be divided into 4 submodules (Asturias et al., 1999): Tail (yellow), Middle (green), Head (blue) and Cdk (red). This model is based on previous structural studies e.g. (Kang et al., 2001), taking into account recent work, all summarized in (Guglielmi et al., 2004). The (non-essential) subunits studied here are shown in bold and were completely deleted with the exception of MED19 for which a partial deletion was used. (**B**) Microarray signal intensities of cyccΔ (vertical axis) compared to wt reference (horizontal axis). The intensities of all genes are shown, after background subtraction, normalization and merging of replicate culture dye-swap hybridizations. Genes determined significantly up- and down-regulated in this mutant are colored red and green, respectively. (**C**) Scatter plot of intensities from one of the wt control (vertical axis) versus wt reference (horizontal axis) experiments. Genes are colored as in the cyccΔ example.*

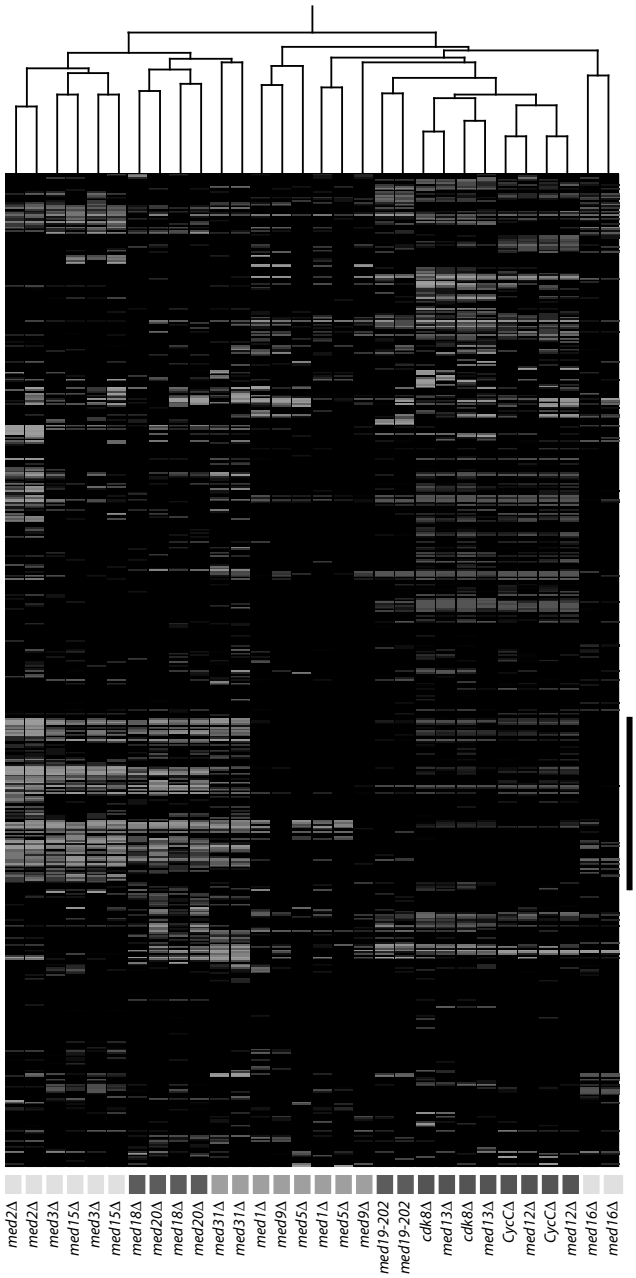

Figure 3.2: Expression-profiling reveals Mediator subunit relationships.
*Diagram of all 927 genes (clustered vertically) exhibiting significant changes specific to one or more of the deletion strains (clustered horizontally). Results are shown for the replicate microarray hybridizations individually. Change in gene expression compared to wt is depicted in red (up), green (down) or black (no change). The black bar indicates some of the genes that behave in opposite ways, depending on which subunit is deleted (**Figure 3.3**).*

results of *med2*Δ are interpreted as the combined effect of losing Med2, Med3 and Med15. Med16 has also been ascribed an anchoring role for Tail components (Li et al., 1995). Upon deletion of *MED16*, the remaining Tail components are still recruited into initiation complexes as a separate entity (Zhang et al., 2004). This explains the lack of overlap between *med16*Δ and other Tail subunit deletions (**Figure 3.2**).

Deletion of any of the four components of the negative regulatory Cdk submodule results in virtually identical expression profiles (**Figure 3.2**). It is also noteworthy that partial deletion of the Middle component *MED19* results in a profile that is similar to loss of any Cdk submodule component (**Figure 3.2**). This suggests functional and or physical interaction between the Middle and Cdk submodules through Med19.

Med31 is another evolutionary conserved Mediator subunit, only recently determined to be part of *S.cerevisiae* Mediator (Guglielmi et al., 2004; Linder and Gustafsson, 2004). The *med31*Δ profile indicates that Med31 has a largely positive role in transcription with similar transcripts affected as in deletion of Tail components *MED15*, *MED3*, *MED2* or Head components *MED20* and *MED18*. The similarities in deleting any of these six positive components is quite surprising given their proposed disparate positions within the complex (**Figure 3.1a**). This may reflect functional and or physical interactions that only take place in some of the distinct conformations described for Mediator (Davis et al., 2002; Naar et al., 2002; Taatjes et al., 2002).

Antagonistic submodules within Mediator

The expression-profiling analysis provides

several starting points for investigating individual subunits, their functional interactions and putative target genes. A major finding is the antagonistic nature of different subunits (**Figure 3.2**, bar). This is also apparent upon comparison of all pair-wise overall correlations (**Figure 3.3a**). A large degree of anti-correlation is observed between the Cdk submodule and the positively acting components from the Tail (Med15, Med2, Med3), Head (Med20, Med18) and Middle (Med31). This indicates that different subunits within the same complex can have opposite roles on the same genes.

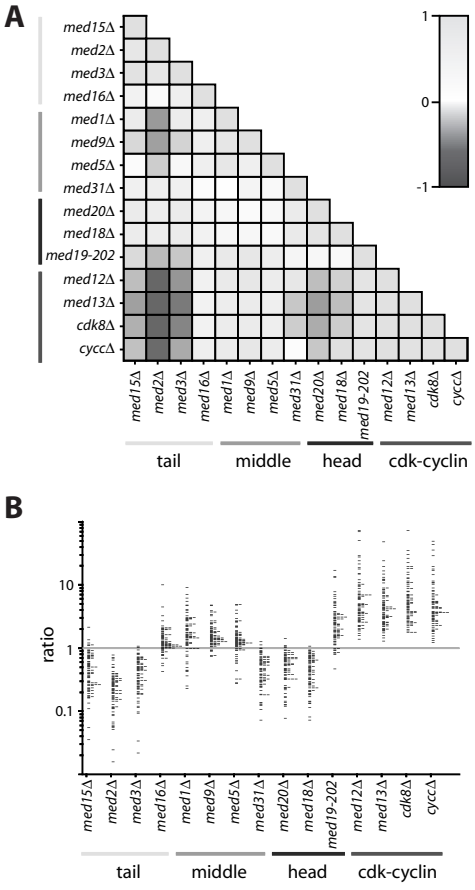

Figure 3.3: Functional antagonism between Mediator subunits. *(**A**) Pearson's correlation matrix for merged expression profiles of individual subunits, with yellow for high correlation and blue representing anti-correlation. (**B**) Oppositely behaving genes, selected for showing significant decrease in expression in med2Δ, med3Δ or med15Δ, significant increase in expression in cdk8Δ, cycCΔ, med12Δ or med13Δ and no significant changes in wt versus wt controls. The expression ratio for each gene (vertical axis) is depicted for each mutant strain (horizontal axis).*

Finding functionally antagonistic subunits supports the notion that Mediator is capable of directly processing positive and negative signaling pathways (Jiang et al., 1998). Assuming that different subunits are responsible for transmitting either positive or negative signals, it should be possible under some conditions to find genes that behave in opposite ways depending on which subunit is deleted. This is already evident in **Figure 3.2** (bar). **Figure 3.3b** shows a collection of 78 genes with differential behavior depending on which subunit is deleted. This likely represents a conservative estimate of such genes as only particular growth conditions will result in an expression level that can both become increased or decreased. The contrarily behaving genes determined under the growth conditions assayed here have a large overlap with environmental stress response genes (42 out of 78, $p=10^{-17}$) (Gasch et al., 2000) and also overlap with genes involved in carbohydrate metabolism (Gene Ontology category, 14 out of 78, $p=8.10^{-8}$).

Expression-profiling epistasis reveals that the Mediator Tail is downstream of *CDK8-CYCC*

Two alternative models can account for the functional antagonism observed between the negative Cdk submodule and the various positively acting subunits in the Tail, Head or Middle (**Figure 3.4a,b**). Taking the Tail effects exhibited by *med2*Δ as an example, both submodules can act independently of each other, in parallel pathways that converge on the contrarily behaving target genes (**Figure 3.4a**). Alternatively, the Cdk submodule may negatively influence the Tail, which acts positively on target genes, together forming a serial pathway (**Figure 3.4b**).

Such pathway relationships have previously been determined by genetic epistasis experiments, using phenotypes as read-out. Here we determine which model is correct by interpreting the expression-profile as a phenotype. If the Tail and Cdk submodules have independent contributions to transcription (**Figure 3.4a**), then a double mutant with components of both submodules deleted should exhibit a mixed expression-profile. **Figure 3.4c** shows that this is not the case. *Med2*Δ is completely dominant over *cdk8*Δ or *cycc*Δ, with

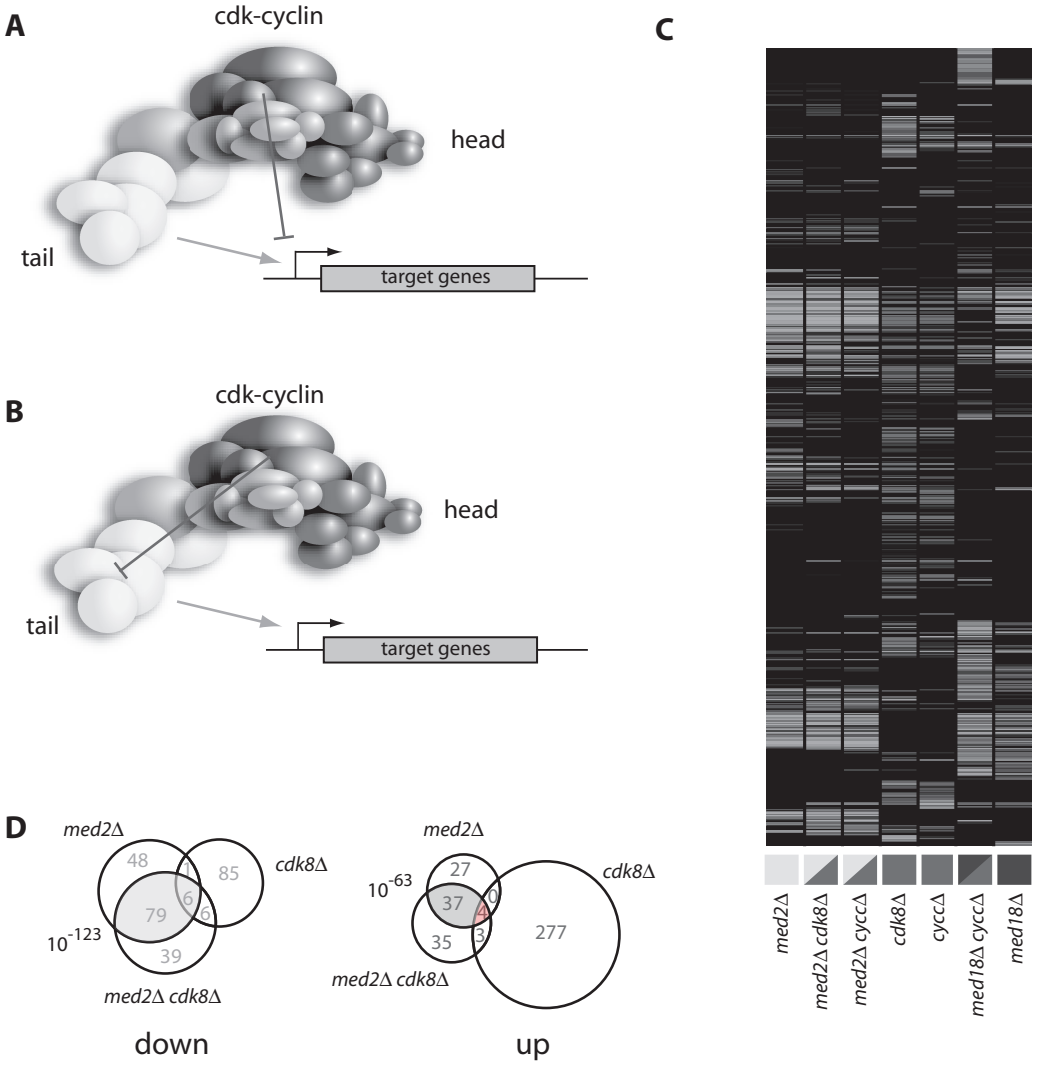

Figure 3.4: Epistasis with expression-profiles reveals that MED2 and MED18 function downstream of CDK8-CYCC.

*(**A**) A parallel pathway model of how the Cdk submodule (negative contribution) and Tail submodule (positive contribution) may independently act on genes that behave in opposite ways depending on which component is deleted. (**B**) A serial pathway model whereby the Cdk submodule exerts negative regulation indirectly, through influencing the activity of the Tail. (**C**) Expression-profiles of single and double gene deletion strains demonstrate that the model shown in B is correct, with dominance of med2Δ and med18Δ over Cdk subunit deletions when combined. Merged profiles of the independent replicate dye-swaps are shown, as is otherwise described for **Figure 3.2**. (**D**) Venn diagrams of genes with significant changes in med2Δ and cdk8Δ single deletions versus med2Δ cdk8Δ double deletion, either for genes with decreased expression (left) or increased expression (right). p-values are for the significance of the shaded overlaps. Additional Venn diagrams are shown in **S2**.*

virtually no additional effects observed when deleting the Cdk subunits in *med2*Δ (**Figure 3.4c**). Venn diagrams of genes with significantly changed expression show a very high degree of overlap between the *cdk8*Δ *med2*Δ double mutant and *med2*Δ on its own (**Figure 3.4d** see Figure S2 for a Venn diagram with *cycc*Δ mutants). Conversely, hardly any overlap is observed between the double mutant and *cdk8*Δ or *cycc*Δ alone (**Figure 3.4c,d** and S2). As is also confirmed below, these results demonstrate that epistasis analyses can be performed using expression-profiles as detailed molecular phenotypes. In this case it shows that the effects of the Tail, as exemplified by *med2*Δ,

are downstream of the negative regulatory Cdk submodule components *CDK8* and *CYCC*.

The antagonistic interplay between the Cdk submodule and the positive Head component *MED18* (**Figure 3.2,3**) was also investigated in the same way. Similarly to *med2*Δ, *med18*Δ is also dominant over *cycc*Δ in the *med18*Δ *cycc*Δ double deletion (**Figure 3.4c**, S2). This implies that the activity of the Head component Med18 is also largely downstream of the negative regulatory Cdk submodule.

CDK8-dependent phosphorylation of Med2

What is the mechanism underlying the negative regulatory pathways indicated within Mediator by the expression-profiling analyses described above? During the course of our studies it was demonstrated that Med2 can be phosphorylated by Cdk8 *in vitro* and that loss of Med2 phosphorylation is observed upon radioactive labeling of *cdk8*Δ cells (Hallberg et al., 2004). We determined whether Med2 phosphorylation by Cdk8 also takes place under the growth conditions studied here. Med2 phosphorylation can be observed in the form of a mobility shift of Med2 (**Figure 3.5a**), indicating that only a fraction of Med2 is phosphorylated. As expected (Hallberg et al., 2004), Med2 phosphorylation is dependent on the presence of *CDK8* or its cyclin partner *CYCC* (**Figure 3.5a**). Most of the phosphorylated form of Med2 disappears in *cdk8*Δ or *cycc*Δ cells (**Figure 3.5a,b**). Substitution of serine 208 with alanine confirms that Med2 phosphorylation by Cdk8 also occurs at this position (Hallberg et al., 2004) in the cultures investigated here (**Figure 3.5b**). Interestingly, phosphatase treatment or alanine substitution results in more loss of the phosphorylated form of Med2 than observed in *cdk8*Δ or *cycc*√(**Figure 3.5**). This may indicate the presence of another kinase which can phosphorylate Med2 at serine 208. Additional evidence for this proposal is presented later.

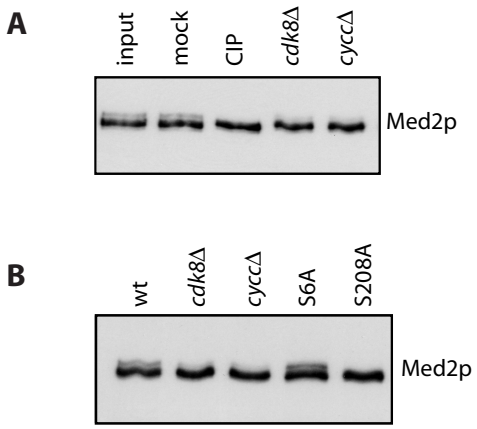

Figure 3.5: CDK8-dependent phosphorylation of Med2 on serine 208.
*(**A**) Immunoblot for Tap-tagged Med2. Lanes 1, 4 and 5 are protein extracts from wt, cdk8Δ and cyccΔ respectively. Lane 1 is also the input for the Med2 immunoprecipitation that was mock- (lane 2) or phosphatase treated (lane 3). (**B**) Immunoblot for Tap-tagged Med2 with protein extracts derived from wt and mutant strains as indicated. Med2 has two potential Cdk phosphorylation sites. Only alanine substitution of serine 208 results in loss of the upper, phosphorylated form of Med2 (lane 5 versus lane 4).*

Med2 phosphorylation by Cdk8 offers a possible mechanism for the antagonistic epistatic relationship whereby *med2*Δ is dominant over *cdk8*Δ or *cycc*Δ (**Figure 3.4c**). In this model (**Figure 3.4b**), negative regulation by Cdk8 through Med2 phosphorylation cannot be exerted in the absence of Med2, explaining why no effects of *cdk8*Δ are observed in the *cdk8*Δ *med2*Δ double deletion. Previously described functional consequences of Med2 phosphorylation by Cdk8 are not clear and do not necessarily fit with this model. In a genome-wide survey, four yeast 2-micron plasmid genes were described as significantly upregulated in a Med2 serine substitution mutant (*med2-S208A*) (Hallberg et al., 2004). These genes have not been described as upregulated upon inactivation of Cdk8, which is a requirement for considering such genes as negatively regulated through Cdk8 phosphorylation of Med2.

Med2 phosphorylation by Cdk8 represses the Rsc1/Aft1 low iron response regulon

To investigate the functional consequences of Med2 phosphorylation by Cdk8, expression-profiles were generated for *med2-S208A* cells. Deletion of any Cdk submodule component results in flocculation (data not shown). To prevent this from masking putative target genes, strains were grown under reduced flocculation conditions (Experimental Procedures). Taking dye-bias

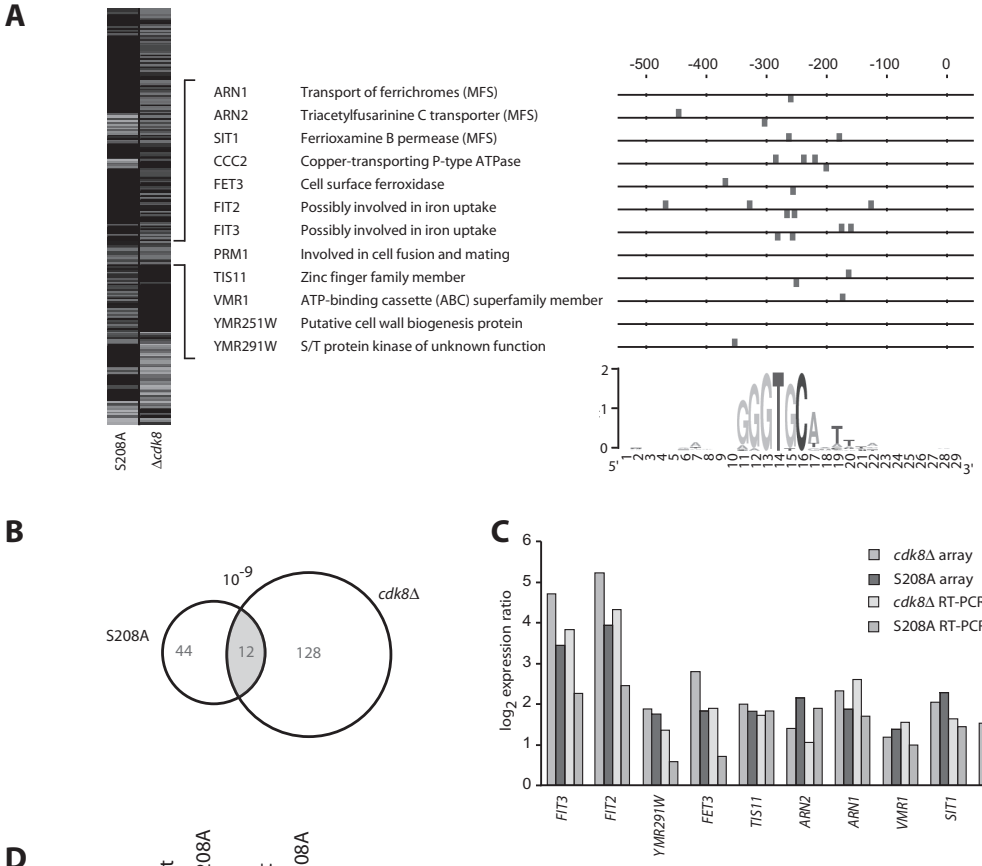

Figure 3.6: Cdk8 phosphorylation of Med2 at serine 208 represses the low iron response regulon.
*(**A**) All genes with significantly changed expression in either cdk8Δ or med2-S208A are depicted. Genes that become upregulated both in cdk8Δ and upon mutation of the Cdk8 phosphorylation site in Med2 are listed. A search for a shared motif within the 600 nucleotide region upstream of the translation start results in the depicted motif found at the positions indicated by the red boxes. (**B**) Venn diagram of genes with significantly increased expression in cdk8Δ and med2-S208A strains. (**C**) Microarray expression ratios of Rsc1 binding site genes listed in A for cdk8Δ (blue) and med2-S208A (red) as a log2 ratio versus reference wt. RT-PCR expression ratios are depicted in light blue (cdk8Δ) and light red (med2-S208A). (**D**) Spot plate growth assay with serial dilutions of wt and med2-S208A cells under normal (left) and iron-depleted conditions (+ 1mM of the iron chelator Ferrozine, right). The Ferrozine plate has an apparent decrease in spot size (top to bottom) for both wt and mutant strains compared to normal plates. This is caused by loss of contrast between yeast growth and plate background which is pink in the presence of Ferrozine.*

and same versus same wt control experiments into account, ANOVA resulted in 56 genes with significantly upregulated expression as a consequence of the *med2-S208A* point mutation (**Figure 3.6a**).

To determine whether any of these genes are upregulated as a consequence of Med2 phosphorylation by Cdk8, the *med2-S208A* expression-profile was compared to a *cdk8Δ* expression-profile generated under the same conditions. At least twelve genes are significantly upregulated as a consequence of *CDK8* deletion and upon mutation of the Cdk8 phosphorylation site in Med2 (**Figure 3.6a**). The degree of upregulation varies from 2- to 30-fold and is

well-matched between the two mutant strains (**Figure 3.6c**). Independent verification by RT-PCR yielded a similar result (**Figure 3.6c**). Given the demonstration that Cdk8 can phosphorylate Med2 on serine 208 *in vitro* (Hallberg et al., 2004) and that this phosphorylation is also observed to be *CDK8*-dependent here (**Figure 3.5**), these genes are most likely direct regulatory targets of this phosphorylation event.

The overlap between the two expression profiles is not complete. Genes affected only by Cdk8 inactivation (**Figure 3.6b**, right), probably represent the consequences of Cdk8 phosphorylation of other proteins. As is also discussed later, the presence of genes upregulated in *med2-S208A*, but not upon inactivation of Cdk8 (**Figure 3.6b**, left), suggests the existence of another kinase which can phosphorylate Med2.

A search for DNA sequence motifs upstream of the genes with shared upregulation in *cdk8*Δ and *med2-S208A*, yielded the surprising finding that 10 of the 12 genes carry one or more copies of a similar six nucleotide motif within the region 500 nucleotides upstream of their translation start site (**Figure 3.6a**). Comparison of this motif to a collection of binding motifs recently determined by genome-wide transcription factor location analysis (Harbison et al., 2004), yielded a perfect match for the transcription factor Rcs1/Aft1. Rcs1/Aft1 is required for activation of a set of genes when *S. cerevisiae* encounters low iron conditions (Yamaguchi-Iwai et al., 1996). The 10 genes with shared upregulation in *cdk8*Δ and *med2-S208A* and which carry the Rcs1/ Aft1 motif, encompass the majority of genes strongly and reproducibly induced under low iron (Shakoury-Elizeh et al., 2004).

Med2 serine 208 mutation restores growth under low iron

The high degree of overlap between the Rcs1/Aft1 regulon and genes negatively regulated through Med2 phosphorylation by Cdk8, indicates that expression-profiling analysis is not only capable of uncovering epistatic regulatory cascades (**Figure 3.2-4**), but can also effectively determine the consequences of protein phosphorylation (**Figure 3.6a,b**). Models of how Cdk8 represses the Rcs1/ low iron response through Med2 phosphorylation are discussed below. One prediction is that *med2-S208A* may be better able to cope with reduced iron concentrations. Under standard conditions *med2-S208A* grows slower that wt (**Figure 3.6d**), likely as a consequence of altered gene expression. However, when assayed under low iron conditions, slow growth of *med2-S208A* is no longer apparent in comparison to wt (**Figure 3.6d**). Under conditions whereby growth is limited by low iron, *med2-S208A* grows as efficiently as wt, probably because the low iron response is constitutively active. This supports the idea that Cdk8 phosphorylation at Med2 serine 208 is involved in repressing the low iron response and provides additional evidence that the regulatory pathways determined here are functionally relevant.

DISCUSSION

In this study we have initially applied expression-profiling to analyze structure-function relationships between Mediator subunits. This results in finding several antagonistic components co-existing within the same complex. Expression-profiling is then applied to determine epistatic relationships, demonstrating that the Tail component *MED2* and the Head component *MED18*, are downstream of the negative regulatory Cdk submodule. The functional consequences of one of these pathways is mapped further, revealing that inactivation of the Cdk8 kinase and mutation of one of its target residues, on Med2, both result in upregulation of a set of genes normally activated by a single transcription factor, Rcs1/Atf1, in response to low iron. These findings have implications for our understanding of transcription regulation as well as for studying regulatory pathways in general.

Specificity in transcription regulation

Current models consider gene-specific transcriptional activators as important determinants of high specificity through their control of small groups of functionally related genes. Genome-wide analyses have previously shown that altering the activity of global

coregulators affects expression of significantly larger sets of genes e.g. (Holstege et al., 1998). In agreement with this, combinatorial use of various cofactors has also been proposed to contribute towards specificity (Hochheimer and Tjian, 2003). Med2 phosphorylation by Cdk8 has a very specific effect. This is demonstrated by finding a single transcription factor regulon in the overlap between *cdk8*Δ and *med2-S208A* profiles (**Figure 3.6**). A high degree of specificity can therefore also be achieved through subtle modifications of the general transcription machinery. It is unlikely that such a mechanism is restricted to the low iron response in yeast. The regulation described here suggests novel mechanisms to over-rule, by-pass or work cooperatively with activators.

Mediator as a signaling pathway processor

The presence of both negative and positive subunits within Mediator is well-documented (Hampsey, 1998; Myers and Kornberg, 2000). We show here that such subunits can act antagonistically on the same genes (**Figure 3.2,3**). The effects of Med2 phosphorylation by Cdk8 supports an activator by-pass model whereby Mediator is capable of directly processing cellular signals, into a single specific transcriptional response, locally on promoters.

Cdk8 is an important contributor to the signal-processing capacity of Mediator. It is intriguing that the Cdk8 submodule is antagonistic with several different parts of Mediator (**Figure 3.2,3**). As is evident from the epistasis analysis, *CDK8* is upstream of at least both *MED2* and *MED18* (**Figure 3.4**). Our results do not exclude that Cdk8 repression is also achieved through phosphorylation of gene-specific activators (Chi et al., 2001; Fryer et al., 2004; Nelson et al., 2003). In fact, many of the genes derepressed in *cdk8*Δ but not affected in *med2-S208A*, are targets of the stress response transcription factor Msn2/4 (data not shown), which is regulated through Cdk8-dependent phosphorylation (Chi et al., 2001).

That *med2*Δ is completely dominant over *cdk8*Δ is an important observation (**Figure 3.4**). This implies that those gene-specific transcription factors regulated through Cdk8-mediated phosphorylation are also all dependent on an intact Mediator Tail. This may simply reflect the importance of Tail interactions for almost all activators tested so far in *S.cerevisiae* e.g. (Fishburn et al., 2005; Myers et al., 1999; Park et al., 2000; Zhang et al., 2004). Alternatively, the dominance of *med2*Δ over *cdk8*Δ may indicate that different mechanisms of Cdk8-mediated repression are functionally linked through use of the Tail. Our findings lead to the conclusion that the action of gene-specific transcriptional activators are repressed through at least two Cdk8-dependent mechanisms in *S. cerevisiae*; Mediator phosphorylation (this study) and activator phosphorylation (Chi et al., 2001; Nelson et al., 2003).

Downstream mechanisms

Rcs1/Aft1 is required for activation of the low iron response regulon (Yamaguchi-Iwai et al., 1996). That its target genes can become upregulated under the growth conditions assayed here indicates that to some degree Rcs1/Aft1 is already present at these promoters. This agrees with the ability to determine Rcs1/Aft1 genomic binding sites under other not severely iron-deprived conditions (Harbison et al., 2004). That only a fraction of Med2 seems phosphorylated under standard conditions (**Figure 3.5**) may indicate that Cdk8-bearing Mediator is also already present at repressed genes. In this model, Med2 phosphorylation does not influence Mediator recruitment but rather prevents activator-driven structural changes in Mediator (Taatjes et al., 2002), which are perhaps required for recruitment of RNA polymerase II (Davis et al., 2002; Naar et al., 2002). An alternative model is that Med2 phosphorylation prevents recruitment of Mediator. Our efforts to discriminate between these alternatives by chromatin immunoprecipitation have as yet been confounded by signals too low above background for confident interpretation.

Upstream of Cdk8

How is the activity of Cdk8 towards Med2 regulated? Components of the Cdk8 submodule are degraded in response to diverse environmental changes, resulting in derepression (Cooper et al., 1997; Holstege et al., 1998). This suggests

that targeted degradation of Cdk8 on only a few promoters may regulate its activity at specific genes. Ras/PKA dependent phosphorylation of the Cdk submodule component Med13, modulates Cdk8-mediated repression on some genes (Chang et al., 2004). Ras/PKA is the first example of a signal transduction pathway that targets Mediator, providing important evidence for the role of Mediator as a direct signaling pathway processor. Signaling-dependent phosphorylation followed by targeted degradation of the Cdk submodule forms a plausible hypothesis for upstream control of the antagonistic Mediator pathway described here.

It has recently been shown that mammalian Mediator complexes can also harbor a Cdk other than Cdk8 (Sato et al., 2004). Here we make two observations that support the presence of an alternative kinase in *S. cerevisiae*, which can also phosphorylate Med2 on serine 208. In the first place, loss of the phosphorylated form of Med2 is complete upon phosphatase treatment or upon mutation of serine 208, but incomplete upon *CDK8* or *CYCC* deletion (**Figure 3.5**). Secondly, the expression-profile of *med2-S208A* shows effects that are not exhibited by *cdk8*Δ (**Figure 3.6**). Together this suggests that an alternative kinase can phosphorylate Med2, perhaps at different genes and therefore with different consequences.

Expression-profiling regulatory cascades

Expression-profiling transcription factor loss-of-function is generally employed to identify putative target genes. Here we present three additional applications of expression-profiles; structure-function analysis of a large multisubunit complex (**Figure 3.2**), genetic epistasis using expression-profiles as phenotype (**Figure 3.4**) and pin-pointing the specific effects of regulatory kinase activity (**Figure 3.6**). Comparative analysis of expression-profiles derived from collections of gene deletion strains is a powerful way of determining functional relationships (Hughes et al., 2000a). The microarray analyses presented here shows that concentrating on a single multi-subunit complex for such studies can be similarly revealing for structure-function relationships.

The microarray structure-function analysis results in finding functionally antagonistic components within a single complex. Previously, epistatic relationships between pathway components have been determined by phenotype analysis of (combinations of) mutations. Here we show that expression-profiles can also be used as a detailed molecular phenotype for uncovering epistatic relationships between pathway components. This is useful if no growth or conditional phenotype is known. Because changes in gene expression of all genes are taken into account, expression-profiles may also be more revealing than a single phenotype, as the latter may reflect changes in expression of only a few genes.

The third application of expression-profiles exhibited here is similarly innovative. By comparing kinase inactivation with mutation of the target amino acid, the functional consequences of kinase activity can be precisely mapped. Overlaps in such profiles are a requirement for demonstrating that a particular kinase is responsible for regulating genes with changed expression upon mutation of the target amino acid. On their own, such overlaps cannot necessarily be interpreted as conclusive of a direct link. In this case, the previous report of *in vitro* kinase activity of Cdk8 for Med2 (Hallberg et al., 2004), is important for supporting the conclusion of a direct effect.

In summary, our results demonstrate that regulatory pathways as well as the specific effects of protein modification can be charted using expression-profiles. Here, this leads to the identification and mapping of an antagonistic signal-transduction cascade within a key transcription complex. This provides unexpected insight into how specificity in gene regulation is achieved and supports a direct signaling pathway processor model for Mediator function.

EXPERIMENTAL PROCEDURES

Yeast strains and growth

All strains are isogenic to S288c (**Table 3.1**). Deletion strains were initially from the Saccharomyces genome deletion consortium (Giaever et al., 2002), obtained through Euroscarf (Frankfurt). Aneuploidy was observed upon expression-profiling *med12*Δ, *med18*Δ, *med19*Δ.

Partially reverted flocculance was found in *med13*Δ and *cdk8*Δ. New deletions were therefore constructed for all these genes. Reports about *med19*Δ viability are contradictory and we were unable to generate viable *med19*Δ. A *MED19* truncation was therefore made using the kanamycin cassette from pFA6a-13myc-kanMX6 (Longtine et al., 1998), resulting in a carboxy-terminal truncation (amino acids 1-119), (Rosenblum-Vos et al., 1991). Tandem affinity purification (Tap) tags were introduced carboxy-terminal of Med2, at the original locus (Puig et al., 2001) and verified for the absence of growth defects (glucose, 37°C and galactose). Tap-tagged Med2 strains with *CDK8* and *CYCC* disruptions, the double deletions, as well as sequence verified alanine substitutions of *MED2*, were constructed using an *URA3*-cassette (Reid et al., 2002). All experiments were performed in SC medium with 2% glucose. For microarray analysis, two independent colonies were inoculated and overnight cultures were diluted in fresh medium to an optical density at 600 nm (OD600) of 0.05 (200 ml cultures, 250 rpm shaking incubator). Cells were harvested by centrifugation (4000 rpm, 3 min.) at OD600 of 0.3 to 0.35 and pellets were frozen in liquid nitrogen. Cultures were similarly grown for protein analysis. For the experiments shown in Figure 6, cells were incubated under higher agitation (330 rpm) with the addition of glass beads (3 mm diameter). For analysis of growth under iron deprivation, serial dilutions of cells were spotted onto SC 2% glucose plates with and without 1 mM Ferrozine.

Microarray hybridization and RT-PCR

RNA isolation, labeling and hybridization were performed as described, including the use of external control RNAs for verifying the absence of global shifts in mRNA populations (van de Peppel et al., 2003). 300 ng of each cDNA was hybridized for 16–20 h at 42 °C on microarrays with duplicate spots for each 70-mer oligo (Yeast Genome ArrayReady, Qiagen) (van de Peppel et al., 2003). All mutant strain experiments were performed with two independent cultures hybridized in dye-swap against wt reference (Figure S1). As a control for assessing highly variant transcripts, 9 wt strains were cultured in parallel with different deletion strains and hybridized against the wt reference. All strains were *MAT*a, except *med2*Δ, which was reported only viable as *MAT*α (Giaever et al., 2002). An additional experiment was therefore carried out with wt *MAT*α versus ref wt (*MAT*a) and analyzed as described below for the mutant strains. This yielded 20 mating type specific genes which were excluded from further analyses. For RT-PCR, total RNA samples from two independent cultures were treated with DNase using an RNAeasy kit (Qiagen). RT-PCR products were quantified in the presence of SYBR-green with a 7900HT (Applied Biosystems) and normalized using *TUB1* expression levels as an internal reference. The average log2 ratio from the two

Table 3.1 Strains and genotypes

name	#	genotype
Wt Mata	BY4741[1]	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*
Wt Matα	BY4742[1]	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*
Wt	YPH499[3]	*MATa; ura3-52; his3Δ-200; ade2-101; trp1Δ-63; lys2-801; leu2Δ-1*
*med15*Δ	Y01742[1]	BY4741, *yol051w::kanMX4*
*med2*Δ	Y13701[1]	BY4742, *ydl005c::kanMX4*
*med3*Δ	Y04393[1]	BY4741, *ygl025c::kanMX4*
*med16*Δ	Y01976[1]	BY4741, *ynl236w::kanMX4*
*med5*Δ	Y04518[1]	BY4741, *ygl151w::kanMX4*
*med1*Δ	Y05489[1]	BY4741, *ypr070w::kanMX4*
*med9*Δ	Y05385[1]	BY4741, *ynr010w::kanMX4*
med19-202	YNK017[2]	BY4741, *rox3-202:kanMX4*
*med20*Δ	Y06611[1]	BY4741, *yhr041c::kanMX4*
*med18*Δ	YNK018[2]	BY4741, *ygr104c::kanMX4*
*med31*Δ	Y04494[1]	BY4741, *ygl127c::kanMX4*
*med12*Δ	Y05799[1]	BY4741, *ycr081w::kanMX4*
*med13*Δ	YJP413[2]	BY4741, *ydr443c::kanMX4*
*cdk8*Δ	YJP415[2]	BY4741, *ypl042c::kanMX4*
*cycc*Δ	Y05351[1]	BY4741, *ynl025c::kanMX4*
*med2*Δ *cdk8*Δ	YJP204[2]	BY4742, *ydl005c::kanMX4; ypl042c*
*med2*Δ *cycc*Δ	YJP344[2]	BY4742, *ydl005c::kanMX4; ynl025c*
*med18*Δ *cycc*Δ	YJP407[2]	BY4741, *ydr443c::kanMX4; ynl025c*
Med2-Tap	YEB014[2]	YPH499, *YDL005c*:TAP-K.l.TRP1
Med2-Tap *cdk8*Δ	YJP139[2]	YPH499, *YDL005c*:TAP-K.l.TRP1; *ypl042c*
Med2-Tap *cycc*Δ	YJP336[2]	YPH499, *YDL005c*:TAP-K.l.TRP1; *ynl025c*
Med2:S208A	YJP244[2]	BY4742, *med2S208A*
Med2:S208A-Tap	YJP354[2]	BY4742, *med2S208A*::TAP-K.l.URA3
Med2:S6A-Tap	YJP359[2]	BY4742, *med2S6A*::TAP-K.l.URA3

[1] deletion consortium (Giaever et al., 2002)
[2] this study
[3] (Sikorski and Hieter, 1989)

independent cultures relative to wt is plotted. Primer sequences are available upon request.

Data analysis

After scanning (G2565AA Agilent scanner, 100% laser power, 30% PMT), raw data was extracted using Imagene 4.0 (Biodiscovery). After print-tip Loess normalization (Yang et al., 2002), the intensity-dependent variance of each slide was stabilized in 30 iterations using variance stabilization normalization (VSN 1.3.2) (Huber et al., 2002) in R (http://www.r-project.org/). VSN has since been changed which may make the initial Loess step superfluous. Fold-changes are shown as an average of the duplicate spots from a single microarray (Figure 2) or as an average of the four measurements derived from the two independent cultures hybridized on two microarrays in dye-swap (merged profile, all other Figures). For each mutant individually, the replicate profiles were compared to the collection of 9 wt profiles through the common reference using ANOVA (R/MAANOVA version 0.95-3). In a fixed effect analysis, sample, array, spot and dye effects were modeled. *p*-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times. Genes with $p<0.05$ and an average fold-change over the four measurements of at least 1.6 were considered significant. Hierarchical clustering was performed with GeneSpring 6.1 (Agilent) using standard (cosine) correlation as distance. Pearson's correlation were calculated in Microsoft Office Excel 2003, based on all genes with significantly changed expression in one or more mutant strains. *p*-values for overlapping gene sets were determined with a hypergeometric test. The DNA motif search was through Regulatory Sequence Analysis Tools, oligo analysis (van Helden, 2003).

Protein extracts

Protein extracts were made from 15 OD units of cells using sirconium beads (200ul, 0.5 mm; BioSpec) in lysis buffer; 50 mM Hepes pH7.5, 150 mM NaCl, 1 mM EDTA pH8, 1% Triton, 0.1% Sodiumdeoxycholate, 1% SDS, 1 mM PMSF, 50 mM NaF, 1mM NaVO3 and protease inhibitors (complete tablets, Roche), with a minibead beater (3 min., Disruptor Genie; Scientific Industries). Immunoprecipitations using IgG-sepharose (Amersham) were performed with 100 µl protein extract (2 hrs, 4°C). Phosphatase treatment was carried out on immunoprecipitated material with 40 units of calf intestinal phosphatase (Roche). Antibodies against ProtA (PAP) were from Sigma.

Microarray database accession numbers

Complete descriptions of arrays, protocols as well as data have been submitted to the public microarray database ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under the experiment accession number: E-UMCU-20.

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REFERENCES

Akoulitchev, S., Chuikov, S., and Reinberg, D. (2000). TFIIH is negatively regulated by cdk8-containing mediator complexes. Nature *407*, 102-106.

Asturias, F. J., Jiang, Y. W., Myers, L. C., Gustafsson, C. M., and Kornberg, R. D. (1999). Conserved structures of mediator and RNA polymerase II holoenzyme. Science *283*, 985-987.

Blazek, E., Mittler, G., and Meisterernst, M. (2005). The mediator of RNA polymerase II. Chromosoma *113*, 399-408.

Bourbon, H. M., Aguilera, A., Ansari, A. Z., Asturias, F. J., Berk, A. J., Bjorklund, S., Blackwell, T. K., Borggrefe, T., Carey, M., Carlson, M., et al. (2004). A unified nomenclature for protein subunits of mediator complexes linking transcriptional regulators to RNA polymerase II. Mol Cell *14*, 553-557.

Chang, Y. W., Howard, S. C., and Herman, P. K. (2004). The Ras/PKA signaling pathway directly targets the Srb9 protein, a component of the general RNA polymerase II transcription apparatus. Mol Cell *15*, 107-116.

Chi, Y., Huddleston, M. J., Zhang, X., Young, R. A., Annan, R. S., Carr, S. A., and Deshaies, R. J. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. Genes Dev *15*, 1078-1092.

Conaway, J. W., Florens, L., Sato, S., Tomomori-Sato, C., Parmely, T. J., Yao, T., Swanson, S. K., Banks, C. A., Washburn, M. P., and Conaway, R. C. (2005). The mammalian Mediator complex. FEBS Lett *579*, 904-908.

Cooper, K. F., Mallory, M. J., Smith, J. B., and Strich, R. (1997). Stress and developmental regulation of the yeast C-type cyclin Ume3p (Srb11p/Ssn8p). Embo J *16*, 4665-4675.

Davis, J. A., Takagi, Y., Kornberg, R. D., and Asturias, F. A. (2002). Structure of the yeast RNA polymerase II holoenzyme: Mediator conformation and polymerase interaction. Mol Cell *10*, 409-415.

Fishburn, J., Mohibullah, N., and Hahn, S. (2005). Function of a Eukaryotic Transcription Activator during the Transcription Cycle. Molecular Cell *18*, 369.

Fryer, C. J., White, J. B., and Jones, K. A. (2004). Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol Cell *16*, 509-520.

Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell *11*, 4241-4257.

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., *et al.* (2002). Functional profiling of the Saccharomyces cerevisiae genome. Nature *418*, 387-391.

Guglielmi, B., van Berkum, N. L., Klapholz, B., Bijma, T., Boube, M., Boschiero, C., Bourbon, H. M., Holstege, F. C., and Werner, M. (2004). A high resolution protein interaction map of the yeast Mediator complex. Nucleic Acids Res *32*, 5379-5391.

Hallberg, M., Polozkov, G. V., Hu, G. Z., Beve, J., Gustafsson, C. M., Ronne, H., and Bjorklund, S. (2004). Site-specific Srb10-dependent phosphorylation of the yeast Mediator subunit Med2 regulates gene expression from the 2-microm plasmid. Proc Natl Acad Sci U S A *101*, 3370-3375.

Hampsey, M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. Microbiol Mol Biol Rev *62*, 465-503.

Harbison, C. T., Gordon, D. B., Lee, T. I., Rinaldi, N. J., Macisaac, K. D., Danford, T. W., Hannett, N. M., Tagne, J. B., Reynolds, D. B., Yoo, J., *et al.* (2004). Transcriptional regulatory code of a eukaryotic genome. Nature *431*, 99-104.

Hengartner, C. J., Myer, V. E., Liao, S. M., Wilson, C. J., Koh, S. S., and Young, R. A. (1998). Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Mol Cell *2*, 43-53.

Hochheimer, A., and Tjian, R. (2003). Diversified transcription initiation complexes expand promoter selectivity and tissue-specific gene expression. Genes Dev *17*, 1309-1320.

Holstege, F. C., Jennings, E. G., Wyrick, J. J., Lee, T. I., Hengartner, C. J., Green, M. R., Golub, T. R., Lander, E. S., and Young, R. A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell *95*, 717-728.

Huber, W., von Heydebreck, A., Sultmann, H., Poustka, A., and Vingron, M. (2002). Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics *18 Suppl 1*, S96-104.

Hughes, T. R., Marton, M. J., Jones, A. R., Roberts, C. J., Stoughton, R., Armour, C. D., Bennett, H. A., Coffey, E., Dai, H., He, Y. D., *et al.* (2000a). Functional discovery via a compendium of expression profiles. Cell *102*, 109-126.

Hughes, T. R., Roberts, C. J., Dai, H., Jones, A. R., Meyer, M. R., Slade, D., Burchard, J., Dow, S., Ward, T. R., Kidd, M. J., *et al.* (2000b). Widespread aneuploidy revealed by DNA microarray expression profiling. Nat Genet *25*, 333-337.

Jiang, Y. W., Veschambre, P., Erdjument-Bromage, H., Tempst, P., Conaway, J. W., Conaway, R. C., and Kornberg, R. D. (1998). Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways. Proc Natl Acad Sci U S A *95*, 8538-8543.

Kang, J. S., Kim, S. H., Hwang, M. S., Han, S. J., Lee, Y. C., and Kim, Y. J. (2001). The structural and functional organization of the yeast mediator complex. J Biol Chem *276*, 42003-42010.

Lee, T. I., and Young, R. A. (2000). Transcription of eukaryotic protein-coding genes. Annu Rev Genet *34*, 77-137.

Lee, Y. C., Park, J. M., Min, S., Han, S. J., and Kim, Y. J. (1999). An activator binding module of yeast RNA polymerase II holoenzyme. Mol Cell Biol *19*, 2967-2976.

Lewis, B. A., and Reinberg, D. (2003). The mediator coactivator complex: functional and physical roles in transcriptional regulation. J Cell Sci *116*, 3667-3675.

Li, Y., Bjorklund, S., Jiang, Y. W., Kim, Y. J., Lane, W. S., Stillman, D. J., and Kornberg, R. D. (1995). Yeast global transcriptional regulators Sin4 and Rgr1 are components of mediator complex/RNA polymerase II holoenzyme. Proc Natl Acad Sci U S A *92*, 10864-10868.

Linder, T., and Gustafsson, C. M. (2004). The Soh1/MED31 protein is an ancient component of Schizosaccharomyces pombe and Saccharomyces cerevisiae Mediator. J Biol Chem *279*, 49455-49459.

Liu, Y., Kung, C., Fishburn, J., Ansari, A. Z., Shokat, K. M., and Hahn, S. (2004). Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. Mol Cell Biol *24*, 1721-1735.

Liu, Y., Ranish, J. A., Aebersold, R., and Hahn, S. (2001). Yeast nuclear extract contains two major forms of RNA polymerase II mediator complexes. J Biol Chem *276*, 7169-7175.

Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-961.

Myers, L. C., Gustafsson, C. M., Hayashibara, K. C., Brown, P. O., and Kornberg, R. D. (1999). Mediator protein mutations that selectively abolish activated transcription. Proc Natl Acad Sci U S A *96*, 67-72.

Myers, L. C., and Kornberg, R. D. (2000). Mediator of transcriptional regulation. Annu Rev Biochem *69*, 729-749.

Naar, A. M., Taatjes, D. J., Zhai, W., Nogales, E., and Tjian, R. (2002). Human CRSP interacts with RNA polymerase II CTD and adopts a specific CTD-bound conformation. Genes Dev *16*, 1339-1344.

Nelson, C., Goto, S., Lund, K., Hung, W., and Sadowski, I. (2003). Srb10/Cdk8 regulates yeast filamentous growth by phosphorylating the transcription factor Ste12. Nature *421*, 187-190.

Park, J. M., Kim, H. S., Han, S. J., Hwang, M. S., Lee,

Y. C., and Kim, Y. J. (2000). In vivo requirement of activator-specific binding targets of mediator. Mol Cell Biol *20*, 8709-8719.

Pavri, R., Lewis, B., Kim, T. K., Dilworth, F. J., Erdjument-Bromage, H., Tempst, P., de Murcia, G., Evans, R., Chambon, P., and Reinberg, D. (2005). PARP-1 determines specificity in a retinoid signaling pathway via direct modulation of mediator. Mol Cell *18*, 83-96.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods *24*, 218-229.

Reid, R. J., Sunjevaric, I., Keddache, M., Rothstein, R., and Kedacche, M. (2002). Efficient PCR-based gene disruption in Saccharomyces strains using intergenic primers. Yeast *19*, 319-328.

Roeder, R. G., Malik, S., Roeder, R. G., Conaway, J. W., Florens, L., Sato, S., Tomomori-Sato, C., Parmely, T. J., Yao, T., Swanson, S. K., *et al.* (2005). Transcriptional regulation and the role of diverse coactivators in animal cells. FEBS Lett *579*, 909-915.

Rosenblum-Vos, L. S., Rhodes, L., Evangelista, C. C., Jr., Boayke, K. A., and Zitomer, R. S. (1991). The ROX3 gene encodes an essential nuclear protein involved in CYC7 gene expression in Saccharomyces cerevisiae. Mol Cell Biol *11*, 5639-5647.

Sato, S., Tomomori-Sato, C., Parmely, T. J., Florens, L., Zybailov, B., Swanson, S. K., Banks, C. A., Jin, J., Cai, Y., Washburn, M. P., *et al.* (2004). A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. Mol Cell *14*, 685-691.

Shakoury-Elizeh, M., Tiedeman, J., Rashford, J., Ferea, T., Demeter, J., Garcia, E., Rolfes, R., Brown, P. O., Botstein, D., and Philpott, C. C. (2004). Transcriptional remodeling in response to iron deprivation in Saccharomyces cerevisiae. Mol Biol Cell *15*, 1233-1243.

Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19-27.

Sims, R. J., 3rd, Mandal, S. S., and Reinberg, D. (2004). Recent highlights of RNA-polymerase-II-mediated transcription. Curr Opin Cell Biol *16*, 263-271.

Spahr, H., Khorosjutina, O., Baraznenok, V., Linder, T., Samuelsen, C. O., Hermand, D., Makela, T. P., Holmberg, S., and Gustafsson, C. M. (2003). Mediator influences Schizosaccharomyces pombe RNA polymerase II-dependent transcription in vitro. J Biol Chem *278*, 51301-51306.

Taatjes, D. J., Naar, A. M., Andel, F., 3rd, Nogales, E., and Tjian, R. (2002). Structure, function, and activator-induced conformations of the CRSP coactivator. Science *295*, 1058-1062.

van de Peppel, J., Kemmeren, P., van Bakel, H., Radonjic, M., van Leenen, D., and Holstege, F. C. (2003). Monitoring global messenger RNA changes in externally controlled microarray experiments. EMBO Rep *4*, 387-393.

van Helden, J. (2003). Regulatory sequence analysis tools. Nucleic Acids Res *31*, 3593-3596.

Vincent, O., Kuchin, S., Hong, S. P., Townley, R., Vyas, V. K., and Carlson, M. (2001). Interaction of the Srb10 kinase with Sip4, a transcriptional activator of gluconeogenic genes in Saccharomyces cerevisiae. Mol Cell Biol *21*, 5790-5796.

Wang, G., Balamotis, M. A., Stevens, J. L., Yamaguchi, Y., Handa, H., and Berk, A. J. (2005). Mediator requirement for both recruitment and postrecruitment steps in transcription initiation. Mol Cell *17*, 683-694.

Yamaguchi-Iwai, Y., Stearman, R., Dancis, A., and Klausner, R. D. (1996). Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. Embo J *15*, 3377-3384.

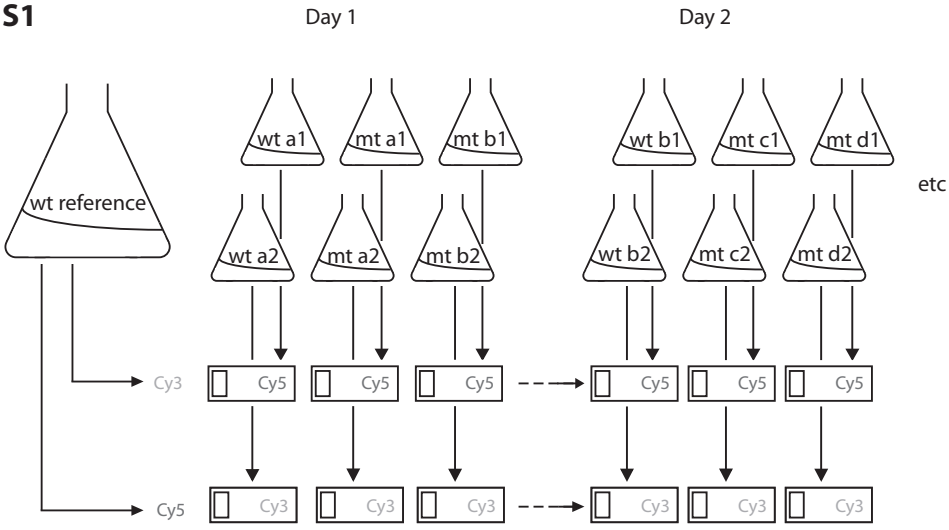
Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., and Speed, T. P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res *30*, e15.

Yudkovsky, N., Ranish, J. A., and Hahn, S. (2000). A transcription reinitiation intermediate that is stabilized by activator. Nature *408*, 225-229.

Zhang, F., Sumibcay, L., Hinnebusch, A. G., and Swanson, M. J. (2004). A triad of subunits from the Gal11/tail domain of Srb mediator is an in vivo target of transcriptional activator Gcn4p. Mol Cell Biol *24*, 6871-6886.

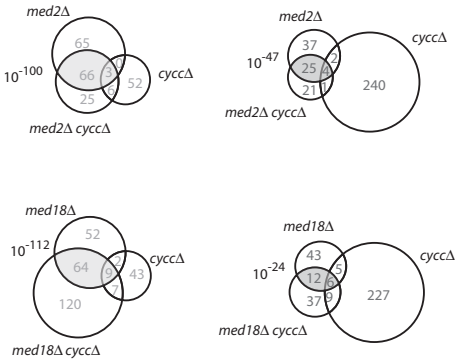
Supplemental figures

S1



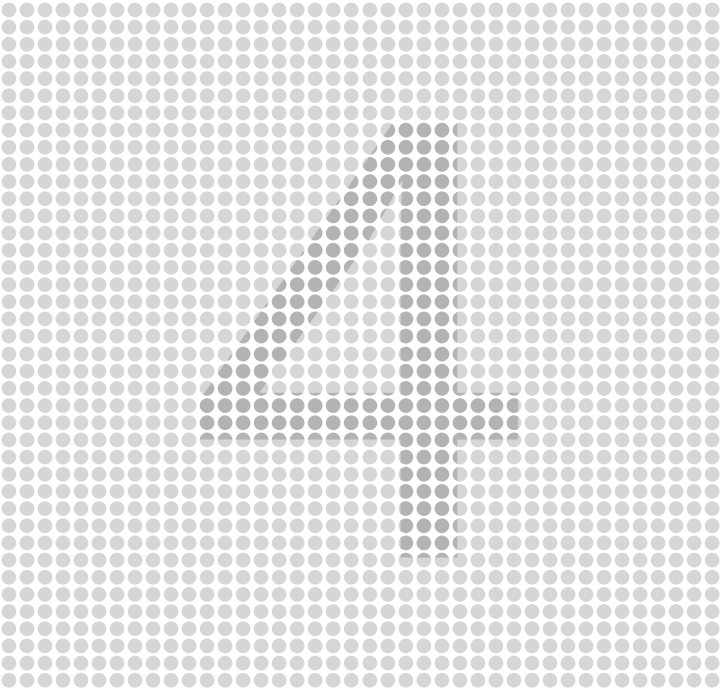
Expression-profiling experiment design

S2



down up

CYCC is upstream of *MED2* and *MED18*



Cdk8-dependent phosphorylation of Med15

Cdk8-dependent phosphorylation of Med15

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SUMMARY

Mediator is a multi-protein complex that functions as a co-regulator of transcription by RNA polymerase II. One co-regulatory role of the 25-subunit complex is to form a molecular bridge between gene-specific transcription factors and RNA pol II. The Kinase module and its catalytic component Cdk8, have been shown previously to have an important function in negative regulation of transcription by Mediator. Besides the phosphorylation of several gene-specific transcription factors, Cdk8 was recently found to target a subunit within the Tail module of core-Mediator. Here we investigated whether Cdk8 phosphorylates additional proteins within the Tail module. We show that Med15 is phosphorylated in vivo and that this is dependent on the presence of Cdk8 or its cyclin partner CycC. In vitro kinase assays also show Cdk8-dependent phosphorylation of Med15. To determine the functional consequences of Med15 phosphorylation by Cdk8, two approaches are underway to map the exact phosphorylated residues. The first is identification of phosphorylation sites by targeted mutagenesis and the second is by a quantitative mass-spectrometry approach.

INTRODUCTION

Regulation of transcription plays a key role in the control of cell growth, differentiation and the response to environmental changes. In eukaryotes, transcription of protein coding genes is regulated by complex mechanisms that require gene-specific regulators, co-regulators, general transcription factors (TFIIB, -D, -E, -F, and -H) and RNA polymerase II (Lee and Young, 2000). In addition, recruitment of chromatin regulating complexes such as SAGA and Swi/Snf facilitate proper regulation of transcription, by making promoter regions accessible for the various protein complexes (Levine and Tjian, 2003).

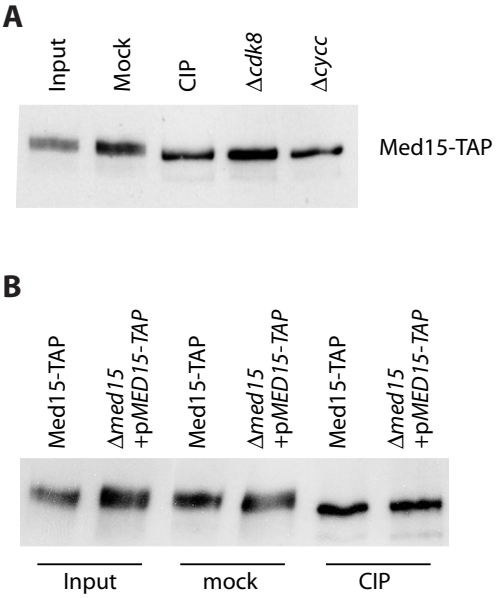
One of the recruited co-regulator complexes is Mediator. In *S.cerevisiae*, Mediator consists of 25 proteins and the complex is well conserved among eukaryotes (Boube et al., 2002; Guglielmi et al., 2004; Linder and Gustafsson, 2004). Mediator interacts with gene-specific transcription factors as well as RNA Polymerase II (RNA Pol II) and is essential for the expression of virtually all protein-coding genes in yeast (Bjorklund and Gustafsson, 2005; Holstege et al., 1998). Mediator can be structurally and functionally divided into four sub-structures, namely the Tail, Middle, Head and Kinase module (Asturias et al., 1999; Dotson et al., 2000). The Tail module is suggested to be the main interacting surface for gene-specific transcription factors (Myers et al., 1999), whereas the Middle and Head module facilitate interactions with RNA Pol II (Chadick and Asturias, 2005). The fourth and less stably associated Kinase module has an important role in transcriptional repression (Hengartner et al., 1998; Holstege et al., 1998) and can phosphorylate several gene-specific transcription factors as well as components of the general transcription machinery (Loyer et al., 2005).

A wide variety of transcription factors have been identified previously to interact with Mediator (Blazek et al., 2005). Biochemical experiments show that these interactions occur with different subunits within the Tail module (Park et al., 2000). This is supported by structural studies in which mammalian Mediator is incubated with different

gene-specific transcription factors (TF) and monitored by single particle electron microscopy (Taatjes et al., 2002; Taatjes et al., 2004). These studies show that the TF-Mediator interactions induce structural changes of Mediator. Together with other studies this suggested that Mediator is recruited by gene-specific transcription factors and forms a molecular bridge with RNA Pol II to regulate transcription (Bryant and Ptashne, 2003; Leroy et al., 2006).

However, recruitment of Mediator does not always correlate with activation of transcription. Genome-wide location analysis of Mediator shows that this complex is present upstream of both active and inactive genes (Andrau et al., 2006; Zhu et al., 2006). Furthermore, the existence of negative regulating subunits and subunits with enzymatic effects does not explain that Mediator functions only as a molecular bridge between gene-specific transcription factors and RNA Pol II. This leads to the proposal that signal transduction pathways may target Mediator and regulate its activity.

Previously, microarray structure-function analyses of cells lacking individual Mediator subunits identified different modules of Mediator that antagonistically regulate the same set of target genes. The Kinase module represses a large fraction of genes that are dependent on the Tail module. In addition, epistasis experiments revealed that subunits from the Tail are epistatic to subunits from the Kinase module and resulted in the identification of Med2 phosphorylation by Cdk8 (Hallberg et al., 2004; van de Peppel et al., 2005). These findings illustrated that a single phosphorylated residue of Med2 can specifically affect the expression of genes activated by a single gene-specific transcription factor. The proposed model derived from these observations is that the Kinase module can regulate the transcriptional activity of Mediator via phosphorylation of a subunit from the Tail. However, it still remains open if Cdk8 can target additional Mediator subunits and can result in a similar regulation. To further investigate the regulatory role of Cdk8 phosphorylation, we examined whether Cdk8 can phosphorylate additional subunits within the Tail module.



RESULTS and DISCUSSION

Med15 is phosphorylated in the presence of Cdk8/CycC

We explored the phosphorylation events initiated by Cdk8, focusing on whether Cdk8 can phosphorylate additional Tail subunits (Med3 and Med15). Investigation of the phosphorylation status of Med15 showed that in wildtype cells, Med15 migrates as a diffuse band on a protein gel, whereas upon removal of Cdk8, CycC or treatment with phosphatase, it migrates as a faster, non-diffuse protein band (**Figure 4.1a**). This shows that all Med15 present in the cell is phosphorylated, and that this phosphorylation depends on the presence of Cdk8/CycC. So far, we did not observe any migration difference of Med3 upon removal of Cdk8 (data not shown). Different methods such as mass-spectrometry have to be applied for more thorough investigation of post-translational

Figure 4.1: Cdk8-dependent phosphorylation of Med15 in vivo
*(**A**) Immunoblot of Med15 from total lysates of wildtype (lane 1) and cells lacking Cdk8 (lane 4) or CycC (lane 5). Immunoprecipitated Med15 was treated without (lane2) and with calf intestinal phosphatase (CIP) (lane3). (**B**) Phosphatase treatment of endogenous expressed Med15 (lane 1,3,5) or expressed from a plasmid (lane 2,4,6). Samples were loaded on a 10% SDS-PAGE gel*

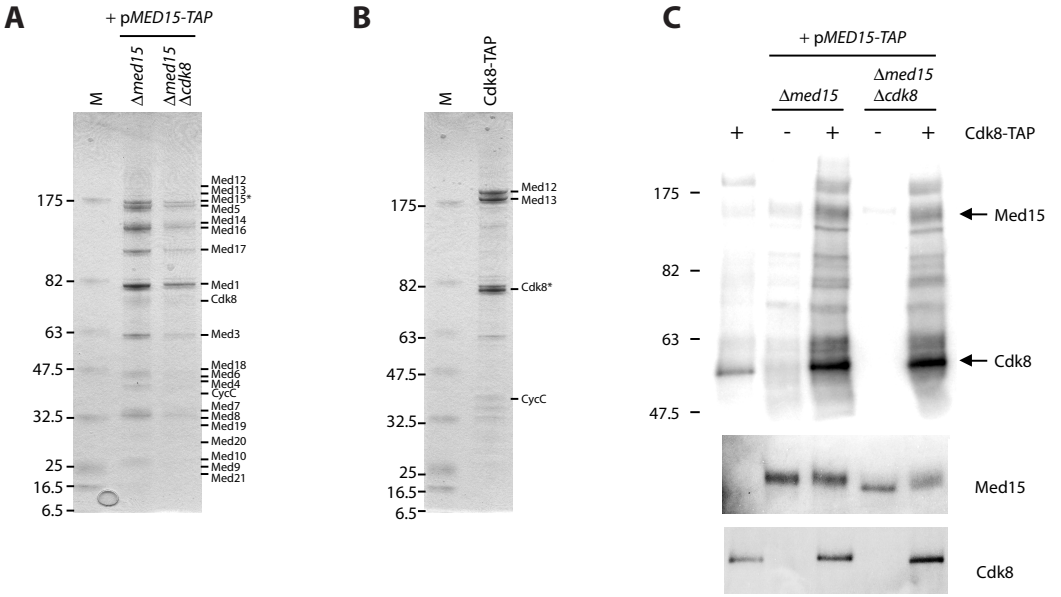


Figure 4.2: Cdk8 phosphorylates Med15 in vitro.
*(**A**) Med15 TAP-tag purified material from wildtype and cells lacking Cdk8 loaded on a 4-12% protein gel and Coomassie stained. The Mediator proteins purified from wildtype cells and identified by Mass-spectrometry are highlighted on the right. M: molecular weight marker. (**B**) Cdk8 TAP- purification. Bands differential with those in (**A**) were analysed in mass-spectrometry. (**C**) in vitro kinase assays with TAP-purified substrate from (**A**) and kinase from (**B**). Autoradiogram illustrates incorporation of ^{32}P in TAP-purified substrates (top). Immunoblot with anti-CBP illustrates that similar amounts of Med15 and Cdk8 were used in each kinase reaction (middle/bottom). Upon incorporation of ^{32}P in cdk8 cells, Med15 migrates as a diffuse band (compare lane 4 and 5 in middle panel). Individual reactions were resolved by SDS-PAGE (10%). Western blots (middle and bottom) were performed on the same nitrocellulose membrane as the autoradiogram (top).*

modifications of Med3.

Med15 was previously identified to affect transcription both positively and negatively. It was found to be required for proper activation of galactose metabolising genes and therefore necessary for efficient galactose utilization (Suzuki et al., 1988). In addition, Med15 was identified as a general activator of basal transcription, able to bind gene-specific transcription factors such as VP16, Gal4 and Gcn4 (Park et al., 2000). Besides its positive effects on transcription, *MED15* mutations have been identified in genetic screens for negative regulators (Fassler and Winston, 1989; Yu and Fassler, 1993). Med15 is well conserved among other yeast species whereas clear conservation in higher eukaryotes is lacking. Some reports have previously suggested mammalian homologues for Med15 (Boube et al., 2002; Novatchkova and Eisenhaber, 2004). Boube *et al.* suggested that Med23 (hSur2) is the mammalian homologue of Med15. This was based on two short homologous regions that are relatively well conserved among eukaryotes. Med23 was found to interact with the adenovirus E1A viral transcriptional activator and forms a sub-complex with Med16 and Med24 (Boyer et al., 1999). In addition, Med23 is required for binding and transactivation through C/EBPbeta (Mo et al., 2004). Recently, Novatchkova *et al* showed that the amino terminus of Med15 is homologous to the KIX-domain of CBP/p300 co-activators and suggested that Arc105 is the mammalian homologue of Med15 (**Figure 4.3**). In addition to the KIX-domain, Arc105 has similar highly glutamine rich regions as Med15. Arc105 is essential for transcriptional activation by TGFβ Activin/Nodal/Smad2/3 signalling (Kato et al., 2002).. Together, this suggests that both proposed mammalian homologues of Med15 have important functions in transcriptional regulation and interact with various activators. Structural studies should elucidate the structural position of both proteins within mammalian Mediator.

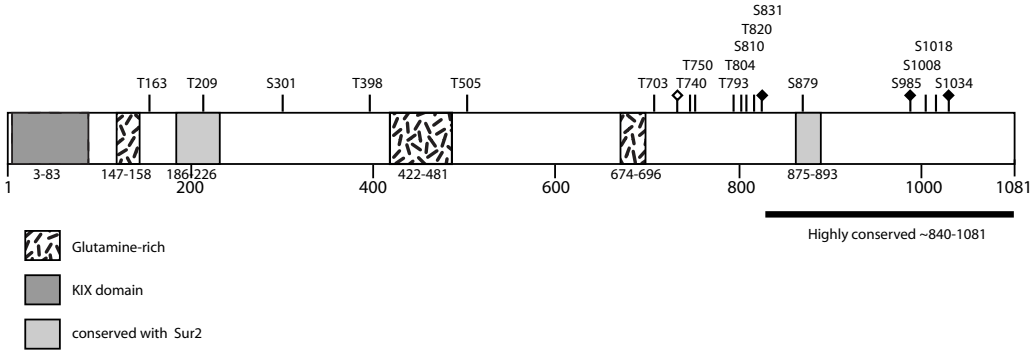


Figure 4.3: Sequence and domain structure of yeast Med15

The sequence and domain structure Med15 in S. cerevisiae. Higher eukaryotes lack orthologues of the Tail subunits Med2 and Med3 whereas the conservation of Med15 has been previously under debate. Boube et al. investigated the conservation of Mediator subunits by using genomic sequences of various eukaryotic species (Boube et al., 2002). By this approach they were able to identify several new orthologues of Mediator proteins and suggested that Sur2 is the mammalian counterpart of Med15 based on 2 small conserved domains. Recently, Novatchkova et al suggested that Med15 is a member of the GACKIX-domain superfamily (Gal11/Arc105/CBP-KIX domain) containing an amino-terminal KIX domain. Sequence alignment indicated that Arc105 is the mammalian orthologue. Both proteins bind transcriptional activators and contain highly glutamine-rich regions (Novatchkova and Eisenhaber, 2004). The KIX domain of CBP/p300 acts as a docking site for various transcriptional activators (Radhakrishnan et al., 1997; Wei et al., 2003). In addition, the carboxy terminal part of the sequence (black bar) is highly conserved among other Saccharomyces species (S. cerevisiae, S. paradoxus, S. bayanus, S. castellii). All potential phosphorylation sites (S/T-P) and their positions are highlighted. Initial mass-spectrometry analysis identified 4 residues that were phosphorylated dependent on the presence of Cdk8. Three phosphorylated residues were identified on S/T-P (black diamonds) whereas one was identified on S-S-T (amino acids 728-730) (white diamond). Quantitative mass-spectrometry is currently underway and may elucidate the Cdk8-dependent phosphorylated residues on Med15.

Med15 is phosphorylated by Cdk8/CycC in vitro.

To investigate whether Cdk8 can phosphorylate Med15 *in vitro*, we first purified Med15-TAP from wildtype cells and cells lacking Cdk8 (**Figure 4.2a**). Clear Coomassie-stained bands of the TAP-purified material were analysed by mass-spectrometry and identified all Mediator subunits with the exception of Med2, Med11, Med22 and Med31. Med11, Med22 and Med31 are among the smallest subunits of Mediator and likely migrated off the protein gel. In addition, a clear protein band at the theoretical mass size of Med2 was absent, and thus has not been analysed. The TAP-purifications resulted in the extraction of the majority of Mediator subunits, including Med15 and were used as substrates for the *in vitro* kinase assay.

To be used as the kinase for the *in vitro* assay, Cdk8 was similarly purified from yeast cells using a TAP-tag purification. The purification of Cdk8-TAP resulted in the extraction of all proteins from the Kinase module (**Figure 4.2b**). The Coomassie stained protein gel furthermore illustrates that proteins from core-Mediator are sub-stochiometrically present in the Cdk8-TAP purified material (**compare 4.2a and 4.2b**) and is in agreement with previous purifications (Borggrefe et al., 2002; Samuelsen et al., 2003).

Next, we performed an *in vitro* kinase assay using the TAP-purified Med15 from both wildtype and *cdk8* cells. **Figure 4.2c** (top panel) shows that incubation of Med15 with Cdk8 resulted in the incorporation of radiolabelled phosphate. The phosphorylation of Med15 by Cdk8 was also observed on a western blot and resulted in a similar fuzzy migration pattern as wildtype (**Figure 4.2c**, middle). This illustrates that Med15 can be phosphorylated by Cdk8 *in vitro*. In addition to Med15, various other proteins were phosphorylated *in vitro* and suggest that Cdk8 can target additional proteins present in the TAP-purified material. The phosphorylation of Med15 purified from wildtype cells illustrates that unphosphorylated residues of Med15 are targeted by Cdk8, indicating a heterogeneous population of phosphorylated Med15 which is further supported by the diffuse pattern on the western blot (**Figure 4.1a, b and 4.2c**).

Together, this leads to the proposal that Med15 is heterogeneously phosphorylated dependent

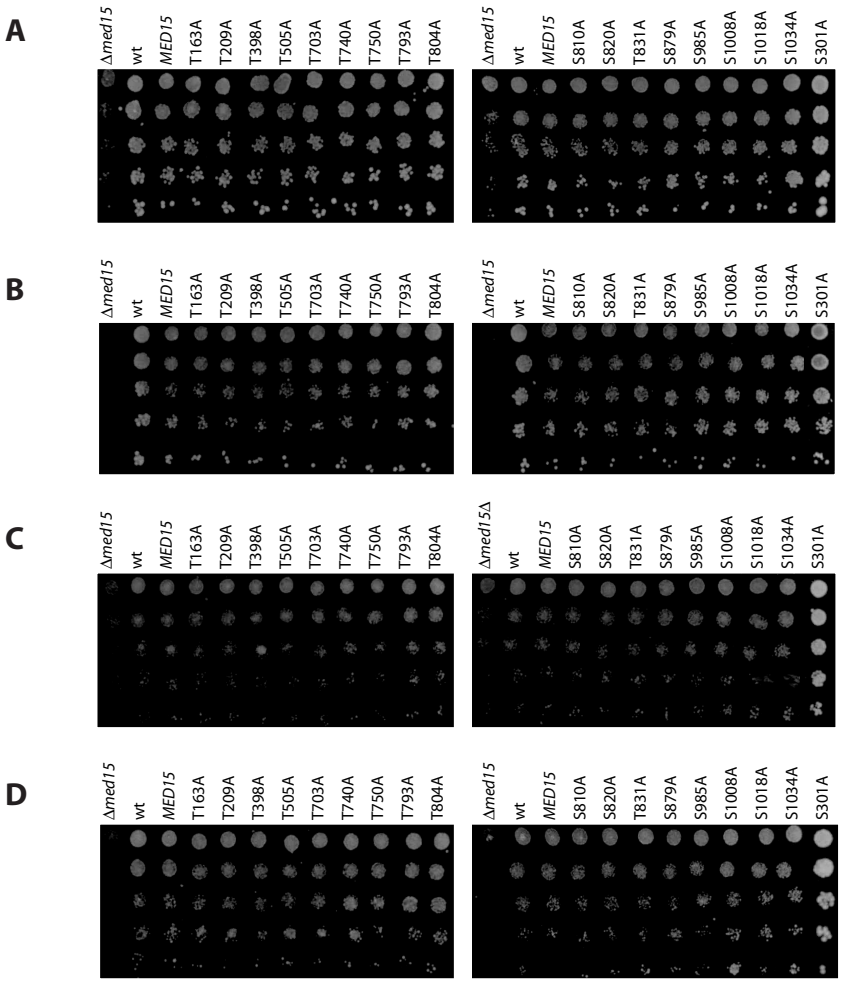


Figure 4.4: Phenotype analysis of alanine substituted residues of Med15.
*All strains were grown overnight in SC-Trp and spotted in five-fold serial dilutions on SC-Trp plates containing (**A**) 2% glucose 30°C, (**B**) 2% glucose 37°C, (**C**) 2% galactose 30°C or (**D**) raffinose 30°C. Plates were grown 3-5 days until appropriate colonies appeared.*

on the presence of Cdk8 and suggests that Med15 is phosphorylated on multiple residues.

Mutational analyses of potential phosphorylation sites.

In *S. cerevisiae*, Med15 contains 18 potential CDK phosphorylation sites (S/T-P) (**Figure 4.3**). In order to screen for potential phosphorylation sites, each site was individually mutated and examined for growth defects or altered migration patterns on a protein gel. A low copy number plasmid was constructed containing the protein coding sequence including 600bp promoter sequence and a carboxy terminal Tandem Affinity Purification (TAP) tag, and subsequently transformed in a MED15 deletion strain. First, we verified the expression level and phosphorylation state of the plasmid born Med15 and found that this was comparable to endogenous expressed Med15 (**Figure 4.1b**).

We next mutated each individual phosphorylation site (S/T-P) in Med15 to a non-charged alanine residue (**Figure 4.3**) and compared its migration with wildtype Med15. No difference in migration was observed on a protein gel with the individual point mutants (data not shown). In addition, we examined

each individual mutant for growth defects under various conditions. Mutant strains were spotted in serial dilutions on plates containing minimal medium (SC-Trp) supplemented with glucose, galactose or raffinose as a carbon source and sensitivity of mutants to elevated temperatures was monitored by incubation of cells at 37 ºC. In all four conditions, *med15* cells showed growth defects, which were rescued by the expression of Med15 from a plasmid. Each individual mutated amino acid complemented growth defects similar to wildtype Med15 (**Figure 4.4**).

Altogether, individual phosphorylation mutants of Med15 did not show an altered migration pattern on a protein gel, nor exhibited growth defects under examined conditions. The inability to detect an altered migration pattern of individual Med15 phosphorylation mutants can have two possible reasons. First, Med15 is phosphorylated on multiple residues, and a single mutation cannot be resolved on a protein gel. This is supported with preliminary mass-spectrometry results that identified 4 phosphorylated residues that were absent in cells lacking Cdk8 (**Figure 4.3**). Alternatively, phosphorylation of Med15 could occur on other residues than those mutated here. Mass-spectrometry and quantitative detection of phosphorylation sites are currently underway and may result in the identification of the exact phosphorylated residue. Finally, analysis of phosphorylation mutants by methods such as expression profiles and phenotype analysis may result in the functional consequence of the phosphorylated residues and their involvement in the regulation of Mediator. In addition, the *in vitro* kinase assay indicated that additional proteins present in the TAP-purified material can be targeted by Cdk8. Identification of post-translational modifications of Mediator by mass-spectrometry may elucidate which Mediator proteins that are targeted by Cdk8 *in vivo*.

MATERIALS AND METHODS

Strains, growth, plasmids, antibody

Yeast strains used in this study are listed in table 1. A Gateway compatible destination plasmid was constructed by inserting Med15 promoter (600 bp upstream of translational startsite), a gateway destination cassette, Tandem affinity tag and the ADH1 terminator into pRS314. Med15 promoter was amplified from wild-type yeast (BY4741) using the following oligos: Gal11-prom-f2: TTT TCG GGG TAC CCC GTC ATG CTT TGG CGC GTG CGC ATC and Gal11-prom-r2: TTT TCC CCC GGG CAT AGC AGA TTT AAA AGA AAT AGC GTT TTA ATC C. Med15 entry clone (pE-Med15) was made as previously described (Guglielmi et al., 2004). For TAP-Tag purification, cells were grown in SC-Trp and diluted to OD600=0.4 in YPD. Antibodies against ProtA (Peroxidase anti-peroxidase) were purchased from Sigma and anti-CBP (calmodulin binding protein) were purchased from Upstate.

Site directed mutagenesis.

Med15 alanine substitution mutants were made using site-directed mutagenesis PCR using pE-Med15 as a template. After amplification the products were treated with DpnI (New England BioLabs inc.) and transformed into Stbl2 (Invitrogen) competent bacteria. The resulting plasmid was verified by restriction and sequence analysis. Finally, a LR reaction was carried out with pE-Med15 and the destination plasmid according to supplier's protocol. The resulting mutated expression plasmids were transformed into YJP411 (**Table 4.1**).

Protein lysates, immunoprecipitation and phosphatase treatment

Protein extracts were made from 15 OD units of cells grown in SC-Trp using sirconium beads (200 µl, 0.5 mm; BioSpec) in lysis buffer; 50 mM Hepes pH7.5, 150 mM NaCl, 1 mM EDTA pH8, 1% Triton, 0.1% Sodium deoxycholate, 1% SDS, 1 mM PMSF, 50 mM NaF, protease inhibitors (complete tablets, Roche), with a minibead beater (3 min., Disruptor Genie; Scientific Industries). Immunoprecipitations using IgG-sepharose (Amersham) were performed with 100 µl protein extract (2 hrs, 4°C). Phosphatase treatment was carried out on immunoprecipitated material with 40 units of calf intestinal phosphatase (Roche).

Tandem affinity purification

Yeast cells were grown in 3 liter YPD (starting OD600=0.4) and harvested (OD600=2) by centrifugation (10 min, 5000rpm, 4°C). Cells were washed once with ice-cold 100 ml Buffer-E (20 mM Hepes pH8, 350 mM NaCl, 0.1% Tween and 10% glycerol, pepstatin, leupeptin, aprotinin, benzamidine, 1 mM PMSF and 5 mM NaF). Pellets were resuspended in 5-10 ml buffer-E and poured in a bead beater (Biospec) containing 0.5 mm glass beads (Biospec). Cells were disrupted in 30 cycles of 5'' bead beating and 55'' cooling down. Disrupted cells were briefly centrifuged to remove beads, transferred in ultracentrifuge tubes and centrifuged (60', 35000g, 4°C) in a Beckman SW41 rotor. Supernatant was incubated with 200 µl IgG sepharose (Amersham) for 2 hrs at 4°C

Table 4.1 Strains and genotypes

name	#	genotype
Wt	YPH499	*MATa; ura3-52; his3Δ–200; ade2-101; trp1Δ–63; lys2-801; leu2Δ-1*
med15Δ	YJP411	YPH499, *yol051w::kanMX4*
med15Δ cdk8Δ	YJP417	YPH499, *yol051w::kanMX4; ypl042c::*URA3
Med15-Tap	YEB017	YPH499, *YOL051w*:TAP-K.l.TRP1
Med15-Tap *cdk8Δ*	YJP099	YPH499, *YOL051w*:TAP-K.l.TRP1; *ypl042c::*URA3
Med15-Tap *cyccΔ*	YJP333	YPH499, *YOL051w*:TAP-K.l.TRP1; *ynl025c*
Cdk8-Tap	YEB022	YPH499, *YPL042c*:TAP-K.l.TRP1

and poured into an empty 10 ml Econo-column (Biorad). Beads were washed with 35 ml Buffer-E and 10 ml TEV cleavage buffer (10 mM Tris pH8, 150 mM NaCl, 0.5 mM EDTA pH 8, 0.1% Tween, 1 mM DTT). TEV cleavage was performed in 1 ml TEV cleavage buffer for 90' at 20°C and cleaved material was collected in a new column containing 100 µl of Calmodulin beads (Stratagene), and incubated for 1 hr 4°C with the addition of $CaCl_2$ to neutralize residual EDTA from TEV cleavage buffer. After incubation, beads were washed with 35 ml CBB-buffer (10 mM β-Mercaptoethanol, 10 mM Tris pH8, 150 mM NaCl, 1 mM Mg-Ac, 1 mM Immidazole, 2 mM $CaCl_2$, 0.1% Tween, 10% Glycerol) and eluted 4x with 100 µl CEB-buffer (10mM β–Mercaptoethanol, 10mM Tris pH8, 150mM NaCl, 1mM Mg-Ac, 1mM Immidazole, 2mM EGTA, 0.1% Tween, 10 % Glycerol). Fifty percent of the sample was precipitated (Wessel and Flugge, 1984) and loaded on a NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen) and stained with Coomassie.

Mass Spectrometry analysis

For in gel digestion, an adapted protocol of Wilm et al. was used (Wilm et al., 1996). Briefly, gel bands of interest were cut out and subsequently washed with MQ and MeCN. Pieces were reduced in DTT and then alkylated with iodoacetamide reagent. After thorough washing, pieces were incubated with trypsin and allowed to digest overnight at 37°C. Supernatant of the digest was collected and pieces were washed for 30 min in 5% formic acid at RT. Again, supernatant was collected and both were combined for subsequent nanoLC-MS analysis. Nanoflow-LC tandem mass spectrometry was performed by coupling an Agilent 1100 HPLC (Agilent Technologies) to an LTQ ion trap (Thermo Electron, Bremen, Germany). For peptide LC, trapping columns (1 cm x 100 µm) and analytical columns (20 cm x 50µm) were packed in-house with ReproSil-Pur C18-AQ, 3 µm (Dr. Maisch GmbH, Ammerbuch, Germany). Peptide mixtures were delivered at 3 µl/min on the trapping column for desalting. After flow-splitting down to ~150 nl/min, peptides were transferred to the analytical column and eluted in a gradient of acetonitrile (1%/min) in 0.1M acetic acid. The eluent was sprayed via emitter tips (New Objective), butt-connected to the analytical column.

The mass spectrometer was operated in the data dependent mode to automatically switch between MS, SIM, and MS/MS acquisition. Survey Full scan MS spectra (from *m/z* 350-1500) were acquired in centroid mode after accumulation to a target value of 3E4 in the linear ion trap. When exceeding a threshold value of 1E4 counts, the three most intense ions were isolated for charge state determination by using a "SIM scan" in profile mode at a target value of 1E4 (10 Da mass range). The selected ions were then subsequently fragmented by collisionally induced dissociation by filling the ion trap at a target value of 1E4 with a maximum filling time of 300 ms. From MS/MS data in each LC run, peak lists were created using Bioworks 3.1 software (Thermo Electron, Bremen, Germany). The UniProt/SwissProt (dated 03/17/2006) was used as a database and the taxonomy restricted to *Saccharomyces cerevisiae*.

In vitro kinase assay

Kinase assays were performed with 5µl TAP-purified substrate and 2µl TAP-purified Cdk8 in a volume of 30 µl containing 150 mM NaCl, 10 mM Tris pH8, 10 mM $MgCl_2$, 5 mM NaF, 5 µM ATP and 10 µCi (3.7 x 10^5 Bq) γ-^{32}P-ATP (Perkin Elmer), and incubated at 30°C for 30 minutes and reactions were terminated by the addition of SDS sample buffer and boiled for 3 minutes. Total reactions were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane.

REFERENCES

Andrau, J. C., van de Pasch, L., Lijnzaad, P., Bijma, T., Koerkamp, M. G., van de Peppel, J., Werner, M., and Holstege, F. C. (2006). Genome-wide location of the coactivator mediator: Binding without activation and transient Cdk8 interaction on DNA. Mol Cell *22*, 179-192.

Asturias, F. J., Jiang, Y. W., Myers, L. C., Gustafsson, C. M., and Kornberg, R. D. (1999). Conserved structures of mediator and RNA polymerase II holoenzyme. Science *283*, 985-987.

Bjorklund, S., and Gustafsson, C. M. (2005). The yeast Mediator complex and its regulation. Trends Biochem Sci *30*, 240-244.

Blazek, E., Mittler, G., and Meisterernst, M. (2005). The mediator of RNA polymerase II. Chromosoma *113*, 399-408.

Borggrefe, T., Davis, R., Erdjument-Bromage, H., Tempst, P., and Kornberg, R. D. (2002). A complex of the Srb8, -9, -10, and -11 transcriptional regulatory proteins from yeast. J Biol Chem *277*, 44202-44207.

Boube, M., Joulia, L., Cribbs, D. L., and Bourbon, H. M. (2002). Evidence for a mediator of RNA polymerase II transcriptional regulation conserved from yeast to man. Cell *110*, 143-151.

Boyer, T. G., Martin, M. E., Lees, E., Ricciardi, R. P., and Berk, A. J. (1999). Mammalian Srb/Mediator complex is targeted by adenovirus E1A protein. Nature *399*, 276-279.

Bryant, G. O., and Ptashne, M. (2003). Independent recruitment in vivo by Gal4 of two complexes required for transcription. Mol Cell *11*, 1301-1309.

Chadick, J. Z., and Asturias, F. J. (2005). Structure of eukaryotic Mediator complexes. Trends Biochem Sci *30*, 264-271.

Dotson, M. R., Yuan, C. X., Roeder, R. G., Myers, L. C., Gustafsson, C. M., Jiang, Y. W., Li, Y., Kornberg, R. D., and Asturias, F. J. (2000). Structural organization of yeast and mammalian mediator complexes. Proc Natl Acad Sci U S A *97*, 14307-14310.

Fassler, J. S., and Winston, F. (1989). The Saccharomyces cerevisiae SPT13/GAL11 gene has both positive and negative regulatory roles in transcription. Mol Cell Biol *9*, 5602-5609.

Guglielmi, B., van Berkum, N. L., Klapholz, B., Bijma, T., Boube, M., Boschiero, C., Bourbon, H. M., Holstege, F. C., and Werner, M. (2004). A high resolution protein interaction map of the yeast Mediator complex. Nucleic Acids Res *32*, 5379-5391.

Hallberg, M., Polozkov, G. V., Hu, G. Z., Beve, J., Gustafsson, C. M., Ronne, H., and Bjorklund, S. (2004). Site-specific Srb10-dependent phosphorylation of the yeast Mediator subunit Med2 regulates gene expression from the 2-microm plasmid. Proc Natl Acad Sci U S A *101*, 3370-3375.

Han, S. J., Lee, J. S., Kang, J. S., and Kim, Y. J. (2001). Med9/Cse2 and Gal11 modules are required for transcriptional repression of distinct group of genes. J Biol Chem *276*, 37020-37026.

Hengartner, C. J., Myer, V. E., Liao, S. M., Wilson, C. J., Koh, S. S., and Young, R. A. (1998). Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Mol Cell *2*, 43-53.

Holstege, F. C., Jennings, E. G., Wyrick, J. J., Lee, T. I., Hengartner, C. J., Green, M. R., Golub, T. R., Lander, E. S., and Young, R. A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell *95*, 717-728.

Kato, Y., Habas, R., Katsuyama, Y., Naar, A. M., and He, X. (2002). A component of the ARC/Mediator complex required for TGF beta/Nodal signalling. Nature *418*, 641-646.

Lee, T. I., and Young, R. A. (2000). Transcription of eukaryotic protein-coding genes. Annu Rev Genet *34*, 77-137.

Leroy, C., Cormier, L., and Kuras, L. (2006). Independent recruitment of mediator and SAGA by the activator Met4. Mol Cell Biol *26*, 3149-3163.

Levine, M., and Tjian, R. (2003). Transcription regulation and animal diversity. Nature *424*, 147-151.

Linder, T., and Gustafsson, C. M. (2004). The Soh1/MED31 protein is an ancient component of Schizosaccharomyces pombe and Saccharomyces cerevisiae mediator. J Biol Chem.

Loyer, P., Trembley, J. H., Katona, R., Kidd, V. J., and Lahti, J. M. (2005). Role of CDK/cyclin complexes in transcription and RNA splicing. Cell Signal *17*, 1033-1051.

Mo, X., Kowenz-Leutz, E., Xu, H., and Leutz, A. (2004). Ras induces mediator complex exchange on C/EBP beta. Mol Cell *13*, 241-250.

Myers, L. C., Gustafsson, C. M., Hayashibara, K. C., Brown, P. O., and Kornberg, R. D. (1999). Mediator protein mutations that selectively abolish activated transcription. Proc Natl Acad Sci U S A *96*, 67-72.

Novatchkova, M., and Eisenhaber, F. (2004). Linking transcriptional mediators via the GACKIX domain super family. Curr Biol *14*, R54-55.

Park, J. M., Kim, H. S., Han, S. J., Hwang, M. S., Lee, Y. C., and Kim, Y. J. (2000). In vivo requirement of activator-specific binding targets of mediator. Mol Cell Biol *20*, 8709-8719.

Radhakrishnan, I., Perez-Alvarado, G. C., Parker, D., Dyson, H. J., Montminy, M. R., and Wright, P. E. (1997). Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator: coactivator interactions. Cell *91*, 741-752.

Samuelsen, C. O., Baraznenok, V., Khorosjutina, O., Spahr, H., Kieselbach, T., Holmberg, S., and Gustafsson, C. M. (2003). TRAP230/ARC240 and TRAP240/ARC250 Mediator subunits are functionally conserved through evolution. Proc Natl Acad Sci U S A *100*, 6422-6427.

Suzuki, Y., Nogi, Y., Abe, A., and Fukasawa, T. (1988). GAL11 protein, an auxiliary transcription activator for genes encoding galactose-metabolizing enzymes in Saccharomyces cerevisiae. Mol Cell Biol *8*, 4991-4999.

Taatjes, D. J., Naar, A. M., Andel, F., 3rd, Nogales, E.,

and Tjian, R. (2002). Structure, function, and activator-induced conformations of the CRSP coactivator. Science *295*, 1058-1062.

Taatjes, D. J., Schneider-Poetsch, T., and Tjian, R. (2004). Distinct conformational states of nuclear receptor-bound CRSP-Med complexes. Nat Struct Mol Biol *11*, 664-671.

van de Peppel, J., Kettelarij, N., van Bakel, H., Kockelkorn, T. T., van Leenen, D., and Holstege, F. C. (2005). Mediator expression profiling epistasis reveals a signal transduction pathway with antagonistic submodules and highly specific downstream targets. Mol Cell *19*, 511-522.

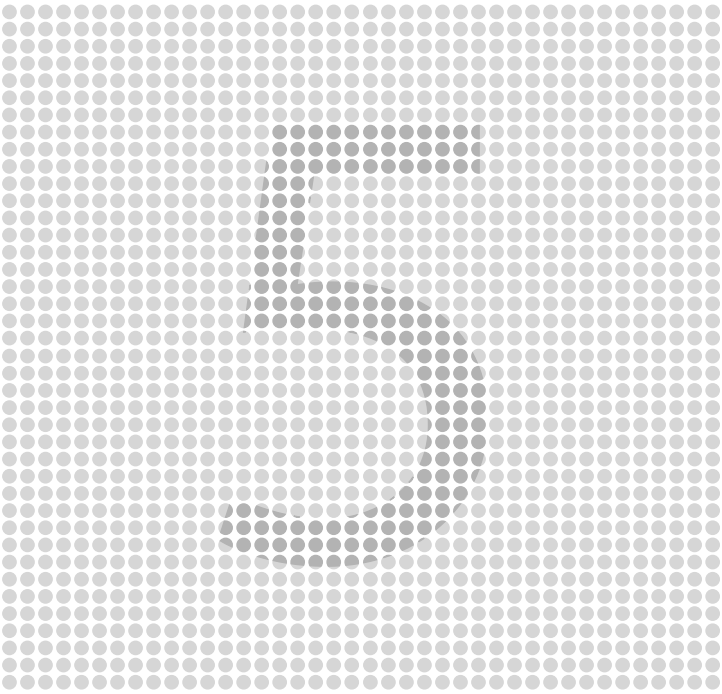
Wei, Y., Horng, J. C., Vendel, A. C., Raleigh, D. P., and Lumb, K. J. (2003). Contribution to stability and folding of a buried polar residue at the CARM1 methylation site of the KIX domain of CBP. Biochemistry *42*, 7044-7049.

Wessel, D., and Flugge, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem *138*, 141-143.

Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996). Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature *379*, 466-469.

Yu, G., and Fassler, J. S. (1993). SPT13 (GAL11) of Saccharomyces cerevisiae negatively regulates activity of the MCM1 transcription factor in Ty1 elements. Mol Cell Biol *13*, 63-71.

Zhu, X., Wiren, M., Sinha, I., Rasmussen, N. N., Linder, T., Holmberg, S., Ekwall, K., and Gustafsson, C. M. (2006). Genome-wide occupancy profile of mediator and the Srb8-11 module reveals interactions with coding regions. Mol Cell *22*, 169-178.



Discussion

In the last decade, the development of genome-wide approaches, i.e. studying all genes and/or proteins in parallel, has exponentially increased the rate at which data is being generated within the life sciences. *S. cerevisiae* was the first eukaryotic genome sequenced (Goffeau et al., 1996) and is an intensively studied model organism for biological processes such as transcription regulation, signal transduction, cell cycle and metabolism. It shares the complex internal cell structure of higher eukaryotes, it has a relative small genome size, and most aspects of transcription regulation are well conserved. Furthermore, easy manipulation and cultivation makes yeast an ideal eukaryotic model organism for applying and developing genome-wide approaches (Botstein and Fink, 1988; Dujon, 1996; Korona, 2004; Kumar and Snyder, 2001).

This thesis describes a genome-wide analysis of the yeast Mediator complex using microarrays. Several lines of evidence suggest that Mediator can function as a target of signal transduction pathways and is itself regulated through post-translational modifications. This discussion describes the information we can gain from systems approaches, in particular expression-profiling, and the use of quantitative data in genome-wide approaches to more accurately and better predict functional processes within living organisms. Furthermore, we discuss the function of Mediator and the regulatory events initiated by its Kinase module.

SYSTEMS BIOLOGY AND THE GENERATION OF QUANTITATIVE DATA

The availability of whole genome sequences of a large variety of organisms and the identification of the stored genetic information is increasing our understanding of how cells use and regulate their genetic information. Integration of genome-wide data such as genomic sequences, mRNA expression data, DNA-protein interactions, protein-protein interactions, sub-cellular localisation and protein complex compositions, enables prediction of regulatory processes and functions of uncharacterised genes or proteins (Tanay et al., 2004). Furthermore, bioinformatics approaches such as whole genome comparisons can now identify more accurately gene coding regions and regulatory elements present on DNA (Frazer et al., 2003). The recent development of RNA interference in higher eukaryotes will make it possible to perform some of such genome-wide studies in higher eukaryotes (Vanhecke and Janitz, 2005). Comparison of data obtained from yeast with experiments performed in higher eukaryotes can now offer more insight in the regulatory processes and development of diseases in higher eukaryotes (Rubin et al., 2000).

Systems biology focuses on obtaining a quantitative description of complete biological systems and modelling this (Ideker et al., 2001). Quantitative and absolute measurements in genome-wide approaches will allow better functional predictions and enhance future drug-development (Butcher et al., 2004). In chapter 2, we describe the use of external RNA controls for the normalisation of two-colour microarray experiments. Current normalisation methods assume that the majority of transcripts are invariant and result in reported expression ratios relative to the average expression ratio of all genes (Yang et al., 2002). However, inactivation of components generally required for transcription can result in large unbalanced changes and general transcription defects (Holstege et al., 1998). Therefore, proper normalisation requires a set of transcripts that are invariant between samples. The use of synthetic transcripts spiked into the samples can overcome this and is the preferred method to use. For proper performance, these controls have to meet certain criteria. First, they should mimic the range of expression of all transcripts. Second, the sequence should be unique to overcome cross hybridisation with endogenous transcripts. Third, reporter oligos/spots should be distributed over the whole surface of the array. Currently, large efforts are being made by an international consortium to design a large set (more than 100) of artificial and prokaryotic sequences that can be used in custom as well commercially available microarray platforms (ERCC, 2005). This will ultimately result in more accurate measurements, better comparisons of experiments performed by different labs and more accurate descriptions for

systems biology.

External control normalisation allows the determination of global mRNA changes between different conditions relative to a reference sample. However, the absolute amount of individual transcripts is the result of a balance between mRNA synthesis and decay. Therefore, the challenge in future studies will be to include genome-wide methods such as *in vivo* labelling of mRNA, which can measure actual transcription rates and decay and will result in better understanding of the complex transcriptional processes taking place within the cell at a given time (Cleary et al., 2005; Garcia-Martinez et al., 2004).

Besides the proof of principle, we illustrated that global mRNA and unbalanced changes occur in diverse experimental conditions commonly used and exemplify that such changes do not only occur upon disruption of components of the general transcription machinery. However, accurate quantification of extracted total RNA is crucial for proper use of these external RNA controls and therefore not always applicable. Microarray applications using small amounts of RNA extracted from biopsies or retrieved from LCM (Laser Capture Micro dissection) are not always suitable for external control normalisation. In addition, target gene identification or the selection of the most responsive transcripts does not necessarily need external control normalisation.

Besides functioning as a good tool for normalisation of two colour microarray experiments, we have found additionally that these controls can function well for quality assurance purposes of microarray experiments (van Bakel and Holstege, 2004). Artefacts evolved from experimental procedures such as RNA degradation or inappropriate cDNA synthesis can easily be detected and results in more accurate and higher data quality.

EXPRESSION PROFILING APPLICATIONS

Since its development 10 years ago (Schena et al., 1995), microarrays have been widely used to measure relative transcript levels in biological processes such as cell cycle regulation (Spellman et al., 1998), response to environmental stress or small compounds (Blower et al., 2002; Butcher and Schreiber, 2005; Gasch, 2002), to study the effects of gene disruption (Hughes et al., 2000) and classify tumors (Golub et al., 1999; van 't Veer et al., 2002). The expression-profiling experiments described in this thesis reveal several new applications and illustrate the power of systems approaches.

Structure/Function analysis

Previously, structural and biochemical experiments illustrated that Mediator is composed of four sub-modules (Dotson et al., 2000; Kang et al., 2001). Interestingly, hierarchical clustering of the expression profiles from individual gene deletions resulted in a similar modular organisation (chapter 3). Expression profiles of cells lacking any subunit from the Tail were virtually identical. Similarly, the expression-profiles of cells lacking any subunit from the Kinase module clustered together, as did deletions of the Head module components Med18 and Med20. As was discussed in chapter 3, the expression-profiling results agree well with previous biochemical purifications of Mediator lacking individual components (Lee et al., 1999; Myers et al., 1999).

Besides confirming previously known relationships, several new functional dependencies are also suggested by the microarray analyses. Until now little was known about Med12 and Med13. They are stably associated with Cdk8/CycC and form together the Kinase module. The identical profiles of cells lacking any subunit of the Kinase module would suggest that all subunits are necessary for the catalytic activity of Cdk8 or required for proper association of the Kinase module with core-Mediator. Moreover, partial deletion of the Head component Med19 resulted in a similar profile and might suggest the presence of a novel interaction surface of the Kinase module and the c-terminal region of Med19. In support of this, all five proteins were previously identified in three different genetic screens and have a similar flocculent phenotype (Rosenblum-Vos et al., 1991; Song et al., 1996; Tabtiang and Herskowitz, 1998). Further biochemical analysis should determine whether the c-terminal region of Med19 is indeed necessary for the association of the Kinase module

and whether a direct interaction with the Kinase module occurs.

The modular structure obtained from the expression profiles and a new potential interaction illustrate that microarrays can identify proteins that interact or are in close proximity within large protein complexes.

Discovery of new regulatory pathways and epistasis analysis

In the past, various Mediator subunits were analysed using different strains, growth conditions and microarray platforms (Becerra et al., 2002; Beve et al., 2005; Han et al., 2001; Holstege et al., 1998; Myers et al., 1999). As a result, comparison of data is complicated and only resulted in the effects of the disrupted subunit and/or the response to different treatments. The expression profiles described in this thesis, all performed using similar growth and experimental conditions, illustrates the power of a systematic approach.

Mediator was previously identified as a co-regulator that has both positive and negative effects on transcription. Results described in chapter 3 support this and indicate that the examined subunits from the Head and Tail have mainly a positive effect on gene transcription as exemplified by the number of genes down-regulated upon disruption, whereas all subunits from the Kinase module negatively affect transcription.

Importantly, transcription of a set of genes was dependent on the Tail and Head module and repressed by the Kinase module. This indicated that different modules within Mediator act antagonistically on the transcription of a specific set of genes and lead to the discovery of signal transduction within Mediator.

It is possible that more genes are regulated via similar mechanisms but were not identified in this analysis. Detection of both activation and repression of transcription requires a basal expression level that might be absent in the condition tested here. Furthermore, these genes might serve as good targets for the investigation of activation/derepression mechanisms of Mediator in the conditions used in chapter 3.

To further analyse the antagonistic relation between the Tail and the Kinase module, we applied epistasis analysis using microarray expression profiles as a transcriptional phenotype. Epistasis is the genetic interaction between two or more genes that control a single phenotype (Cordell, 2002). When a mutation of gene A masks a mutation of gene B, this is interpreted as that gene A is epistatic to gene B or in other words that gene A functions downstream of gene B. Results described in chapter 3 illustrates that transcriptional phenotypes produced by microarray expression profiles are good alternatives and have several advantages over classical phenotypes. The existence of a phenotype is crucial for the analysis of epistatic pathways though not always easily defined in complex biological processes and genetic networks. Microarray expression profiles, however, report the effects of mutations on the expression of all genes. Furthermore, pathway relationships can be analysed within different growth conditions and can result in finding additional pathway components.

The expression-profiling epistasis analyses presented in chapter 3 therefore illustrate that microarrays can also be used to determine pathway relationships (Anholt et al., 2003; Van Driessche et al., 2005).

Pin-pointing precise effects of protein modifications

Signal transduction pathways are cascades of proteins containing enzymatic activity and each modifying the activity of downstream targets. Besides the analysis of pathway components, we have investigated the effects of post-translational modifications using microarrays. The overlap of expression profiles between loss of the Cdk8 kinase and alanine substitution of one of its target substrates allowed very precise identification of the downstream effects of this phosphorylation event.

Ten of the twelve genes affected in both *med2-S208A* and *cdk8* contain a Rcs1/Aft1 binding site (Harbison et al., 2004) and were previously described as activated upon iron limitations (Shakoury-Elizeh et al., 2004). This suggests that this phosphorylation event is necessary to suppress the activity of the Rcs1/Aft1 transcription factor.

This finding has important implications for the understanding of how specificity in transcription regulation is achieved and is discussed later.

However, removal of Cdk8 or CycC indicated residual Med2 phosphorylation and suggests a redundant kinase that is able to phosphorylate Med2. Consistent with these results was the lack of full overlap between the *med2-S208A* and the absence of Cdk8. A similar cyclin dependent kinase to Cdk8 and also involved in transcription is Pho85. Expression-profiling of a Pho85 deletion mutant, revealed a promising overlap with *med2-S208A*. However, Med2 phosphorylation was not determined to be affected upon deletion of Pho85 and therefore need further investigations.

Together, the work presented in chapter 3 indicates that microarrays and the use of a systematic approach is a powerful tool for the analysis of structure/function relationships, analysis of pathways and investigations of post-translational modifications.

MEDIATOR AS A SIGNAL TRANDUCER

Two interesting questions arise from the results discussed in this thesis. First, how do signal transduction pathways target and regulate Mediator? Second, what are the consequences of the phosphorylation of Tail subunits by Cdk8/CycC?

Upstream of Cdk8

The current model that Mediator simply functions as a molecular bridge between gene specific transcription factors and the basal transcription machinery doesn't explain the presence of Mediator subunits with enzymatic activity and subunits negatively affecting transcription. As a consequence, it has been proposed that signal transduction pathways are able to target Mediator and thereby affect and regulate its activity (Denis and Green, 1996; Jiang et al., 1998; Kuchin et al., 2000).

Our studies were not aimed at identification of signalling pathways affecting Mediator directly. However, we could identify functional interactions within Mediator that can affect the activity of the Kinase module, and thus regulating downstream processes. Expression profiles of cells containing a truncation in Med19 were similar as deleting any subunit from the Kinase module. This suggests that Med19 interacts with subunits from the Kinase Module and/or is required for the activity of the Kinase module (as described before). Furthermore it suggests that besides affecting subunits from the Kinase module directly, signalling pathways may target subunits from core-Mediator and subsequently affect the activity of Cdk8 (**Figure 5.1a**). How might signalling pathways target Mediator and how does this regulate the activity of Cdk8?

Besides the phosphorylation of mammalian Med1 by the MAPK pathway and yeast Med13 by PKA (Chang et al., 2004; Pandey et al., 2005), a systematic analysis of yeast protein phosphorylation using protein microarrays identified additional signalling pathways which are able to target Mediator subunits *in vitro* (Med3 by Pho85/Pcl1, Med9 by Pho85/Pho80; Med13 by Tpk3 and Swe1) (Ptacek et al., 2005). The phosphorylation of Med3 and Med9 by Pho85 was dependent on different cyclin partners (Pcl1 and Pho80) (Ptacek et al., 2005) and would suggest different signalling pathways (Carroll and O'Shea, 2002). Moreover, Tpk3 (component of PKA) and Swe1 (regulates G2/M transition) have been found to phosphorylate Med13. Swe1 expression is cell-cycle regulated, with accumulation beginning in S phase (Asano et al., 2005) and suggests a regulation of Mediator during the cell cycle. Moreover, deletion of Cdk8 or Swe1 both cause filamentous and invasive growth (La Valle and Wittenberg, 2001; Nelson et al., 2003). Together this illustrates that several signalling pathways can target Mediator by post-translational modifications thereby influencing the regulatory activity of Mediator (**Figure 5.1**).

From the data described in this thesis and previously by others we can't distinguish whether the Kinase module is inactivated but present, or whether it dissociates from core-Mediator upon activation (**Figure 5.1b**). Previously, biochemical purifications of Mediator indicated that the Kinase module is only sub-stochiometrically present (Sato et al., 2004). Furthermore, Cdk8 is depleted from yeast cells after logarithmic growth (Holstege et

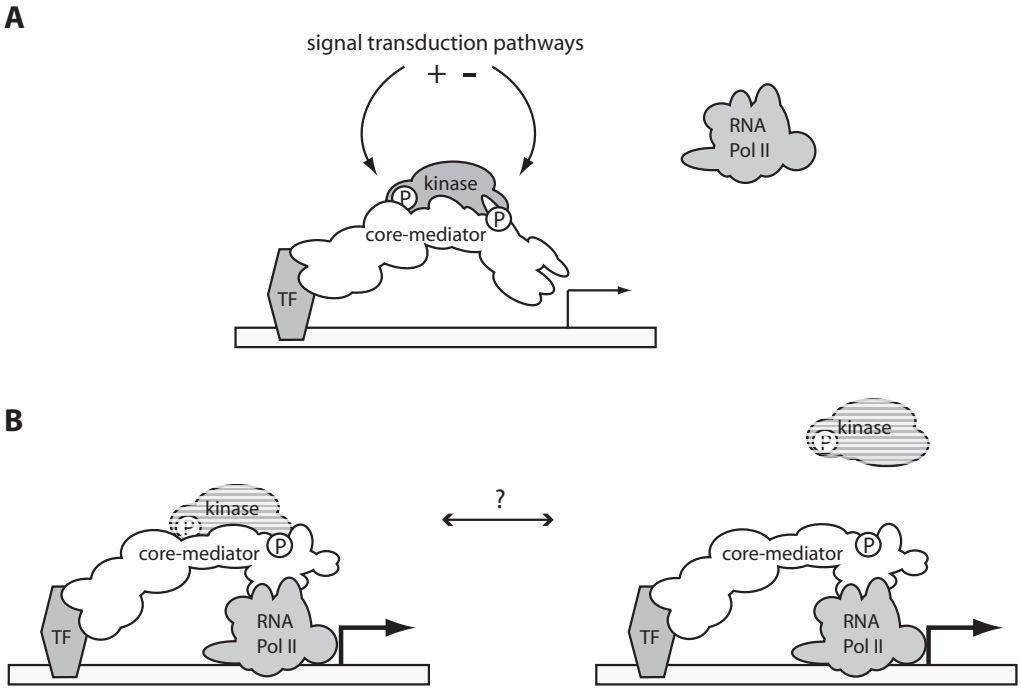


Figure 5.1: Proposed model of how signal transduction pathways affect activity of Mediator.
*Several signal transduction pathways have been proposed to target Mediator and affect its role in transcription regulation (see text for details). In addition, Cdk8/CycC has been shown to have important roles in the negative regulatory effects on transcription. (**A**) This suggests that core-Mediator or subunits from the Kinase module are targeted by signal transduction pathways and regulate the activity of the Kinase module. (**B**) Upon inactivation or dissociation of the Kinase module, a conformational change of Mediator may enable the recruitment of RNA pol II leading to activation.*

al., 1998) and would therefore suggest that the Kinase module dissociates from core-Mediator. On the other hand, genome-wide location analysis of Mediator shows that both core-Mediator and the Kinase module co-localize and are present upstream of inactive and active genes (Andrau et al., 2006; Zhu et al., 2006) and would suggest that the Kinase module is continuously present but inactivated when necessary.

It can't as yet be excluded whether the Kinase module is specifically present during specific stages of the transcription cycle or cell cycle. The identification of Swe1 (Ptacek et al., 2005) and the fact that subunits from the Kinase module encode negative regulators of genes expressed early in meiotic development (Strich et al., 1989) would suggest an additional regulatory role during the cell cycle, and may explain the transient association of the Kinase module in reChIP experiments (Andrau et al., 2006). Analysis of Mediator recruitment during activation/repression conditions and the use of synchronised cells should elucidate the specific mechanism and presence of the Kinase module during the transcription and cell cycle.

Downstream of Cdk8

As described before, the association of the Kinase module with core-Mediator is an important aspect for the activity of Mediator. Therefore, the question remains: what are the downstream effects of the Kinase module and in particular of its catalytic component Cdk8?

In this work we have identified Mediator components as targets of Cdk8 that can result in highly specific effects on transcription. One single amino acid modified by Cdk8 selectively influenced the activity of a single transcription factor Rcs1/Aft1 showing that Mediator can have highly specific effects on transcription. In current models of transcription regulation, specificity of transcription is determined by gene specific transcription factors (Lemon and Tjian, 2000;

Ptashne, 2005). A widely diverse set of gene-specific transcription factors are recruited to specific regulatory elements located upstream of the transcription start site. Subsequently, activation or inhibition of the general transcription machinery regulates expression.

Results described in this thesis illustrate that Mediator can additionally modulate the specificity of transcription by post-translational modifications initiated by Cdk8 and raises the question of how this phosphorylation event regulates specificity in transcription. Two mechanisms might explain this. First, phosphorylation of Med2 may induce a conformational change of Mediator making it unable to be recruited by particular gene-specific transcription factors (**Figure 5.2**). Alternatively, phosphorylation of Med2 may influence the activation and recruitment of downstream factors such as RNA Pol II and the basal transcription machinery. This latter model agrees with previous structural studies performed with mammalian Mediator. Binding of gene-specific transcription factors to human Mediator triggers specific conformational changes and phosphorylation of Med2 may result in a Mediator conformation that does not allow recruitment of additional co-regulatory complexes, RNA Pol II and general transcription factors.

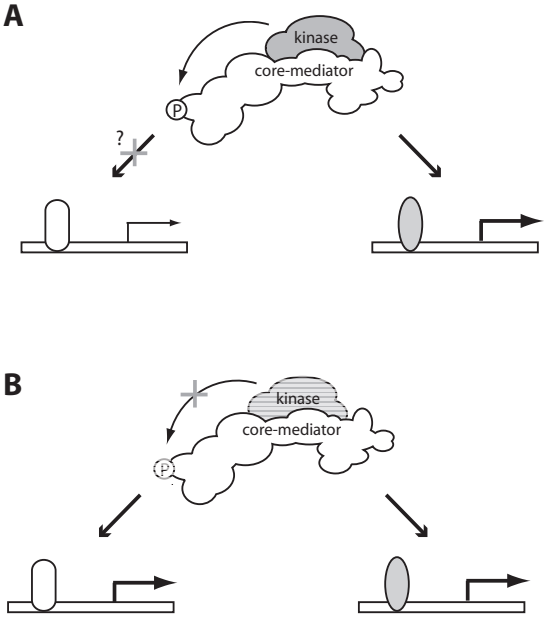


Figure 5.2: Phosphorylation of Tail module results in gene specific repression.
*From the data described in this thesis we propose a model in which Cdk8/CycC phosphorylates Tail subunits resulting in gene specific repression. (**A**) One possible mechanism is that phosphorylated Med2 can selectively modulate the activation of Rcs1/Aft1 whereas this doesn't influence the activation by other gene-specific transcription factors. This specificity would be the result of the inability of Mediator to be recruited by the transcription factor or structural changes that disable proper assembly of additional co-activators or the general transcription machinery. (**B**) Upon inactivation or dissociation of the Kinase module (see **Figure 5.1**), removal of the phosphorylated residue would restore activation of transcription. In addition, dephosphorylation of Med2 seems to be essential to activate transcription and would therefore suggest a requirement for phosphatases for activation of transcription within the proposed model.*

Previously, several studies illustrated that Mediator interacts with various gene specific transcription factors via the Tail module (Han et al., 1999; Kim et al., 2004; Park et al., 2000). Moreover, biochemical and structural analysis demonstrated that different activators have different interaction domains within the Tail (Park et al., 2000; Taatjes et al., 2002; Taatjes et al., 2004) which suggests that Mediator is a multi-protein complex able to interact with a wide variety of gene specific transcription factors. Together this might explain how modifications within the Tail of Mediator can result in specificity of transcription. Upon modification of Mediator, the TF-Mediator interaction can't be established and transcription is inhibited. Analysis of mutants that maintain the integrity and association of Tail subunits should confirm in the future the exact interaction surfaces with transcription factors and whether the described phosphorylation events affect interactions with gene specific transcription factors.

Although we have as yet not been able to show that Med15 is an *in vivo* target of Cdk8/CycC, the diffuse phosphorylation pattern observed in wild-type cells might indicate a similar mechanism (chapter 4). Specific Med15 phosphorylated residues might be required for a specific set of transcription factors able to modulate the activity of individual gene specific transcription factors.

Besides the phosphorylation of Med2 and Med15, previous studies illustrated additional mechanisms of how Cdk8 can control the activity of gene-specific transcription factors and the

general transcription machinery. Phosphorylation of the transcription factors Gcn4 and Ste12 by Cdk8 resulted in degradation of the transcription factors whereas phosphorylated Msn2 is translocated to the cytoplasm (Chi et al., 2001; Nelson et al., 2003) and indicates that besides the phosphorylation of Mediator subunits, Cdk8 initiates specific regulatory events directly on gene-specific transcription factors. In addition, this exemplifies the negative regulatory roles of Cdk8 in transcription regulation. On the other hand, Gal4 and Sip4 phosphorylation by Cdk8 is required for proper activation of its target genes (Hirst et al., 1999; Vincent et al., 2001). Furthermore, previous studies illustrated that Cdk8 can target proteins from the general transcription machinery, CTD of RNA Pol II and the cyclin partner of the catalytic activity of TFIIH (Loyer et al., 2005). Together with results described before, this indicates that the Kinase module can affect different processes during activation and initiation of transcription by post-translational modification of its target substrates.

Microarray analyses of previous identified phosphorylation sites would give more understanding in the role of Mediator and its regulation by signalling pathways and might elucidate regulatory cascades and more in depth function of each Mediator subunit and its contribution to transcription regulation.

Final remarks

Altogether, recent data shows that Mediator is not only a molecular bridge between transcription factors and the general transcription machinery as thought shortly after its discovery. As discussed, several lines of evidence suggest that Mediator is a crucial complex that is targeted by signal transduction pathways. These new findings illustrate the complexity of transcriptional regulation. Proper identification of all post-transcriptional modifications is necessary to fully understand the signal cascades leading to transcription activation/repression and how Mediator functions. Another open question is why the Kinase module transiently associated with Mediator on DNA and what exact mechanism regulates its presence and/or activity? The results described here have revealed that the mechanism of Mediator function is more complex then a molecular bridge and provides several starting points for mechanistically analysis of both up- and downstream processes.

REFERENCES

Andrau, J. C., van de Pasch, L., Lijnzaad, P., Bijma, T., Koerkamp, M. G., van de Peppel, J., Werner, M., and Holstege, F. C. (2006). Genome-Wide Location of the Coactivator Mediator: Binding without Activation and Transient Cdk8 Interaction on DNA. Mol Cell *22*, 179-192.

Anholt, R. R., Dilda, C. L., Chang, S., Fanara, J. J., Kulkarni, N. H., Ganguly, I., Rollmann, S. M., Kamdar, K. P., and Mackay, T. F. (2003). The genetic architecture of odor-guided behavior in Drosophila: epistasis and the transcriptome. Nat Genet *35*, 180-184.

Asano, S., Park, J. E., Sakchaisri, K., Yu, L. R., Song, S., Supavilai, P., Veenstra, T. D., and Lee, K. S. (2005). Concerted mechanism of Swe1/Wee1 regulation by multiple kinases in budding yeast. Embo J *24*, 2194-2204.

Becerra, M., Lombardia-Ferreira, L. J., Hauser, N. C., Hoheisel, J. D., Tizon, B., and Cerdan, M. E. (2002). The yeast transcriptome in aerobic and hypoxic conditions: effects of hap1, rox1, rox3 and srb10 deletions. Mol Microbiol *43*, 545-555.

Beve, J., Hu, G. Z., Myers, L. C., Balciunas, D., Werngren, O., Hultenby, K., Wibom, R., Ronne, H., and Gustafsson, C. M. (2005). The structural and functional role of Med5 in the yeast Mediator tail module. J Biol Chem *280*, 41366-41372.

Blower, P. E., Yang, C., Fligner, M. A., Verducci, J. S., Yu, L., Richman, S., and Weinstein, J. N. (2002). Pharmacogenomic analysis: correlating molecular substructure classes with microarray gene expression data. Pharmacogenomics J *2*, 259-271.

Botstein, D., and Fink, G. R. (1988). Yeast: an experimental organism for modern biology. Science *240*, 1439-1443.

Butcher, E. C., Berg, E. L., and Kunkel, E. J. (2004). Systems biology in drug discovery. Nat Biotechnol *22*, 1253-1259.

Butcher, R. A., and Schreiber, S. L. (2005). Using genome-wide transcriptional profiling to elucidate small-molecule mechanism. Curr Opin Chem Biol *9*, 25-30.

Carroll, A. S., and O'Shea, E. K. (2002). Pho85 and signaling environmental conditions. Trends Biochem Sci *27*, 87-93.

Chang, Y. W., Howard, S. C., and Herman, P. K. (2004). The Ras/PKA signaling pathway directly targets the Srb9 protein, a component of the general RNA polymerase II transcription apparatus. Mol Cell *15*, 107-116.

Chi, Y., Huddleston, M. J., Zhang, X., Young, R. A.,

Annan, R. S., Carr, S. A., and Deshaies, R. J. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. Genes Dev *15*, 1078-1092.

Cleary, M. D., Meiering, C. D., Jan, E., Guymon, R., and Boothroyd, J. C. (2005). Biosynthetic labeling of RNA with uracil phosphoribosyltransferase allows cell-specific microarray analysis of mRNA synthesis and decay. Nat Biotechnol *23*, 232-237.

Cordell, H. J. (2002). Epistasis: what it means, what it doesn't mean, and statistical methods to detect it in humans. Hum Mol Genet *11*, 2463-2468.

Denis, G. V., and Green, M. R. (1996). A novel, mitogen-activated nuclear kinase is related to a Drosophila developmental regulator. Genes Dev *10*, 261-271.

Dotson, M. R., Yuan, C. X., Roeder, R. G., Myers, L. C., Gustafsson, C. M., Jiang, Y. W., Li, Y., Kornberg, R. D., and Asturias, F. J. (2000). Structural organization of yeast and mammalian mediator complexes. Proc Natl Acad Sci U S A *97*, 14307-14310.

Dujon, B. (1996). The yeast genome project: what did we learn? Trends Genet *12*, 263-270.

ERCC (2005). Proposed methods for testing and selecting the ERCC external RNA controls. BMC Genomics *6*, 150.

Frazer, K. A., Elnitski, L., Church, D. M., Dubchak, I., and Hardison, R. C. (2003). Cross-species sequence comparisons: a review of methods and available resources. Genome Res *13*, 1-12.

Garcia-Martinez, J., Aranda, A., and Perez-Ortin, J. E. (2004). Genomic run-on evaluates transcription rates for all yeast genes and identifies gene regulatory mechanisms. Mol Cell *15*, 303-313.

Gasch, A. P. (2002). Yeast genomic expression studies using DNA microarrays. Methods Enzymol *350*, 393-414.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., *et al.* (1996). Life with 6000 genes. Science *274*, 546, 563-547.

Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., *et al.* (1999). Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science *286*, 531-537.

Han, S. J., Lee, J. S., Kang, J. S., and Kim, Y. J. (2001). Med9/Cse2 and Gal11 modules are required for transcriptional repression of distinct group of genes. J Biol Chem *276*, 37020-37026.

Han, S. J., Lee, Y. C., Gim, B. S., Ryu, G. H., Park, S. J., Lane, W. S., and Kim, Y. J. (1999). Activator-specific requirement of yeast mediator proteins for RNA polymerase II transcriptional activation. Mol Cell Biol *19*, 979-988.

Harbison, C. T., Gordon, D. B., Lee, T. I., Rinaldi, N. J., Macisaac, K. D., Danford, T. W., Hannett, N. M., Tagne, J. B., Reynolds, D. B., Yoo, J., *et al.* (2004). Transcriptional regulatory code of a eukaryotic genome. Nature *431*, 99-104.

Hirst, M., Kobor, M. S., Kuriakose, N., Greenblatt, J., and Sadowski, I. (1999). GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase SRB10/CDK8. Mol Cell *3*, 673-678.

Holstege, F. C., Jennings, E. G., Wyrick, J. J., Lee, T. I., Hengartner, C. J., Green, M. R., Golub, T. R., Lander, E. S., and Young, R. A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell *95*, 717-728.

Hughes, T. R., Marton, M. J., Jones, A. R., Roberts, C. J., Stoughton, R., Armour, C. D., Bennett, H. A., Coffey, E., Dai, H., He, Y. D., *et al.* (2000). Functional discovery via a compendium of expression profiles. Cell *102*, 109-126.

Ideker, T., Galitski, T., and Hood, L. (2001). A new approach to decoding life: systems biology. Annu Rev Genomics Hum Genet *2*, 343-372.

Jiang, Y. W., Veschambre, P., Erdjument-Bromage, H., Tempst, P., Conaway, J. W., Conaway, R. C., and Kornberg, R. D. (1998). Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways. Proc Natl Acad Sci U S A *95*, 8538-8543.

Kang, J. S., Kim, S. H., Hwang, M. S., Han, S. J., Lee, Y. C., and Kim, Y. J. (2001). The structural and functional organization of the yeast mediator complex. J Biol Chem *276*, 42003-42010.

Kim, T. W., Kwon, Y. J., Kim, J. M., Song, Y. H., Kim, S. N., and Kim, Y. J. (2004). MED16 and MED23 of Mediator are coactivators of lipopolysaccharide- and heat-shock-induced transcriptional activators. Proc Natl Acad Sci U S A *101*, 12153-12158.

Korona, R. (2004). Experimental studies of deleterious mutation in Saccharomyces cerevisiae. Res Microbiol *155*, 301-310.

Kuchin, S., Treich, I., and Carlson, M. (2000). A regulatory shortcut between the Snf1 protein kinase and RNA polymerase II holoenzyme. Proc Natl Acad Sci U S A *97*, 7916-7920.

Kumar, A., and Snyder, M. (2001). Emerging technologies in yeast genomics. Nat Rev Genet *2*, 302-312.

La Valle, R., and Wittenberg, C. (2001). A role for the Swe1 checkpoint kinase during filamentous growth of Saccharomyces cerevisiae. Genetics *158*, 549-562.

Lee, Y. C., Park, J. M., Min, S., Han, S. J., and Kim, Y. J. (1999). An activator binding module of yeast RNA polymerase II holoenzyme. Mol Cell Biol *19*, 2967-2976.

Lemon, B., and Tjian, R. (2000). Orchestrated response: a symphony of transcription factors for gene control. Genes Dev *14*, 2551-2569.

Loyer, P., Trembley, J. H., Katona, R., Kidd, V. J., and Lahti, J. M. (2005). Role of CDK/cyclin complexes in transcription and RNA splicing. Cell Signal *17*, 1033-1051.

Myers, L. C., Gustafsson, C. M., Hayashibara, K. C., Brown, P. O., and Kornberg, R. D. (1999). Mediator protein mutations that selectively abolish activated transcription. Proc Natl Acad Sci U S A *96*, 67-72.

Nelson, C., Goto, S., Lund, K., Hung, W., and Sadowski,

I. (2003). Srb10/Cdk8 regulates yeast filamentous growth by phosphorylating the transcription factor Ste12. Nature *421*, 187-190.

Pandey, P. K., Udayakumar, T. S., Lin, X., Sharma, D., Shapiro, P. S., and Fondell, J. D. (2005). Activation of TRAP/mediator subunit TRAP220/Med1 is regulated by mitogen-activated protein kinase-dependent phosphorylation. Mol Cell Biol *25*, 10695-10710.

Park, J. M., Kim, H. S., Han, S. J., Hwang, M. S., Lee, Y. C., and Kim, Y. J. (2000). In vivo requirement of activator-specific binding targets of mediator. Mol Cell Biol *20*, 8709-8719.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., *et al.* (2005). Global analysis of protein phosphorylation in yeast. Nature *438*, 679-684.

Ptashne, M. (2005). Regulation of transcription: from lambda to eukaryotes. Trends Biochem Sci *30*, 275-279.

Rosenblum-Vos, L. S., Rhodes, L., Evangelista, C. C., Jr., Boayke, K. A., and Zitomer, R. S. (1991). The ROX3 gene encodes an essential nuclear protein involved in CYC7 gene expression in Saccharomyces cerevisiae. Mol Cell Biol *11*, 5639-5647.

Rubin, G. M., Yandell, M. D., Wortman, J. R., Gabor Miklos, G. L., Nelson, C. R., Hariharan, I. K., Fortini, M. E., Li, P. W., Apweiler, R., Fleischmann, W., *et al.* (2000). Comparative genomics of the eukaryotes. Science *287*, 2204-2215.

Sato, S., Tomomori-Sato, C., Parmely, T. J., Florens, L., Zybailov, B., Swanson, S. K., Banks, C. A., Jin, J., Cai, Y., Washburn, M. P., *et al.* (2004). A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. Mol Cell *14*, 685-691.

Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science *270*, 467-470.

Shakoury-Elizeh, M., Tiedeman, J., Rashford, J., Ferea, T., Demeter, J., Garcia, E., Rolfes, R., Brown, P. O., Botstein, D., and Philpott, C. C. (2004). Transcriptional remodeling in response to iron deprivation in Saccharomyces cerevisiae. Mol Biol Cell *15*, 1233-1243.

Song, W., Treich, I., Qian, N., Kuchin, S., and Carlson, M. (1996). SSN genes that affect transcriptional repression in Saccharomyces cerevisiae encode SIN4, ROX3, and SRB proteins associated with RNA polymerase II. Mol Cell Biol *16*, 115-120.

Spellman, P. T., Sherlock, G., Zhang, M. Q., Iyer, V. R., Anders, K., Eisen, M. B., Brown, P. O., Botstein, D., and Futcher, B. (1998). Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization. Mol Biol Cell *9*, 3273-3297.

Strich, R., Slater, M. R., and Esposito, R. E. (1989). Identification of negative regulatory genes that govern the expression of early meiotic genes in yeast. Proc Natl Acad Sci U S A *86*, 10018-10022.

Taatjes, D. J., Naar, A. M., Andel, F., 3rd, Nogales, E., and Tjian, R. (2002). Structure, function, and activator-induced conformations of the CRSP coactivator. Science *295*, 1058-1062.

Taatjes, D. J., Schneider-Poetsch, T., and Tjian, R. (2004). Distinct conformational states of nuclear receptor-bound CRSP-Med complexes. Nat Struct Mol Biol *11*, 664-671.

Tabtiang, R. K., and Herskowitz, I. (1998). Nuclear proteins Nut1p and Nut2p cooperate to negatively regulate a Swi4p-dependent lacZ reporter gene in Saccharomyces cerevisiae. Mol Cell Biol *18*, 4707-4718.

Tanay, A., Sharan, R., Kupiec, M., and Shamir, R. (2004). Revealing modularity and organization in the yeast molecular network by integrated analysis of highly heterogeneous genomewide data. Proc Natl Acad Sci U S A *101*, 2981-2986.

van 't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T., *et al.* (2002). Gene expression profiling predicts clinical outcome of breast cancer. Nature *415*, 530-536.

van Bakel, H., and Holstege, F. C. (2004). In control: systematic assessment of microarray performance. EMBO Rep *5*, 964-969.

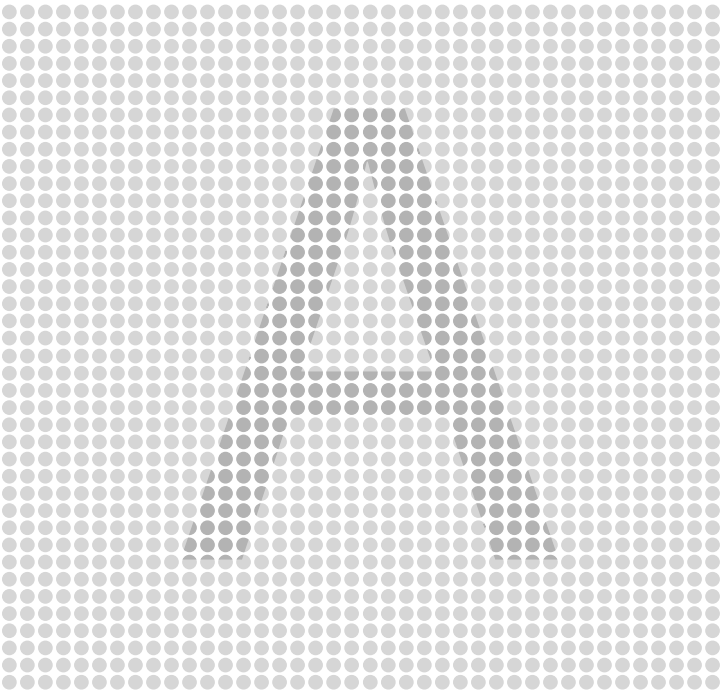
Van Driessche, N., Demsar, J., Booth, E. O., Hill, P., Juvan, P., Zupan, B., Kuspa, A., and Shaulsky, G. (2005). Epistasis analysis with global transcriptional phenotypes. Nat Genet *37*, 471-477.

Vanhecke, D., and Janitz, M. (2005). Functional genomics using high-throughput RNA interference. Drug Discov Today *10*, 205-212.

Vincent, O., Kuchin, S., Hong, S. P., Townley, R., Vyas, V. K., and Carlson, M. (2001). Interaction of the Srb10 kinase with Sip4, a transcriptional activator of gluconeogenic genes in Saccharomyces cerevisiae. Mol Cell Biol *21*, 5790-5796.

Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., and Speed, T. P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res *30*, e15.

Zhu, X., Wiren, M., Sinha, I., Rasmussen, N. N., Linder, T., Holmberg, S., Ekwall, K., and Gustafsson, C. M. (2006). Genome-wide occupancy profile of mediator and the srb8-11 module reveals interactions with coding regions. Mol Cell *22*, 169-178.



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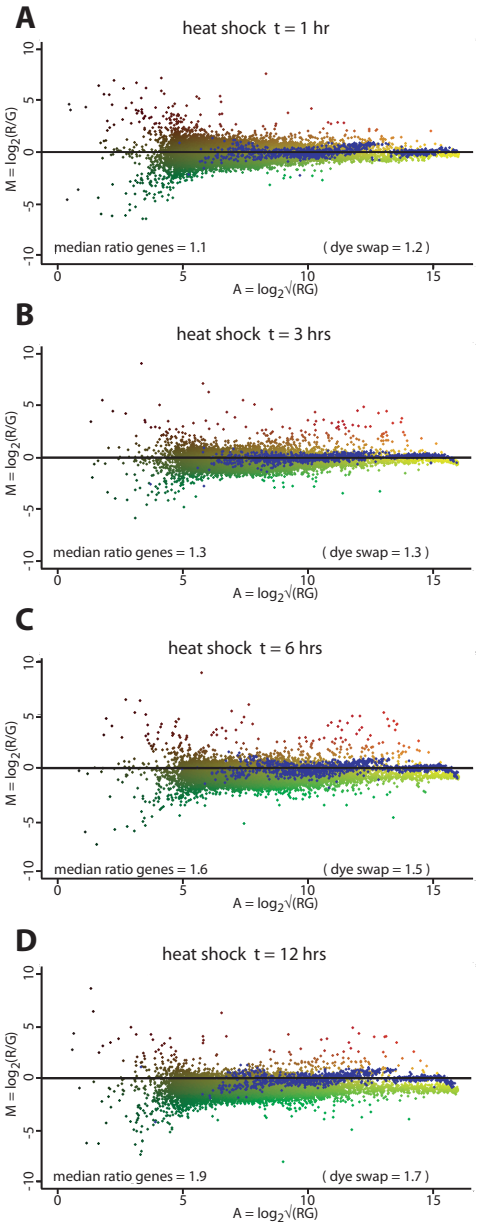


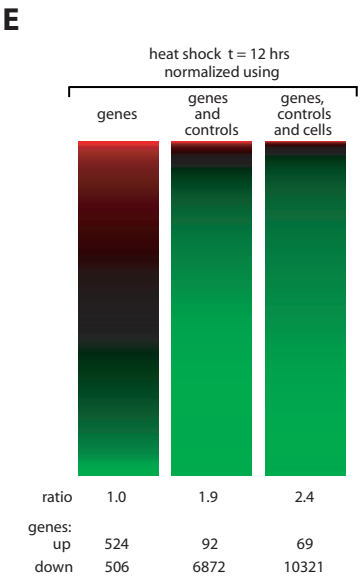


Figure 2.2: Global messenger RNA changes during mammalian heat shock.
*Human umbilical vein endothelial cells were heat-shocked and RNA was isolated at the timepoints shown (**A–D**). The result of hybridization of each sample (R) against that of the non-heatshocked reference cells (G) is shown. Each graph is an MA scatterplot (where M= log2(R/G) and A = log2√(RG); Yang et al., 2002). The y axis shows the log2 ratio (mean spot intensity minus mean local area background) after normalization using genes and controls (see Methods). The values plotted on the x axes are derived from the intensities of both channels. The median change for all genes is indicated, as well as the result of a corresponding dye-swap experiment. (**E**) Comparison of different normalization strategies. From left to right are the results of three strategies applied to the 12-h timepoint. Each column is made up of 16,735 coloured lines, stacked vertically. Each line represents the change for a single gene. Upregulated genes are shown in red and downregulated genes are shown in green. Numbers below the bars indicate the median change of all genes after normalization (as a ratio) and how many genes are reported as being upregulated or downregulated if an arbitrary twofold cut-off is applied. The first column shows the result of Lowess normalization per sub-grid using endogenous genes (see Methods). The second column shows the result of incorporating external controls into the normalization strategy by carrying out normalization to equivalent amounts of total RNA (normalization using genes and controls; see Methods). This leads to a markedly altered perception of the changes that have taken place, with many more transcripts reported as being downregulated. The third column shows the result when the slight drop in the total RNA content*

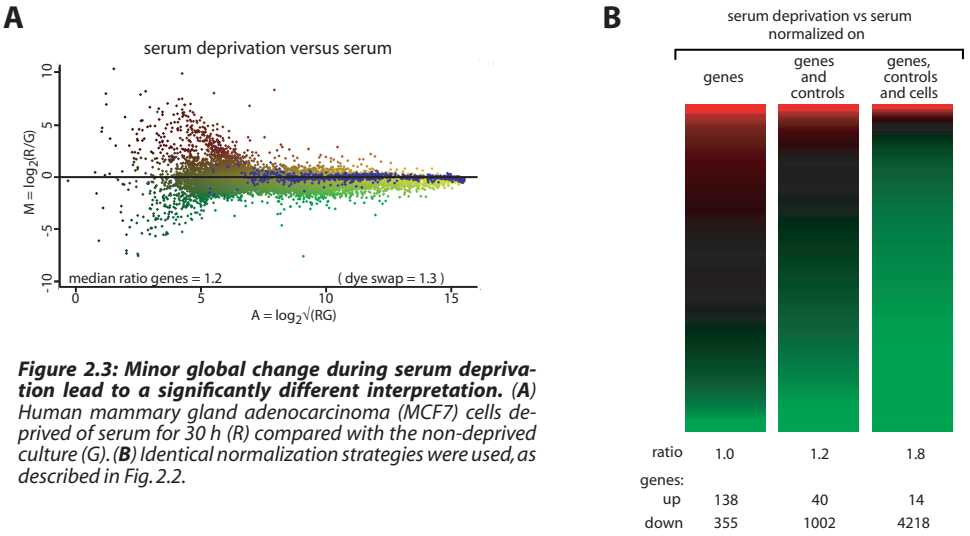


Figure 2.3: Minor global change during serum deprivation lead to a significantly different interpretation. *(**A**) Human mammary gland adenocarcinoma (MCF7) cells deprived of serum for 30 h (R) compared with the non-deprived culture (G). (**B**) Identical normalization strategies were used, as described in Fig. 2.2.*

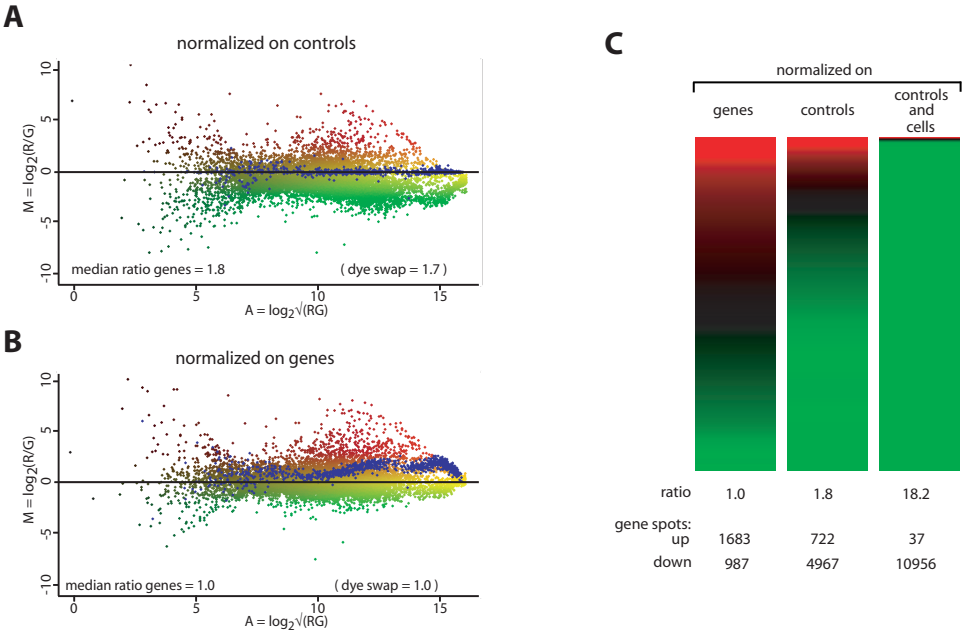


Figure 2.4: Yeast stationary phase culture compared with mid-log phase culture.
*(**A**) MA scatterplot (where M = log2(R/G) and A = log2√(R/G)) after Lowess normalization for each subgrid using controls (see Methods). (**B**) Lowess normalization for each subgrid using genes (see Methods). The aberrant pattern of external control spots (blue) occurred because messenger RNA levels had not changed uniformly across the entire range of expression levels (along the x axis). RNA levels for genes with higher expression levels had dropped to a greater degree than those of genes expressed at lower levels. Due to saturation of the signals in the scanned images, this effect seemingly decreases for a small group of genes expressed at the highest levels, resulting in the curve of the control spots to the far right of the graph. (**C**) Comparison of different normalization strategies. The left and middle columns correspond to the graphs shown in (**B**) and (**A**), respectively. The drop in total RNA yields per cell (see Methods) can also be taken into account (right column). Because each probe was spotted twice on the yeast arrays, the numbers reflect how many spots have changed. R, stationary phase culture; G, mid-log phase culture.*

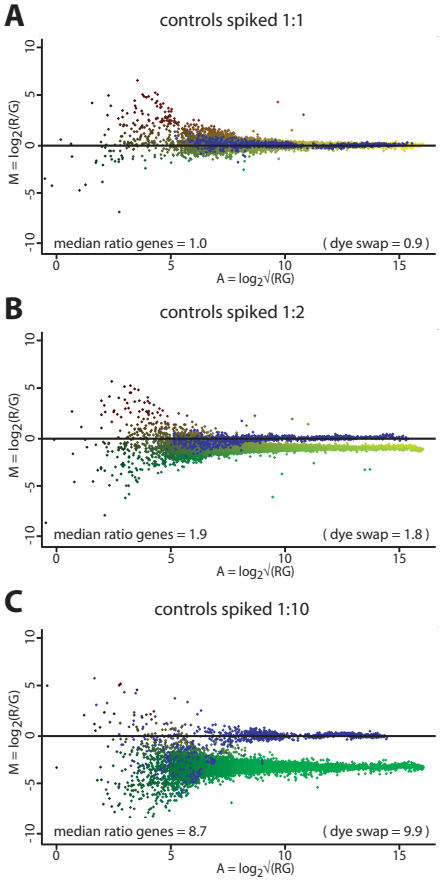


Figure: 2.5 External controls can accurately detect global transcript changes.
*The external control mix was added at a ratio of (**A**) 1:1, (**B**) 1:2 and (**C**) 1:10 to paired aliquots of a single yeast total RNA preparation.*

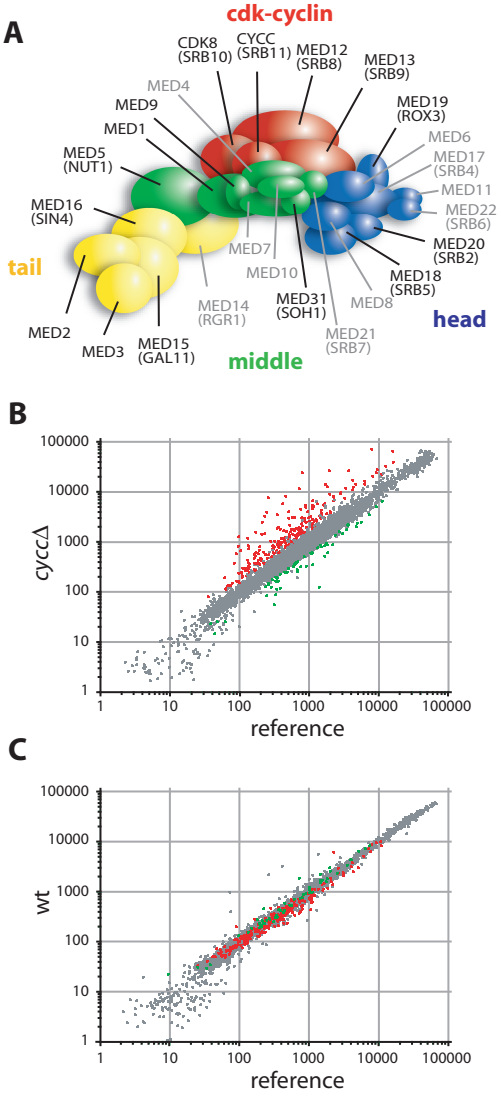


Figure: 3.1: Mediator structure-function by expression-profiling.
*(**A**) Mediator consists of 25 subunits which can be divided into 4 submodules (Asturias et al., 1999): Tail (yellow), Middle (green), Head (blue) and Cdk (red). This model is based on previous structural studies e.g. (Kang et al., 2001), taking into account recent work, all summarized in (Guglielmi et al., 2004). The (non-essential) subunits studied here are shown in bold and were completely deleted with the exception of MED19 for which a partial deletion was used. (**B**) Microarray signal intensities of cyccΔ (vertical axis) compared to wt reference (horizontal axis). The intensities of all genes are shown, after background subtraction, normalization and merging of replicate culture dye-swap hybridizations. Genes determined significantly up- and down-regulated in this mutant are colored red and green, respectively. (**C**) Scatter plot of intensities from one of the wt control (vertical axis) versus wt reference (horizontal axis) experiments. Genes are colored as in the cyccΔ example.*

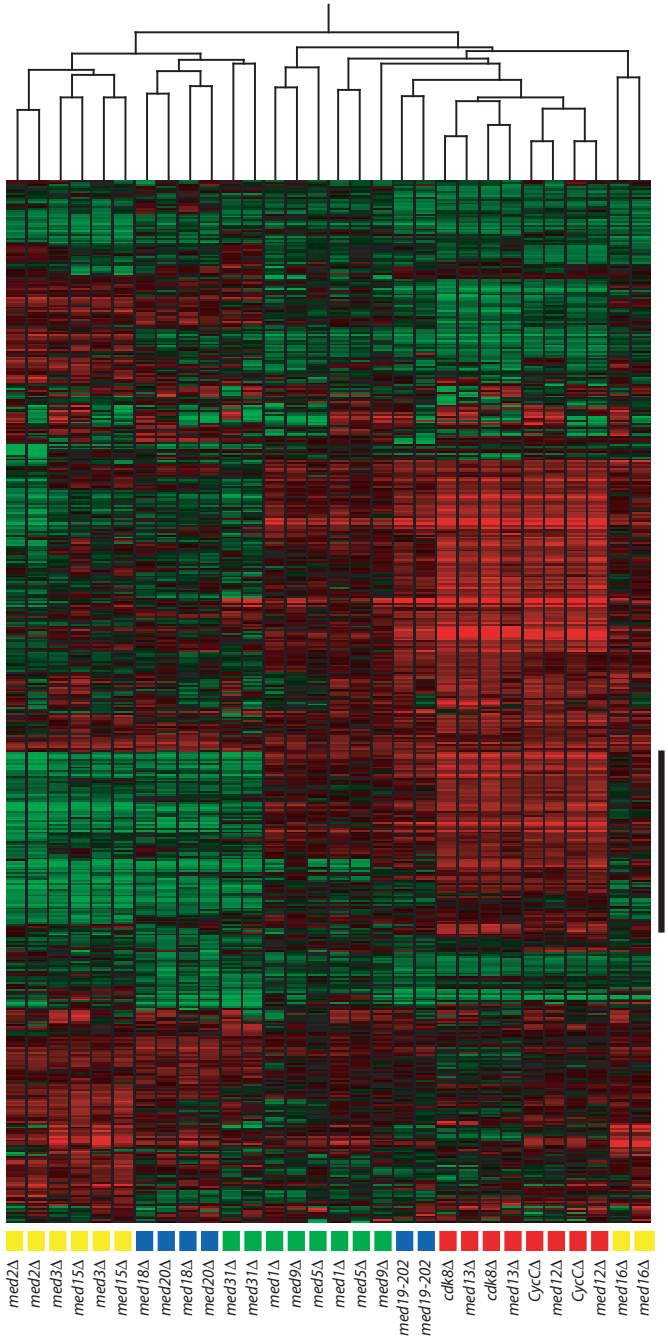


Figure 3.2: Expression-profiling reveals Mediator subunit relationships
Diagram of all 927 genes (clustered vertically) exhibiting significant changes specific to one or more of the deletion strains (clustered horizontally). Results are shown for the replicate microarray hybridizations individually. Change in gene expression compared to wt is depicted in red (up), green (down) or black (no change). The black bar indicates some of the genes that behave in opposite ways, depending on which subunit is deleted (Figure 3.3).

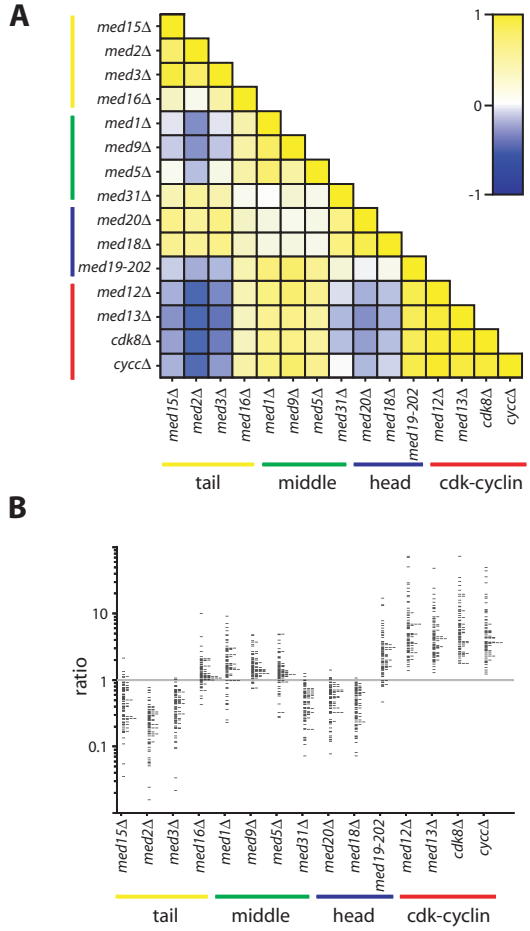


Figure 3.3: Functional antagonism between Mediator subunits. *(**A**) Pearson's correlation matrix for merged expression profiles of individual subunits, with yellow for high correlation and blue representing anti-correlation. (**B**) Oppositely behaving genes, selected for showing significant decrease in expression in med2Δ, med3Δ or med15Δ, significant increase in expression in cdk8Δ, cycCΔ, med12Δ or med13Δ and no significant changes in wt versus wt controls. The expression ratio for each gene (vertical axis) is depicted for each mutant strain (horizontal axis).*

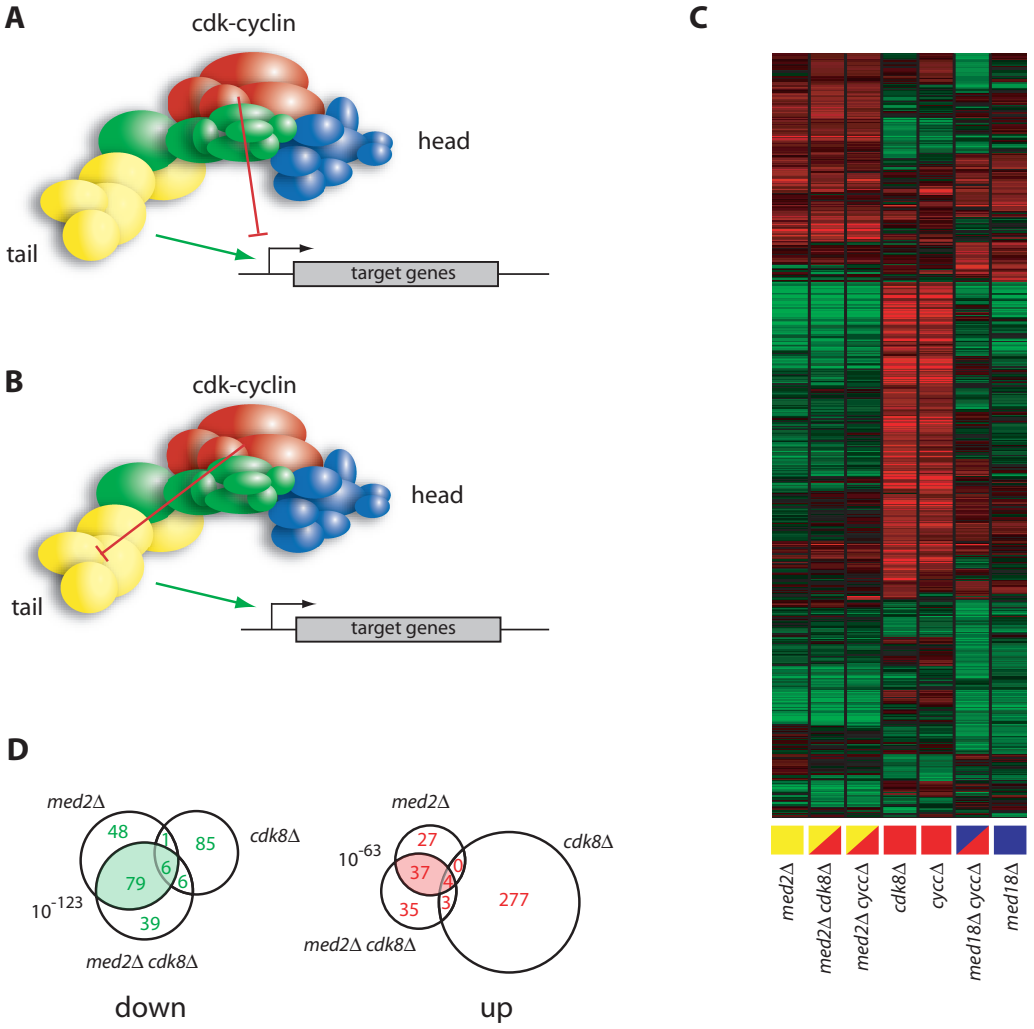
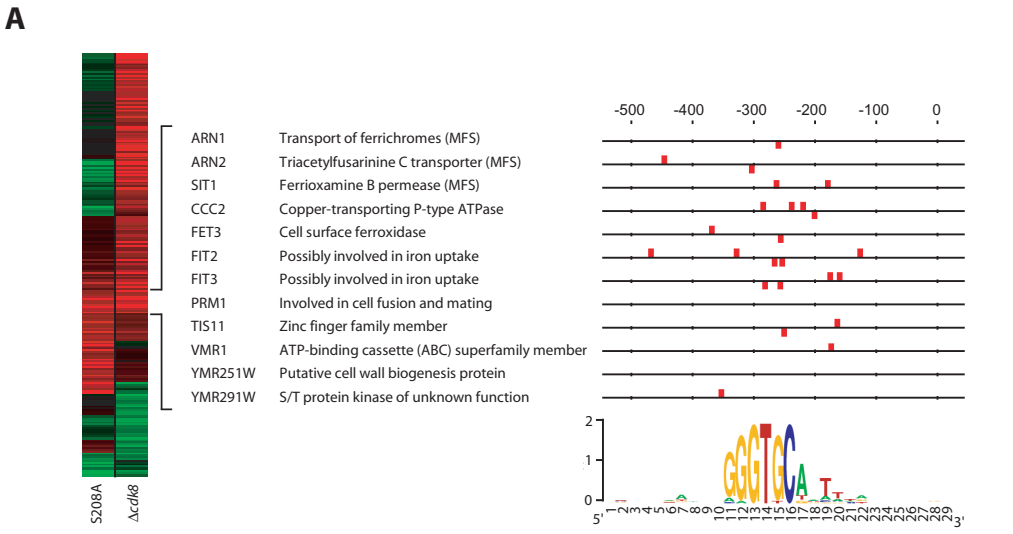
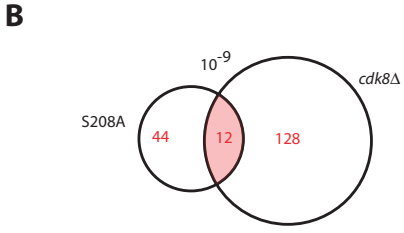
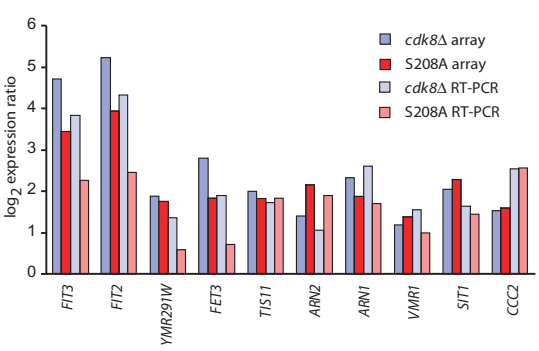


Figure 3.4: Epistasis with expression-profiles reveals that MED2 and MED18 function downstream of CDK8-CYCC. *(**A**) A parallel pathway model of how the Cdk submodule (negative contribution) and Tail submodule (positive contribution) may independently act on genes that behave in opposite ways depending on which component is deleted. (**B**) A serial pathway model whereby the Cdk submodule exerts negative regulation indirectly, through influencing the activity of the Tail. (**C**) Expression-profiles of single and double gene deletion strains demonstrate that the model shown in B is correct, with dominance of med2Δ and med18Δ over Cdk subunit deletions when combined. Merged profiles of the independent replicate dye-swaps are shown, as is otherwise described for **Figure 3.2**. (**D**) Venn diagrams of genes with significant changes in med2Δ and cdk8Δ single deletions versus med2Δ cdk8Δ double deletion, either for genes with decreased expression (left) or increased expression (right). p-values are for the significance of the shaded overlaps. Additional Venn diagrams are shown in **S2**.*







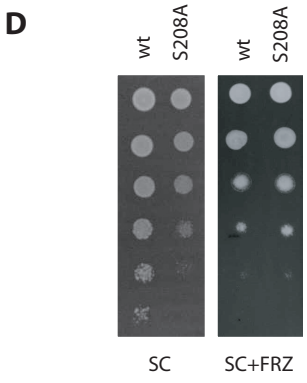
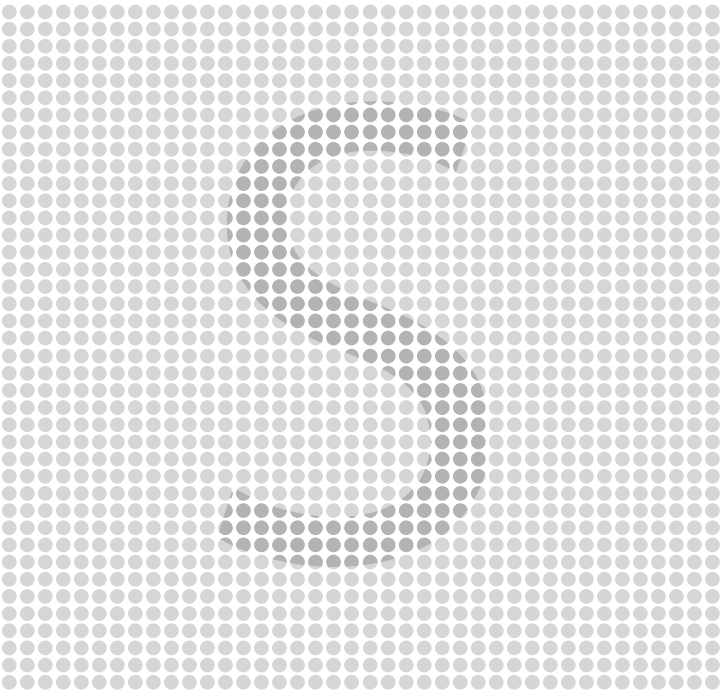


Figure 3.6: Cdk8 phosphorylation of Med2 at serine 208 represses the low iron response regulon.
*(**A**) All genes with significantly changed expression in either cdk8Δ or med2-S208A are depicted. Genes that become upregulated both in cdk8Δ and upon mutation of the Cdk8 phosphorylation site in Med2 are listed. A search for a shared motif within the 600 nucleotide region upstream of the translation start results in the depicted motif found at the positions indicated by the red boxes. (**B**) Venn diagram of genes with significantly increased expression in cdk8Δ and med2-S208A strains. (**C**) Microarray expression ratios of Rsc1 binding site genes listed in A for cdk8Δ (blue) and med2-S208A (red) as a log2 ratio versus reference wt. RT-PCR expression ratios are depicted in light blue (cdk8Δ) and light red (med2-S208A). (**D**) Spot plate growth assay with serial dilutions of wt and med2-S208A cells under normal (left) and iron-depleted conditions (+ 1mM of the iron chelator Ferrozine, right). The Ferrozine plate has an apparent decrease in spot size (top to bottom) for both wt and mutant strains compared to normal plates. This is caused by loss of contrast between yeast growth and plate background which is pink in the presence of Ferrozine.*



Summary / Samenvatting
Curriculum Vitae
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Summary

Transcription regulation is an essential process that enables living organisms to develop, to respond to extra-cellular signals and to environmental changes. In *S. cerevisiae* more than 300 proteins are required for accurate transcription regulation. This thesis focuses on one of the more central transcription regulatory complexes, known as Mediator. Mediator is highly conserved from yeast to human and is required for the expression of nearly all protein-coding genes. Initially, Mediator was suggested to form a molecular bridge between gene-specific transcription factors and RNA Polymerase II. The presences of negatively and positively acting subunits as well as enzymatic activity does not agree with this simple model and suggest that Mediator may function as a signal processor that integrates signalling pathways and the regulation of transcription. The objective of the work described in this thesis was to investigate the role of Mediator subunits by systematically analysing the effects of deleting individual subunits by microarray expression profiling.

Previous microarray experiments showed that the expression of nearly all transcripts is affected upon disrupting essential subunits of Mediator or RNA Polymerase II. To detect such global expression changes, normalization of microarray experiments requires a set of transcripts that do not vary between the different samples. In **chapter 2** we describe the development and use of external RNA controls for normalization of microarray expression profiling experiments. In control experiments, we show that external RNA controls can accurately report global mRNA changes. Furthermore, we demonstrate that conditions such as heat-shock or starvation can affect global transcript levels. This illustrates that global changes occur more frequently than is currently assumed in microarray studies. Finally, a yeast stationary phase experiment exemplified the importance and power of external RNA controls when large global changes occur, especially when global transcript changes are unbalanced.

In **chapter 3** we applied DNA microarrays to examine the effects of deleting individual Mediator subunits. The results reveal that the microarray expression profiles correlate well with the structural organization of individual subunits leading to the proposal that expression-profiling is useful for structure-function analyses. In addition, different modules within Mediator were found to have antagonistic effects on the transcription of the same set of genes. Epistasis analyses, with microarray expression profiles as transcriptional phenotypes, demonstrate that *MED2* and *MED18* are epistatic to *CDK8*. The functional consequences of one of these pathways is mapped further and reveals that Cdk8-mediated phosphorylation of Med2 affects the transcription of a single transcription factor regulon. Together, this shows that intra-complex phosphorylation mediated by Cdk8 can regulate specificity of transcription regulation. This demonstrates that microarray expression-profiling can uncover new signal transduction pathways, delineate their components and precisely determine the consequences of single amino acid modifications.

In **chapter 4** we further investigated the regulation of Mediator activity by Cdk8. We show that Med15, another subunit from the Tail, is a potential target of Cdk8. Med15 migrates as a higher mobility, non-diffuse band on a protein gel when Cdk8 is absent. This illustrates that Med15 is phosphorylated dependent on Cdk8. In addition we show that Cdk8 can phosphorylate Med15 *in vitro*. Precise mapping of the Cdk8-dependent phosphorylated residues by mass-spectrometry is currently underway. Upcoming experiments should illustrate whether Med15 phosphorylation results in similar regulatory events as with Med2.

Chapter 5 is a general discussion of the results that are described in this thesis and discusses possible mechanisms through which Mediator can integrate signal transduction pathways and regulation of transcription.

Samenvatting

Regulatie van transcriptie is een essentieel proces voor ieder levend organisme om zich verder te ontwikkelen, voor de beantwoording van extracellulaire signalen en te reageren op veranderingen in het milieu waar het leeft. In *Saccharomyces cerevisiae* (bakkersgist) zijn meer dan 300 verschillende eiwitten vereist voor een nauwkeurige regelgeving van transcriptie. Dit proefschrift concentreert zich op één van de meer centralere transcriptie regulerende complexen, die bekent staat als Mediator. Mediator in gist bestaat uit 25 verschillende eiwitten, is geconserveerd in gist en hogere eukaryoten en is essentieel voor de expressie van bijna alle eiwitcoderende genen. Kort na de ontdekking werd gedacht dat Mediator hoofdzakelijk een verbinding maakt tussen genspecifieke transcriptiefactoren en RNA Polymerase II. De aanwezigheid van remmende en actieverende subeenheden binnen Mediator evenals de aanwezigheid van enzymatische activiteit gaat niet met dit eenvoudige model akkoord en suggereert dat Mediator als een target kan functioneren van signaal routes en deze signalen integreert met transcriptie. De doelstelling van het werk dat beschreven staat in dit proefschrift was de rol van de verschillende subeenheden van Mediator te onderzoeken door gebruik te maken van deletie mutanten en DNA microarrays.

Voorafgaande microarray experimenten van cellen waarbij essentiële eiwitten van Mediator of RNA Polymerase II afwezig waren, lieten zien dat de expressie van alle genen werd beïnvloed. Om dergelijke globale expressie veranderingen te herkennen, vereist de normalisatie van microarray experimenten een reeks transcripten die gelijk blijven tussen de te vergelijken monsters. In **hoofdstuk 2** beschrijven wij de ontwikkeling en het gebruik van externe RNA controles voor de normalisatie van microarray expressieprofielen. In controle experimenten tonen wij aan dat externe RNA controles globale mRNA veranderingen nauwkeurig kunnen detecteren. Verder laten wij zien dat kleine globale expressie veranderingen optreden wanneer menselijke cellen een hitte-schok krijgen of worden verhongerd. Dit illustreert dat globale mRNA veranderingen zich vaker voordoen dan momenteel in microarray studies wordt verondersteld. Een voorbeeld waarbij het belang en de kracht van deze externe RNA controles nogmaals wordt onderstreept is een gist stationaire fase experiment. Hier treden grote globale mRNA veranderingen op welke niet gebalanceerd zijn.

In **hoofdstuk 3** worden microarrays gebruikt om de gevolgen te onderzoeken van deleties van de individuele subeenheden van de Mediator. De resultaten laten zien dat de microarray expressieprofielen goed correleren met de structurele organisatie van de individuele subeenheden binnen het eiwit complex en stelt voor dat expressieprofielen gebruikt kunnen worden voor structuur-functie analyses. Bovendien werden verschillende modules binnen Mediator gevonden die tegengestelde gevolgen hadden voor de transcriptie van dezelfde reeks genen. Epistase analyses, met microarray expressieprofielen als fenotype, tonen aan dat *MED2* en *MED18* epistatisch zijn tot *CDK8*. De functionele gevolgen van één van deze wegen werd verder in kaart gebracht en liet zien dat Cdk8-afhankelijke fosforylatie van Med2 de transcriptie van één enkele transcriptiefactor regulon beïnvloedt. Dit suggereert dat intra-complex fosforylatie, die door Cdk8 wordt bemiddeld, specificiteit van transcriptieregelgeving kan regelen. Dit toont aan dat microarray expressieprofielen nieuwe signaaltransductie routes aan het licht kunnen brengen, hun componenten in kaart brengen en de precieze gevolgen van aminozuur veranderingen kunnen bepalen.

In **hoofdstuk 4** hebben wij de regulatie van de activiteit van Mediator door Cdk8 verder onderzocht. Hier laten wij zien dat Med15, een ander eiwit in de staart van Mediator, een potentiële target is van Cdk8. Med15 migreert sneller en als een niet diffuse band op een eiwit gel wanneer Cdk8 afwezig is. Dit illustreert dat de fosforylatie van Med15 afhankelijk is van Cdk8. Daarnaast laten wij zien dat Cdk8 Med15 kan fosforyleren *in vitro*. De precieze identificatie van de Cdk8-afhankelijke gefosforyleerde residu's door massa-spectrometrie is momenteel aan de gang. Komende experimenten zouden moeten illustreren of fosforylatie van Med15 resulteert in een zelfde regelgevende gebeurtenis als met Med2.

Hoofdstuk 5 is een algemene discussie van de resultaten die in dit proefschrift worden beschreven en bespreekt mogelijke mechanismen hoe Mediator signaaltransductie routes en regelgeving van transcriptie kan integreren.

Curriculum vitae

Jeroen van de Peppel werd geboren op 25 november 1973 te Oss. In 1992 behaalde hij zijn HAVO diploma aan het Comenius College te Uden. Vervolgens begon hij zijn studie aan het HLO van de Fontys Hogeschool te Eindhoven. Tijdens deze studie werd onderzoekservaring opgedaan tijdens een stage op het Statens Serum Instituut te Kopenhagen, Denemarken onder begeleiding van Dr. Jørgen Skov Jensen. Een tweede onderzoekstage werd gedaan bij het Eijkman Winkler instituut, UMCU onder begeleiding van Dr. Andre Verheul en Prof. Dr. Harm Snippe. Het HLO diploma werd behaald in juni 1996. Van juni 1997 tot juni 2001 heeft hij gewerkt als research analist / junior onderzoeker bij Lionbioscience te Heidelberg, Duitsland, onder begeleiding van Dr. Hartmut Voss en Prof. Dr. Martin Hofmann. Vervolgens werd het promotie-onderzoek, zoals beschreven in dit proefschrift, gedaan in de periode van augustus 2001 tot augustus 2006 onder begeleiding van Prof. Dr. Frank C.P. Holstege bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht.

List of Publications

Andrau JC, van de Pasch L, Lijnzaad P, Bijma T, Groot Koerkamp M, van de Peppel J, Werner M, Holstege F.
Genome-wide location of the coactivator Mediator: binding without activation and transient Cdk8 interaction on DNA.
Mol. Cell. 2006 Apr 21;22(2):179-192

van de Peppel J, Kettelarij N, van Bakel H, Kockelkorn TT, van Leenen D, Holstege FC.
Mediator expression profiling epistasis reveals a signal transduction pathway with antagonistic submodules and highly specific downstream targets.
Mol. Cell. 2005 Aug 19;19(4):511-22.

J. van de Peppel, F.C.P. Holstege
Multifunctional genes. News & Views
Molecular Systems biology, 29 March 2005, doi:10.1038/msb4100006

van de Peppel J, Kemmeren P, van Bakel H, Radonjic M, van Leenen D, Holstege FC.
Monitoring global messenger RNA changes in externally controlled microarray experiments.
EMBO Rep. 2003 Apr;4(4):387-93.

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xxx.

Jeroen