Mediator Promotes CENP-A Incorporation at Fission Yeast Centromeres.

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ABSTRACT

At *Schizosaccharomyces pombe* centromeres, heterochromatin formation is required for *de novo* incorporation of the histone H3 variant CENP-A<sup>Cnp1</sup>, which in turn directs kinetochore assembly and ultimately chromosome segregation during mitosis. Non-coding RNAs (ncRNAs) transcribed by RNA polymerase II (Pol II) directs heterochromatin formation via the RNAi machinery, but also through RNAi-independent RNA processing factors. Control of centromeric ncRNA transcription is therefore a key factor for proper centromere function. We here demonstrate that Mediator directs ncRNA transcription and regulates centromeric heterochromatin formation in fission yeast. Mediator co-localizes with Pol II at centromeres and loss of the Mediator subunit Med20 causes a dramatic increase in pericentromeric transcription and desilencing of the core centromere. As a consequence, heterochromatin formation is impaired both via the RNAi dependent and independent pathways, resulting in loss of CENP-A<sup>Cnp1</sup> from the core centromere, defect kinetochore function, and a severe chromosome segregation defect. Interestingly, the increased centromeric transcription observed in *med20Δ* appears to directly block CENP-A<sup>Cnp1</sup> incorporation, since inhibition of Pol II transcription can suppress the observed phenotypes. Our data thus identify Mediator as a crucial regulator of ncRNA transcription at fission yeast centromeres and add another crucial layer of regulation to centromere function.
INTRODUCTION

The kinetochore serves as the attachment point for spindle microtubules during mitotic and meiotic cell divisions. It is assembled on a specialized chromatin domain called the centromere, which is subject to epigenetic control (16). In *Schizosaccharomyces pombe* the centromere contains a central core region that is flanked by two types of repeat regions, the innermost region (imr) contains several tRNA genes, whereas the outermost region (otr) harbors the *dh* and *dg* elements. These flanking pericentromeric repeat regions are organized into heterochromatin and are transcriptionally silent (45). The central core region functions as a platform for formation of the kinetochore structure and it contains the unique histone H3 variant CENP-A<sup>Cnp1</sup> (6). CENP-A<sup>Cnp1</sup> incorporation is critical for centromere function and depletion of it leads to loss of other kinetochore proteins and to chromosome segregation defects (43).

Heterochromatin organization and transcription silencing at the centromere is a regulated process, which involves transcription of the centromeric repeat regions by Pol II to produce non-coding RNA (ncRNA) (9, 13). Mutations in genes encoding Pol II subunit can impair production of siRNA and heterochromatin formation (14, 26). The ncRNA is complemented to double strand RNA by the RNA dependent RNA polymerase (Rdp1) (37, 52). The RNase III helicase, Dcr1, cleaves the double strand RNA into small interfering RNA (siRNA), which interferes with the transcription of the centromeric repeat regions themselves (12). The siRNAs are bound by the RNA-induced transcription-silencing (RITS) complex, which is recruited to heterochromatin by interactions with H3-lysine-9-methylation (H3K9me) (40, 50, 54). RITS in turn
stimulates recruitment of Clr4, which methylates H3K9, thus creating a positive feedback loop with increasing levels of H3K9me (62). The H3K9me mark is recognized and bound by Swi6, which is critical for heterochromatin formation and spreading in fission yeast (3, 39, 57). Furthermore, RNAi also promotes termination of Pol II transcription in the pericentromeric regions and thereby prevents stalling of replication forks due to collisions of RNA and DNA polymerase (61). Recent data have also suggested a novel regulatory pathway in which the exosome RNA degradation machinery acts in parallel to the RNAi pathway to mediate heterochromatin formation (49).

Pol II also transcribes the central core region of centromeres, but the resulting ncRNA transcripts do not contribute to siRNA production, but are instead rapidly degraded by the exosome (11). The functional importance and the control of central core region transcription is not yet known and whereas RNAi dependent regulation of pericentromeric transcription has been studied in detail, it is not known how core centromere transcription is regulated. An important co-regulator of Pol II-dependent transcription at many other genomic locations is the evolutionary conserved Mediator complex (7). This multiprotein complex serves as a bridge between gene specific regulatory proteins bound to upstream elements and the general transcription machinery (53). There is a wealth of genetic data connecting Mediator to gene regulation in eukaryotic cells and a temperature sensitive mutation in the essential Mediator component \textit{MED17/SRB4}, abolishes nearly all Pol II dependent transcription in \textit{S. cerevisiae} (55). Mediator is not only required for gene activation, but genetic data that have also connected Mediator to gene repression. The repressive effect of Mediator has
primarily been linked to a subgroup of Mediator proteins, Med12, Med13, Cdk8 (cyclin-dependent kinase 8), and CycC (Cyclin C), which forms a specific module (the Cdk8 module) in the Mediator complex and block interactions with Pol II (18, 31). However, it is not correct to divide the Mediator into activating and repressive modules, since most Mediator subunits, including the Cdk8 module, display both positive and negative effects on gene transcription (15, 21). The Med8, Med18, and Med20 proteins constitute another Mediator submodule that can affect transcription in both positive and negative directions (36). The MED18/SRB5 and MED20/SRB2 genes were originally identified in budding yeast as suppressors of a cold sensitive phenotype associated with a truncation of the C-terminal domain in Rpb1, the largest subunit of Pol II (41). Structural studies have demonstrated that Med18-Med20 forms a stable heterodimer that is tethered to the Mediator complex via the C-terminus of Med8 (34). The TATA-box binding protein (TBP) interacts directly with the Med18-Med20 heterodimer, causing structural changes in the Mediator complex that could regulate interactions with Pol II (8, 34). The Med18-Med20 heterodimer also interacts directly with Pol II via the Rpb4 and Rpb7 subunits (8). The multifaceted interactions described between Med18-Med20 and the basal transcription machinery, may explain why loss of these two Mediator components leads to both positive and negative effects on global gene regulation in fission yeast (36). Even if most focus has been on the role for Mediator in the regulation of mRNA encoding genes, there are recent reports that link Mediator to the control of Pol II dependent transcription of ncRNA. In mouse embryonic stem cells, Mediator interacts with an Ada-Two-A-containing histone acetyltransferase and regulates a set of ncRNA genes.
(32) and in *Arabidopsis thaliana*, Mediator recruits Pol II to ncRNA gene promoters (30).

Here we demonstrate that Mediator is a regulator of ncRNA transcription at fission yeast centromeres and required for heterochromatin formation via an RNAi-independent pathway. Loss of the Mediator component Med20 impairs transcription silencing and heterochromatin formation. As a consequence, CENP-A<sup>Cnp1</sup> is lost from the centromere, which disturbs kinetochore function, and causes chromosome segregation defects. Based on our studies, we suggest a model, in which strict transcriptional control of the core centromeric region is required for proper CENP-A<sup>Cnp1</sup> chromatin assembly.
MATERIALS AND METHODS

Yeast strains and growth conditions
Spot tests were performed on complete media (YEA) with either 10 μg/ml TBZ or 1 g/l 5-fluoroorotic acid (5-FOA) using 10 × dilution steps. The cells were incubated at 25 °C for 3 - 5 days. Liquid cultures were grown in complete media (YEL) at 25 °C, 30 °C, or 36 °C. Strains were sporulated on SPAS-plates. Overexpression was performed using pREP81x-vector (4) in Edinburgh Minimal Medium - Leucine (EMM - Leu) with and without thiamine (5 μg/ml). A list of yeast strains used in this study is available upon request.

Cell cycle synchronization
Cell cultures containing the cdc25-22 mutation were prepared at 25 °C (OD ~0.2). The cells were blocked at 36 °C for 4 hrs and then released by cooling to 25 °C, and incubated at 25 °C. At each time point ~10⁹ cells were collected for ChIP, RNA analysis, immunostaining, and septation index counting (n = 100).

Fluorescence Microscopy
Immunofluorescent staining was carried out following a procedure previously described (17). Antibodies used were α-GFP (ab290, Abcam), α-tubulin (T5168, Sigma Aldrich), Alexa Fluor 488 goat anti—mouse IgG (A11001, Invitrogen) and Alexa Fluor 594 Chicken Anti-Rabbit IgG (A21442, Invitrogen). Chromosome segregation defects counted as chromosome lagging follows a previously described classification (46). CENP-A-CFP cells were grown to O.D. =
0.5. When indicated, Actinomycin D (SIGMA) was added to a final concentration of 10 ug/ml and cells were incubated for another 60 min at 30 °C. GFP and CFP fluorescence microscopy were performed using a Carl Zeiss Axiovert 200M fluorescence microscope. CENP-A signal were counted blindly. Statistical significance level was calculated using two-sample z-test.

**Tiling array transcriptome, Chip-Seq and ChIP analysis**

Tiling array analysis was performed as previously described (63) using the *S. pombe* Tiling 1.0FR Array (900647, Affymetrix). Raw data and signal files have been deposited on the NCBI GEO website (GSE32310, GSE35527). Expression profiles for med8-598, med18Δ, med20Δ and med12Δ were obtained from (36). List of segregation-associated factors were obtained from Go Terms (GO:0007059, GO:0000775, GO:0031497) in *S. Pombe* (taxon 4896) from [http://www.ebi.ac.uk/QuickGO/](http://www.ebi.ac.uk/QuickGO/). ChIP assays were performed as described previously (64). ChIP-Seq was performed using an Illumina Genome Analyzer according to manufacturer’s instruction. The quality check of the short reads was done by generating QC statistics with FastQC ([http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc](http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc)). Read alignments to the NCBI assembly of the *S. pombe* genome were performed using Bowtie (version 0.12.7)(33) allowing up to two mismatches. Tiling array and ChIP-seq raw data and signal files have been deposited on the NCBI GEO website (GSE35527). Primer sequences are available on request. Antibodies used were, α-tubulin (T5168, Sigma Aldrich), α-Histone H3 (ab1791, abcam), α-H3K9me (ab1220, abcam), α-sw16 (BAM-63-101, Cosmo Bio), α-GFP (for immunoblotting: A10260, Invitrogen; and for ChIP: ab290, Abcam), α-RNA Pol II CTD
(ab5408, abcam), and α-myc (M4439, Sigma Aldrich). Ratios of IP/input are depicted in the figures after subtracting ratios obtained with non-antibody controls.

**RNA extraction and detection**

Total RNA was extracted by a standard hot phenol method (60). Small RNA molecules were isolated from total RNA using mirVana miRNA Isolation Kit (Ambion) and analyzed as previously described (13). Probe sequences are available on request. The results were normalized to a U6 RNA-control and calculated based on two biological repeats. Reverse Transcription PCR (RT-PCR) was performed with SuperScript® II Reverse Transcriptase (18064-022, Invitrogen) using random primers according to the manufacturer’s recommendation. RT reactions without reverse transcriptase were used as controls to monitor possible DNA contaminations. qPCR experiments and analysis of the results were performed as stated in ChIP-methods, but normalized to act+. The ura4+ transcript levels were visualized on a 1% agarose gel. Primer sequences are available on request.
RESULTS

Mediator is required for centromere function.

We tested if Mediator affects centromere function in fission yeast, by monitoring growth of Mediator mutant strains in the presence of the anti-microtubule drug Tiabendazole (TBZ). For comparison, we used a dcr1Δ deletion mutant, which impairs centromere function and displays TBZ sensitivity (47). We found three Mediator mutants, med8-598, med18Δ, and med20Δ that were sensitive to TBZ, even if the effects were milder than those seen with dcr1Δ. Some of the Mediator components investigated, e.g. Med1, Med12, Med13, and Med27, had no apparent effect on TBZ sensitivity, whereas other components, e.g. Med15 and Med31, had only a modest effect (Figure 1A, data not shown). Interestingly, the three TBZ sensitive mutants are functionally related and encode proteins (Med8, Med18, Med20) that form a sub-module in the Mediator complex (34, 36). Of these three proteins, loss of Med20 has the mildest general effects on Mediator function. Furthermore, the med8-598 and med18Δ mutations both lead to concomitant loss of the Med20 protein from the Mediator complex and we therefore decided to focus on Med20 for our further studies (36).

We used chromatin immunoprecipitation (ChIP) and monitored H3K9me and Swi6 occupancy to analyze effects on centromeric heterochromatin formation. In agreement with previous reports, dcr1Δ and a strain carrying a mutation in the Rpb2 subunit of Pol II (rpb2-m203) displayed a strong reduction in H3K9me and Swi6 binding in the otr and imr regions of chromosome 1 (26, 57). Interestingly, the levels of both H3K9me and
Swi6 were also significantly reduced in the med20Δ cells, indicating impaired heterochromatin formation (Figure 1B). Mutations in the RNAi machinery that cause problems in forming a proper heterochromatin structure at centromeres, ultimately affect kinetochore assembly and chromosome segregation (23). We therefore monitored the distribution of α-tubulin and DNA in med20Δ cells during mitosis. Loss of Med20 caused a severe defect in chromosome segregation (Figure 1C and 1D), with a frequency of chromosome lagging of about 28.2% in anaphase cells (n = 124) compared to 1.7%, in a wild type control (n = 116). We also deleted med20 in a strain containing a disposable minichromosome with an adenine marker (1). Loss of Med20 resulted in a strong increase in minichromosome loss, further supporting the chromosome lagging phenotype (data not shown). In mammalian cells, Mediator interacts with cohesin (24). To address if the med20Δ deletion could influence cohesin distribution at fission yeast centromeres, we performed a ChIP analysis and analyzed the distribution of Rad21, a component of the cohesin complex. We observed no significant changes in Rad21 core centromeric occupancy in the med20Δ deletion strain (Figure 1E). Similarly, Rad21 localization as determined by immunoflorescent microscopy was also unaffected (data not shown).

The effect of Med20 on centromere function is partially distinct from the RNAi machinery.

We hypothesized that the effects of med20Δ on centromere function could be explained by changes in pericentromeric ncRNA transcription and siRNA production. However, even if med20Δ caused a significant reduction of siRNA levels produced from dh transcripts (36% of wt levels), the effect was not nearly as strong as that observed in a
$dcr1^+$ deletion mutant. Furthermore, siRNA production from $dg$ was not significantly impaired, whereas this RNA species was completely abolished upon loss of Dcr1 (Figure 1F). The changes in siRNA production were therefore mild compared to the strong effects of the $med20^+$ deletion on chromosome segregation. Therefore, even if the RNAi machinery could contribute to the observed phenotypes, it appeared likely that Med20 also affected chromosome segregation through a mechanism that was distinct from the RNAi pathway.

Mediator interacts directly with centromeres.

To investigate if Mediator interacted directly with the centromere region, we used ChIP followed by DNA sequencing (ChIP-Seq) (Figure 2A and B). We found a number of distinct Mediator peaks, and more importantly, a pattern of binding that was similar to Pol II. We observed Mediator and Pol II binding to the pericentriomeric repeat regions, but also lower levels of protein binding to central core region ($cnt$). We also monitored Pol II binding in the $med20\Delta$ strain and observed a significant increase in the pericentromeric region, but also modestly higher Pol II binding at the core centromere (Figure 2A). Compared to many other genomic regions, the levels of Mediator and Pol II were relatively low in the centromeric region. We hypothesized that this could be due to periodic variations and that Mediator only interacted with the centromeric regions during a short phase of the cell cycle. In support of this idea, transcription of the $otr$ and $imr$ region primarily occurs in S-phase, when the DNA is exposed during replication (10). We therefore also investigated Mediator and Pol II occupancy during cell cycle progression.
when cells were synchronized with cdc25-22 block-release experiments. Even if the variations were relatively mild (about 2-fold), both Mediator and Pol II bound to the dh repeat region, with a peak in S-phase (Figure 2C and D). The periodic variation supported the ChIP-Seq data and demonstrated that Mediator is directly recruited to the centromeric region. Loss of Med20 did not change the periodicity of Mediator binding (Figure 2C), but did cause a distinct increase of Pol II occupancy in the med20Δ strain (Figure 2D). In agreement with increased Pol II binding, loss of Med20 also caused a dramatic 25 – 50 fold up regulation of transcript levels at both dh and imr (Figure 2F and 2G). We could still discern a cell cycle dependent periodicity of imr transcription in the med20Δ strain, but the levels of transcripts were much higher (Figure 2G). We therefore conclude that loss of Med20 derepresses pericentromeric transcription, without affecting Mediator recruitment.

Deletion of med20+ disrupts centromeric silencing.

To further analyze silencing defects at different centromeric locations, we used a set of S. pombe strains, in which the ura4+ marker had been integrated at three different regions of the fission yeast centromere (otr, imr, or cnt) (1). Growth on 5-Fluoroorotic Acid (FOA) was used as an indicator of ura4+ silencing. In agreement with our measurements of pericentromeric transcription (Figure 2F and G), med20Δ caused derepression of the ura4+ marker gene transcription in the otr- and imr-regions. Interestingly, we also noted derepression of ura4+ in the cnt-region. This finding was distinctly different from that seen with RNAi mutants, which disrupt silencing of otr and imr, but not the cnt region (57). Another Mediator mutant, med12Δ, that did not influence TBZ sensitivity, had no
effect on ura4+ transcription at the locations tested (Figure 3A). To further verify our findings, we used reverse transcription-PCR and demonstrated that ura4+ was transcribed from both the pericentromeric repeats and the centromeric core region when med20+ was deleted (Figure 3B).

Even if our findings so far pointed towards a direct effect of Mediator on centromere function, we could not exclude that indirect effects on gene transcription contributed to the observed phenotypes. Mediator is a general regulator of Pol II transcription and deletion of med20+ could therefore affect transcription of genes involved in chromosome segregation and centromere function. To address this possibility, we used genome-wide expression data for med20Δ as well as for med8ts, med12Δ, med15Δ, and med18Δ (36, 38). We compared how the Mediator mutations affected transcription of a group of 233 genes required for centromere function and chromosome segregation (Figure 3C, data not shown). Only 7 of the 233 genes were up (4 genes) or down (3 genes) regulated more than 1.5 fold in med20Δ. Five out of the 7 affected genes were also changed in other mediator mutants, e.g. med12Δ, that did not display TBZ sensitivity (data not shown) and only two of the genes with changed transcription levels were unique to med20Δ, cdc20+/slp1+ and sir2+. It appeared unlikely that changes in the expression of these two genes could explain the observed effects of med20Δ on centromere function. The cdc20+ gene is required for the spindle checkpoint, but has not been linked to centromeric silencing (29), and deletion of sir2+ leads to impaired silencing of imr, but only weakly reduces silencing of otr, and does not affect the cnt region (20, 51). To directly address the contribution of cdc20+ and sir2+ to the phenotypes observed in med20Δ, we
overexpressed these genes in med20Δ strains with a ura4+ marker introduced at the otr, imr, or cnt region. Overexpression increased the transcript levels of cdc20+ and sir2+, but neither of the two genes could restore centromeric transcription silencing in med20Δ at any of the locations investigated (Figure 2D). Impaired transcription of cdc20+ or sir2+ could therefore not explain the centromeric silencing defect observed in med20Δ.

Tiling array analysis of centromeric ncRNA transcription

To better characterize the effects of Mediator on centromeric transcription, we performed transcriptome analysis with tiling arrays and monitored centromeric ncRNA transcription in med20Δ cells. We also profiled transcription in cells where both med20+ and dcr1+ had been deleted, since it allowed us to monitor transcripts that would otherwise have been processed by the RNAi machinery (57). In the med20Δ strain, transcript levels were increased in the centromeric region as compared to a wild type (wt) control (Figure 3E). The upregulated transcripts corresponded to ncRNA, which are processed into siRNA (13). We observed a significant increase of the same ncRNA transcripts in the dcr1Δ strain (data not shown) and the levels were increased even further in the dcr1Δ/med20Δ double mutant (Figure 3E). These observations were verified using quantitative PCR with primers covering the otr and imr transcripts (Figure 3F). Our findings therefore supported the observations done with synchronized cells (Figure 2F and G), and demonstrated that med20+ is required for repression of centromeric transcripts that are subsequently used for siRNA production.

We could not observe any clear effects on cnt region transcription in the tiling array
transcriptome analysis, which we expected to be a consequence of cnt transcripts being rapidly degraded by the exosome (11). To address this possibility, we used reverse transcription PCR and monitored cnt transcription. We could not detect cnt transcripts in wt cells. Similarly, cnt transcripts were not observed in cells where med20+ or the exosome component rrp6Δ had been deleted. However, when med20+ was deleted in an rrp6Δ mutant background, cnt transcripts were detected (Figure 3G). Based on these results, we conclude that Med20 is required for transcription silencing also at the core centromere regions, but that the produced transcripts are rapidly degraded by the exosome. In contrast, the increased levels of pericentromeric transcripts (dh and imr) observed in the med20Δ strain, were not significantly affected by deletion of rrp6+. (Figure 3G).

**CENP-A^Cnp1^ incorporation is impaired in med20Δ cells.**

De novo recruitment of CENP-A^Cnp1^ to centromeres depends on pericentromeric heterochromatin formation (19). The chromosome segregation defects and impaired transcription silencing associated with med20Δ therefore prompted us to investigate effects on CENP-A^Cnp1^ localization (58). An endogenously expressed CENP-A^Cnp1^-CFP fusion protein was used to visualize CENP-A^Cnp1^ positioning (2). In wt cells, a distinct CENP-A^Cnp1^–CFP signal was observed from kinetochores, but the signals were much weaker in the med20Δ strain and many cells did not have a localized CENP-A^Cnp1^ signal (Figure 4A). Impaired recruitment of CENP-A^Cnp1^ to centromeres was confirmed by ChIP analysis, in which CENP-A^Cnp1^ levels were reduced more than 5-fold compared to a wt control (Figure 4B). The effect appeared specific to CENP-A^Cnp1^, since deposition of
histone H3 in the surrounding pericentromeric regions was unaffected by loss of Med20 (data not shown). In budding yeast, failure of CENP-A\textsuperscript{Cse4} to localize to centromere leads to CENP-A\textsuperscript{Cse4} degradation (22, 48). The situation appears to be similar in fission yeast, since immunoblotting revealed a strong reduction of CENP-A\textsuperscript{Cnp1} protein levels in the \textit{med20Δ} strain (Figure 4C), even though the mRNA levels for CENP-A\textsuperscript{Cnp1} were unaffected (Figure 4D). We thus concluded that Med20 is required for incorporation of CENP-A\textsuperscript{Cnp1} at centromeres.

We speculated that the increased transcription observed in \textit{med20Δ} directly block CENP-A\textsuperscript{Cnp1} chromatin assembly. This model would connect the observed effect on transcription, with the chromosome segregation defects found in \textit{med20Δ}. To address this possibility, we used the transcription inhibitor Actinomycin D (dactinomycin), which inhibits RNA polymerases in eukaryotic cells (5). In wt and \textit{dcr1Δ} cells, Actinomycin D caused a slight reduction of CENP-A\textsuperscript{Cnp1} incorporation. In contrast, the levels of CENP-A\textsuperscript{Cnp1} were significantly increased in \textit{med20Δ} cells and the percentage of cells with a localized CENP-A\textsuperscript{Cnp1} signal increased from 21\% to 32\% (p<0.001, Figure 4E). These results demonstrate that the effect seen in \textit{med20Δ} is at least partially transcription dependent and suggest that Mediator-dependent repression of centromeric transcription is essential for CENP-A\textsuperscript{Cnp1} chromatin assembly.
In this work, we demonstrate that the Mediator complex is required for proper centromere function in *S. pombe*. Loss of the Med20 subunit leads to upregulation of centromeric ncRNA transcription, impaired heterochromatin formation, and reduced CENP-A\textsuperscript{Cnp1} incorporation. CENP-A\textsuperscript{Cnp1} is required for proper kinetochore assembly (44), explaining the dramatic defect on chromosome segregation, with lagging chromosomes seen in the med20\(\Delta\) strain. The phenotypes are in some aspects different and the effects are even more pronounced than those observed for mutations in the RNAi machinery (56).

Based on our results, we would like to propose a tentative model for Mediator-dependent CENP-A\textsuperscript{Cnp1} incorporation at centromeres (Figure 5). As demonstrated here, the Mediator complex binds to the centromere region and regulates Pol II binding and activity. The effect on Pol II transcription is most likely direct, since the Med18-Med20 heterodimer has been shown to physically interact with both TBP and Pol II (8, 34). It is thus possible that Med20 may repress TBP and/or Pol II recruitment during certain phases of the cell cycle, thereby affecting preinitiation complex formation at centromeric transcription units. Interestingly, the interactions between Med18-Med20 and Pol II are mediated by the polymerase subunits Rpb4 and Rpb7 in budding yeast. This observation may be of relevance, since we have previously reported that a mutation in the *rpb7\(^+\)* gene can impair centromeric transcription and RNAi-directed chromatin silencing in fission yeast (14).

Our data reveal that Mediator-dependent transcription regulation is critical for establishment of the correct chromatin structure in both the pericentromeric and
centromeric regions. Deregulated transcription may affect chromatin structure in at least two different ways. First, efficient siRNA production from the *dh* region transcripts, may require precise regulation of ncRNA transcription, which would explain why the observed increase in ncRNA transcript levels results in lower, and not higher, levels of siRNA. Second, loss of regulation leading to higher transcription levels may in itself physically disturb heterochromatin formation. Either by recruiting chromatin-remodeling complexes in a manner, which is not properly coordinated with cell cycle progression or by interfering with the CENP-A<sup>Cnp1</sup> loading machinery.

According to our model, Mediator-dependent transcription repression is also important for the correct incorporation of CENP-A<sup>Cnp1</sup> at the centromere core region. Disruption of *med20<sup>+</sup>* impairs repression of Pol II activity, leads to a strong up regulation of the centromere core region transcription, which in turn causes decreased CENP-A<sup>Cnp1</sup> incorporation (Figure 5). Here, direct effects on transcript levels are more difficult to observe. In fact, the exosome appears to efficiently degrade the centromere core region transcripts as they are being formed, suggesting that it is the transcription-process *per se* that influences CENP-A<sup>Cnp1</sup> incorporation and not the ncRNA products formed. Eventually, loss of CENP-A<sup>Cnp1</sup> results in a dysfunctional kinetochore and chromosome segregation defects. Overall CENP-A<sup>Cnp1</sup> levels are dramatically decreased. We believe that this effect is similar to that seen in *S. cerevisiae*, where CENP-A<sup>Cse4</sup> is rapidly degraded when it is not present in chromatin.
The idea that the basal transcription machinery may be involved in centromere assembly in fission yeast is consistent also with studies of the transcription elongation factor Spt6. Deletion of the spt6+ gene leads to similar centromeric defects as med20Δ, i.e. strongly increased levels of Pol II and non-coding RNA and impaired heterochromatin formation with reduced H3K9me and Swi6. In addition siRNAs produced from the centromeric dh and dg repeats are decreased (28). In view of the effects reported here for Med20 deficiency, it will be interesting to see whether CENP-A Cnp1 assembly is affected also in spt6Δ cells.

Mediator may also affect centromere function by additional pathways. One factor required for proper CENP-A Cnp1 incorporation is Hrp1. This is a CHD1 ATP-dependent chromatin-remodeling factor, which has been shown to interact directly with the centromere during DNA replication in early S-phase (11, 58). Interestingly, we have recently demonstrated that Hrp1 is also a transient component of the Mediator complex in fission yeast (27). This observation raises the interesting possibility that Mediator not only regulates ncRNA transcription in the central core region, but also affects the activity of Hrp1 at specific centromeric nucleosomes, i.e. Mediator can effect CENP-A Cnp1 incorporation by partially independent mechanisms. In fact, a similar mechanism may also exist in higher cells, since human Mediator has been shown to functionally interact with the Hrp1 homologue, Chd1 (35).

In Arabidopsis thaliana, a med20+ homologue regulates production of long non-coding scaffold RNA molecules from heterochromatin, which are subsequently used by the
RNAi machinery (30). This observation raises the interesting possibility that Mediator has a conserved role in heterochromatin formation and that its role for centromere function may be conserved also in other eukaryotes. However, there are also species-specific differences, since loss of Med20 led to reduced pericentromeric ncRNA transcripts and Pol II occupancy in *A. thaliana*, whereas the opposite effects was observed when *med20* was deleted in fission yeast. In future studies, it will be important to investigate effects of Mediator on mammalian centromeres, which similar to fission yeast also depend on the RNAi machinery for proper centromere function (25). Comparative studies in budding yeast may also be relevant. Centromeric transcription is required for incorporation of the *S. cerevisiae* CENP-A homologue, Cse4, even if budding yeast centromeres are distinctly different from those in fission yeast and human cells (42). Interestingly, previous studies have identified the mediator component Med11 (Cse2) as required for proper chromosome segregation in *S. cerevisiae* (59), which could indicate that Mediator regulates centromeric transcription and Cse4 incorporation also in budding yeast. Further studies are required to establish what seems to be an evolutionary conserved role for Mediator in regulation of centromere function and chromosome inheritance in the different branches of the eukaryotic kingdom.
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FIGURE LEGENDS

Figure 1. Mediator components are required for chromosome segregation. (A) TBZ sensitivity test of Mediator mutants. Ten-fold dilution series of the indicated strains were spotted on YEA with and without TBZ followed by incubation at 25 °C. (B) H3K9me and Swi6 occupancy in the centromeric repeat regions (dh and imr) of chromosome I. (C) Images showing DNA content and spindle microtubules in anaphase cells of wt, med20Δ, dcr1Δ, and clr4Δ at 30 °C. Immunofluorescent staining of tubulin, DAPI staining, and merged images are shown as indicated. Scale bar = 5 µm. (D) Diagram showing the percentage of lagging chromosomes in anaphase cells of wt, med20Δ, dcr1Δ, and clr4Δ at 30 °C (n = 100 to 125 per strain). (E) Rad21 occupancy in the core centromeric regions (cnt) of chromosome I and in a control locus (the fbp1 gene). (F) Northern blot showing siRNA levels using dh- and dg-probes. U6 RNA is used as loading control. Relative siRNA fold changes are indicated below each lane. The location of probes are indicated as black dots in Figure 3C.

Figure 2. Mediator occupancy and transcription at centromeres during cell cycle progression. (A and B) Occupancy profiles for Pol II and Mediator (Med7-Myc) at centromere 1. ChIP-Seq data are shown as reads per million (RPM). The centromere structure is shown aligned to the x-axis. In panel A, Pol II occupancy in wt cells (red profile) are compared to that in a med20Δ mutant strain (black profile). (C and D) ChIP analyses of Med7-myc (panel C) and Pol II (panel D) occupancy in the otr (dh) region. ChIP ratios were normalized to no-antibody controls. (E) Septation index for the
experiments in panels C and D. Peak indicating S-phase. (F and G) Quantification of transcript levels by qPCR from the dh and imr regions in synchronized cells. Samples were normalized to act1+. (H) Septation index for the experiments in panels F and G. Error bars indicate standard deviations from at least three independent experiments.

Figure 3. Loss of Med20 causes a defect in centromeric silencing. (A) Ten-fold dilutions of the indicated strains were spotted on YEA, with and without 5-FOA. The locations of the introduced ura4+ markers are indicated after the strain names. (B) RT-PCR showing transcript levels of the ura4+ marker (upper band) and a mutated version of ura4+ (ura4-DSE) transcribed from the endogenous locus (lower band). Reactions without reverse transcriptase (-RT) are shown as controls. (C) A Venn diagram showing how genes involved in centromere function and chromosome segregation overlap with genome-wide transcription changes (cut off 1.5-fold) in the med20Δ strain. (D) RT-PCR analysis of ura4+-marker located in otr-, imr- and cnt-regions. cdc20+ and sir2+ are expressed from a pREP81X vector. Minus (-) indicates without induction and plus (+) indicates with gene induction. Empty vector is shown as negative control. (E) Tiling array analysis show transcription changes in med20Δ normalized to wt; and med20Δ/dcr1Δ normalized to dcr1Δ, in the chromosome I centromere region. A schematic figure of the centromere is shown aligned to the x-axis. Black bars represent previously identified siRNA regions (10). Dots represent siRNA probe location for the Northern blot analyses in Figure 1F. (F) Quantification by qPCR of dh and imr transcripts. Samples are normalized to act1+. Bars show standard deviations of at least three independent cultures. (G) RT-PCR showing transcript levels in the centromere.
regions (dh, imr, and cnt) in wt, med20Δ, rrp6Δ, and med20Δ/rrp6Δ strains. Actin and –RT are shown as controls.

Figure 4. Deletion of med20+ leads to loss of CENP-A<sup>Cnp1</sup>. (A) Localization of CENP-A<sup>Cnp1</sup> fused to CFP in wt, med20Δ, and dcr1Δ cells. Scale bar = 5 μm (B) ChIP analysis of CENP-A<sup>Cnp1</sup> occupancy over the cnt region. Bars show standard deviation. (C) The CENP-A<sup>Cnp1</sup> protein was detected by western blotting using a GFP antibody. Tubulin was used as the loading control. (D) Quantification by qPCR of CENP-A<sup>Cnp1</sup> transcript levels. Bars indicate standard deviation. (E) Diagram showing the effect of Actinomycin D on the percentage of cells with an identifiable CENP-A<sup>Cnp1</sup> signal in the indicated strains (n > 160 per strain). CENP-A<sup>Cnp1</sup> localization was blindly scored and the statistical significance (** p < 0.01, *** p < 0.001) was calculated as described in Material and Methods.

Figure 5. A suggested model for the function of Mediator at centromeres. The Mediator Complex binds to the core centromere region and regulates Pol II binding and activity. Med20 represses Pol II binding to the cnt-region, which promotes incorporation of CENP-A<sup>Cnp1</sup> at the core centromere, forming a silenced chromatin structure. Disruption of med20<sup>+</sup> (indicated in yellow) causes unregulated binding of Pol II to the centromere. The continued transcription impairs the incorporation of CENP-A<sup>Cnp1</sup>, which leads to a dysfunctional kinetochore and chromosome segregation defects.
A

wt

dcr1Δ
med8-598
med18Δ
med20Δ
med1Δ
med31Δ
med15Δ
med27Δ
med20Δ/dcr1Δ

YEA
+TBZ

B

H3K9me/H3 Occup.

Swi6 Occupancy

dh
imr

wt
dcr1Δ
med20Δ
dcr1Δ/med20Δ
rpβ2-203
med12Δ

C

DAPI
Tubulin
Merged

wt
dcr1Δ
Δ

cnt
fbp1

wt
dcr1Δ
Δ

Percent (%)

D

E

Rad21 Occupancy

wt
dcr1Δ
cnt

F

Control

Control

dh

dg

wt
dcr1Δ
Δ

wt
dcr1Δ
Δ

1
0.4
0.0
-100
0.1
-25
-100

A

wt otr
wt imr
wt cnt
med20Δ otr
med20Δ imr
med20Δ cnt
med20Δ/dcr1Δ imr
med12Δ otr
ago1Δ otr

B

+RT
-RT

C

Centromere Function, Chromosome Segregation

D

wt otr
wt imr
wt cnt
med20Δ otr
med20Δ imr
med20Δ cnt
med20Δ/dcr1Δ imr
med12Δ otr
ago1Δ otr

E

med20Δ/dcr1Δ / dcr1Δ

F

Relative expres.

G

WT med20Δ rpb2-m203 med20Δ/dcr1Δ med12Δ

med20Δ / wt
Wild type med20Δ

Pol II

Mediator

Nucleosome

CENP-A Nucleosome

Transcript
ERRATUM

Mediator Promotes CENP-A Incorporation at Fission Yeast Centromeres

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