CSL protein regulates transcription of genes required to prevent catastrophic mitosis in fission yeast

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ABSTRACT

For every eukaryotic cell to grow and divide, intricately coordinated action of numerous proteins is required to ensure proper cell-cycle progression. The fission yeast *Schizosaccharomyces pombe* has been instrumental in elucidating the fundamental principles of cell-cycle control. Mutations in *S. pombe* 'cut' (cell untimely torn) genes cause failed coordination between cell and nuclear division, resulting in catastrophic mitosis. Deletion of *cbf11*, a fission yeast CSL transcription factor gene, triggers a 'cut' phenotype, but the precise role of Cbf11 in promoting mitotic fidelity is not known. We report that Cbf11 directly activates the transcription of the acetyl-coenzyme A carboxylase gene *cut6*, and the biotin uptake/biosynthesis genes *vht1* and *bio2*, with the former two implicated in mitotic fidelity. Cbf11 binds to a canonical, metazoan-like CSL response element (GTGGGAA) in the *cut6* promoter. Expression of Cbf11 target genes shows apparent oscillations during the cell cycle using temperature-sensitive *cdc25-22* and *cdc10-M17* block-release experiments, but not with other synchronization methods. The penetrance of catastrophic mitosis in *cbf11* and *cut6* mutants is nutrient-dependent. We also show that drastic decrease in biotin availability arrests cell proliferation but does not cause mitotic defects. Taken together, our results raise the possibility that CSL proteins play conserved roles in regulating cell-cycle progression, and they could guide experiments into mitotic CSL functions in mammals.

KEYWORDS

Schizosaccharomyces pombe, cut, catastrophic mitosis, premature cytokinesis, transcription factor, periodic gene expression, biotin

INTRODUCTION

Studies in fission yeast, *Schizosaccharomyces pombe*, have yielded fundamental insights into the principles of cell-cycle control.[1] The *S. pombe* cell cycle consists of a prolonged period of growth (G2 phase) followed by a rapid sequence of mitosis, a very short G1 phase, and DNA replication (S phase). Under standard laboratory conditions, division septum formation takes place while the two daughter nuclei are already in S phase.[2,3] In fission yeast, as in other eukaryotes, numerous genes play a role in the coordination of cell and nuclear division. When this coordination is perturbed, a catastrophic mitosis may occur, resulting in the so-called 'cut' (for cell untimely torn) phenotype, in which the nucleus, whose membrane does not break down during fission yeast mitosis, is split by the division septum before completion of mitosis.[4] 'Cut' mutant phenotypes have been described for genes whose products function in various aspects of chromosome biology, such as chromosome condensation or sister chromatid separation.[5] Curiously, mutations in some lipid metabolism genes also produce the 'cut' phenotype, although little is known why or how mitotic fidelity is compromised in these mutants.[6]

The gene expression program of the fission yeast cell cycle comprises some 500 genes, the expression of which oscillates periodically with the cell-cycle phase. Many of these periodic genes are regulated at the transcriptional level, and several transcription factors regulating particular subsets of periodic genes have been identified.[7--9] These factors include Fkh2, Sep1, Mbx1 and the recently identified RFX protein Sak1, that regulate genes involved in mitosis and cytokinesis,[10--14] Ace2 regulating cell separation genes,[15] the MBF complex, Nrm1 and Yox1 controlling DNA replication genes,[16--18] the histone gene regulator Ams2,[19] or Toe1 and Toe2 implicated in the pyrimidine-salvage pathway and division septum formation, respectively.[20] Importantly, the transcription factors driving periodic expression of many cell cycle-regulated genes are yet to be identified.

CSL transcription factors regulate development, cell fate determination and cell-cycle progression in Metazoa, mostly via the Notch signalling pathway.[21] Fungal CSL proteins also exist,[22,23] with Cbf11 and Cbf12, the CSL proteins of fission yeast, also functioning as transcription factors.[24] Cbf11 and Cbf12 have been implicated in the control of cell-cycle progression,[25,26] and regulate directly and indirectly the expression of ~80 cell-cycle periodic genes.[26] Cbf11 also regulates several lipid metabolism genes.[26] Interestingly, cells lacking *cbf11* frequently undergo catastrophic mitosis and display the 'cut' phenotype.[25] Furthermore, the penetrance of the 'cut' phenotype in *Δcbf11* cells is affected by growth media composition.[26] However, the molecular mechanism underlying this severe cell-cycle defect is not known.

In this study, we identify Cbf11 target genes contributing to the 'cut' phenotype of *Δcbf11* cells, and describe the dynamics of their regulation using different cell synchronization methods. We show that Cbf11 is an activator of genes required for proper coordination of cell and nuclear division to prevent catastrophic mitosis.

RESULTS

Mutations in *cbf11* or *cut6* both cause catastrophic mitosis

Our previous analyses revealed that deletion of *cbf11* (*Δcbf11*) causes impaired coordination of cell and nuclear division, resulting in catastrophic mitosis and the 'cut' phenotype (Fig. S1, and refs. 25, 26). The 'cut' phenotype was also described in cells bearing a temperature-sensitive mutation in *cut6* (*cut6-621*), an essential gene encoding an acetyl-CoA/biotin carboxylase, when grown at 36°C. The precise mechanism of how Cut6 promotes mitotic fidelity is not known.[6] The *cut6-621* cells display the 'cut' phenotype even at the semi-restrictive temperature of 30°C (Fig. S1). Notably, we identified *cut6* as one of the putative Cbf11 target genes,[26] among which *cut6* was actually the only gene associated with the 'cut' phenotype.[27] This finding raises the possibility that the catastrophic mitosis observed in *Δcbf11* cells is mediated by *cut6*.

Cbf11 binding to DNA oscillates after *cdc25-22* block-release

One study identified the *cut6* gene as being periodically expressed during the cell cycle with maximum expression in S phase,[8] which in fission yeast coincides with cytokinesis (septation).[2] Our previous genome-wide chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) identified a region in the *cut6* promoter bound by Cbf11. Within that region we found a short sequence element recognized specifically by Cbf11 *in vitro*.[26] We carried out ChIP from cells synchronized using the *cdc25-22* temperature-sensitive allele and confirmed that Cbf11 bound to the *cut6* promoter *in vivo* and, notably, maximum binding occurred in S phase/cytokinesis (Fig. 1A and Fig. S2A).

Previous ChIP-seq experiments in unsynchronized cell cultures (which contain mostly G2 cells) revealed rather low binding of Cbf11 to DNA.[26] It is therefore possible that increased DNA binding in S phase/cytokinesis is a more general property of Cbf11. To test this hypothesis we performed ChIP-seq from cells progressing synchronously through S phase/cytokinesis after *cdc25-22* block-release. Indeed, we found higher Cbf11 occupancy at most of the 284 previously identified Cbf11 target loci in S phase/cytokinesis compared to unsynchronized cells, even though the degree of change in Cbf11 occupancy was locus-specific (Fig. 1B). We also identified novel loci bound by Cbf11, which showed little Cbf11 binding in unsynchronized cells but markedly increased Cbf11 occupancy in S

phase/cytokinesis (Fig. 2A). Interestingly, genes adjacent to these novel Cbf11-bound loci typically showed increased expression in S phase (Fig. 2B, expression data taken from ref. 7).

Two of these novel putative Cbf11 target genes are linked to biotin metabolism: *vht1* and *bio2* encode a biotin (vitamin H) transporter[28] and a biotin synthase,[29] respectively. Moreover, *vht1* is expressed periodically during the cell cycle, with maximum expression in early G2 phase.[30,31] Since the Cut6 enzyme requires biotin for its function, we selected these two genes for further analysis. The promoter of *vht1* contains two Cbf11 response elements (GTGGGAA),[24,25] both corresponding to peaks of Cbf11 ChIP-seq signal; a variant GTGAGTA motif is located in the centre of the Cbf11 ChIP-seq peak in the *bio2* promoter (data not shown). As expected, maximum Cbf11 binding to *vht1* and *bio2* promoters was detected in S phase/cytokinesis by cell-cycle timecourse ChIP (Fig. 1A and Fig. S2A). We conclude that the DNA binding activity of Cbf11 is modulated after *cdc25-22* block-release synchronization and peaks at S phase/cytokinesis.

Cbf11 is required for oscillatory expression of *cut6, vht1* and *bio2* after *cdc25-22* block-release

Next we wanted to determine how Cbf11 affects expression of its target genes and how this regulation is related to cell-cycle progression. We carried out a longer timecourse experiment, spanning two cell cycles synchronized by *cdc25-22* block-release for wild-type and *Δcbf11* cells, and measured *cut6, vht1* and *bio2* mRNA levels using reverse transcription followed by quantitative PCR (RT-qPCR). The expression of *cut6, vht1* and *bio2* in wild-type cells oscillated, with peaks at S phase/cytokinesis or early G2 phase, although the expression peak was considerably less pronounced in the second synchronous cell-cycle (Fig. 1C and Fig. S2B). Notably, the mRNA levels of these genes were decreased in *Δcbf11* cells compared to wild type and any oscillations of expression were lost. The *cdc22* ribonucleotide reductase large subunit gene, a well-studied periodic gene with peak expression at the G1/S boundary,[32] showed periodic expression irrespective of Cbf11 presence. Thus, Cbf11 functions as an activator of expression of the *cut6, vht1* and *bio2* genes, likely via directly regulating their transcription. Furthermore, this regulation shows oscillatory dynamics following *cdc25-22* block-release synchronization.

The apparently periodic expression of Cbf11 target genes might be synchronization artefact

Each cell synchronization method has its limitations and may introduce artefacts.[33] Notably, unlike for the *cdc22* control G1/S-phase gene, the amplitude of oscillatory expression of Cbf11 target genes in the second synchronous cell cycle following *cdc25-22* block-release was markedly diminished (Fig. 1C and Fig. S2B). Therefore, we asked whether these oscillations of Cbf11 targets reflect truly cell-cycle periodic expression or rather artefacts caused by the

synchronization procedure which involves a heat shock. We employed diverse additional cell synchronization methods: *cdc10-M17* block-release, which uses a temperature-sensitive MBF complex mutant to synchronize cells in G1; hydroxyurea block-release, which synchronizes cells in S phase by reversibly blocking dNTP synthesis; and lactose gradient centrifugation, which selects a subpopulation of small early-G2 cells.[2,33] We used these techniques to perform RT-qPCR timecourse analyses of *cut6*, *vht1*, *bio2* and *cdc22* expression during at least two synchronous cell cycles in wild-type cells (Fig. 3 and Fig S3). Cbf11 target genes showed oscillatory expression following *cdc10-M17* block-release that was similar to the *cdc25-22* timecourse pattern (Fig. 3A and Fig. S3A; note that for *cdc10-M17* synchronization the very first S phase after release is not accompanied by septation). No clear oscillatory expression was observed for Cbf11 targets after hydroxyurea block-release (Fig. 3B and Fig. S3B). The expression of *bio2* appeared constant in lactose gradient-synchronized cells, while the expression of *cut6* and *vht1* markedly increased only during the second synchronous cell cycle (Fig. 3C and Fig. S3C). Thus, each synchronization method produced a distinct pattern of Cbf11 target gene expression. By contrast, the expression of the *cdc22* control was clearly periodic in all cases. In summary, Cbf11 targets show oscillatory expression following some but not all synchronization methods tested, and the apparent cell-cycle periodicity of their regulation by Cbf11 might be an artefact.

Cbf11 is a direct regulator of *cut6* expression

Next, we tested whether the putative Cbf11 response element we identified in the *cut6* promoter (GTGGGAA)[26] could mediate regulation of gene expression by Cbf11. We inserted a 30 bp sequence flanking this Cbf11 response element upstream of a minimal promoter in the pREPORT vector.[34] This construct did indeed drive β-galactosidase expression in a Cbf11-dependent manner. No reporter activation was observed when the core 6 nucleotides of the CSL response element were mutated into a XhoI site (GTGGGA → cTcGag) (ref. 34 and data not shown).

We then introduced the same Cbf11 response element mutation into the native *cut6* promoter. The levels of *cut6* mRNA were downregulated in the resulting *Pcut6MUT* strain, to the same extent as in *Δcbf11* cells (Fig. 4A). No further reduction in *cut6* mRNA occurred when the two mutations were combined, confirming that the identified Cbf11 response element is both necessary and sufficient for Cbf11 to activate *cut6* expression (Fig. 4A). Taken together, these findings indicate that Cbf11 is a direct transcriptional activator of *cut6*.

cbf11* interacts genetically with *cut6

The penetrance of the 'cut' phenotype is markedly lower when *Δcbf11* cells are grown in the EMM minimal medium compared to the rich YES medium.[26] Notably, this is also true for the *cut6-621* mutant grown at semi-restrictive

temperature (Fig. 4B), supporting possible implication of *cut6* in the catastrophic mitosis occurring when Cbf11 is missing. While a similar media-dependent trend was observed even for wild-type cells, it was much less pronounced and not statistically significant ($p > 0.05$; one-sided paired t-test).

The *cut6-621* mutation conferred a mild growth defect even at permissive temperature (data not shown). The *Δcbf11 cut6-621* double mutant displayed a severe synthetic-sick phenotype at permissive temperature (Fig. 4C). The hypomorphic mutation in *cut6* combined with the absence of the Cbf11 activator might thus reduce the overall Cut6 activity to a level no longer tolerated by the cell.

The *Δcbf11* cell culture showed strong growth retardation compared to wild type (Fig. 4D). Since cell-cycle duration in synchronized *Δcbf11* cells was very similar to wild type (Fig. 1C), this population-level growth defect was likely attributable to high cell mortality in *Δcbf11* cultures, possibly due to catastrophic mitosis. Unexpectedly, the *Pcut6MUT* strain, featuring *Δcbf11*-like decreased *cut6* expression (Fig. 4A) did not show impaired growth (Fig. 4D), or higher occurrence of catastrophic mitosis compared to wild type (Fig. 5A). Decreased *cut6* mRNA levels alone are thus not sufficient to cause the 'cut' phenotype of *Δcbf11* cells.

We also tested whether *cut6* overexpression could rescue some of the defects observed in *Δcbf11* cells. We replaced the native *cut6* promoter with a strong constitutive *adh1* promoter in both wild-type and *Δcbf11* backgrounds; the resulting *cut6OE* strains showed a ~5 fold increase in *cut6* mRNA levels (data not shown). While *cut6* overexpression had little effect in a wild-type background, the *cut6OE* allele conferred a modest growth improvement (mean doubling time ± SD, n = 3: *Δcbf11* 227 ± 5 min, *Δcbf11 cut6OE* 208 ± 14 min; note the differences in growth curve slope in Fig. 4E) and significantly decreased the frequency of mitotic defects in a *Δcbf11* background (Fig. 5A; $p = 0.01$; one-sided unpaired t-test). These results further demonstrate an important functional link between Cbf11 and Cut6.

Multiple Cbf11-regulated genes are required to prevent catastrophic mitosis

The Cut6 enzyme is presumably synthesized as an inactive precursor and depends upon the addition of a biotin cofactor for activity.[35] In fission yeast, *vht1* and *bio2* are the only known biotin uptake and biosynthesis genes, and both are regulated by Cbf11 (Fig. 1). It is thus plausible that the cellular biotin pool is decreased in the *Δcbf11* mutant, which might affect the functionality of Cut6. To test this hypothesis, we analysed the cellular content of biotinylated proteins in wild-type, *Δcbf11* and *Pcut6MUT* strains (Fig. 5B, C). Notably, biotinylated, and thus likely active, Cut6 levels were significantly decreased in *Δcbf11* cells ($p = 0.023$; one-sided unpaired t-test; compared to wild type), but not in *Pcut6MUT* cells.

Adding biotin into the medium or overexpressing the *vht1* biotin transporter gene did not rescue the mitotic defects of *Δcbf11* cells (data not shown). Curiously, when the *Pcut6MUT* mutation was combined with the deletion of *vht1*, the double mutant but not the single mutant strains showed increased occurrence of catastrophic mitosis ($p = 0.011$ and $p = 0.014$ for double mutant compared to *Δvht1* and *Pcut6MUT*, respectively; one-sided unpaired t-test), which was further exacerbated by deletion of *cbf11* (Fig. 5A; $p = 0.031$; one-sided unpaired t-test). However, the dramatic decrease in total biotinylated protein levels (including Cut6) in *Δvht1* cells (Fig. 5B) was not sufficient for increased occurrence of catastrophic mitosis (Fig. 5A). Deletion of *bio2*, either alone or in combination with the *Pcut6MUT* allele, did not trigger any increase in the frequency of catastrophic mitosis (Fig. 5A). Together, these data indicate that the Cbf11 targets *cut6* and *vht1* are important to prevent catastrophic mitosis, but their decreased expression in *Δcbf11* cells can only partially explain the observed mitotic defects. We therefore conclude that other Cbf11-regulated genes are also involved in the coordination of cytokinesis with mitosis.

Complete biotin removal does not lead to catastrophic mitosis

To further examine the impact of biotin availability on mitotic fidelity we performed a biotin depletion experiment. Wild-type cells were grown in the rich YES medium and then shifted to either standard EMM (1 mg/l biotin) or biotin-free EMM. Cultures were then diluted every 5 hours and samples were taken for analysis. Over the course of 20 hours, cells in the biotin-free medium gradually ceased proliferation (Fig. S4A), became small (Fig. S4B, C), and arrested with DNA content corresponding either to G1 or G2 (Fig. S4D). Importantly, we did not observe increased occurrence of catastrophic mitosis at any timepoint in the absence of biotin (Fig. S4C and data not shown), suggesting that a drastic drop in biotin availability leads to cell-cycle arrest rather than mitotic defects.

DISCUSSION

Fission yeast CSL proteins are important for multiple aspects of cell-cycle progression and contribute to the regulation of periodic genes.[20,25,26] In the present study, we show that Cbf11 directly regulates the transcription of several genes required to prevent catastrophic mitosis (Fig. 5D).

Notably, the metazoan Notch/CSL pathway also regulates cell-cycle progression and the expression of some periodic genes.[36--39] Furthermore, deregulation of the Notch pathway triggers catastrophic mitosis in mammalian cells, although any specific roles of CSL proteins in this process have not been investigated.[40,41] Detailed analysis of *cut6* regulation by Cbf11 revealed that the responsiveness of the *cut6* gene to Cbf11 is mediated by a promoter sequence motif

(GTGGGAA) matching the canonical response element of metazoan CSL transcription factors (GTGRGAA).[42,43] Moreover, we found similar sequence motifs within the Cbf11-bound regions of the *vht1* and *bio2* promoters. These results document a striking degree of functional conservation among CSL family members across large evolutionary distances, and they may give valuable clues about cell cycle-related roles of CSL proteins in Metazoa.

The precise role of Cut6 in coordinating cytokinesis with mitosis is not known. Cut6 is an acetyl-CoA/biotin carboxylase, the rate-limiting enzyme of fatty acid biosynthesis, but a hypothetical regulatory role of Cut6 in cell-cycle progression has also been proposed.[6] During the closed mitosis of both *S. pombe* and the budding yeast, fatty acid synthesis is required for nuclear membrane expansion and, subsequently, spindle elongation and proper chromosome segregation.[44,45] Chemical inhibition of the fatty acid synthase Fas1 subunit results in the 'cut' phenotype in fission yeast.[45] The synthesis and/or addition of new phospholipids into the nuclear membrane is highly localized in the budding yeast.[46,47] Interestingly, Cut6 interacts with Sad1, a spindle pole body (SPB; yeast centrosome) component.[48] Thus, an SPB-localized Cut6 pool could hypothetically contribute to and/or regulate the production of new nuclear membrane, possibly in a Cbf11-dependent manner, to support proper mitotic progression. Recently, multiple Cbf11 target genes, including *cut6*, *vht1* and *bio2*, were shown to be regulated by Mga2, a transcription factor (analogous to mammalian SREBP-1) controlling oxygen-responsive lipid homeostasis in *S. pombe*.[49] However, it is not yet clear whether or how Cbf11 and Mga2 cooperate to regulate lipid metabolism and/or cell-cycle progression. Intriguingly, it has recently been reported that mammalian cells remodel their membrane lipid composition during the cell cycle, and these periodic changes are important for proper cell-cycle progression.[50--52]

Curiously, the mitotic defects of the *Δcbf11* and *cut6-621* mutants were alleviated when cells were grown in the minimal EMM medium (Fig. 4B and ref. 26). We have shown previously that *Δcbf11* phenotypes can be suppressed by the deletion of *pka1*, which encodes the catalytic subunit of the PKA protein kinase, a positive regulator of cell proliferation in response to nutrients.[26] PKA counteracts the anaphase-promoting complex/cyclosome (APC/C), which is required for proper progression through mitosis. APC/C mutants also show the 'cut' phenotype. Notably, PKA inactivation or cultivation in EMM can also rescue APC/C 'cut' mutants.[5,53,54] It would be interesting to test directly whether Cbf11 and Cut6 are functionally linked to APC/C.

We show that Cbf11 regulates *vht1* and *bio2*, the only known fission yeast biotin uptake and biosynthesis genes. Biotin (vitamin H) deficiency underlies several human diseases and is teratogenic in animal models.[35,55] Notably, Vht1 has

recently been shown to play a role in the resistance to bortezomib, a proteasome inhibitor and an anti-cancer drug that causes aberrant mitosis and 'cut' phenotype in fission yeast, likely via inhibiting degradation of securin and cyclin B and thus blocking the metaphase--anaphase transition.[56,57] Our results show that complete removal of biotin from the medium leads to cell proliferation arrest without any noticeable mitotic defects (Fig. S4). However, this is a rather drastic disturbance, and it is still possible that less severe perturbations of biotin metabolism contribute to decreased mitotic fidelity seen in the *Δcbf11* and *Δvht1 Pcut6MUT* strains (Fig. 5A).

We also show that Cbf11 exerts its regulatory activity in a seemingly cell cycle-modulated manner, peaking at S phase/cytokinesis after *cdc25-22* block-release (Fig. 1). However, when we followed Cbf11 target gene expression during two synchronized cell cycles, oscillations in mRNA levels were mostly present only in the first cycle. Previous transcriptomic studies identified *cut6* and *vht1* as cell-cycle periodic genes peaking at S and early G2 phase, respectively.[7--9,30] Nevertheless, the periodicity of *cut6* expression was only claimed by one of these studies.[8] Our extensive testing of three other synchronization methods revealed that expression patterns of Cbf11 target genes were not consistent across different methods, with only the *cdc10-M17* block-release results resembling the initial observations from *cdc25-22* block-release (Fig. 3). As different synchronization methods can alter cell physiology and gene expression in different ways,[2,33] it is possible that any observed oscillations in mRNA levels of *cut6*, *vht1* and *bio2* are synchronization artefacts. Importantly, this regulation of gene activity is Cbf11-dependent (Fig. 1C). A potential explanation is that Cbf11 targets might become transiently upregulated due to the heat shock employed in both *cdc25-22* and *cdc10-M17* block-release. Interestingly, *cbf11* interacts genetically with *sty1*,[26] which encodes the main fission yeast stress-activated protein kinase (homolog of mammalian p38). Sty1 is essential for transcriptional response to heat shock,[58] and contributes to the regulation of cell-cycle progression.[59] The expression of *cbf11* remains largely unchanged under various stresses, including heat shock.[58] It is therefore possible that any hypothetical stress-dependent control over Cbf11 activity would be achieved at the post-translational level. Indeed, two phosphorylation sites, S104 and S117, have been identified in Cbf11,[60--62] although it is not known whether their phosphorylation status changes after stress and which kinase(s) are involved. Another potential explanation for the oscillating expression of Cbf11 target genes relates to altered nuclear volume following some synchronization methods. The ratio between nuclear volume and the volume of chromosomes is important for mitotic fidelity,[44,45] and increased nuclear volume (with normal DNA content and compaction) found in the *cdc25-22* mutant background can suppress chemically-induced catastrophic mitosis.[45] Thus, hypothetically, altered nuclear size during the first cell cycle following *cdc25-22* (and

possibly *cdc10-M17*) block-release synchronization might result in aberrant regulation of lipid biosynthesis genes. This misregulation would then be resolved in subsequent cell cycles, as cells attain normal proportions. In this scenario, Cbf11 would be needed for coupling the sensing of nuclear volume to the production of new membrane components.

In summary, we have shown that Cbf11 is an activator of several genes, including the Cut6 acetyl-CoA/biotin carboxylase, that are required for mitotic fidelity. Cbf11 regulates Cut6 via a canonical, metazoan-like CSL response element, suggesting functional conservation of the CSL protein family during evolution. Our results indicate potential mechanisms of how Cbf11 and its target genes act to prevent catastrophic mitosis, that will be addressed by future studies. Such research will shed further light into the intricacies of transcriptional control supporting cell-cycle progression.

MATERIALS AND METHODS

Yeast culture

Fission yeast cells were grown according to standard procedures at 30 or 32°C,[2,63] unless stated otherwise; temperature-sensitive strains were grown at 25°C. For *cdc25-22* and *cdc10-M17* block-release experiments, cell cultures grown in YES were synchronized at 36°C for 4 hours and released by rapid cooling to 25°C. For hydroxyurea block-release experiments, cell cultures grown in YES at 32°C were synchronized in the presence of 12 mM hydroxyurea for 4 hours and then released by washing and resuspending into fresh YES.[2] The protocol for cell synchronization by lactose gradient centrifugation was derived from ref. 2. Briefly, exponentially growing cells were centrifuged (200 × g, 3 min) through two consecutive 10-40% lactose gradients (volumes 45 and 10 ml, respectively; lactose was dissolved in YES). Approximately 10^9-10^{10} cells were layered on top of the first gradient. The collected fraction of small, early-G2 cells was washed and resuspended into fresh YES. Cell-cycle progression was monitored by flow cytometry and/or microscopy. Under standard cultivation conditions, the S phase coincides with the division septum formation in fission yeast.[2] Growth curves were measured in the VarioSkan Flash instrument (Thermo Scientific) using 12-well dishes and 1.4 ml culture volumes. For biotin depletion experiments, precultures were grown in YES and then cells were washed and resuspended into either standard EMM (1 mg/l biotin) or biotin-free EMM. The list of fission yeast strains used in this study is provided in Table S1.

Microscopy

Cells were fixed in 70% ethanol, rehydrated in water, stained with DAPI (1 μg/ml) and photographed using the Olympus CellR system. To quantify the occurrence of catastrophic mitosis, at least 200 cells (exponential growth phase) per sample were scored. Cell length measurements were performed in imageJ[64] and at least 46 cells were scored per sample.

Plasmids and strain construction

The lists of oligonucleotides and plasmids used in this study are provided in Tables S2 and S3, respectively. Transcription reporter plasmids were based on pREPORT-U (ref. 34) and had oligonucleotides (cut6r-fwd & cut6r-rev; cut6mutr-fwd & cut6mutr-rev) containing CSL response elements inserted upstream of the minimal promoter. The β-galactosidase assays were performed as described previously.[34]

For *vht1* overexpression, the *vht1* ORF was amplified by PCR (primers mp165 & mp166), cloned into pGEM-T Easy (Promega), validated by sequencing, and subcloned into the BamHI/SalI sites of pDUAL-FFH51.[65] The resulting plasmid pMP118 was linearised with NotI and integrated into the *leu1-32* locus. Vht1 functionality was tested by complementation of a *Δvht1* strain.[28]

For *cut6* overexpression, the native *cut6* promoter was replaced by the strong constitutive *adh1* promoter by one-step integration of a PCR-amplified replacement cassette using the pFA6a-natMX6-PADH1-3HA vector template (primers mp222 & mp223).[66] Successful integration was verified by PCR (primers mp159 & mp160 & mp224) and *cut6* overexpression was confirmed by RT-qPCR.

The procedure for the *cut6* promoter mutagenesis was adapted from ref. 67. The *ura4* gene (promoter, ORF, terminator) was amplified from wild-type (JB32) genomic DNA using primers mp161 & mp162 and inserted into the pCR-2.1 vector by TA cloning (Invitrogen). Subsequently, regions flanking the Cbf11 response element (located 28 bp upstream of the *cut6* exon 1) were amplified using primers mp157 & mp158 and mp159 & mp160, respectively, and inserted in the pCR-2.1-ura4 plasmid in the HindIII/BamHI and NotI/XbaI sites, respectively, yielding plasmid pMP110. Primers mp158 and mp159 each contained an internal XhoI site, allowing *ura4* gene excision from the pMP110 construct. Removing *ura4* resulted in a DNA insert identical to the endogenous *cut6* promoter region, except for the Cbf11 response element that was replaced with an XhoI site (pMP113). The HindIII/XbaI fragment of pMP110 was then used

to knock-out the Cbf11 response element in the *cut6* promoter in a *ura*$^-$ haploid background by homologous recombination, producing a *ura*$^+$ strain (MP340). The integration was checked by PCR. Next, the HindIII/XbaI fragment of pMP113 was used to substitute the *ura4* cassette in the genome for the mutated Cbf11 response element (i.e., XhoI site). Clones resistant to 5'-fluoroorotic acid (*ura*$^-$) were further checked by PCR and sequencing (MP342).

Deletion of the *bio2* gene was performed using the pCloneHyg1 system (primers mp198 & mp199; mp200 & mp201).[68] Deletion was verified by PCR (primers mp202 & mp144; mp33 & mp203).

All other strains used in this study were constructed by standard genetic crosses.

qPCR and RT-qPCR

Total RNA was extracted from cells using the MasterPure Yeast RNA Purification Kit (Epicentre) and converted to cDNA using random primers and the RevertAid™ Reverse Transcriptase kit (Fermentas). Quantitative PCR was performed using the MESA GREEN qPCR MasterMix Plus for SYBR (Eurogentec) and the LightCycler 480 II instrument (Roche). For RT-qPCR, *act1* (actin) was used as a reference gene. The primers used are listed in Table S2.

Chromatin immunoprecipitation and deep sequencing (ChIP-seq)

Five hundred millilitre cultures of an untagged strain and strains expressing TAP-tagged Cbf11 were grown in YES, synchronized by *cdc25-22* block-release and collected in S phase (coincides with peak of septation in both wild-type and *Δcbf11* cells). Chromatin immunoprecipitation was performed as described previously;[26] two loci not bound by Cbf11 were used as controls for ChIP-qPCR (see Table S2 for the respective chromosome coordinates). Sequencing libraries were prepared using the NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina and NEBNext Multiplex Oligos for Illumina (New England Biolabs) according to manufacturer's instructions, and sequenced on an Illumina MiSeq instrument (12-fold multiplexing per run). Two independent biological repeats were performed. Sequencing reads were aligned to the reference fission yeast genome (release 12) using BWA 0.6.1,[69] processed with the samtools 0.1.18 package,[70] and inspected using IGV browser 2.0.23 and the igvtools package.[71] ChIP-seq data are available in the ArrayExpress database under accession number E-MTAB-2725.

Western blotting

Proteins were separated on a 7.5% Tris-glycine-SDS gel, transferred to a nitrocellulose membrane and probed with either a streptavidin-HRP polymer (S2438, Sigma; 1:1,000 dilution) or mouse monoclonal anti-PSTAIR antibody (anti-Cdc2 loading control; P7962, Sigma; 1:8,000 dilution), as appropriate. A goat-anti-mouse HRP-conjugated secondary antibody (sc-2031, Santa Cruz; 1:3,000) was used where required. Detection was performed using the ECL Prime Western Blotting Detection Reagent (Amersham) and the ImageQuant LAS 4000 instrument (GE Healthcare). ECL signals were quantified using ImageJ.[64] To determine the position of Cut6 on polyacrylamide gels, cells expressing tagged Cut6 from an integrated pDUAL-FFH1c plasmid were used.[72]

Flow cytometry

For analysis of DNA content by flow cytometry, ethanol-fixed cells were treated with RNase and stained with 4 µg/ml propidium iodide as described previously,[26] and analysed using the CytoFLEX instrument and CytExpert software version 1.2.11.0 (Beckman-Coulter). At least 1,000 singlet cells were measured per sample.

Data processing and statistics

The R software environment for statistical computing and graphics (https://www.r-project.org/) was used for data processing, statisti.

ABBREVIATIONS

ChIP-seq -- chromatin immunoprecipitation followed by deep sequencing

cut -- cell untimely torn

EMM -- Edinburgh minimal medium

RT-qPCR -- reverse transcription followed by quantitative real-time PCR

YES -- yeast extract with supplements

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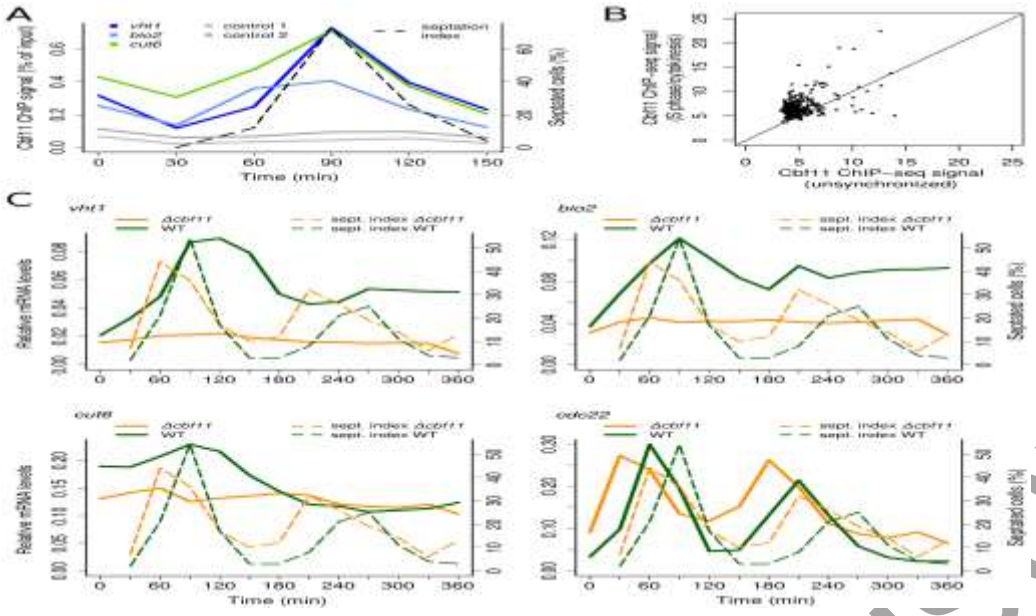

Figure 1. Cbf11 regulates gene expression in an apparently cell-cycle phase-specific manner after *cdc25-22* block-release.(A) Timecourse ChIP analysis of Cbf11 binding to *cut6*, *vht1* and *bio2* promoters and control unbound loci in cells synchronized in late G2 by *cdc25-22* block-release. S phase coincides with division septum formation (see 'septation index'). Results representative of two independent experiments are shown. **(B)** Comparison of normalized Cbf11 ChIP-seq signal (area under ChIP-seq peak) from unsynchronized[26] and S-phase/cytokinesis cells synchronized by *cdc25-22* block-release. Pooled data from two independent experiments are shown. **(C)** Timecourse RT-qPCR analysis of *cut6*, *vht1* and *bio2* mRNA levels in wild-type and *Δcbf11* cells synchronized by *cdc25-22* block-release. The *cdc22* gene is a positive control, peaking at the G1/S boundary.[32] Results representative of two independent experiments are shown.

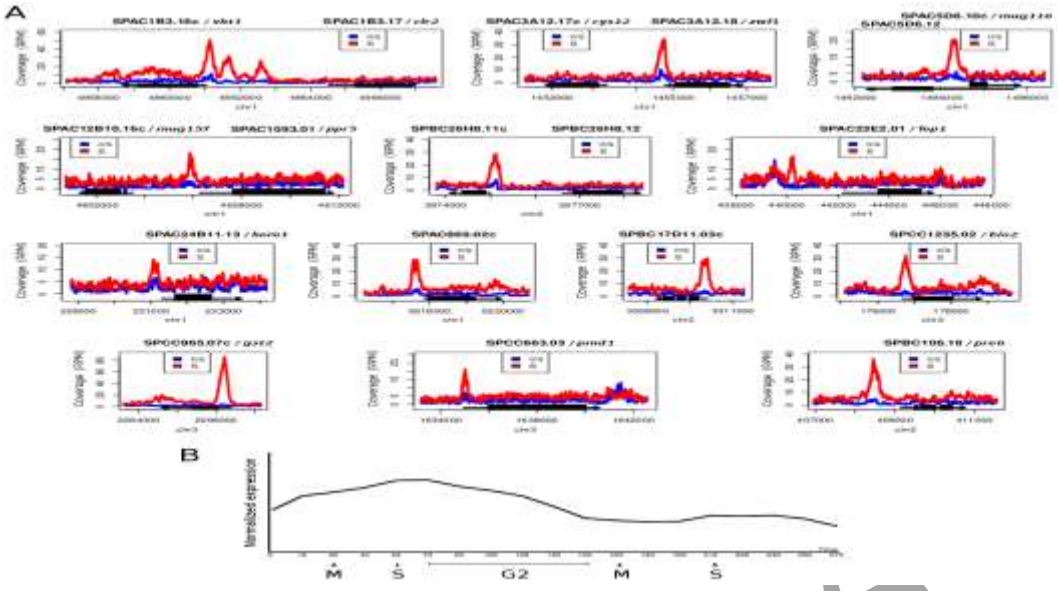

Figure 2. Genes with markedly increased Cbf11 binding to their promoters during S phase/cytokinesis following *cdc25-22* block-release.(A) Pooled data from two independent ChIP-seq experiments are shown. 'n/s' - not synchronized, 'S' - S phase/cytokinesis. **(B)** Average expression levels of genes from (A) during two cell cycles, post *cdc25-22* block-release. Data were taken from ref. 7. Expression peaks around S/G2.

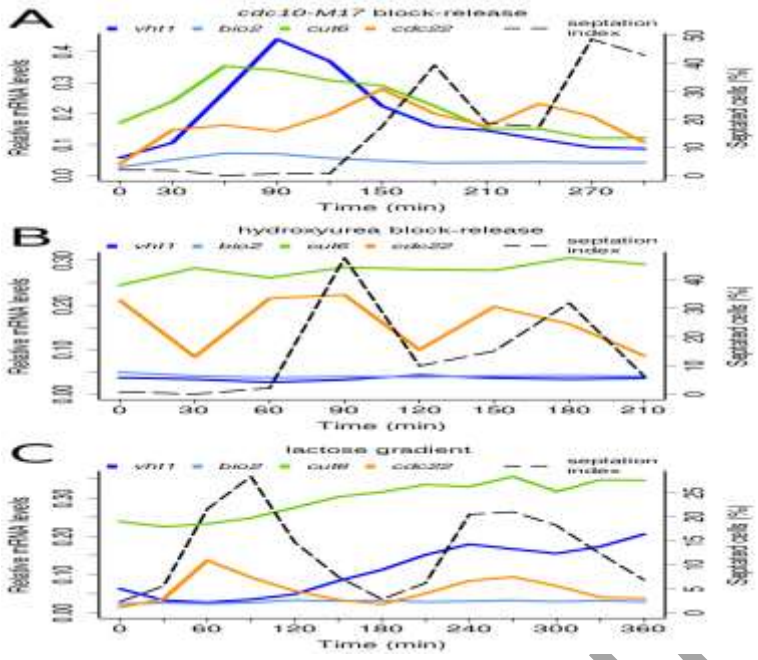

Figure 3. Expression patterns of Cbf11 target genes depend on synchronization method.

Timecourse RT-qPCR analysis of *cut6*, *vht1* and *bio2* mRNA levels in wild-type cells synchronized by *cdc10-M17* block-release **(A)**, hydroxyurea block-release **(B)**, and lactose gradient centrifugation **(C)**. S phase coincides with division septum formation (see 'septation index'); note that for the synchronization method used in panel (A) the very first S phase is never accompanied by septation. The *cdc22* gene is a positive control, peaking at the G1/S boundary.[32] Note that *cdc22* is induced by hydroxyurea treatment even at time 0 in (B).[7] Results representative of three independent experiments are shown.

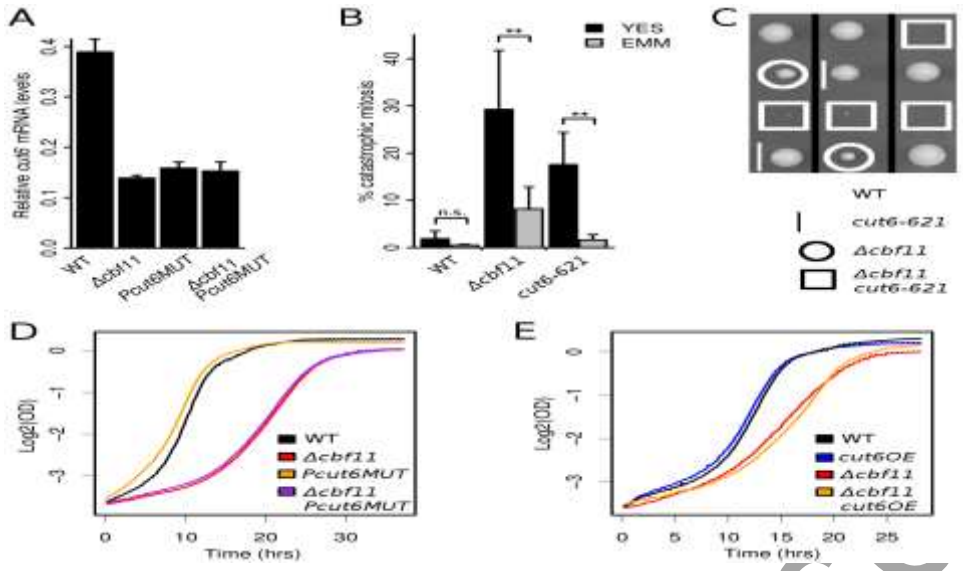

Figure 4. Cbf11 is a direct activator of *cut6* expression. (A) RT-qPCR analysis of *cut6* expression. Mean values ± SD from 3 independent experiments are shown. **(B)** Differential occurrence of catastrophic mitosis in cells grown in YES and EMM. Mean values ± SD from 6 independent experiments are shown. Significance was determined by one-sided paired t-test (** $p \leq 0.01$; 'n.s.' $p > 0.05$). **(C)** Deletion of *cbf11* shows a severe synthetic sick genetic interaction with *cut6-621* at permissive temperature. Segregants from three representative meioses are shown. **(D)** The *Δcbf11* strain, but not the *Pcut6MUT* promoter mutant strain, showed reduced growth compared to wild type. Results representative of ≥3 independent experiments are shown. **(D)** Effect of *cut6* overexpression on wild-type and *Δcbf11* cell proliferation. Results representative of 3 independent experiments are shown.

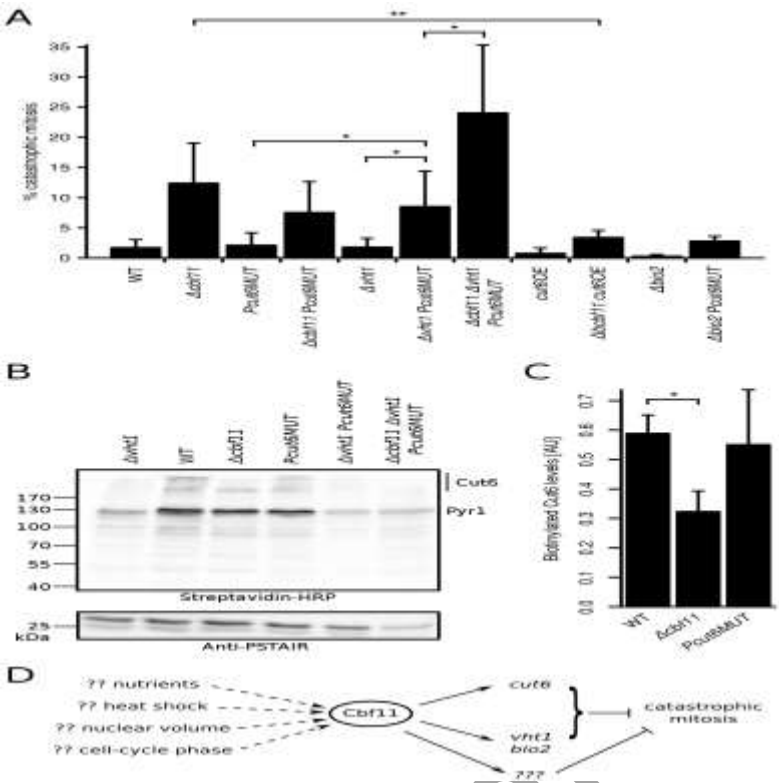

Figure 5. Cbf11-regulated genes are needed to prevent catastrophic mitosis.(A) Cells were fixed, stained with DAPI and the occurrence of catastrophic mitosis was determined. Mean values ± SD from ≥3 independent experiments are shown. Significance was determined by one-sided unpaired t-test (* $p \leq 0.05$; ** $p \leq 0.01$). **(B)** Western blots of whole cell extracts were probed with streptavidin-HRP to visualize biotinylated proteins, and with anti-PSTAIR antibody as a loading control. The position of Cut6 (two bands) and the expected position of pyruvate carboxylase Pyr1 are indicated. Results representative of 3 independent experiments are shown. **(C)** Densitometric quantification of Cut6 ECL signals from (B). Mean values ± SD of 3 independent repeats are shown. Significance was determined by one-sided paired t-test (* $p \leq 0.05$). **(D)** Model of gene expression regulation by the Cbf11 transcription factor. Cbf11 binds to target promoters and activates transcription of genes required to prevent catastrophic mitosis. Speculative inputs influencing Cbf11 activity are indicated on the left and discussed in the main text.