



Original Article

Oca2 of *Schizosaccharomyces pombe* Regulates Pol II Transcriptional Termination

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ABSTRACT: We describe a *Schizosaccharomyces pombe* protein kinase, Oca2, which is involved in transcription termination of a subset of genes. Lack of Oca2 results the accumulation of Pol II over termination regions. Oca2 phosphorylates *S. pombe* homologue of Ctk2, the putative cyclin subunit of fission yeast Ctk1, *in vitro*. In *S. cerevisiae*, Ctk1 phosphorylates serine 2 of the CTD of RNA polymerase II (Pol II), a modification associated with transcriptional elongation, termination and co-transcriptional pre-mRNA processing. Both, Oca2 and Ctk2 localize predominantly to terminator regions, and in *oca2Δ* cells Ctk2 localization to terminators is lost. Oca2 mediated recruitment of Ctk2 may be a prerequisite for correct Pol II release from certain termination sites.

KEYWORDS: transcriptional termination, CTD phosphorylation, polyadenylation factors.

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INTRODUCTION

The mechanism of transcription termination by RNA polymerase II (Pol II) has been under intense scrutiny in recent years.¹ A major level of gene regulation occurs through the co-transcriptional processing of pre-mRNA as it is synthesised by elongating Pol II.² A key coordinator in this process is the C-terminal domain of the large PolIII subunit Rpb1, which serves as a binding scaffold for a variety of nuclear factors (Cramer et al., 2001). Several CTD kinases have been identified. The TFIIF associated Cdk7/cyclinH complex is responsible for S5-phosphorylation of the CTD in metazoan (Kin28/Ccl1 in budding yeast, Mcs6/Mcs2/Pmh1 in fission yeast). S2 of the CTD is phosphorylated by the metazoan positive transcription elongation factor P-TEFb, consisting of the catalytic subunit Cdk9 and the regulatory subunit cyclinT1.

S. cerevisiae has two possible homologues of P-TEFb: the essential Bur1/Bur2 complex,³ and the non-essential CTD-K1 complex comprising Ctk1, Ctk2 and Ctk3.^{4,5} Both factors have been implicated in transcription elongation and phosphorylate the CTD *in vitro*.⁷⁻⁹ However, *bur1* and *ctk1* mutant phenotypes suggest different roles during transcription. *bur1* mutants show reduced cross-linking of Pol II towards the 3' end of the gene. In contrast, lack of Ctk1 has little effect on the distribution of Pol II, but results in reduced S2 phosphorylation levels and impaired recruit-

ment of polyadenylation factors towards the 3' end of the gene.¹⁰ Ctk1 genetically interacts with the polyadenylation factor Pti1, and deletion of Ctk1 affects poly (A) site choice of particular transcripts.¹¹

We have sought to obtain further molecular components in the Pol II termination process by employing a genetic screen for termination factors in *S. pombe*. The basis of our original screen was to insert a synthetic intron containing termination signals into the *ura4* gene.¹² The phenotype of this mutant strain is *ura-*, as Pol II terminates in the intronic termination sequence and thus prevents *ura4* expression. Mutants, that cause RNA Pol II to read-through the intronic terminator and so splice out the intron sequence, were selected based on their *ura+* phenotype. Several mutant genes were obtained by this genetic approach and have resulted in the identification of polyadenylation factors chromatin remodeling factors in Pol II termination.¹⁴

In this report we describe another termination factor identified using this genetic screen. We show that the previously uncharacterized kinase Oca2 is involved in Pol II termination of a subset of genes. Loss of Oca2 leads to build up of RNA Pol II over the poly(A) site region. Oca2 phosphorylates *S. pombe* homologue of Ctk2 and both proteins localize to the 3'end of genes. In *oca2Δ* cells, Ctk2 no longer localizes to terminator regions. This suggests that Oca2 is required for the final stages of Pol II termination through its ability to recruit Ctk2 to 3' ends.

MATERIALS AND METHODS

S. pombe strains and medium

The following strains were employed: WT (A2, *h+ ade6-M216 his3-D1 leu1 1-32 ura4-D1*,¹³ *Oca2Δ* (*h+ ade6-M216 his3-D1 leu1 1-32 ura4-D18 Oca2Δ::kanMX6*); *Oca2-HA* (*h+ ade6-M216 his3-D1 leu1 1-32 ura4-D18 Oca2-HA::kanMX6*); *ctk2* (*h+ ade6-M216 his3-D1 leu1 1-32 ura4-D18 ctk2::kanMX6*); *Ctk2-myc* (*h+ ade6-M216 his3-D1 leu1 1-32 ura4-D18 Ctk2-myc::kanMX6*). Techniques for growth conditions, maintenance and genetic procedures used in this work have been previously described.¹⁹ Standard genetic procedures were applied for deletion and tagging of *oca2* and *ctk2* using respective plasmid pFa6a-KanMX6.²²

Isolation of *m47* mutant, cloning of *oca2+* and RNA analysis

Isolation of *m47* mutants used in the complementation studies, construction of genomic library and transformation procedure are all previously described.¹³ Northern blots and transcriptional run-on (TRO) analyses were also performed as described.

Preparation of *S. pombe* whole cell protein extracts and Kinase assay

Total cell extracts were prepared as described previously.²⁰ To purify *Oca2-HA*, total protein extract from *Oca2-HA* expressing strain (expression was verified by western blot using anti HA antibody, data not shown) was prepared as described and incubated with anti-HA agarose beads and the *Oca2* kinase was eluted from the beads with 0.1 mg/ml of HA-peptide (Roche) in buffer C (20 mM Tris-Hcl pH 7.5, 150 mM NaCl, 0.01% NP-40, 10% glycerol).

To identify *Oca2* substrate, total protein extracts were prepared from wild type *S. pombe* cells and fractionated by a Superose 6 column. The fractions were subjected to kinase assay with *Oca2*. The positive fractions were further purified through monoQ column and collected as flow through (FT) fraction and monoQ elute. This fraction was again subjected to the kinase assay and the positive substrates identified by mass spectrometry.

To perform a specific kinase assay with *Ctk2*, *Ctk2* was affinity purified using protein extract from cells expressing *Ctk2-myc*. Immunoprecipitation was carried out with anti-myc agarose (Sigma) and beads were washed 3x with IP buffer (20 mM tris-Hcl pH 8.0, 200mM NaCl, 0.05% NP-40) and 2x with kinase buffer (10 mM HEPES, pH 7.5, 75 mM KCl, 5mM MgCl₂). The reaction was carried out with beads enriched with *Ctk2-myc* containing 2x kinase buffer, 1mM ATP, 5μ Ci of γ ³²P-ATP (Amersham) and purified *Oca2-HA* kinase at 30°C for 1hr. The reaction was stopped by addition of SDS sample buffer. All kinase reactions were performed as described previously.²¹

Chromatin Immunoprecipitation (ChIP) analysis

ChIP analysis was performed as described previously.²³ Anti HA antibody was purchased from Covance and anti *Ctk2* antibody was developed in the lab. Immunoprecipitated DNA was quantified using real-time PCR with a QuantiTect SYBR green kit (Qiagen).

RESULTS

Identification of *oca2+* as a Pol II termination factor

Our genetic screen for Pol II termination factors employed the *S. pombe*¹² strain U1, which is deleted for the endogenous *ura4* gene, but contains an integrated and modified *ura4* with an inserted synthetic intron additionally containing termination signals. The phenotype of this modified strain is *ura-*, as Pol II terminates in the intronic termination sequence and thus prevents *ura4* expression. Termination mutants that cause Pol II to read-through the *ura4* intronic terminator and so splice out intronic sequence, were selected based on their *ura+* phenotype (Figure 1A). This approach has identified a number of uncharacterized termination mutant and one such mutant strain, *m47*, was subjected to both nascent and steady state analysis using transcription run on (TRO) and Northern blot analyses respectively. TRO analysis was performed by transforming both U1 and *m47* with TRO reporter plasmid pNU which expresses a hybrid *nmt1-ura4* gene using the strong *nmt* promoter and *ura4* termination signals. As shown by TRO analysis (Figure 1B), *m47* mutant failed to terminate over probe 3 causing read-through signals into the downstream sequences (probe 4-5). But in marked contrast, transcription terminated efficiently over the *ura4* poly (A) signals region in U1. In steady state analysis (Figure 1C), *ura4* U1 gave a single truncated transcript (Term) corresponding to RNA terminated within synthetic intron. In contrast *m47* gave overall reduced transcript levels, but with significant read through products (RT) some corresponding to spliced mature *ura4* mRNA. Other aberrant *ura4* transcripts were also detectible presumably corresponding to miss-spliced products.

In an attempt to identify which gene was mutated in *m47* strain, an extensive complementation screening was employed by transforming *m47* with *S. pombe* genomic library. Although no authentic gene was identified that provide information about the original mutation of *m47* mutant gene, a particular genomic plasmid was found to complement the original mutant, switching its phenotype from *ura+* back to *ura-* (isolated by back selection with 5-FOA). Since this complementing *S. pombe* DNA sequence was found to be unchanged in the *m47* mutant as compared to the wild type sequence, we infer that this complementing DNA probably acts as a suppressor of *m47*. Sequence analysis revealed the presence of a single open reading frame (SPCC1020.10) within this complementing sequence, which codes for a predicted serine/threonine

protein kinase of 650 amino acids. During the course of our studies SPCC1020.10 was independently identified as *oca2+* in a screen for *S. pombe* genes causing over expression-mediated cell division arrest¹⁵. Northern blot analysis of *m47* transformed with the genomic library *poca2* confirmed that the *m47* read-through profile reverts to a U1 profile with no significant read-through (RT) or *ura4* mRNA signal detectible (Figure 1C, lane 4).

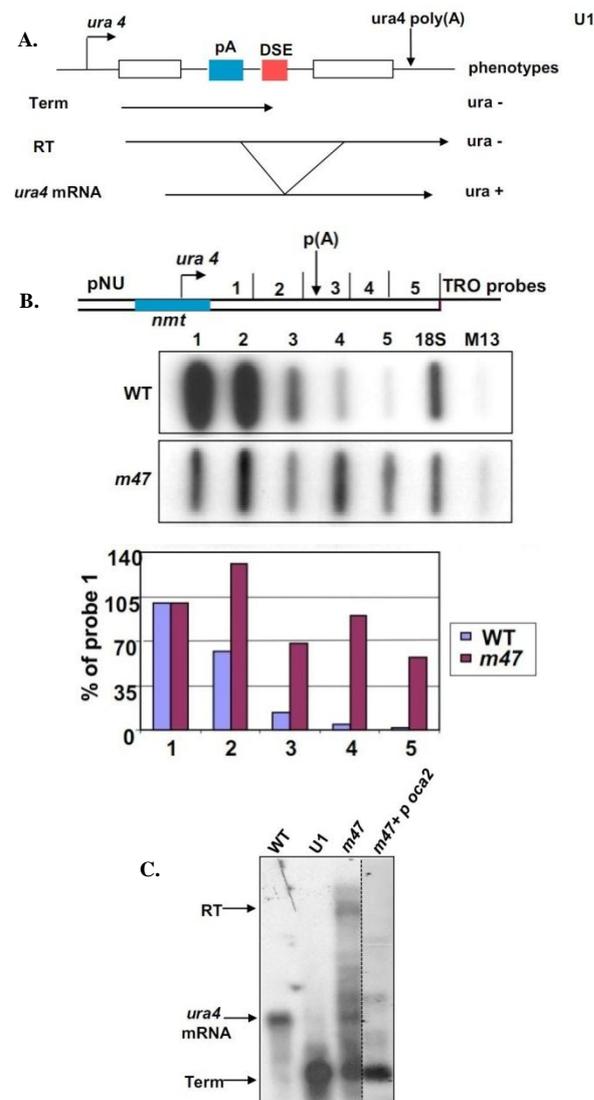


Figure 1. Isolation of Pol II transcription termination mutant *m47* and its complementing gene *oca2+*. (A) Diagram integrated *ura4* construct in U1 strain showing synthetic intron in *ura4* with added terminator elements (pA and DSE) (3). Expected *ura4* transcripts are indicated for U1 or termination defective cells. (B) Northern blot of *ura4* mRNA detected from WT *S. pombe* (transformed with *ura4+* transcript), U1, *m47* and *m47* strains complemented with plasmid containing *oca2*. Respective transcripts were indicated by arrow. (C) TRO analysis of pNU plasmid as indicated transformed into U1 and *m47* cells. The TRO reporter plasmid pNU contains the *ura4* gene transcribed from *nmt1* promoter. Locations of TRO probes are indicated. The signal from each probe was corrected for M13 background level and U content and normalized to probe 1 (taken as 100%). Quantitation of TRO signals is shown below TRO panels. TRO analysis was repeated multiple times and data are the mean percentage of 3 independent experiments, \pm SD.

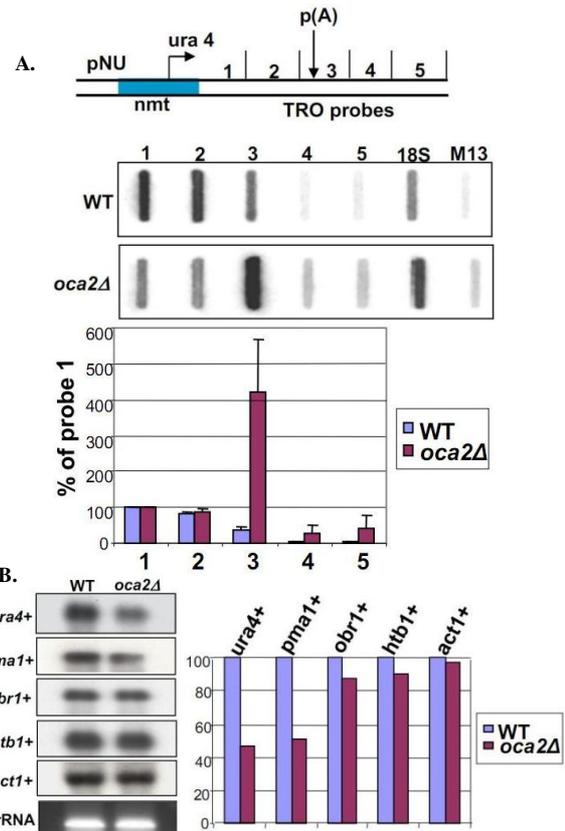


Figure 2. Deletion of *Oca2* affects Pol II transcriptional termination. (A) TRO analysis of wild type (WT) and *Oca2* strains transformed with TRO reporter plasmid. TRO signals were analyzed as in figure 1B. This is representative image of 3 independent experiments and quantitation was presented as a mean percentage, \pm SD. (B) Northern blot analysis of total RNA extracted from WT and *Oca2* cells. Filters hybridised with random-primer labelled probes for mRNAs as indicated. Ethidium bromide staining of rRNA provides loading control. Quantitated RNA levels are shown graphically in the panel below with the WT level 100%.

Oca2 affects nascent and steady state mRNA levels

To investigate the potential role of *Oca2* on Pol II termination we generated an *S. pombe* deletion strain (*oca2Δ*) by inserting a KanMX6 marker gene into the chromosomal locus of the *oca2+* gene. Disruption of *oca2* is non-lethal, but *oca2Δ* cells have a slow-growth phenotype in minimal media as compared to isogenic wild type cells (data not shown). To investigate the role of *oca2+* in RNA Pol II transcription we performed a transcription run on (TRO) analysis. The *oca2Δ* and its isogenic wild type strain were transformed with the plasmid pNU as a TRO reporter gene. In TRO analysis, the *oca2Δ* strain gives an aberrant transcription profile over the *ura4* reporter gene with a substantial build-up of Pol II over probe 3, which corresponds to the normal site of *ura4* Pol II termination (Figure 2A). Apparently, in the absence of *oca2+*, Pol II is stalled at the poly(A) site. This could be either due to a slower release of Pol II from the termination site as a result causing less read-through signals into downstream sequences (probes 4-5). This result suggests that *oca2+* may play a role in promoting release of Pol II from the *ura4* termination site. It should also be noted that as compared to the endogenous 18S rRNA TRO signal, the levels of *ura4* nascent transcription in *oca2Δ* strain over the genic probes 1 and 2 are significantly reduced (about 4 fold).

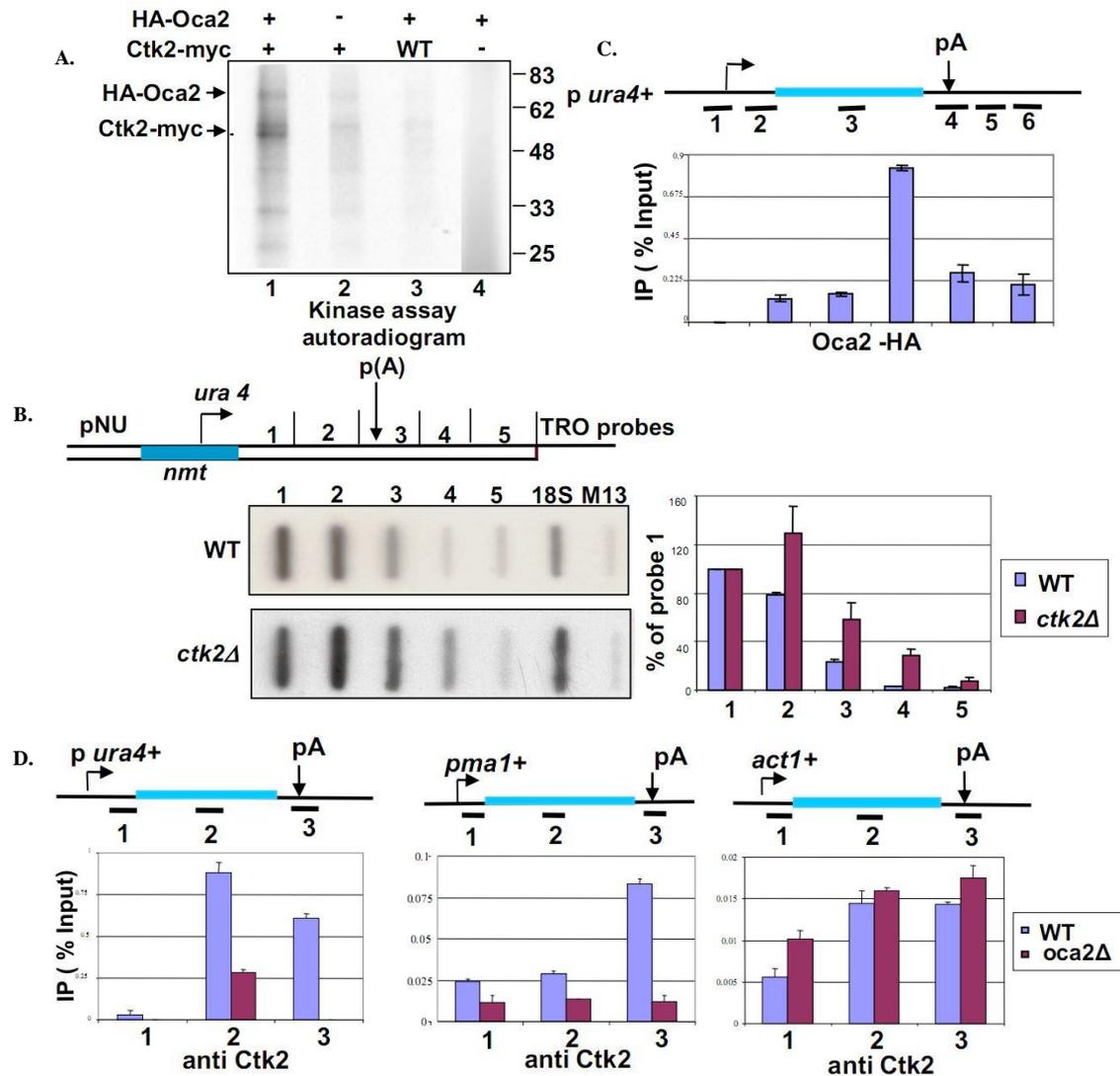


Figure 3. *S. pombe* Ctk2 is involved in transcriptional termination. (A) In vitro kinase reaction with affinity purified Oca2-HA from *S. pombe* extract. Left panel: Oca2-HA was incubated with affinity purified Ctk2-myc (lane 1) under in vitro phosphorylation conditions. Lane 2 contains Ctk2-myc alone, lane 3 contains Oca2-HA with mock myc-affinity purified Ctk2 from untagged strain. Lane 4 contains Oca2-HA alone. (B) Ctk2 is required for efficient transcriptional termination. TRO analysis of pNU transformed WT and *ctk2Δ* strains was performed as described in Figure 1C. TRO analysis was repeated multiple times and data are the mean percentage of 3 independent experiments, \pm SD. (C) Oca2 is localized at the 3' end of *ura4* gene. ChIP was performed with a strain containing HA-tagged Oca2 with anti-HA antibody to examine Oca2 localisation on *ura4*. Immunoprecipitated chromatin was analyzed by real time PCR on *ura4* gene with primers as shown by bars below the gene. (D) Oca2 is required for recruitment of Ctk2 at the 3' end of gene. ChIP was performed with WT and *oca2Δ* strains using anti-ctk2 antibody that recognizes Ctk2 in *S. pombe* to examine the association of *ctk2* on genes as indicated. Quantitation of PCR analysis of immunoprecipitated chromatin was performed on *ura4+* (left), *pma1+* (middle) and *act1+* (right) genes. PCR products were represented by bars below each gene map. All the experiments were repeated 3 times and data were presented as a mean percentage, \pm SD.

We then tested the effect of *oca2Δ* on endogenous mRNA levels for a number of *S. pombe* genes (Figure 2B). As compared to equivalent levels of rRNA, northern blots of 6 different mRNA showed either no change or at most 50% reduction in total cellular levels. Significantly, *ura4* mRNA levels from the pNU plasmid were reduced by 50%. The stability of *ura4* mRNA may compensate for the more drastic reduction in *ura4* nascent transcript levels. A similar 50% reduction in *pma1* mRNA levels was also observed. In contrast, three other endogenous mRNAs, *obr1*, *htb1*, *act1* showed no change in mRNA levels between the wild type and *oca2Δ* strains. In conclusion these results indicate that the Oca2 kinase is required for correct expression of a subset of *S. pombe* genes,

where it may operate to promote release of Pol II from the termination region of the gene in question.

Oca2 phosphorylates Ctk2 and Ctk2 is involved in transcriptional termination

The data presented above indicates a connection between Oca2 and some aspect of Pol II transcriptional termination. The amino acid sequence of Oca2 shows the characteristic sequence motifs of a serine/threonine protein kinase. Therefore, we sought to obtain further information about the function of Oca2 by identifying its substrates. In our preliminary attempt to identify Oca2 substrates by mass-spectroscopy revealed that Ctk2 as a substrate of Oca2. Therefore, we tested affinity purified Oca2 for

kinase activity. For this purpose we created an *S. pombe* strain with the *ctk2* gene tagged by a (C-terminal) myc epitope. Expression of the Ctk2-myc fusion protein was verified by western blot analysis using an anti-myc antibody (data not shown). Ctk2-myc immuno-purified from *S. pombe* total cell extracts, and incubated together in an *in vitro* kinase assay with Oca2. As shown in Figure 3A, phosphorylation of a polypeptide with the mobility expected for Ctk2-myc was observed (right panel, lane 1). ³²P-Labeling of this protein was not observed in control reactions that either did not contain Oca2-HA (lane 2) or containing mock-affinity purified Ctk2 from wild type (untagged) *S. pombe* (lane 3) or Oca2 alone (lane 4). We also observed increased autophosphorylation of Oca2 in the presence of Ctk2, as judged from the appearance of ³²P-labelled polypeptide with the size of Oca2-HA. Overall, these results establish that Oca2 has protein kinase activity *in vitro* towards Ctk2.

To establish a role of Ctk2 in transcriptional termination, *ctk2* was generated by disrupting the chromosomal copy of *ctk2* using KanMX6. Both isogenic WT and *ctk2* were transformed with TRO reporter pNU and TRO analysis was performed. Compare to WT, *ctk2* strain revealed increased TRO signals beyond the normal site of Pol II termination of the *ura4* gene (probe 3 and 4) (Figure 3B). We also noted increased signal over probe 2. From this result we conclude that Ctk2 is involved in transcriptional termination.

Both Oca2 and Ctk2 are associated with gene terminator region

To demonstrate a more direct involvement of Oca2 in Pol II transcription termination, we performed chromatin immuno-precipitation (ChIP) experiments using Oca2-HA strain and anti-HA antibody. We looked at the distribution of Oca2 across the *ura4*⁺ gene, whose expression we showed above is Oca2 dependent. Oca2 distinctively accumulates over the *ura4* poly(A)/terminator region (Figure 3C, primers 3). No signal was detectable upstream of the *ura4* promoter (primers 1), whereas low of label of Oca2 signal was observed in the body of the *ura4* gene (primers 2 and 3) and downstream of the terminator region (primers 4 and 5). The fact that Oca2 is associated with the *ura4* terminator region strongly suggests that it plays a direct role in the Pol II transcriptional termination process.

We also investigated the chromosomal association of Ctk2 by ChIP analysis using the anti Ctk2-antibody was performed on the genes *ura4*, *pma1* and *act1* with PCR primers specific for the promoter, ORF and terminator regions of these genes in the presence and absence of Oca2. We found that in wild type cells, Ctk2 accumulated predominantly over the coding and the terminator regions of the *ura4*⁺, *pma1*⁺ and *act1*⁺ genes (Figure 3D). Strikingly however, in the *oca2Δ*

strain, cross linking of Ctk2 to all parts of the *ura4*⁺ and *pma1*⁺ genes was significantly reduced. In contrast, the *oca2Δ* insensitive *act1*⁺ gene showed no reduction of Ctk2 association. These data show that Ctk2 localizes predominantly to ORFs and terminator regions of selected genes and chromosomal localization of Ctk2 to the terminator regions is dependent on Oca2, which itself is also present at these terminator regions. This in turn would recruit polyadenylation factors that are required for termination and release of RNA Pol II from termination sites. Overall our results support a role for Oca2 kinase in Pol II termination of selected *S. pombe* genes.

DISCUSSION

Our genetic screen for transcription termination factors in *S. pombe* led to the identification of the uncharacterized protein kinase Oca2. We have established a role of Oca2 in transcription termination. We found that loss of Oca2 leads to a significant build up of Pol II over the terminator region of selected genes. In ChIP analysis, Ctk2 and Oca2 show a similar distribution across genes, with no cross linking signal to promoter regions, but increased accumulation towards the terminator region. In the absence of Oca2, Ctk2 is no longer detectable at terminator regions, suggesting that Ctk2 recruitment to selected gene terminators is dependent on Oca2. Based on these observations, we propose that Oca2 is required for Pol II termination of selected *S. pombe* genes. A possible model could be that Oca2 recruits Ctk2, possibly in a phosphorylated form, to terminator regions. The localization of Oca2 and Ctk2 near poly(A) sites would result in recruitment of polyadenylation factors, some of which are required for Pol II termination.

The expression of only a subset of genes depends on Oca2, which might explain why Oca2 is non-essential. However, *S. pombe* contains two further protein kinases of unknown function, Ppk8 (SPAC22G7.08) and Hal4/Sat4/PPk10 (SPAC29A4.16)¹⁶ which are related in sequence to Oca2, and therefore could have overlapping functions. Oca2 is related to the *S. cerevisiae* kinases Hrk1, Npr1, Ydl025c, Prr2 and Sat4, which collectively belong to a fungus-specific subfamily of kinases. Many of these kinases are involved in regulating plasma membrane transport systems in response to environmental changes.²⁴ We tested *S. cerevisiae* *npr*, *hrk1*, *prr2* and *ydl025c* mutant strains in TRO analysis using the *CYC-1* reporter,¹⁷ but did not detect any significant defects in transcription termination (data not shown). However, we cannot rule out an involvement of Npr1 in transcription termination of other genes, similar as Oca2 is required for the expression of certain genes only. Interestingly, Hrk1 has been implicated in the activation of the H(+)-ATPase Pma1 in response to glucose,¹⁸ one of the genes affected by loss of Oca2. In addition, Hrk1 has also been found to bind to the CTDK-1 phosphorylated CTD.²⁵

Future work is required to further characterize the role of Oca2 in transcription termination and to demonstrate both in vitro and in vivo interaction of Oca2 and Ctk2. Here we reported that Oca2 phosphorylates ctk2 in vitro but further investigations are required to establish Ctk2 is a substrate of Oca2 in vivo. It also remains to be shown mutant Oca2 kinase can phosphorylate Ctk2 and identification of ser/thr residue that is phosphorylated by Oca2. Furthermore effect of mutation of those residues on transcription termination and recruitment of Ctk2 to the 3' ends of selected genes are dependent on phosphorylation. Because of a connection between Oca2 and Ctk2, Oca2 may also regulate CTD phosphorylation needs to be investigated and also recruitment of other polyadenylation factors to the 3' end of selected genes. It remains to be established how different genes in *S. pombe* select different CTD kinases to modulate their transcription cycles. However it is an attractive possibility, that the elucidation of these different pathways in *S. pombe* will provide insight into the regulation of CTD phosphorylation and hence transcription in all eukaryotes.

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