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Cdk-related kinase 9 regulates RNA polymerase II mediated transcription in *Toxoplasma gondii*

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ABSTRACT

Cyclin-dependent kinases are an essential part of eukaryotic transcriptional machinery. In Apicomplexan parasites, the role and relevance of the kinases in the multistep process of transcription seeks more attention given the absence of full repertoire of canonical Cdks and cognate cyclin partners. In this study, we functionally characterize *T. gondii* Cdk-related kinase 9 (TgCrk9) showing maximal homology to eukaryotic Cdk9. An uncanonical cyclin, TgCyclin L, colocalizes with TgCrk9 in the parasite nucleus and co-immunoprecipitate, could activate the kinase *in-vitro*. We identify two threonines in conserved T-loop domain of TgCrk9 that are important for its activity. The activated TgCrk9 phosphorylates C-terminal domain (CTD) of TgRpb1, the largest subunit of RNA polymerase II highlighting its role in transcription. Selective chemical inhibition of TgCrk9 affected serine 2 phosphorylation in the heptapeptide repeats of TgRpb1-CTD towards 3′ end of genes consistent with a possible role in transcription elongation. Interestingly, TgCrk9 kinase activity is regulated by the upstream TgCrk7 based CAK complex. TgCrk9 was found to functionally complement the role of its yeast counterpart Bur1 establishing its role as an important transcriptional kinase. In this study, we provide robust evidence that TgCrk9 is an important part of transcription machinery regulating gene expression in *T. gondii*.

1. Introduction

Toxoplasma gondii is a protozoan parasite of phylum Apicomplexa with ability to infect any warm-blooded vertebrate and found in nearly one third of human population [1]. This parasite with evident medical and veterinary importance displays a complex life cycle with multiple hosts and different morphological stages. Its survival and propagation greatly depend on changes of gene expression and their tight regulation during different life cycle stages indicating important roles of transcription and mRNA processing in parasite growth and development.

Cyclin-dependent kinases (Cdks) are a family of serine/threonine protein kinases represented by several members with distinct as well as overlapping functions in cell cycle and transcriptional regulations among other cellular processes [2]. As observed, Cdks are broadly categorized based on whether it controls cell cycle or transcription. Mammalian Cdks, Cdk1 to Cdk6 have primary role in cell cycle control while Cdk8 to Cdk11 participate in transcriptional regulation with the exception of Cdk7 which functions to control both cell cycle as Cdk activating kinase (CAK) and transcription as a part of TFIIH general transcription factor [3–6].

Co-ordinated control of gene expression is undertaken at the level of transcription of specific mRNAs by RNA polymerase II (RNAPII), in a multistep, sequential pattern with distinct phases: preinitiation, initiation, promoter clearance, elongation, RNA processing, and termination. The carboxy-terminal domain (CTD) of the largest subunit of RNAPII, Rpb1, is a key regulatory unit in this process [7]. The CTD consists of heptapeptide repeats of canonical sequence YSPTSPS, where sequential and coordinated phosphorylation of serines by series of Cdks is central to transcription regulation [8]. This phosphorylation predominantly takes place at the conserved serine 2 (Ser2) and serine 5 (Ser5) residues in the heptapeptide repeats [8]. Transcription initiation and promoter clearance is mediated by phosphorylation at serine 5 by Cdk7 (yeast ortholog-kin28), essentially as a part of TFIIH basal transcription factor [8]. This phosphorylation event is also accompanied with co-transcriptional mRNA capping [9]. Phosphorylation of serine 2 mediated by Cdk9 (yeast ortholog-Bur1/Ctk1), is an event downstream of transcription initiation which makes way for RNAPII mediated elongation [10–12]. Cdk9 mediated phosphorylation of serine 2 at promoter proximal sites facilitate release of RNAPII from initiation complexes [13]. In fact, the extent of serine 2 phosphorylation increases towards 3′

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end of genes which promote binding of termination and RNA processing factors [14]. The process of transcription elongation is regulated by Cdk9 in conjunction with its cyclin partner, cyclin T [15]. This Cdk9-Cyclin T complex is referred to as positive transcription elongation factor b (P-TEFb) [9,16]. In *Saccharomyces cerevisiae*, the functions of P-TEFb was initially thought to be carried out by the essential Bur1/Bur2 complex and the nonessential Ctk1/Ctk2/Ctk3 complex, with both Bur1 and Ctk1 protein kinases sharing sequence similarities to Cdk9 from higher eukaryotes [17]. Later, metazoan CDK proteins Cdk12 and Cdk13 [18,19], were identified and renamed to be orthologs of yeast Ctk1 as compared to Cdk9 which was found to be a functional counterpart of yeast Bur1 kinase [20].

Recent study has uncovered several divergent Cdk-related kinases (Crks) in *T. gondii* genome based on presence of conserved kinase domain and cyclin-binding sequence [21]. The possible Crks involved in transcription regulation were indicated, however, their functional role remains to be investigated. TgCrk7 (TgCdk7) has been demonstrated to have a central role in *T. gondii* transcription initiation and nascent transcript maturation [22]. The other transcription related Cdk that has been identified in *Toxoplasma* is Cdk11 homolog, named TgCrk1 [21]. While transcriptional involvement of TgCrk1 is yet to be unraveled but its role in mitosis especially in parasite cytokinesis have been reported [21]. Like Cdk11, a homolog of Cdk9, an important transcriptional kinase and a potential substrate of Cdk7 is speculated to be present in *T. gondii*, however it remains uncharacterized.

In eukaryotes, Cdk narrative remains incomplete without the mention of its partner cyclin which functions as the regulatory co-factor. *Toxoplasma* has been found to harbor only a limited set of cyclins with a few including TgCycH, TgCycL and TgCycY expressing abundantly in the tachyzoite stage. We have recently demonstrated the role of *Toxoplasma* cyclin H homolog in conjunction with TgCrk7 in transcription initiation [22]. A putative homolog of transcriptional kinase, Cdk11, TgCrk1 has been shown to partner with TgCyclin L to undertake successful mitotic progression and cytokinesis [21]. Interestingly, the cyclin's ability to partner more than one CDK or *vice versa* was observed for CycH which has been demonstrated to interact with both TgCrk7 and TgCrk2 (TgCdk2) supporting the much suggested flexibility in cyclin-Cdk pairings in *Toxoplasma* [22,23]. Similar, functional plasticity is anticipated with respect to the other identified cyclins, including TgCyclin L and TgCyclin Y and remains to be experimentally verified in presence of appropriate Cdks.

The functional heptapeptide unit of Rpb1 CTD is progressively phosphorylated as transcription proceeds, particularly at serine 5 and serine 2 residues by the appropriate Cdk coinciding with specific phase in the transcriptional cycle. The CTD (1631-1847aa) of TgRpb1 contains nine putative heptapeptide YSPxSPx (where x can be any amino acid) sequences instead of a consensus YSPTSPS sequence with observable conservation at serine 5 and serine 2 residues [22]. The flexibility at positions 4 and 7 in the heptapeptide repeats are reported in many organisms [8].

We have previously identified the role of TgCrk7 in RNAPII mediated transcription exercised through phosphorylation of TgRpb1-CTD at serine 5 [22]. TgCrk which phosphorylates the conserved serine 2 and its relevance in transcription is yet to be examined.

Regulation of transcriptional elongation is carried out by a Cdk9 homolog in eukaryotes mediated through the phosphorylation of serine 2 of Rpb1-CTD. This is conventionally accomplished by Cdk9 in presence of cyclin regulatory partner cyclin T. While a Cdk9 homolog (TgCrk9) could be identified in *Toxoplasma*, an absence of a canonical cyclin T, posed an interesting question with respect to the mechanism of action of this Cdk homolog. Whether this TgCrk9 shows a cyclin independent activity or partners with any of the abundantly expressing TgCyclins (CycH, CycL or CycY) in the tachyzoite stage, required investigation. Therefore, in this study we characterize TgCrk9, a prospective transcriptional kinase, and its potential cyclin partner with possible involvement in RNAPII transcriptional regulation.

Here, we provide robust biochemical evidence along with *in vivo* observations for the presence of an active Cdk9 homolog in *T. gondii*. TgCrk9 which gets activated in presence of cyclin partner, TgCycL, displays CTD kinase activity suggesting a role in transcription control. Two threonine residues in the T-loop domain of TgCrk9 were found to be crucial for the kinase activity. Interestingly, in addition to the cyclin partner, TgCrk9 is regulated through the upstream TgCrk7 based CAK complex. Selective chemical inhibition of TgCrk9 led to a diminished enrichment of phospho-serine 2 at 3′ end of constitutively expressed *PCNA1*, *Bip*, *β-Tubulin* and *α-Tubulin* genes indicating a possible role in transcriptional elongation. Moreover, selective chemical inhibition of TgCrk9 which was observed at lower dosages than that inhibiting host Cdk9 homolog, effectively controlled parasite progression. Our observations were further validated as TgCrk9 could functionally complement the essential Cdk9 homolog in yeast (ScBur1). On the whole the data highlight both the conserved as well as divergent mechanisms operating in tandem to effectively regulate RNAPII transcription in *T. gondii*.

2. Materials and methods

2.1. Parasite culture

T. gondii RH strain parasites were grown in Human foreskin fibroblasts (HFF, CRL1634, H27, ATCC) as described [24]. Freshly harvested parasites were purified by filtration through 3.0 μm-Nucleopore filter.

2.2. DNA manipulation

Sequences encoding ORF of full length TgCrk9 (excluding signal sequence), TgCycL, TgCycY were amplified by PCR using *T. gondii* cDNA and specific primers (Supplementary Table 1) and cloned into pET-21a vector between *BamH*I-*Xho*I, *Nde*I-*Eco*RI and *Nde*I-*Xho*I sites respectively. The resulting recombinant clones were sequenced. TgCrk7, TgCycH, TgMat1 and TgRpb1-CTD constructs were used from the previous study [19]. TgCrk9 threonine mutants, TgCrk9-T1-His (T250A), TgCrk9-T2 (T257A), TgCrk9-T1&T2 (T250A & T257A) and TgCrk7 kinase dead mutant, TgCrk7dM (K44A) were generated using specific set of primers (Supplementary Table 1) following Stratagene (210515) site directed mutagenesis protocol.

2.3. Purification of recombinant proteins

His and GST-tagged recombinant proteins were purified as described previously [25]. The following proteins were used in the study: TgCrk9-His, TgCycL-His, TgCrk9-T1-His, TgCrk9-T2-His, TgCrk9-T1&T2-His, His-TgRpb1-CTD, GST-TgCrk7, GST-TgCrk7dM, GST-TgCycH and His-TgMat1. TgCycL-His and His-TgMat1 were purified from the inclusion bodies as described previously [22].

2.4. Antibody production

Mouse antiserum against TgCrk9 and TgCycL were prepared by primary injection with 30 μg of purified protein in Freund's complete adjuvant (Sigma) followed by four boosts of 30 μg each in Freund's incomplete adjuvant (Sigma) at 2-weeks intervals. A polyclonal rabbit antiserum also was raised against TgCycL by immunizing rabbits with 200 μg of recombinant protein mixed with appropriate Freund's adjuvant. Serum was collected after three weeks of immunization. Mouse anti-TgCrk7, anti-TgCycH and anti-TgRpb1 polyclonal antibodies were generated as described [22].

Following antibodies were commercially procured: anti-RNAPII CTD repeat P-Ser5 (ab5131, Abcam), anti-RNAPII CTD repeat P-Ser2 (ab5095, Abcam), control IgG (ab46540, Abcam), anti-His (H1029, Sigma), anti-GAPDH (ab9485, Abcam), anti-mouse Alexa flour 488 (A-11001, ThermoFisher Scientific) and anti-Rabbit Alexa flour 594 (A-

11037, ThermoFisher Scientific).

2.5. Immunofluorescence (IFA) analysis

Monolayers of HFF were grown on coverslips in a 6-well plate and infected with parasites. Post 16 to 24 h of infection, the infected cells were fixed in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, blocked in 1% BSA and incubated sequentially with primary and secondary antibody. The following primary antibodies were used at dilution 1:200: mouse polyclonal anti-TgCrk9, rabbit polyclonal anti-TgCycL, mouse polyclonal anti-TgCycH, mouse polyclonal anti-TgCycY and mouse polyclonal anti-TgCrk7. Alexa-conjugated secondary antibodies (Alexa Fluor-488 or Alexa Fluor-594) were used at a dilution of 1:1000. Stained parasites on the coverslips were mounted with Antifade containing DAPI (4′,6-diamidino-2-phenylindole) (P36931, ThermoFisher Scientific) on glass slide and viewed on a Leica Confocal microscope with 100× objective. Images were collected and processed using LAS X software and were further processed in Adobe Photoshop software.

2.6. Yeast two-hybrid analysis

Yeast two-hybrid assay was performed as per the manufacturer's guidelines (630489, Clontech). Bait plasmids were constructed by cloning full length ORF of TgCycL, TgCycH and TgCycY into the pGBKT7 vector whereas pray plasmids were constructed by cloning full length ORF of TgCrk9 and TgCrk7 into the pGADT7 vector. Respective bait and pray plasmids were cotransformed into *S. cerevisiae* strain Y2HGold using the lithium acetate method and plated onto SD plates lacking Leu (leucine) and Trp (tryptophan). The colonies were subsequently transfer to medium lacking His (histidine), Leu and Trp and for higher stringency onto SD plates lacking Ade (Adenine), His, Leu and Trp. For spot assays, serial dilutions were prepared (OD_{600}) as 1, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} and plated onto SD/-Leu/-Trp, SD/-His/-Leu/-Trp and SD/-Ade/-His/-Leu/-Trp plates.

2.7. Immunoprecipitation (IP) assay

The IP from parasite proteins was performed using IP kit (26149, Pierce) as described by the manufacturer. Briefly, filter purified parasites were collected, washed in PBS and lysed in the lysis buffer with protease and phosphatase inhibitors on ice for 1 h. Protein extract was obtained by centrifugation at 21,000 ×*g* for 10 min at 4 °C. Protein normalized parasite lysates were immunoprecipitated using appropriate antibody crosslinked to agarose at 4 °C, overnight. The immunoprecipitates were recovered after centrifugation, and the eluted proteins were subjected to Western blot analysis.

2.8. Kinase assay

Kinase assays were performed as described previously [26]. Briefly, 0.5 μg to 1.0 μg of TgCrk9/TgRpb1-CTD/TgCrk9-T1/TgCrk9-T2/TgCrk9-T1&T2 were assayed at 30 °C for 30 min in a 30 μl kinase reaction buffer (50 mM Tris-HCl pH 7.5, 10 mm $MgCl_2$, 0.1 mM EDTA, 200 μM ATP and 5 μCi γ-32P ATP) supplemented with various combinations of 0.5 μg to 1.0 μg of TgCycL/TgCycH/TgCycY/TgCAK (TgCrk7/dM-TgCycH-TgMat1) proteins or parasite lysate in absence or presence of 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB, D1916; Sigma) or flavopiridol (FP, F3050; Sigma). Following the incubation, the reactions were stopped by the addition of 5× SDS loading buffer and boiled. The reactions were resolved on a 10 to 12% SDS-PAGE gels and visualized by phosphorimager. Parasite lysate for kinase assay was prepared from *T. gondii* treated with DMSO or DRB or flavopiridol for 6 h using IP extraction buffer. Kinase reactions were performed using 10 μg total protein lysate.

2.9. Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described [22] with the following modifications. Parasites grown in HFF cells for 24 h were cross-linked at room temperature for 10 min with 1% formaldehyde. The cross-linked DNA was sheared followed by IP using respective antibodies at 4 °C overnight. Protein G agarose beads were added to the IP reaction tubes and nutated at 4 °C for an additional hour. DNA was further subjected to a treatment with proteinase K for 2 h followed by phenol: chloroform purification. The control IgG antibody was used as a negative control. Immunoprecipitated DNA from DRB or flavopiridol treated parasites was quantified and normalized to total RNAPII levels.

2.10. RT-qPCR analysis

Total RNAs from DMSO or DRB or flavopiridol inhibitor treated parasites were isolated by TRIzol (15596-026, Invitrogen). DNase I treated RNAs were used to generate cDNA using SuperScipt III reverse transcriptase (12574018, Invitrogen). Real-time quantitative PCR was performed on the 7500 ABI apparatus using cDNA samples with SYBR green PCR Master Mix. The nascent RNA levels were determined as described previously [27]. Briefly, the nascent RNA levels of *IMC1*, *MIC2*, *PCNA1* and *α-Tubulin* were determined using primers targeting specific exon-intron or intron-exon junctions, which allowed quantitative analysis of newly synthesized pre-mRNA. Amplification from exonic region (exon 4 and exon 5) of pre-existing (basal) mRNA of the *IMC1* which is expected to be unaltered was used as control. The reactions were performed in triplicates, for each sample using the following cycle conditions: 95 °C, 15 min followed by 40 cycles of 94 °C, 30 s; 55 °C, 40 s and 68 °C, 50 s. Relative transcript levels were calculated by the $\Delta\Delta C_T$ (where C_T is threshold cycle) method.

2.11. Proliferation of T. gondii

Parasites inside the parasitophorous vacuoles (PVs) were counted as described [28]. HFF cells grown on coverslips were infected with *T. gondii* at a multiplicity of infection (moi) of 5. Following 2 h of infection, parasite infected cells were subjected to inhibitor treatment for 24 h. The coverslips were then washed with PBS and fixed using 4% paraformaldehyde and scanned under the microscope. One hundred PVs were randomly selected, and parasite replication was monitored by counting the number of tachyzoites per PV.

2.12. Apoptosis measurement

Apoptosis analysis of HFF cells treated with DRB or flavopiridol for 24 h was undertaken by flow cytometry using annexin-fluorescein and propidium iodide staining according to standard protocols (556547, BD Biosciences).

2.13. Yeast complementation

For complementation assays, yeast strains with chromosomal copy deletion of Sc*ctk1*, Sc*ctk2* showing cold sensitive phenotype were used. Sc*bur1* and Sc*bur2* chromosomal copy deletion mutant strains carrying the wild-type copy in a plasmid with *URA* marker were utilized [29,30]. Full-length *T. gondii* genes (*crk9*, *crk9-T1*, *crk9-T2*, *crk9-T1&T2* and *cycL*) and *S. cerevisiae* genes (*ctk1*, *ctk2*, *bur1* and *bur2*) were amplified by PCR using either *T. gondii* cDNA or rescued plasmids from the respective *S. cerevisiae* mutant strains and specific primers (Supplementary Table 1) and cloned into pYES3/CT yeast expression vector. All constructs were confirmed by sequencing. Cold sensitive strains were transformed with plasmid carrying *S. cerevisiae* and *T. gondii* genes of interest and selected at the restrictive temperature (12 °C). Yeast *bur1* and *bur2* mutant strains were similarly transformed with plasmid carrying *T. gondii* gene of interest or *S. cerevisiae* control gene or empty

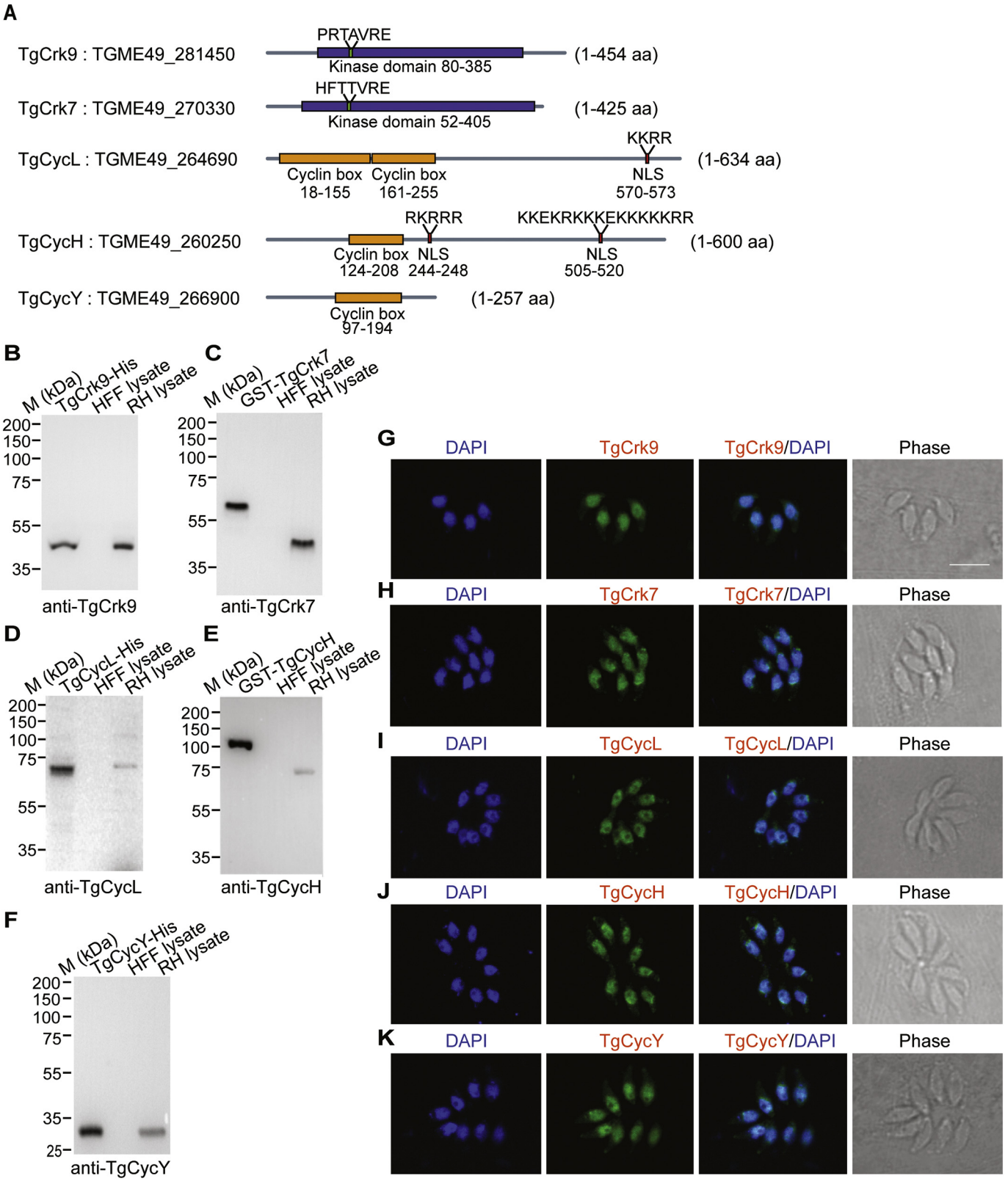

Fig. 1. *T. gondii* genome encodes transcriptional Cdk-related kinases (Crks) and cyclins. (A) Schematic diagrams of full length TgCrk9 (TGME49_281450) and TgCrk7 (TGME49_270330): conserved kinase domain and cyclin binding motif are shown. Schematic diagrams of full length TgCycL (TGME49_264690), TgCycH (TGME49_260250) and TgCycY (TGME49_266900): conserved cyclin domain and nuclear localization signal/s are shown. (B–F) Expression of TgCrks and TgCyclins in tachyzoite stage of parasite was analyzed by Western blotting with anti-TgCrk9, anti-TgCrk7, anti-TgCycL, anti-TgCycH and anti-TgCycY polyclonal antibodies. Polyclonal antibodies recognized recombinant (TgCrk9-His ~45 kDa, GST-TgCrk7 ~72 kDa, TgCycL-His ~67 kDa, GST-TgCycH ~92 kDa and TgCycY-His ~30 kDa) and native protein (TgCrk9 ~45 kDa, TgCrk7 ~46 kDa, TgCycL ~67 kDa, TgCycH ~65 kDa and TgCycY ~30 kDa). Antibodies did not show cross-reactivity with host HFF cell proteins. (G–K) IFA images in tachyzoite stage show predominant nuclear localization of TgCrks (Crk9, Crk7) and TgCyclins (CycL, CycH and CycY). Scale bar 5 μm.

vector under tryptophan selection and C-terminal his-tag for detection. Transformants were selected on SD –Trp plates with or without 5-Fluoroorotic Acid (5-FOA) as described [31]. Western blot analysis was performed to check for the expression of the complemented genes in respective transformants using anti-His antibody. *ScGAPDH* expression levels were checked in all the transformants as a control.

3. Results

3.1. T. gondii Crk9, Crk7, CycL, CycH and CycY genes express in the tachyzoite stage

In order to determine the cyclin-Cdk pair involved in *Toxoplasma* transcription control, we performed sequence homology analysis and enlisted important Cdk-related kinases TgCrk9 and TgCrk7 (TgCdk7) and TgCyclins (TgCycL, TgCyH and TgCycY) which may be responsible in this regard (Fig. 1A). In the earlier report, we demonstrated that TgCycH partners with TgCrk7 to drive RNAPII mediated transcription initiation and subsequent co-transcriptional nascent RNA capping [22]. To determine the potential regulation of RNAPII transcription downstream of initiation, TgCrk9 protein kinase, a Cdk9 homolog, was evaluated in this study. We tested the enlisted cyclin proteins which express in the tachyzoite stage for their ability to interact and activate TgCrk9 kinase activity. TgCrk7 served as a control transcriptional kinase in this experiment.

Sequence analysis showed that the predicted TgCrk9 shares broadly 30 to 35% sequence identity with other eukaryotic homologs including HsCdk9 (human), ScBur1, ScCtk1 (yeast), PfCrk3 (*Plasmodium*) and TbCrk9 (*Trypanosoma*). TgCrk9 like its eukaryotic counterparts harbors the conserved cyclin binding domain, T-loop as well as P-loop. The cyclin-binding motif (PSTAIRE in Cdk1, PITALRE in Cdk9 and PITQLRE in yeast Bur1) is represented by PRTAVRE in TgCrk9 (Fig. 1A). Phosphorylation inhibitory TY (threonine and tyrosine) residues are present in the P-loop. The observation is consistent with transcriptional kinase TgCrk7 which shares these features including cyclin binding and inhibitory TY motifs (Fig. 1A).

T. gondii cyclins showed divergence with respect to canonical cyclins with conservation mostly limited to the cyclin box. The small and unusual complement of TgCyclins was represented by P-, H-, L- and Y-types. Recent report [21] along with transcriptome data (ToxoDB) suggest that four TgCyclins (TgCycP, TgCycL, TgCycH and TgCycY) express in tachyzoite stage of parasite. Out of these four cyclins, TgCycP is reported to be low abundance and cytosolically localized [21] and therefore, unlikely to have any transcription role. The remaining TgCyclins under investigation (TgCycL, TgCycH and TgCycY) were characterized by the presence of the cyclin box, and nuclear localization signal (Fig. 1A). Cyclin H has already established roles in transcription in eukaryotes as well as in *Toxoplasma* [4,22] whereas cyclin Y is reported to partner a Cdk involved in cell cycle progression [32] with no transcriptional role observed so far. However, given the limited repertoire of available TgCyclins and hence the possibility of a single cyclin partnering more than one Cdk in distinct functional roles, it was worth assessing the transcriptional role of the enlisted cyclins.

The cyclin identified as TgCycL which showed 34% sequence identity with human cyclin L, partners the Cdk11 homolog, TgCrk1 [21]. Interestingly, TgCycL which also shared 25% identity with human cyclin T protein (conventional Cdk9 regulatory cyclin partner), offered more promise as a possible transcriptional cyclin. Therefore, TgCycL appeared to be the probable cyclin partner of TgCrk9, however it required to be experimentally tested. The two Cdks, TgCrk1 and TgCrk9 share < 16% sequence similarity with both being possible substrates of Cdk7 kinase appears to share the regulatory cyclin partner. If proven, this further exemplifies the promiscuity of Cdk-cyclin pairings in *T. gondii*.

To determine the expression of TgCrks and TgCyclins of interest in tachyzoites, specific antibodies were utilized. In order to raise these specific antibodies, full length TgCrk9 (excluding signal sequence), TgCrk7, TgCycL, TgCycH and TgCycY were bacterially expressed. Purified recombinant proteins with either histidine or GST-tag were used to generate specific mouse and rabbit polyclonal antibodies. As shown in Fig. 1B–F, each of the antiserum recognized the recombinant protein and a band of expected size in parasite lysate (TgCrk9: ~45 kDa; TgCrk7: ~46 kDa, TgCycL: ~67 kDa, TgCycH: ~65 kDa and TgCycY: ~30 kDa). TgCrk7 and TgCycH results were consistent with our previous study [22]. None of the polyclonal antibodies showed cross-reactivity with HFF proteins.

Immunofluorescence assay (IFA) was performed using the specific antibodies to define the localization of the identified TgCrks and TgCyclins in tachyzoites. We found that all the TgCrks and TgCyclins tested (TgCrk9, TgCrk7, TgCycL, TgCycH and TgCycY) were localized in the parasite nucleus, supporting their possible involvement in *T. gondii* transcription (Fig. 1G–K).

3.2. T. gondii Crk9 interacts with CycL and not with CycH or CycY

To examine and establish the role of TgCrk9 in transcription regulation, we first attempted to identify its cyclin partner/s. In order to determine the TgCrk9 cyclin partner among the potential cyclins, we performed yeast two-hybrid analysis. A *GAL4AD-TgCrk9* and *GAL4AD-TgCrk7* constructs were cotransformed with either *TgCycL-GAL4BD* or *TgCycY-GAL4BD* or *TgCycH-GAL4BD* and checked for expression of *GALUAS-HIS3* reporter fusion. Interaction between two given proteins was visualized by monitoring growth on medium lacking histidine. Of all the cyclin proteins tested, only TgCycL was able to interact with TgCrk9 under our experimental conditions (Fig. 2A). TgCrk7 interacted with TgCycH and not with TgCycL or TgCycY under same experimental conditions (Fig. 2A), consistent with previous report [22]. Interaction between p53 with T-antigen and Lamin with T-antigen were used as positive and negative control respectively (Fig. 2A bottom panel). The yeast two-hybrid experiments strongly suggest that TgCrk9 interact with TgCycL.

To further support the yeast two-hybrid interaction, IFA experiments were performed with antibodies directed against proteins TgCrk9 and TgCycL in the replicating-tachyzoites. Double-labeling experiments showed that both these proteins co-localize in the parasite nucleus (Supplementary Fig. 1).

To confirm the interaction suggested by the yeast two-hybrid and co-localization experiments in the parasite, we tested whether TgCrk9 and TgCycL co-immunoprecipitate from tachyzoite stage parasite lysate. When rabbit polyclonal antibody was used to immunoprecipitate TgCycL, co-immunoprecipitation of TgCrk9 was observed (Fig. 2B). Identical results were obtained in reciprocal immunoprecipitation using TgCrk9 mouse polyclonal antibody (Fig. 2B). No interaction was observed between TgCdk7 and TgCycL in control co-immunoprecipitation experiments. Based on yeast two-hybrid and co-immunoprecipitation experiments, we confirm the interaction of TgCrk9 and TgCycL.

3.3. TgCycL is required for TgCrk9 kinase activity

Genetic and physical interaction results so far strongly suggested that TgCycL functions as a cyclin partner for the TgCrk9 protein kinase, however it was still to be tested whether this Cdk-cyclin combination resulted in an active kinase or the cyclins (TgCycH and TgCycY) which failed to show any interaction with TgCrk9 could still activate its kinase function. Therefore, we wanted to determine whether addition of any of these cyclins could activate the TgCrk9. In order to test that, *in vitro* kinase assays were performed using recombinant TgCrk9 either in the presence of TgCycH or TgCycL or TgCycY protein (Fig. 3A and B). Human Cdk9 is characterized by autophosphorylation which marks its activation [33]. Similar autophosphorylation was observed for TgCrk9 only in the presence of TgCycL and not with TgCycH and TgCycY (Fig. 3B). The Rpb1 CTD serves as a key substrate for transcriptional

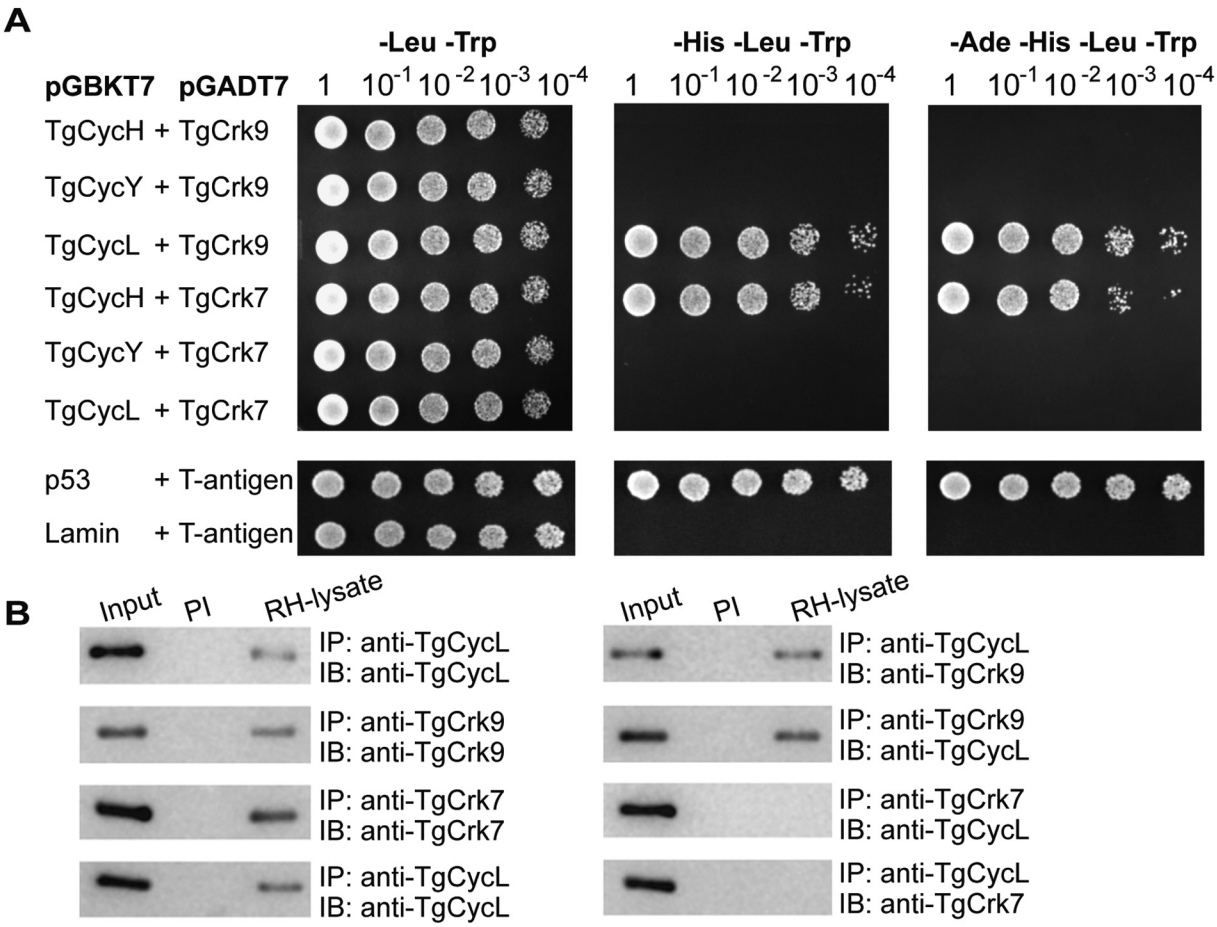

Fig. 2. TgCrk9 interacts with TgCycL. (A) Yeast two-hybrid interaction between the GAL4 DNA-binding domain fused to TgCyclins (TgCycH, TgCycY and TgCycL) and GAL4 activation domain fused to TgCrks (TgCrk9 and TgCrk7). To test for interaction, cotransformed yeasts were spotted in serial dilutions from 1 to 10^{-4} on nutritionally selective SD/-His/-Leu/-Trp or SD/-Ade/-His/-Leu/-Trp plates. (B) IP using anti-TgCycL antibody followed by immunoblotting (IB) with anti-TgCrk9 antibody and *vice-versa* were performed to confirm interaction between TgCrk9 and TgCycL. TgCrk7 could not pulldown TgCycL and *vice-versa* in the respective IP reactions. Efficient self IP was observed for individual antibody.

kinase activity of Cdk9 homologs [8]. Therefore, the activated TgCrk9 was tested for its ability to phosphorylate TgRpb1-CTD. TgCrk9 displayed CTD kinase activity which increases with increasing concentration of the substrate (Fig. 3C). The TgCycL activates TgCrk9 *in vitro*, in terms of both autophosphorylation and substrate phosphorylation.

Phosphorylation of a conserved threonine residue in the T-loop has been found to be crucial for kinase activity of Cdks [3]. Interestingly, there are two threonines (T250 and T257) present in T-loop of TgCrk9, both of which get phosphorylated as per the phosphoproteome data [34]. To evaluate the significance of these threonines in activation of TgCrk9, we substituted threonines 250 and 257 with alanine residues (T250A and T257A) by creating single (TgCrk9-T1 or TgCrk9-T2) and double point mutations (TgCrk9-T1 & T2). Protein expression profiles of wild type and mutant TgCrk9 are shown in Fig. 3A. Wild type and either of the single threonine mutant protein (TgCrk9-T1 and TgCrk9-T2) displayed autophosphorylation in the presence of TgCycL while no phosphorylation was observed in double mutant (TgCrk9-T1&T2) (Fig. 3D). Subsequently, the catalytic activities of wild type and mutant TgCrk9 proteins were assessed using TgRpb1-CTD substrate. Both wild type and single threonine mutants of TgCrk9 were able to phosphorylate CTD while double threonine mutant failed to do so (Fig. 3E). The CTD phosphorylation was further confirmed using Rpb1-CTD-P-Ser2 antibody (Fig. 3E). Therefore, double threonine mutant was catalytically inactive unlike wild type and single threonine mutants, demonstrating that at least one intact threonine in the T-loop is essential for its activity.

3.4. Crk7 based TgCAK activates TgCrk9 in vitro

The metazoan CAK (Cdk7-CycH-Mat1) is responsible for phosphorylation mediated activation of several Cdks including Cdk1 and Cdk2 [35]; Cdk4 and Cdk6 [36]; Cdk9 [37] and Cdk11 [38]. To determine whether a similar CAK homolog in *Toxoplasma* composed of Crk7-CycH-Mat1 ternary complex would activate TgCrk9, kinase reactions were performed using TgCAK with either TgRpb1-CTD (positive control) or TgCrk9. We observed that TgCAK could phosphorylate TgCrk9 as well as its known substrate TgRpb1-CTD (Fig. 4A). CAK complex generally exerts its activating influence through a conserved threonine residue in the T-loop of Cdks. Although threonines 250 and 257 in the T-loop appeared important for TgCrk9 kinase activity, it is important to determine whether these threonines could be a substrate for phosphorylation by TgCAK. Two different approaches were used to test the possibility that TgCAK could phosphorylate and thus activate TgCrk9. First, wild type and mutants (single and double threonine mutants) of TgCrk9 were tested as substrates for TgCAK. As shown in Fig. 4B, phosphorylation of TgCrk9, TgCrk9-T1, TgCrk9-T2 but not TgCrk9-T1&T2 were observed in the presence of TgCAK, while, catalytically dead mutant of TgCrk7 used as kinase component in TgCAK (TgCAKdM) failed to phosphorylate either wild type or mutants of TgCrk9 under the same experimental condition. Phosphorylation of TgCrk9 was not observed in absence of TgCAK. The results suggest that

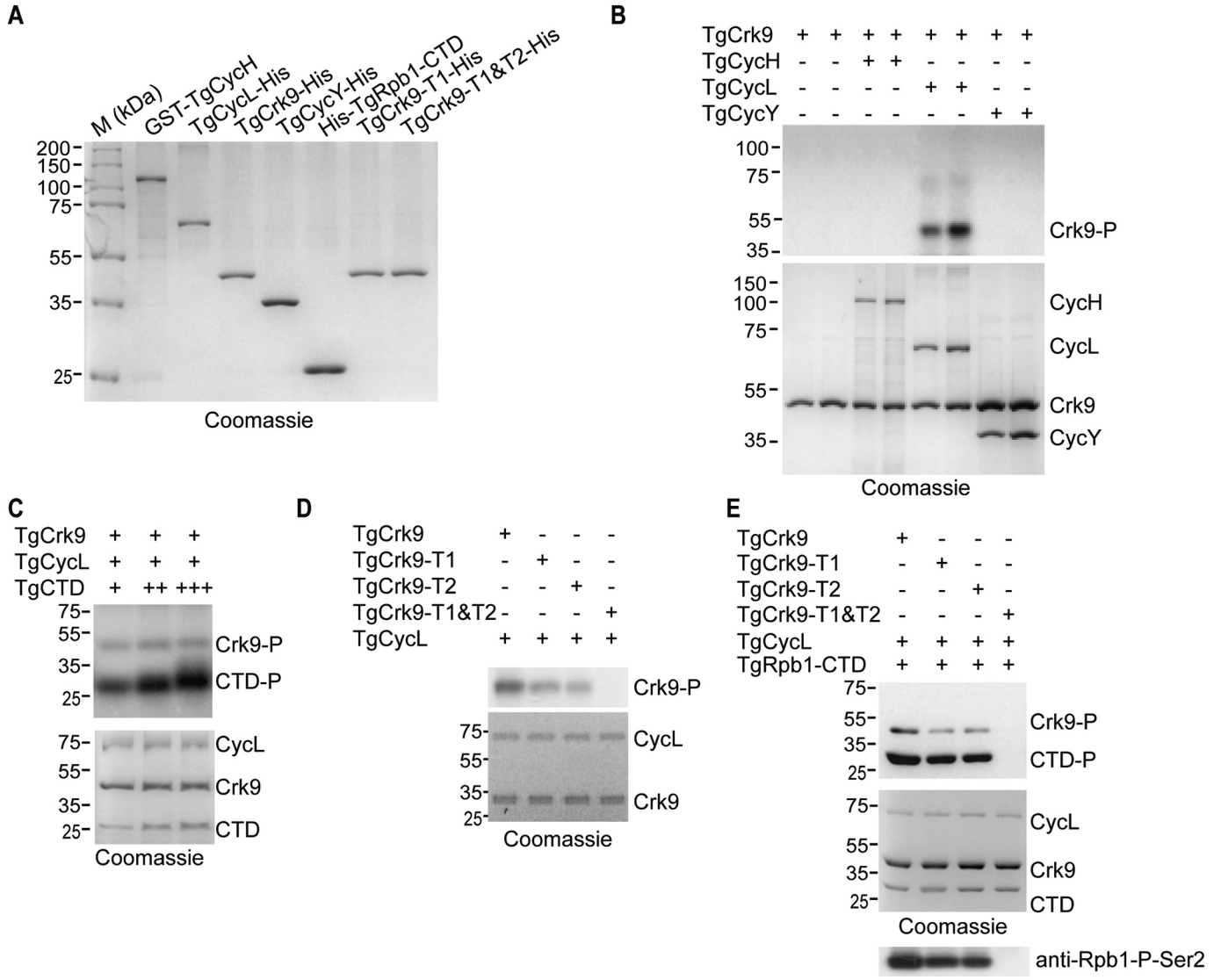

Fig. 3. TgCrk9 is an active kinase which can phosphorylate TgRpb1-CTD. (A) Coomassie gel showing purified recombinant GST-TgCycH, TgCycL-His, TgCrk9-His, TgCycY-His, His-TgRpb1-CTD, TgCrk9-T1 (T250A), and TgCrk9-T1&T2 (T250A & T257A) used in the kinase assays. (B) TgCrk9 was autophosphorylated in the presence of TgCycL suggesting TgCrk9 activation. TgCycH or TgCycY failed to activate TgCrk9. For each protein, effect of increasing concentration was tested (Crk9: lane no. 1 & 2; CycH: lane no. 3 & 4; CycL: lane no. 5 & 6 and CycY: lane no. 7 & 8). (C) TgRpb1-CTD was phosphorylated in a concentration dependent manner, in the presence of activated TgCrk9 (TgCrk9/TgCycL). (D) Both the TgCrk9 single threonine mutants, TgCrk9-T1 and TgCrk9-T2 were activated in the presence of TgCycL like wild-type TgCrk9 whereas double threonine mutant, TgCrk9-T1&T2 failed to activate upon addition of TgCycL. (E) Activated single point mutant of TgCrk9 displayed CTD kinase activity whereas double point mutant failed to do so, suggesting phosphorylation of at least one of the threonines in T-loop of TgCrk9 are required for its kinase activity. Coomassie stained gel (lower panel) show loading of all the proteins. Identical reactions were subjected to Western blot analysis using anti-Rpb1-CTD-P-Ser2 antibody which could efficiently detect P-Ser2 CTD.

threonines 250 and 257 of TgCrk9 may be target sites of TgCAK action. Subsequently, the TgCrk9-TgCycL complex was tested as a substrate instead of a TgCrk9 monomer in presence of TgCAK. Phosphorylation of TgCrk9 in presence of cognate TgCycL was further enhanced upon addition of TgCAK (Fig. 4C). We also tested whether TgCrk9-TgCycL kinase activity might be cooperatively regulated by TgCAK. In this case, kinase activity of wild type and mutant variants of TgCrk9-TgCycL was examined using a substrate, TgRpb1-CTD in the absence or presence of TgCAK. CTD kinase activity of all the variants of TgCrk9-TgCycL was stimulated in presence of TgCAK as compared to its absence (Fig. 4D). Interestingly, the level of phosphorylation of TgRpb1-CTD by TgCrk9-TgCycL wild type as well as mutants in presence of TgCAK was observed to be similar (Fig. 4D). The densitometry analysis of the representative figure (Fig. 4D) shows two fold stimulation in CTD kinase activity of wild type TgCrk9 in the presence of TgCAK as compared to its absence (Fig. 4E). Similarly, a threefold stimulation of CTD kinase activity was observed in case of threonine mutants in the presence of TgCAK as opposed to its absence (Fig. 4E). Interestingly, in absence of TgCAK, level of CTD phosphorylation by wild type TgCrk9 is nearly two fold more as compared mutants. However, in the presence of TgCAK similar levels of CTD phosphorylation were observed for all the variants of TgCrk9 (Fig. 4E). Our observations clearly indicate that TgCrk9 may be regulated by an upstream TgCAK complex possibly mediated through the threonines in the T-loop. Similar molecular mechanism regulating TgCrk9 activity may be operating *in vivo* and requires investigation.

3.5. TgCrk9 inhibition confirms phosphorylation of TgRpb1-CTD at the conserved serine 2 residue in the parasite

To determine whether TgCrk9 activity is important for CTD phosphorylation in the parasite, we used two drugs known to selectively

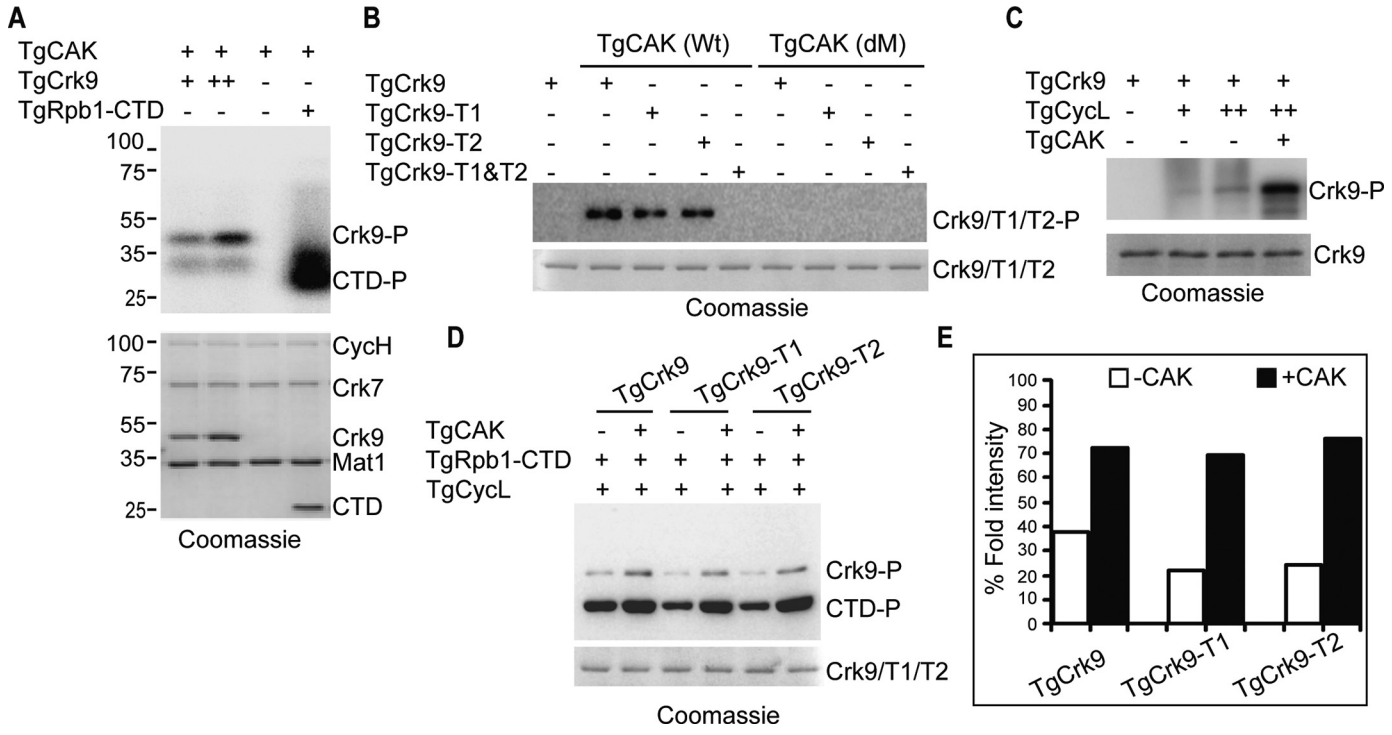

Fig. 4. TgCAK (Tg-Crk7/CycH/Mat1) stimulates TgCrk9 CTD kinase activity *in vitro*. (A) TgCrk9 gets activated in the presence of TgCAK even in absence of TgCycL. TgRpb1-CTD phosphorylation by TgCAK was used as positive control. (B) TgCrk9-T1 and TgCrk9-T2 undergo TgCAK mediated activation like TgCrk9 wild type, however, TgCrk9-T1&T2 fails to get activated. Reactions performed with kinase dead mutant of TgCrk7 (as a part of CAK) failed to activate any of the TgCrk9 variants. Control reaction without CAK did not show TgCrk9 activation. (C) Phosphorylation of TgCrk9 in presence of TgCycL is further stimulated on addition of TgCAK. (D) TgCAK stimulates CTD kinase activity of TgCrk9, TgCrk9-T1 and TgCrk9-T2 in the presence of TgCycL. (E) Densitometric analysis was performed for signals (obtained in Panel D) to quantify the CTD kinase activity of TgCrk9, TgCrk9-T1 and TgCrk9-T2. Image J tool was used for the densitometry analysis. Protein loading is shown by Coomassie stained gel (lower panel).

inhibit mammalian Cdk9; DRB and flavopiridol (FP) [39,40]. In order to examine whether DRB and flavopiridol could also inhibit the TgCrk9 kinase activity, we first checked TgCrk9 kinase activity in presence of different concentrations of inhibitors *in vitro*. We observed diminished phosphorylation of TgCrk9 with concomitant decrease in CTD phosphorylation with increasing concentration of DRB (Fig. 5A). Similar results like DRB were observed upon flavopiridol treatment (Fig. 5B). The concentrations of DRB required to inhibit kinase activity is considerably less for TgCrk9 as compared to mammalian Cdk9 reported elsewhere [40,41]. In case of flavopiridol, the concentration required to inhibit TgCrk9 is only slightly better than the values reported for mammalian Cdk9 [39–41]. To ascertain that TgCrk9 is indeed responsible for TgRpb1-CTD phosphorylation, we performed kinase assays using parasite lysate treated with different concentrations of DRB and flavopiridol. A dose dependent decrease of TgRpb1-CTD phosphorylation was observed upon DRB and flavopiridol treatment (Fig. 5C).

Phosphorylation of Rpb1-CTD in heptapeptide repeats YSPTSPS regulates eukaryotic transcription. Ser5 is phosphorylated in the initiation phase and Ser2 phosphorylation marks the elongation state. TgRpb1-CTD has only two conserved serine residues Ser2 and Ser5 in all identified heptapeptide sequences, indicating possible phosphorylation of these residues during *T. gondii* transcription. Recently, we have shown [22] that Ser5 phosphorylation in the Rpb1-CTD by TgCrk7 is required for transcription initiation in *Toxoplasma*. Cdk9 homologs in eukaryotes regulate transcriptional elongation by RNAPII mediated through phosphorylation of conserved serine 2 residue in the CTD of Rpb1 [8,10]. We wanted to ascertain whether TgCrk9 like its human counterpart has any role in Ser2 phosphorylation mediated transcription regulation in *Toxoplasma*. We performed IP of TgRpb1 from parasite treated with either DMSO or DRB or flavopiridol followed by Western blot with P-Ser2 antibody. We observed reduced band of P-Ser2-TgRpb1 in the inhibitor treated parasites while DMSO treated control remains unaltered (Fig. 5D). We checked the level of P-Ser5 upon TgCrk9 inhibition and observed no apparent change in P-Ser5 at TgRpb1-CTD (Fig. 5D). This was consistent with general observation that P-Ser2 rises downstream of transcription start site subsequent to P-Ser5 enrichment coinciding the entry of RNAPII in the productive elongation phase of transcription. Similar IP experiments were performed to determine the time dependent effect on P-Ser2 upon inhibitor treatment for 30, 60, 120 and 150 min. P-Ser2-CTD was markedly reduced in parasites after 120 min of either DRB or flavopiridol treatment, whereas P-Ser5 was less affected (Fig. 5E).

Taking note of the inhibitory effect of DRB and flavopiridol against TgCrk9, we first tested the effect of 10 μM and 15 μM concentrations of DRB on *T. gondii* proliferation in HFF cells. After 24 h of DRB treatment, substantial inhibition of *T. gondii* growth and proliferation at both the concentrations was observed with more pronounced effect at 15 μM concentration (Fig. 5F). Similarly, we tested the effect of two different concentrations (4 nM and 8 nM) of flavopiridol on parasite multiplication and found that at both the concentrations parasite proliferation was substantially abrogated (Fig. 5G).

In order to assess whether the observations following treatment with DRB and flavopiridol are an outcome of a TgCrk9 inhibitory effect and not a possible indirect effect of inhibitor induced apoptosis of the host cell, we checked for annexin V staining of the HFF cells following DRB and flavopiridol treatment for 24 h. We observed < 8% of HFF cells staining positive for annexin V with either DRB or flavopiridol and about 5% in DMSO treatment (Fig. 5H). These results were consistent with previous studies where similar dosages of DRB and flavopiridol treatment result in < 10% apoptotic cells [41]. Effective inhibition of parasite progression at concentrations selectively targeting the parasite protein without noticeable effect on host counterpart is promising with respect to designing intervention strategies.

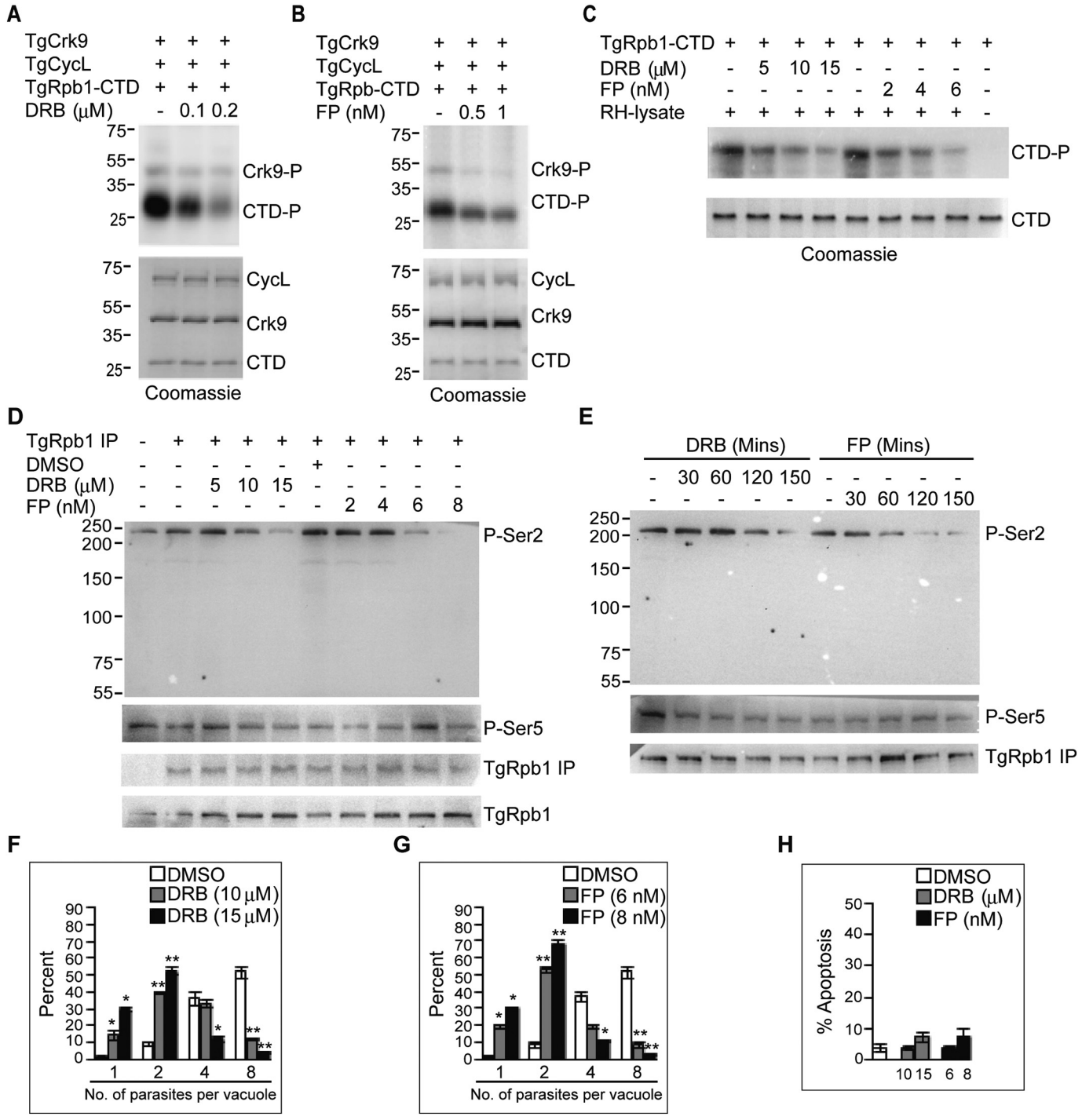

Fig. 5. DRB and flavopiridol (FP) inhibit TgCrk9 kinase activity *in vitro* and *in vivo*. (A and B) TgRpb1-CTD kinase activity of TgCrk9/TgCycL showed reduction in presence of DRB (0.1 μM or 0.2 μM) as well as flavopiridol (0.5 nM or 1 nM) *in vitro*. (C) Kinase assay using parasite lysate treated with DRB or flavopiridol, showed gradual decline in CTD phosphorylation with increasing concentrations of both the inhibitors. Coomassie stained gel show equal loading of TgRpb1-CTD (lower panel). (D) IP of TgRpb1 followed by Western blot analysis of P-Ser2 and P-Ser5 of TgRpb1 in *T. gondii* parasites treated with DMSO or 5, 10, & 15 μM DRB or 2, 4, 6, & 8 nM flavopiridol, shows progressive reduction of P-Ser2 while P-Ser5 is not as affected. No inhibitory effect was observed for DMSO control treated parasites. Similar levels of TgRpb1 in lanes from 2 to 10 suggested equal pull-down of TgRpb1. Similar levels of TgRpb1 (bottom panel) indicates equal parasite proteins used for IP. (E) Parasites were treated for 30, 60, 120 and 150 min with 15 μM DRB or 8 nM flavopiridol and analyzed by IP followed by Western blotting with the indicated antibodies. P-Ser2 levels showed gradual decrease with time whereas there was no apparent change in the P-Ser5 levels. Similar levels of TgRpb1 (bottom panel) indicates equal parasite proteins used for IP. (F and G) DRB and flavopiridol inhibited parasite proliferation in HFF cells. Indicated concentrations of inhibitor treated cells were fixed and observed under microscope. The number of parasites per vacuole were counted and converted to percentage. Error bars, s.e.m. ($n = 3$ independent experiments). *P < 0.005, Student's *t*-tests. (H) Apoptosis induction in HFF cells was assayed after 24 h treatment of the inhibitor using flow cytometry of annexin V-propidium iodide double stained cells. Signals are represented by the means ± SD (error bars) of three biological replicates.

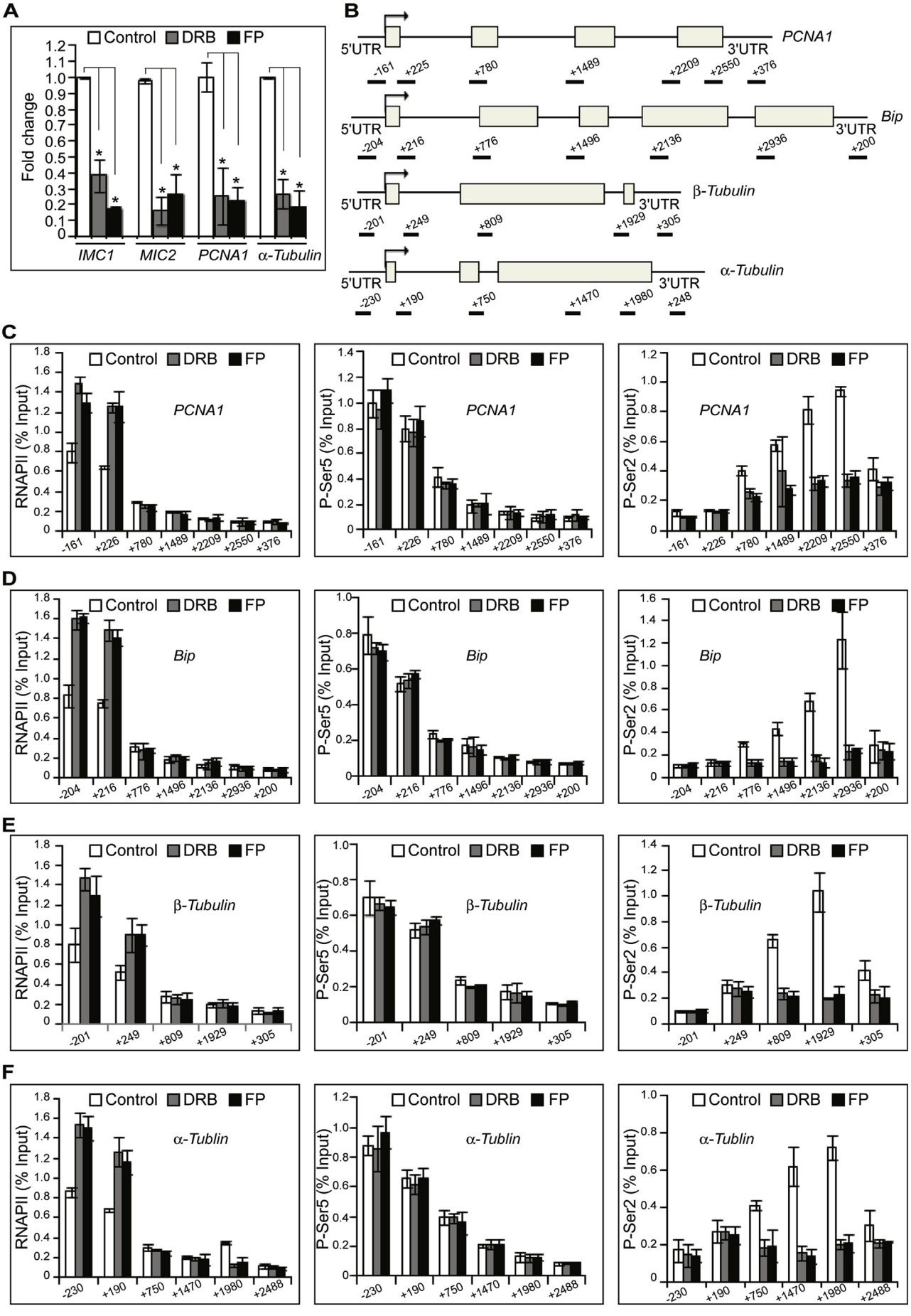

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Fig. 6. *TgCrk9* inhibition results in reduction of pre-mRNA. (A) Quantitative reverse-transcription (qRT)-PCR of nascent RNA (determined using primers targeting exon-intron or intron-exon junctions) from *IMC1*, *MIC2*, *PCNA1* and *α-Tubulin* after 2 h of DRB or flavopiridol treatment. Values were normalized to stable *IMC1* mRNA (primers located in exon 4 and exon 5). (B–F) Schematic of *PCNA1*, *BiP*, *β-Tubulin* and *α-Tubulin* genes with qPCR amplicons used for ChIP analysis indicated underneath (in base pairs) (C–F). Distribution of RNAPII, P-Ser5, P-Ser2 on *PCNA1*, *BiP*, *β-Tubulin* and *α-Tubulin* was determined by ChIP of *T. gondii* parasites treated with 10 μM DRB or 8 nM flavopiridol for 2 h. Values were normalized to total RNAPII. Error bars, s.e.m. ($n = 3$ independent experiments). *P < 0.05, Student's *t*-tests.

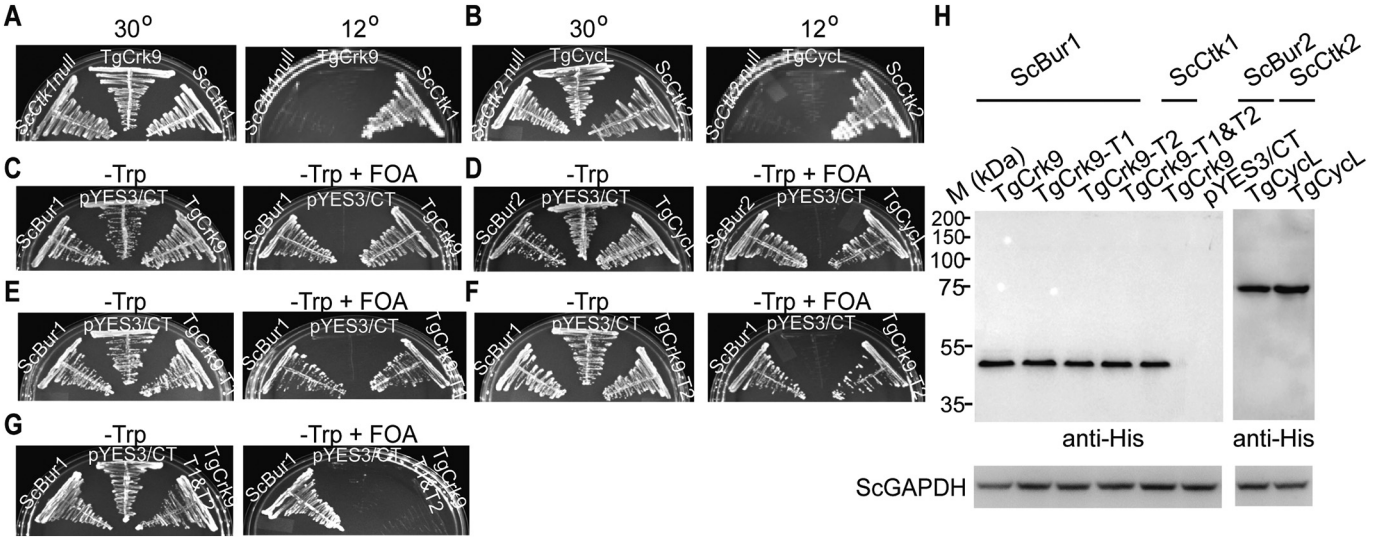

Fig. 7. Tg*crk9* and Tg*cycL* genes functionally complement *S. cerevisiae* counterparts. (A and B) Yeast strains with chromosomal copy deletion of *ctk1*and *ctk2* showing cold sensitive phenotype were transformed with plasmid encoding the Tg*crk9* and Tg*cycL* along with Sc*ctk1* and Sc*ctk2* genes respectively and selected at 12 °C. Neither Tg*crk9* nor Tg*cycL* could rescue cold sensitive mutants of *ctk1* and *ctk2* respectively unlike Sc*ctk1* and Sc*ctk2* which could rescue the respective mutant strain (self-complementation). (C–G) Yeast strains with chromosomal copy deletion of *bur1* were transformed with plasmid encoding the Sc*bur1*, Tg*crk9*, Tg*crk9-T1*, Tg*crk9-T2* and Tg*crk9-T1&T2* genes and selected on -Trp + FOA. Similarly, plasmid encoding Sc*bur2* and Tg*cycL* was transformed in the yeast strain carrying chromosomal copy deletion of *bur2*. Tg*crk9* wild-type and single threonine mutants but not the double threonine mutant were able to rescue *bur1* mutant strain like self-complementation with Sc*bur1* gene. Tg*cycL* was able to rescue the growth of yeast *bur2* mutant strain similar to self-complementation with Sc*bur2* gene. (H) *T. gondii* genes *crk9*, *cycL*, *crk9-T1*, *crk9-T2* and *crk9-T1&T2* were expressed in the respective transformants (Left panel- ScBur1: lane no. 1 to 4 & 6; ScCtk1: lane no. 5; right panel-ScBur2: lane no. 1 and ScCtk2 lane no. 2) as shown by Western blotting using anti-His antibody. *S. cerevisiae GAPDH* was used as loading control (bottom panel).

3.6. Inhibition of TgCrk9 abrogates transcription in parasite

To determine the role of TgCrk9 in RNAPII-driven transcription, we checked the effect of both the inhibitors on formation of nascent RNA at endogenous parasites genes. As newly synthesized pre-mRNA is rapidly spliced in comparison to the relative long half-life of mature mRNA, primers targeting specific exon-intron or intron-exon junctions were used for quantitative analysis of pre-mRNA. Parasites were treated with the 15 μM of DRB and 8 nM of flavopiridol for 120 min, RNA was extracted and pre-mRNA analyzed using RT-qPCR at few highly expressed parasite genes such as *IMC1*, *MIC2*, *PCNA1* and *α-Tubulin*. Reduction of nascent RNA level of all four genes was detected following TgCrk9 inhibition by DRB or flavopiridol (Fig. 6A). These results are consistent with data observed for several human cancer cell lines as well as in mouse embryonic stem cells [41,42].

In the active transcription process release of RNA polymerase II from the promoter is mediated through CTD phosphorylation. Human Cdk9 inhibition by DRB or flavopiridol results in reduced loading of RNAPII Ser2-CTD phosphorylation at 3′ end of the genes [41,42]. In *Toxoplasma*, we observed that DRB and flavopiridol inhibit TgCrk9 kinase activity *in vitro* and decrease Ser2-CTD phosphorylation *in vivo*. To further evaluate the role of TgCrk9 in RNAPII mediated transcription and to investigate how the inhibition of TgCrk9 would correlate with the distribution of TgRNAPII at the prototypical genes namely, *PCNA1*, *Bip*, *β-Tubulin* and *α-Tubulin* (Fig. 6B) ChIP analysis was performed in DRB and flavopiridol treated parasites. We observed enrichment of RNAPII towards the 5′ end of the genes as compared to the gene body and the RNAPII ChIP signals further increased at the 5′-end upon treatment with DRB and flavopiridol (Fig. 6C–F). P-Ser5 ChIP signals accumulate towards 5′ end of the genes and it remains relatively unaffected upon inhibitor treatment (Fig. 6C–F). However, P-Ser2 levels were low at the 5′-end of the genes and steadily increased towards the 3′-end in control parasites whereas in either DRB or flavopiridol treated parasites the levels of P-Ser2 were similar at the promoter and 5′-end but did not increase markedly at 3′-end of the genes (Fig. 6C–F). Taken together, our observations highlight a significant contribution of TgCrk9 in overall RNAPII mediated transcription indicating a possible role in transcription elongation.

3.7. Tgcrk9 and TgcycL genes functionally complement S. cerevisiae bur1 and bur2 respectively

The view that the Cdk9 activity is recapitulated in *S. cerevisiae* by two kinase complexes, Bur1/Bur2 and Ctk1/Ctk2/Ctk3, has been redefined following demonstration of metazoan Cdk12 and Cdk13 to be orthologs of Ctk1. However, equal sequence similarity of TgCrk9 with both Bur1 and Ctk1 (both with 30% sequence identity) and absence of canonical Cdk12/13 like protein kinase has led us to assume that TgCrk9 may reconstitute the activities of both Bur1 and Ctk1 in *Toxoplasma*. Therefore, to test this hypothesis, we performed functional complementation assay using Bur1/Bur2 and Ctk1/Ctk2 mutant strains. In *S. cerevisiae*: Bur1/Bur2 and Ctk1/Ctk2/Ctk3 are kinase complexes [29,30] where, Bur1 and Ctk1 are protein kinases and Bur2 and Ctk2 are their cyclin partners while Ctk3 function is yet to be ascertained. ScBur1 is an essential kinase whereas ScCtk1 and ScCtk2 null mutant are cold sensitive (grows at 30 °C but unable to grow at 12 °C). To

investigate whether *Toxoplasma* Crk9 and CycL (Cdk-cyclin pair) are functional homologs of Bur1 or Ctk1 kinase and Bur2 or Ctk2 cyclin partner respectively, we performed functional complementation assay. Neither Tg*crk9* nor Tg*cycL* could rescue cold sensitive Sc*ctk1* or Sc*ctk2* strain respectively at the restrictive temperature 12 °C (Fig. 7A and B). Sc*bur1* and Sc*bur2* mutant strains were transformed with plasmids expressing *bur1* or Tg*crk9* or pYES3/CT (empty vector) and *bur2* or Tg*cycL* or pYES3/CT respectively. The transformants were selected on FOA. The *bur1* and *bur2* yeast mutants expressing Tg*crk9* and Tg*cycL* grew normally like the self-complemented strain (Fig. 7C and D). These result clearly showed that *T. gondii crk9* and *cycL* could fully functionally complement the function of *S. cerevisiae bur1* and *bur2* respectively. Although *Toxoplasma* Crk9 kinase show ~30% sequence identity to both ScCtk1 and ScBur1, our results suggest that TgCrk9 is more closely related to ScBur1. Further, we tested the Tg*crk9* mutants for their ability to complement Sc*bur1* mutant. The single threonine mutants Tg*crk9-T1* and Tg*crk9-T2* grew normally (Fig. 7E and F) whereas double threonine mutant did not grow on FOA (Fig. 7G). The results confirm that the presence of at least one T-loop threonine is essential for functional activity of TgCrk9.

Expression of wild-type and mutant Tg*crk9* genes (~45 kDa) was confirmed in both Sc*bur1* and Sc*ctk1* mutant strains using Western blot analysis (Fig. 7H). Similarly, Tg*cycL* (~70 kDa) gene expression was verified in Sc*bur2* and Sc*ctk2* mutant strains (Fig. 7H). Inability of Tg*crk9* and Tg*cycL* to complement yeast *ctk1* and c*tk2* mutants respectively was not due to lack of expression of the transformed *Toxoplasma* genes. The results establish that the TgCrk9 and TgCycL are true functional counterparts of ScBur1 and ScBur2 respectively. Taken together; yeast complementation results confirmed TgCrk9 as a true Cdk9 homolog in *T. gondii*.

4. Discussion

T. gondii, an obligate intracellular parasite leads a complex life with a need to adapt to multiple hosts facilitated by its ability to multiply in uniquely flexible ways. Protein kinases especially Cdk-related kinases (Crks) may have a crucial role in regulating and coordinating the successful progression of this parasite. In this study, we have examined the role of an important TgCrk, elucidating its role in RNAPII mediated transcription highlighting some distinctive features. We demonstrate that TgCrk9 is an active kinase and identify its regulatory cyclin partner. In order to recognize the right cyclin associated with Crk9, we tested all the potential transcription related TgCyclins (TgCycH, TgCycL and TgCycY). The cyclins were assessed for their ability to interact and activate TgCrk9 kinase. Among the cyclins tested, only TgCycL could combine with TgCrk9 and activate its kinase function. Interaction between TgCrk9 and TgCycL could be verified both *in vitro* and *in vivo*. The activated TgCrk9 was able to phosphorylate CTD of TgRpb1. The CTD kinase activity of TgCrk9, mediated through a conserved serine 2 in the heptapeptide repeat of TgRpb1 was found to be inhibited by specific inhibitors, DRB and flavopiridol. This inhibition was observed at submicromolar (DRB) and subnanomolar (flavopiridol) concentrations that were considerably lower (for DRB) and slightly lower (for flavopiridol) than the dosage required for inhibiting the mammalian counterpart. The chemical inactivation of TgCrk9 in the parasite leads to abrogation of RNAPII mediated transcription resulting in overall reduction in pre-mRNA. TgCrk9 inhibition on the whole affected the *T. gondii* progression with little or no effect on the host viability. The study revealed several unique features in the *Toxoplasma* Crk mediated regulation of transcription. Presence of a limited repertoire of cyclins and lack or absence of conventional cyclin homologs was observed. Two threonines in T-loop of TgCrk9 appeared to be important for its activation. The activation of TgCrk9 could be achieved through either of the intact threonine residue displaying redundancy as well as functional flexibility. It is an exceptional feature as canonically only one conserved T-loop threonine is responsible for Cdk activation. Cyclin independent activation of TgCrk9 by a TgCrk7 based CAK was observed. TgCAK action on TgCrk9 was possibly mediated through the T-loop threonines.

Following identification of regulatory Crk (TgCrk7) involved in transcription initiation by RNAPII in *Toxoplasma* [22], we were interested to investigate the kinase responsible for events downstream of transcription initiation. In *Toxoplasma*, we found a kinase homolog of mammalian Cdk9 [43] and yeast Bur1 [30] which dispenses similar duties in their respective systems. *Toxoplasma* Cdk9 counterpart is characterized by the presence of cyclin binding domain and T-loop among other conserved features and appeared rightly poised to have a role in transcriptional control.

Cdk's need for a cyclin regulatory partner to fully realize its function was met with a challenge in *Toxoplasma* due to its limited repertoire of conventional cyclins. Cdk9 homolog in metazoans usually associates with cyclin T1, however, in absence of a canonical cyclin T1 in *Toxoplasma*, all potential cyclin partners were tested for their regulatory and functional association with TgCrk9. TgCycL emerged as the functional partner both *in vitro* and *in vivo*. TgCycL also interestingly shares nearly 25% identity with human cyclin T possibly reflecting its ability to partner more than one Cdk.

Cdk activity which is primarily regulated by cyclin binding may also be stimulated by binding of additional factors or phosphorylation at a conserved threonine in the T-loop [3]. T-loop phosphorylation causes small but significant structural changes in substrate binding domain which are essential for maximal activity [44]. In *Toxoplasma*, two such threonines in close succession (T250 and T257) have been found. Multiple phosphorylation have also been observed in the T-loop of mammalian Cdk7 [45,46], Cdk9 [47] and Cdk11 [38]. Sequence alignment of the T-loops of TgCrk9, Cdk7, Cdk9 and Cdk11 shows a conserved activating threonine residue, Thr257 in TgCrk9 in addition to another threonine residue, Thr250 unlike other Cdks aligned, which have a serine residue at similar position (Supplementary Fig. 2). In TgCrk9, any one intact threonine was sufficient for activity *in vitro* while a double threonine mutant lose complete kinase activity showing a unique dependence on two redundant phosphorylations within T-loop for Cdk activation. Importance of T-loop threonine phosphorylation in TgCrk9 led us to believe that there is an activating kinase upstream of TgCrk9 as T-loop threonine in Cdks are conventionally regulated through CAK activity [38]. We previously identified a TgCrk7 based CAK homolog which showed RNAPII transcription regulatory activity but failed to display any CAK activity when tested with cell cycle related Cdk2 homolog [22]. This was unlike higher eukaryotes where Cdk7 demonstrates cell cycle regulatory CAK activity [3,38]. There has been several conflicting reports when considering Cdk7 as the responsible CAK for activating downstream Cdk9 in humans [33,47,48], however, recent report has comprehensively demonstrated Cdk7 as the activating CAK for Cdk9 [37] where it acts by phosphorylating the T-loop. Certainly, it was worth checking whether TgCAK is capable of phosphorylating the TgCrk9. Our results demonstrate that TgCrk7 based ternary complex displays CAK like activity towards TgCrk9.

We adopted a chemical inhibition approach to fully comprehend the functions of TgCrk9. We utilized two Cdk9 selective inhibitors, DRB and flavopiridol which have been widely used across species to investigate its role [39]. Both the inhibitors which act by obliterating the ATP binding site of Cdk9 [33], exerted inhibitory effect on TgCrk9 both *in vitro* and *in vivo*, at concentrations lower than seen for mammalian Cdk9. Both the inhibitors affected RNAPII transcription and showed a potent overall anti-parasitic effect against *T. gondii* without affecting the host at the dosage used. In fact, Cdk9 inhibitor flavopiridol had been in clinical trials and has shown promising results against a number of cancers [49,50]. Therefore, flavopiridol and DRB analogs with more selectivity for TgCrk9 may be developed.

Phospho-threonine in the T-loop acts as an organization center helping achieve the right conformation for substrate binding [44,51]. Conserved T-loop threonine has also been observed to be site of autophosphorylation along with other residues [33] which promotes the

kinase activity of Cdks [52]. Since TgCrk9 has also been observed to be autophosphorylated the identified threonines may also serve as sites for autophosphorylation. In addition, TgCrk7 has emerged as both effector as well as a regulatory upstream kinase as demonstrated by its CAK activity towards TgCrk9. So far some reports have suggested that cell cycle Cdks undergo CAK mediated stimulation and does not undergo autophosphorylation while transcription Cdks lack CAK driven activation [48], however TgCrk9 not only displayed autophosphorylation but an upstream Crk7 based CAK exerts stimulatory phosphorylation influencing its overall activity. These peculiarities again indicate unique molecular mechanisms governing these processes.

ChIP analysis showed inefficient enrichment of P-Ser2 at the 3′ end of genes in the *T. gondii* where TgCrk9 has been inhibited which suggests its role in promoting transcription elongation. Moreover, like eukaryotic Cdk9-cyclin T (P-TEFb) which helps release RNAPII pauses often encountered in promoter proximal regions [53] and facilitates gene expression control and recruitment of RNA processing enzymes [54,55], similar role for TgCrk9-TgCycL may be envisioned. Further, it will be interesting to see if it has other canonical and often reported involvement in post translational modification of histone H2B at conserved lysine residue with implications for a larger chromatin modification dependent control of gene expression [56,57]. In addition to Ser2 of RNAPII CTD, another important target of metazoan Cdk9 kinase is Spt5, the largest subunit of DRB sensitivity-inducing factor (DSIF). The DSIF regulates RNAPII activity coordinating the initiation-elongation transition near promoter-proximal regions [58–60]. In *T. gondii* no Spt5 homolog has been reported so far. Upon searching *T. gondii* database (ToxoDB), we identified Spt5 like gene (TGME49_233000) which shares 40% identity with human counterpart (E value 1e-35), carrying characteristics KOW (Kyprides, Ouzounis, Woese) motifs. The possibility of TgSpt5 being potential target of TgCrk9 kinase may not be ruled out and warrants further investigation.

In conclusion, RNAPII dynamics in the parasite appears to be by and large conserved, however, our data has pointed out several peculiarities which possibly confer a certain level of flexibility and convenience to the parasite in leading a multistage, multifaceted complex life cycle spanning different host environments. Our study helps elucidate the significance of Cdk-cyclin signaling in RNAPII mediated transcription in this special class of eukaryotic parasite.

Transparency document

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Author contributions

Conceived and designed the experiments; ASD, PM. Performed the experiments; ASD, PM, AK, RG. Analyzed data; ASD, PM. Contributed reagents/materials; ASD, PM, AK, RG. Wrote the paper; ASD, PM.

Competing interests

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagrm.2018.02.004.

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