The MAT1 cyclin-dependent kinase-activating kinase (CAK) assembly/targeting factor interacts physically with the MCM7 DNA licensing factor

Yan Wang[a], Fan Xu[a], Frederick L. Hall[a,b,c,*]

[a] *Department of Molecular Pharmacology and Toxicology, University of Southern California School of Pharmacy, Los Angeles, CA 90033, USA*
[b] *Gene Therapy Laboratories, USC Keck School of Medicine, 1975 Zonal Ave., KAM 300, Los Angeles, CA 90033, USA*
[c] *Department of Surgery, USC Keck School of Medicine, 1975 Zonal Ave., KAM 300, Los Angeles, CA 90033, USA*

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Abstract MAT1 (ménage à trois1) functions as an assembly/targeting factor of CAK (cyclin-dependent kinase-activating kinase). In a search for MAT1-interacting proteins using yeast two-hybrid system, MCM7 (minichromosome maintenance 7), a member of a family of DNA licensing factors, was identified. The physical interaction between MAT1 and MCM7 was confirmed in vivo in yeast cells and verified with in vitro protein binding assays. Further studies showed the RING-finger motif of MAT1 is not required for the interaction with MCM7, while the C-terminal domain of MAT1 is indispensable. Immunoprecipitation of MCM7 in human osteosarcoma MG63 cells demonstrated that MCM7 associates with the CAK complex in vivo.

Key words: Ménage à trois1; Minichromosome maintenance 7; Licensing factor; Protein–protein interaction; Yeast two-hybrid system; Cell cycle control

1. Introduction

MAT1 (ménage à trois1) was initially identified as the third subunit of the cyclin-dependent kinase (CDK)-activating kinase (CAK), the other two subunits being the catalytic subunit CDK7 and the regulatory subunit cyclin H [1–3]. The structure of MAT1 includes an N-terminal RING-finger motif [1,3,4], a central coiled-coil region [4,5] and a C-terminal cyclin-like box [6]. MAT1 functions as an assembly factor to promote the stability and activation of the CDK7–cyclin H complex [1,3,4]. Each of these structural domains may function to mediate distinct protein–protein interactions [1,3,7,8]. However, the RING-finger motif of MAT1 is not required for this ternary complex formation [1,3], suggesting that it may mediate interactions with other cellular proteins. In the absence of MAT1, the interaction between CDK7 and cyclin H and the activation of the CDK7–cyclin H complex appear to be dependent upon the phosphorylation of a conserved site (Thr170 in the T-loop of human CDK7) [4]. However, the MAT1-mediated stabilization and activation of the CDK7–cyclin H complex is independent on the phosphorylation state of Thr170 [3,4] indicating that MAT1 functions as an activating assembly factor which is able to bypass the requirement for T-loop phosphorylation in the enzymatic activation of CAK.

In addition to its function as an assembly factor, MAT1 also serves as a targeting subunit of CAK to determine its substrate specificity [9,10]. Two major classes of putative CAK substrates have been identified [11]: (i) the CDK subfamily including CDC2 [12], CDK2 [12], CDK3 [13], CDK4 [14] and CDK6 [15]; and (ii) components of the transcriptional machinery including carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II [16], TFIIE [9], TFIIF [9], TBP [10], the transcription factor Oct-1 [17], and the tumor suppressor protein p53 [18,19]. In the presence of MAT1, CDK7–cyclin H exhibits an increase in its ability to phosphorylate the CTD of RNA pol II over CDK2 [9,10]. Moreover, it has been demonstrated that both p53 [18,19] and Oct-1 [17] are phosphorylated by CDK7–cyclin H in a MAT1-dependent manner. Therefore, in a manner similar to the activating/targeting properties initially demonstrated for cyclin A [6,20], MAT1 functions both as a positive regulating subunit and a targeting subunit that ultimately determines the substrate specificity of the multifunctional CAK complex.

To further understand the biochemical function(s) of MAT1, we performed a yeast two-hybrid screen to identify additional human cDNAs encoding MAT1-interacting proteins. Here, we report that one of the cDNAs isolated from this screen encodes human MCM7 (minichromosome maintenance 7), a new member of a growing family of DNA licensing factors known to be involved in the regulation of DNA replication. Further studies confirmed the protein–protein interaction between MAT1 and MCM7 in vitro and in vivo, suggesting an additional function of the CDK7–cyclin H–MAT1 kinase complex.

2. Materials and methods

2.1. Cell line, cell culture, and antibodies

Human MG63 osteosarcoma cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and antibiotics penicillin and streptomycin. Human 293T kidney epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine and antibiotics penicillin and streptomycin. The polyclonal anti-cyclin H (sc-609) and anti-CDK7 (sc-529) antibodies were purchased from Santa Cruz Biotechnology. The polyclonal anti-MCM7 antisera were generously provided by Dr.

*Corresponding author. Fax: (1)-323-442-3618.
E-mail: fhall@genome2.hsc.usc.edu

Abbreviations: CDK, cyclin-dependent kinase; CAK, cyclin-dependent kinase-activating kinase; CTD, carboxyl-terminal domain; MAT1, ménage à trois1; MCM, minichromosome maintenance

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Masatoshi Fujita, Aichi Cancer Center, Nagoya, Japan. Polyclonal antisera recognizing MAT1 were raised in rabbits against the purified recombinant fusion proteins MAT1-6xHIS, which could specifically recognize recombinant and endogenous MAT1 proteins in terms of Western blotting and immunoprecipitation (data not shown).

2.2. Two-hybrid library screening

The Matchmaker two-hybrid system was obtained from Clontech (Clontech Laboratories, Inc., USA), and two-hybrid library screening was carried out as described by the manufacturer [21]. Human full-length MAT1 cDNA encoding 309 amino acids was cloned into the vector $pGBT_9$ as a fusion with the yeast Gal4 DNA binding domain. The library used for screening was a HeLa cDNA library cloned at the *Eco*RI-*Xho*I site of pGADGH as a fusion with the Gal4 activation domain. The $pGBT_9$-MAT1 'bait' and pGADGH cDNA fusion library were co-transformed into the yeast strain HF7c using the lithium acetate procedure. Co-transformants were plated onto selection medium lacking tryptophan, leucine and histidine, and surviving clones were further tested for β-galactosidase activity using a colony lift filter assay according to the manufacturer's protocols. Then the plasmid DNAs were isolated from positive yeast clones, and a PCR strategy was utilized to amplify the cDNA insert at *Eco*RI-*Xho*I sites within the plasmid pGADGH cDNA. The insert cDNAs were subcloned into a TA cloning vector (Invitrogen), and prepared for DNA sequencing analysis by column chromatography (QIAGEN). Based on the results of a NCBI BLAST search, the insert DNA from clone #14 was determined to carry 489 base pairs (bp) which encodes the C-terminal domain of MCM7 cDNA, designated MCM7c.

2.3. Two-hybrid protein–protein interaction assays

The truncated MCM7c cDNA was cloned back into pGADGH at the *Eco*RI-*Xho*I site. In addition, a MAT1 [2] N-terminal fragment (1–576 bp) and a MAT1 C-terminal fragment (196–930 bp) were cloned into $pGBT_9$. The pGADGH-MCM7c construct was co-transformed into yeast strain Y190 with $pGBT_9$-MAT1, $pGBT_9$-MAT1(1–576) or GBT_9-MAT1(196–930), respectively. For the surviving co-transformants, nutrition selection in the presence of 45 mM 3-amino-triazole and a filter β-gal assay were performed as described above.

2.4. Expression and purification of recombinant MAT1 protein

Full-length human MAT1 cDNA was cloned into the bacterial expression vector pET23d (Novogen) as a fusion protein with a C-terminal 6xHIS tag. The recombinant MAT1 proteins were expressed in *Escherichia coli* [22], then purified, renatured, and dialyzed as described previously [23].

2.5. In vitro transcription/translation and binding assay

Total RNAs were extracted from human 293 cells (embryonic kidney epithelial cells) by phase separation using Trizol (Gibco BRL). Human full-length MCM7 cDNA was amplified by RT-PCR and cloned into the pET23d vector (Novogen). In vitro transcription/translations were performed using the TNT coupled reticulocyte lysate system (Promega) with pET23d-MCM7 as a template in the presence of [^{35}S]methionine/cysteine (ICN), as described by the manufacturer [24]. For evaluation of protein binding, MCM7 lysates were incubated with purified renatured recombinant MAT1 proteins at 30°C for 20 min, pre-immune sera or anti-MAT1 antisera were added for another incubation at 4°C for 1 h, and immune complexes were collected by the addition of protein A-Sepharose. The bound proteins were eluted with SDS buffer and subjected to SDS–PAGE and autoradiography.

2.6. Metabolic labeling and immunoprecipitation

Human MG63 osteosarcoma cells grown to 70% confluence were washed with prewarmed 1×PBS, and were preincubated with Dulbecco's modified Eagle's medium without glutamine, methionine, and cysteine (ICN) supplemented with 10% dialyzed fetal bovine serum (Irvine Scientific) at 37°C for 30 min. [^{35}S]Met/Cys (1.4 mCi/180 μl, ICN) was added directly to the culture media to effect a final concentration of 100 μCi/ml, and the cell cultures were incubated at 37°C for 4 h. The cells were lysed followed by centrifugation. The cell lysates were further clarified with protein A-Sepharose, and incubated with either pre-immune sera (control) or specific antibodies at 4°C for 2 h. The resulting immune complexes were collected with protein A-Sepharose, followed by washing with 1×PBS. The bound proteins were eluted in SDS buffer, and subjected to SDS–PAGE and autoradiography.

3. Results

3.1. Identification of MCM7 as a putative MAT1-interacting protein

To identify potential MAT1-interacting proteins, a yeast two-hybrid library screening was employed. Out of 3.6×10^6 double transformants, ~2000 clones showed His-independent growth. Among these 2000 His^+ transformants, 41 positive clones expressed the β-galactosidase activity indicating appreciable protein–protein interaction. After the isolation and sequencing of the insert DNAs from pGADGH cDNA in the positive clones, the resulting DNA sequences were subjected to a NCBI BLAST similarity search. One particular insert DNA contained 489 bp identical to 3′-end of MCM7 cDNA, designated as MCM7c. MCM7 is a new member of the minichromosome maintenance (MCM) family, a growing family of DNA licensing factors involved in the initiation of DNA replication.

3.2. Verification of the in vivo interaction between MAT1 and MCM7 in the yeast two-hybrid system

To confirm the interaction between MAT1 and MCM7c in vivo, and to identify the MAT1 domains that interact physically with MCM7c, a second yeast two-hybrid protein–protein interaction study was carried out. MCM7c was cloned back into pGADGH. Full-length MAT1 (1–930 bp), N-terminal MAT1 (1–576 bp), and C-terminal MAT1 (196–930 bp) were cloned into $pGBT_9$. PGADGH-MCM7c was co-transformed with $pGBT_9$-MAT1, $pGBT_9$-MAT1(1–576) and $pGBT_9$-MAT1(196–930), respectively, into yeast strain Y190. Confirmation tests were performed to detect the false-positive interactions: pGADGH was co-transformed with $pGBT_9$, $pGBT_9$-MAT1, and pLAM5′, respectively, and $pGBT_9$ was co-transformed with pMCM7c. In addition, pTD1 was co-transformed with pVA_3 to serve as a positive control. These

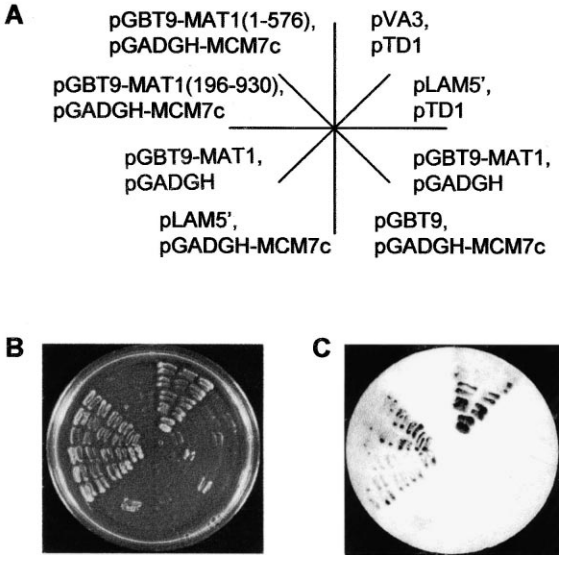

Fig. 1. Yeast two-hybrid assay of the interaction between MAT1 and MCM7. The assay was carried out as described in detail in Section 2. The co-transformants of pairwise constructs were subjected to nutrition selection assay and β-galactosidase activity assay. A: Schematic layout of co-transformed pairwise constructs. B: Growth on the SD/*-trp-Leu-His* plates. C: Filter assay of β-galactosidase activity.

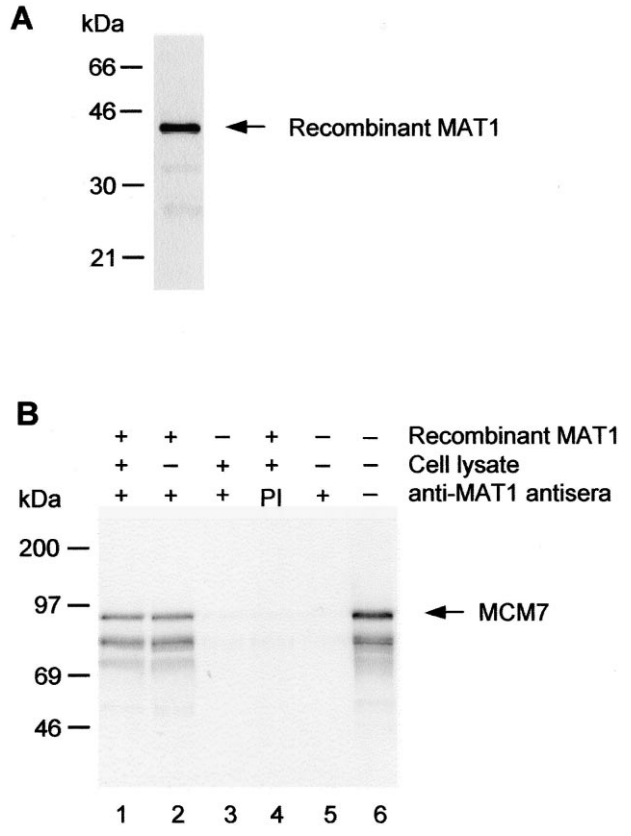

Fig. 2. MAT1 directly interacts with MCM7 in vitro. A: The purified renatured recombinant MAT1 proteins were subjected to SDS–PAGE, and stained with Coomassie blue. B: In vitro [^{35}S]methionine-labeled MCM7 was incubated with recombinant MAT1 in the presence (lanes 1, 4) or absence (lane 2) of cell lysate, followed by the addition of anti-MAT1 antisera (lanes 1, 2) or pre-immune sera (lane 4). The immune complex was precipitated with protein A-Sepharose, and subjected to SDS–PAGE and autoradiography. The specificity of anti-MAT1 antisera was assessed and shown in the presence (lane 3) or absence (lane 5) of cell lysate. Five percent of the MCM7 input is loaded in lane 6. Molecular mass markers are indicated on the left.

tests demonstrated the original positive result and confirmed the absence of false-positive interactions (Fig. 1). Specifically, β-galactosidase and nutrition selection positive clones appeared in the co-transformation of pGBT9-MAT1 and pGADGH-MCM7c (Fig. 1), indicating the specific interaction between MAT1 and MCM7c, which further confirmed the result of the initial two-hybrid library screening. Moreover, positive clones also appeared in the pGADGH-MCM7c co-transformation with pGBT9-MAT1(196–930), but not with pGBT9-MAT1(1–576) (Fig. 1), indicating that MCM7c interacts with the C-terminal MAT1 but not N-terminal domain of MAT1, which includes the RING-finger domain. In the previous report, it is found that the RING finger of MAT1 is not required for CDK7–cyclin H assembly [1,3]. Since RING-finger motif of MAT1 is most likely to mediate protein–protein interactions [3], we speculate that MAT1 interacts with other proteins through this RING-finger motif (see Section 4). Taken together, these experiments demonstrated the C-terminal domain of MAT1 is indispensable for MAT1's binding to MCM7.

3.3. Verification of the physical interaction between MAT1 and MCM7 in vitro

Although the specific physical interaction between MAT1 and MCM7c was detected and confirmed in yeast cells, we wished to verify the interaction between MAT1 and full-length MCM7. To address this issue, protein binding assays were performed with a combination of in vitro translated (IVT) proteins and purified recombinant MAT1 proteins (Fig. 2A). First, IVT full-length MCM7 produced in rabbit reticulocyte extract was incubated with purified renatured recombinant MAT1 in the presence or absence of mammalian cell lysate, which served as a control for interference or facilitation by additional soluble proteins. As shown in Fig. 2B (lanes 1–2), MAT1 bound MCM7 as determined by immunoprecipitation analysis, which is consistent with the results obtained in yeast cells. This binding is found to be independent of cell lysate (compare Fig. 2B, lanes 1 and 2), indicating the direct interaction between MAT1 and MCM7, and additional cellular factors are dispensable. Moreover, to determine the specificity of this interaction, a non-related control protein luciferase was produced by in vitro transcription/translation and included in the protein binding assay. Both IVT luciferase and full-length MCM7 were incubated with purified renatured recombinant MAT1. As shown in Fig. 3 (lane 2), MAT1 bound MCM7, but not luciferase, indicating the specific binding of MAT1 with MCM7.

3.4. Association of MCM7 with CAK complexes (CDK7, cyclin H, MAT1) in mammalian cells

To determine whether MCM7 actually associates with the CAK complex in mammalian cells, a co-immunoprecipitation assay was performed. Human osteosarcoma MG63 cells were metabolically labeled with [^{35}S]methionine/cysteine, followed by immunoprecipitation with a series of antibodies, as indicated in Fig. 4. Anti-MCM7 antibodies efficiently precipitated endogenous MCM7, which migrates on SDS–PAGE at about 86 kDa (Fig. 4, lane 3). Both anti-cyclin H and anti-CDK7 antibodies co-immunoprecipitated an 86 kDa protein, precisely the expected size of endogenous MCM7 (Fig. 4, lanes 4–5), while this protein was not visible in the immunoprecipitation reactions performed with non-immune sera (Fig. 4, lane

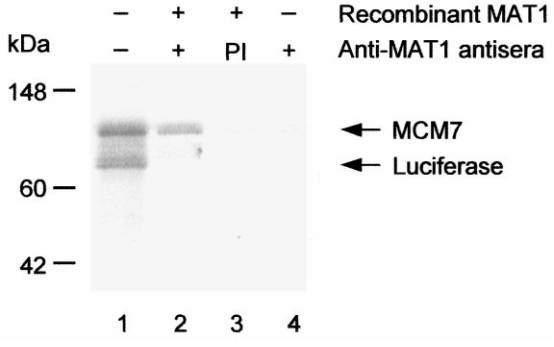

Fig. 3. The interaction between MAT1 and MCM7 is specific. In vitro [^{35}S]methionine-labeled MCM7 and luciferase were mixed and incubated with recombinant MAT1, followed by the addition of anti-MAT1 antisera (lane 2) or pre-immune sera (lane 3). The immune complex was precipitated with protein A-Sepharose, subjected to SDS–PAGE and autoradiography. The specificity of anti-MAT1 antisera was assessed and shown in lane 4. Five percent of the MCM7 and luciferase mixture is loaded in lane 1. Molecular mass markers are indicated on the left.

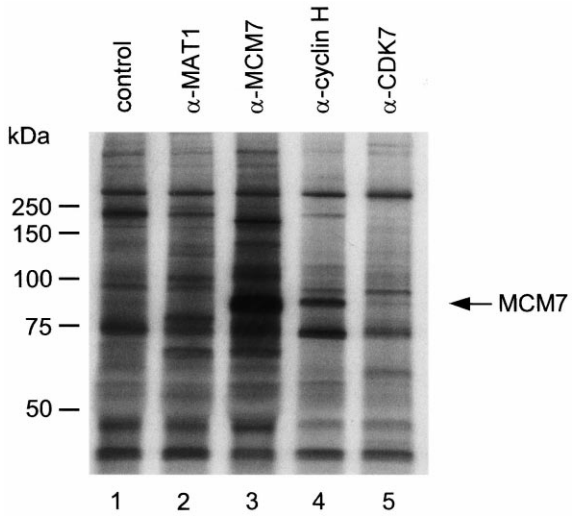

Fig. 4. MCM7 associates with CAK complex in vivo. MG63 cells were metabolically labeled with [^{35}S]methionine, denatured extracts were incubated with pre-immune sera (lane 1), anti-MAT1 antisera (lane 2), anti-MCM7 antisera (lane 3), anti-cyclin H antibodies (lane 4), and anti-CDK7 antibodies (lane 5). The immune complex was precipitated with protein A-Sepharose, subjected to SDS–PAGE and autoradiography. Molecular mass markers are indicated on the left.

1). In contrast to the demonstrable immunoprecipitation of MCM7 by anti-CDK7 and anti-cyclin H antibodies, anti-MAT1 antisera failed to precipitate MCM7 directly (Fig. 4, lane 2) for reasons, which remain speculative. Conceivably, the anti-MAT1 antisera either (i) disrupted the multimeric complexes, (ii) introduced stearic hindrances which precluded efficient precipitation and identification of the multimeric complexes, or (iii) failed to interact adequately with MAT1 which is tightly bound in CAK/MCM7 complexes. Nonetheless, the immunoprecipitation of metabolically labeled 86 kDa protein with anti-CDK7 and anti-cyclin H antibodies indicated that a physical interaction of MCM7 with CAK complexes is observed in vivo.

4. Discussion

In this study, we identified MCM7, a DNA licensing factor [25], as a MAT1-interacting protein using the yeast two-hybrid system. The direct physical interaction between these proteins was subsequently confirmed in yeast cells and in a series of in vitro assays (Figs. 1, 2B, 3). In mammalian cells in vivo, it was further confirmed that MCM7, either directly or indirectly, associates with specific subunits of the CAK complex, i.e. CDK7, cyclin H and MAT1, as detected by immunoprecipitation (Fig. 4). Furthermore, it was demonstrated that the RING-finger domain of MAT1 is not required for its association with MCM7c, while the C-terminal domain of MAT1 is found to be indispensable for this association (Fig. 1).

In a recent study of the association of MCM proteins with the RNA polymerase II (pol II) holoenzyme [26], it was demonstrated that several MCM proteins, including MCM7, co-purify with pol II along with general transcription factors such as TFIIH, indicating MCM7 associates either directly or indirectly with this transcriptional factor. TFIIH is a multisubunit complex consisting of at least nine polypeptides, and all three subunits of CAK, including CDK7, cyclin H and MAT1, are identified as components of the TFIIH complex [16,27]. Therefore, the present finding that MCM7 associates directly with MAT1 in the CAK complex, is in agreement with, and verified by the observation that MCM7 interacts physically with TFIIH.

While the functional significance of the interaction between MCM7 and MAT1 remains to be demonstrated, it is known that MAT1 functions as an assembly factor [1,3,4] and as a targeting factor of CAK [9,10,17], and that the multifunctional CAK has been proposed to be involved in cell cycle control, transcriptional regulation and DNA repair [11]. As the primary targeting factor of CAK, MAT1 is able to modulate CAK activities and functions via its physical and functional interactions with other target proteins, such as transcription factor Oct-1 [17]. In this case, MAT1 interacts with the POU domain of Oct-1, which effectively targets CAK to Oct-1 and results directly in its phosphorylation. Therefore, MAT1 targets and directs CAK activity to the transcription factor Oct-1, as well as the catalytic subunit of S-phase CDKs. Accordingly, the interaction between MAT1 and MCM7 may have implications for the allostatic regulation of MCM7, although this remains speculative at present. Alternatively, the RING finger of MAT1, and indeed many RING-finger proteins, are proposed to function in the ubiquitin degradation pathway [28,29], which may thereby regulate the metabolism of the pol II/TFIIH complexes.

MCM7 is a relatively new member of the MCM protein family (MCM2–7), which performs a critical role in DNA synthesis to ensure DNA is replicated precisely once per cell cycle [30]. The initiation of DNA replication occurs through the association of a set of prereplicative complexes (including the MCM family) with chromatin during G1, followed by activation of S-phase CDKs, which trigger DNA replication and block the assembly of new prereplicative complexes [31]. It is further suggested that the loading of MCM proteins onto chromatin may be regulated by Cdc6p and by CDKs [32]. Moreover, there is evidence that suppression of mitotic CDK activity by a selective CDK inhibitor leads to reloading of MCM7 to chromatin [32]. Therefore, since CAK targets various CDKs as its putative substrates [11], it is conceivable that CAK regulates MCM7 function indirectly through the activation of another associated CDK. In conclusion, the results of the present study suggest that MAT1 and CAK be further implicated in the regulatory biology of DNA replication through their physical interaction with MCM7.

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