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# Interplay between S-CDK and DDK in controlling DNA replication through phosphorylation of yeast Mcm4 N-terminal domain.

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Running head: Mcm4 phosphorylation in DNA replication

Abbreviations: S-CDK: S phase cyclin-dependent kinase; DDK: Dbf4-dependent kinase; preRC: pre-replication complex; MCM: mini-chromosome maintenance

**ABSTRACT**

Cyclin-dependent (CDK) and Dbf4-dependent (DDK) kinases trigger DNA replication in all eukaryotes but how these kinases cooperate to regulate DNA synthesis is largely unknown. Here we show that budding yeast Mcm4 is phosphorylated *in vivo* during S phase in a manner dependent on the presence of five CDK phosphoacceptor residues within Mcm4's N-terminal domain. Mutation to alanine of these five sites (*mcm4-5A*) abolishes phosphorylation and decreases replication origin firing efficiency at 22°C. Surprisingly the loss of function *mcm4-5A* mutation confers cold- and HU-sensitivity to DDK gain of function conditions (*mcm5/bob1* mutation or DDK over-expression), implying that phosphorylation of Mcm4 by CDK somehow counteracts negative effects produced by ectopic DDK activation. Deletion of the S-phase cyclins Clb5,6 is synthetic lethal with *mcm4-5A* and mimics its effects on DDK up mutants. Furthermore, we find that Clb5 expressed late in the cell cycle can still suppress the lethality of *clb5,6*Δ *bob1* cells, whereas mitotic cyclins Clb2, 3 or 4 expressed early cannot. We propose that the N-terminal extension of eukaryotic Mcm4 integrates regulatory inputs from S-CDK and DDK, which may play an important role for the proper assembly or stabilization of replisome-progression complexes.

## INTRODUCTION

Eukaryotic chromosome replication initiates throughout S phase from multiple origins and is controlled in large part by cyclin-dependent kinases (CDKs). CDKs have both positive and negative roles for DNA replication. They promote the initiation of DNA synthesis from competent origins but also prevent the re-assembly of pre-replicative complexes (preRCs) at origins that have already fired. This inhibition of preRC formation by CDKs allows chromosome replication to be coupled to the cell cycle (Diffley and Labib, 2002). PreRC assembly is quite well understood: it begins with origin recognition complex (ORC) binding to origin DNA, followed by recruitment of Cdc6 and Cdt1, which in turn permit loading of the Mcm2-7 complex, the likely replicative helicase (Blow and Dutta, 2005; Takeda and Dutta, 2005). Binding of Cdc6 and Cdt1 is a highly regulated step ensuring that origin licensing is restricted to the G1 phase and that origins do not fire twice during the same cell cycle (Blow and Dutta, 2005). CDKs inhibit origin licensing by targeting several preRC components (Cdc6, Cdt1, Mcm3) for degradation or nuclear exclusion (Liku *et al.*, 2005). Phosphorylation by CDK of Orc2 and Orc6 in *S. cerevisiae* (Nguyen *et al.*, 2001) and Orp2 in *S. pombe* (Vas *et al.*, 2001) also participates in inhibiting preRC formation during the S to M phase period.

Besides preventing preRC assembly CDKs also have a positive, yet less well understood role for origin firing. The maturation of preRCs requires activation of two evolutionary conserved S/T protein kinases: Dbf4-Cdc7 (Dbf4-dependent kinase or DDK) and S-phase CDK (Clb5,6-Cdk1 in *S. cerevisiae* or CycE,A-Cdk2 in higher eukaryotes). These two kinases promote the recruitment of the Sld3-Cdc45 and Sld2-Dpb11 heterodimers, the GINS complex and finally, RPA and DNApolα/primase to the site of initiation (Tanaka and Nasmyth, 1998; Zou and Stillman, 2000; Kamimura *et al.*, 2001; Masumoto *et al.*, 2002; Takayama *et al.*, 2003). Some of these initiation factors (Mcm2-7, Cdc45, GINS) move along with replication forks, indicating that they may be part of the active helicase complex (Aparicio *et al.*, 1997; Labib *et al.*, 2000; Tercero *et al.*, 2000; Takayama *et al.*, 2003). Accordingly, MCM and Cdc45 are both required for DNA unwinding in *Xenopus* egg extracts (Pacek and Walter, 2004). The GINS complex maintains association of MCM helicase with Cdc45 and other replication factors such as the checkpoint mediator Mrc1, the fork-pausing complex Tof1-Csm3 as well as DNA polymerase-associated proteins (Gambus *et al.*, 2006; Kanemaki and Labib, 2006). Thus the replication initiation and progression complexes are large molecular entities that contain numerous potential targets for CDK and DDK, but most studies have first focused on the MCM complex because it is well conserved among eukaryotes and carries helicase activity.

Several *in vivo* phosphorylation sites in the N-terminal region of Mcm2, 4 and 6 have been mapped, which can be phosphorylated *in vitro* either by CDK or DDK (Komamura-Kohno *et al.*, 2006; Masai *et al.*, 2006; Montagnoli *et al.*, 2006; Sheu and Stillman, 2006). However mutation of these sites to either Ala or Glu does not cause lethality, and the importance of these modifications for the proper execution of S phase remains to be evaluated. Recently, a role for DDK-dependent Mcm4 phosphorylation in promoting interaction with Cdc45 was demonstrated (Masai *et al.*, 2006; Sheu and Stillman, 2006). There is also evidence suggesting that Mcm4 phosphorylation by CDK might be inhibitory: in *Xenopus*, Mcm4 hyperphosphorylation by CDK was correlated with decrease of its binding to chromatin (Hendrickson *et al.*, 1996; Findeisen *et al.*, 1999) and the *in vitro* helicase activity of Mcm4-6-7 was inhibited when Mcm4 was phosphorylated by Cdk2 (Ishimi *et al.*, 2000). *In vivo* studies have determined that budding yeast Clb5,6-Cdk1 acts positively on DNA replication by phosphorylating Sld2 (Masumoto *et al.*, 2002) and the DNAPolε subunit Dpb2 (Kesti *et al.*, 2004). An Sld2 mutant in which all CDK phosphoacceptor sites are changed to Ala is lethal, shows strong defects in S-phase progression and it was demonstrated that phosphorylation of Thr84 is solely responsible for stabilizing the Sld2-Dpb11 interaction (Tak *et al.*, 2006). A breakthrough came from the recent discovery that phospho-mimetic forms of Sld2 combined to constitutive Sld3-Dbp11 complex formation can bypass all minimal requirement of CDK for DNA replication (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007). The fact that Sld2 and Sld3 phosphorylation is sufficient implies that phosphorylation of MCM by CDK is not essential for DNA replication. Although not a prime player, the MCM complex is clearly targeted by CDK and DDK in several eukaryotes, where fine-tuning of replisome assembly and helicase activity might be biologically important. In contrast, *Archaea* use only a subset of initiation factors found in eukaryotes and neither orthologs of Sld3, Cdc45, Sld2 and Dpb11 nor of CDK/DDK can be found. The homohexameric MCM complex from *M. thermoautotrophicum* has strong helicase activity *in vitro* whereas MCM complexes or sub-complexes isolated from eukaryotes have, at best, a weak activity (Kelman *et al.*, 1999; Chong *et al.*, 2000; Lee and Hurwitz, 2001; Shin *et al.*, 2003). Interestingly archeal Mcm proteins also lack the S/T-rich N-terminal extensions of eukaryotic Mcm2, 4 and 6, which are proposed targets for regulation by CDK and DDK. Here we provide *in vivo* evidence that phosphorylation of CDK consensus sites within the N-terminus of *S. cerevisiae* Mcm4 contributes to efficient origin firing. We also find that preventing Mcm4 N-ter phosphorylation is severely deleterious when combined to gain of function DDK mutations, suggesting that a proper balance between CDK and DDK activities on the MCM complex is necessary for efficient replisome assembly or progression.

## MATERIAL AND METHODS

### Plasmids and yeast strains

*tetCDC7* plasmid (D577) was described previously (Nougarede *et al.*, 2000) and is a YCplac22(TRP1) derivative. The *DBF4* ORF was inserted into pCM189(*URA3*) (Gari *et al.*, 1997) to obtain the *tetDBF4* plasmid. Mutations of putative CDK phosphorylation sites in ScMcm4 (Ser 7, 17, 32, 69 and 145) were generated using the QuickChange mutagenesis kit (Amersham), substituting serines for alanines. Mutation of Ser7 was marked with a HaeII restriction site, and Ser17 by a KasI site. Integration of all five mutations at the *MCM4* locus to generate the *mcm4-5A* allele was done by direct gene replacement. A *URA3* marker was first integrated 350 bp before the start codon of the *MCM4* ORF. Transformation with a mutant *MCM4* gene fragment and selection with 5-FoA allowed popping out of the *URA3* gene and integration of the mutated allele (strain E1448). The presence of mutations was verified by DNA sequencing. The *bob1-1* allele was marked by replacing nt 25-128 downstream of *MCM5* stop codon by the *K. lactis TRP1* gene. Strains expressing tagged versions of wild type Mcm4 and mutant Mcm4-5A proteins were constructed by replacing the stop codon of the *MCM4* and *mcm4-5A* genes by the TEV-PrA-7His-*Sp.his5* $^{+}$ cassette of plasmid pYM10 (Knop *et al.*, 1999). A 3HA-tagged *CLB5* gene under the control of the *GALS* promoter was generated by inserting the *GALS*-3HA-*nat* cassette of pYM-N32 (Janke *et al.*, 2004) just downstream of the initiator ATG codon of the *CLB5* gene. Chemical inhibition of CDK activity in the *cdc28-as1* strain was done in YEPD medium containing 0.5mM 1-NMPP1 (Bishop *et al.*, 2000). Table 1 lists yeast strains used in this study.

### DNA combing

In vivo BrdU incorporation, DNA combing and detection were performed as described earlier (Lengronne *et al.*, 2001), except that BrdU stretches and DNA fibers were labeled concomitantly with two different fluorophores. BrdU was detected with a rat anti-BrdU antibody (clone BU-75, Sera Lab) and a secondary antibody coupled to Alexa 488 (Molecular Probes), while DNA was revealed with a mouse anti-guanosine antibody (clone GK-21, Argene) and a secondary antibody coupled to Alexa 546 (Molecular Probes). Inter-origin distances (IOD), defined as the distance between the center of two successive co-linear BrdU tracks, were measured and plotted as a distribution (D) of IOD range categories. Box-and-whiskers plots were generated using GraphPadPrism software to visualize the main parameters of the distributions. Hypotheses that two distributions are equal or not were verified using the Mann-Whitney statistical test.

### Phos-Tag™ western-blot analysis

15 µg of whole-cell extract proteins prepared using the TCA method were loaded on standard 6% SDS-PAGE gels (10 x 10 x 0.08 cm) containing 25µM Phos-Tag™ ligand (AAL-107, NARD Institute, Japan) and 50 µM MnCl2, according to (Kinoshita *et al.*, 2006), with special care to avoid any traces of phosphate in buffers or molecular weight markers. Gels were run at 40 mA for 1h30 until bromophenol blue runs out, rinsed twice for 10min in transfer buffer (Tris-glycine, SDS, EtOH) containing 1 mM EDTA to chelate MnCl2, and once in the same buffer without EDTA. Proteins were transferred on Schleicher & Schuell ProTran membrane by semi-dry blotting for 75 min at 0.1 mA/cm2. The Protein A tag was revealed using PAP (peroxydase anti-peroxydase) antibody (Sigma P1291) at dilution 1:4000.

## RESULTS

### The N-terminus of yeast Mcm4 is phosphorylated *in vivo* on CDK sites

The N-terminal domain of eukaryotic Mcm4 contains a cluster of putative CDK phosphorylation sites that is conserved throughout evolution (Fig. 1). This feature is striking in view of the weak overall sequence conservation of this domain. In *S. cerevisiae*, there are two SPxK/R (S7, S145) and three SP motifs (S17, S32, S69) within the first 150 residues. Interestingly all five CDK motifs are preceded by one or two serine residues that, according to (Masai *et al.*, 2006; Montagnoli *et al.*, 2006), could correspond to DDK phospho-acceptors sites. This juxtaposition of potential DDK/CDK phosphoacceptors is a conserved feature of Mcm4 N-termini as all species aligned in Fig. 1 contain at least four of them. The remainder of the protein consists mostly of highly conserved domains ($AAA^+$ helicase motifs) with very few potential CDK sites. We performed phospho-proteomic analysis with an allele of *MCM4* tagged at its C-terminus with Protein A and introduced at the natural locus. However, assessing the *in vivo* phosphorylation status of yeast Mcm4 turned out to be difficult since the protein did not show obvious mobility shift during the cell cycle, in standard or modified western blot conditions (Fig. 2B top panel). Two-dimensional gel electrophoresis and mass spectrometry analyses were also unsuccessful (data not shown), perhaps due to the fact that only a small fraction of cellular MCM molecules is phosphorylated *in vivo* (Sheu and Stillman, 2006). Recently a phosphate ligand (PhosTag™) was introduced, which significantly slows down the migration of phosphoproteins in SDS-PAGE (Kinoshita *et al.*, 2006). Using PhosTag we found very reproducibly that about 10-20% of Mcm4 molecules migrate more slowly, depending on cell cycle position (Fig. 2B bottom panel). These Mcm4-specific (see Fig. 2A) slower migrating species were present in α-factor arrested cells, decreased at 15 min and reached their maximum 30 min after release, concomitant with DNA replication. Better inspection of samples run without PhosTag (Fig. 2B top panel) reveals

a broadening of the Mcm4 band at 30min, which likely corresponds to the phosphorylated forms seen using PhosTag. These forms decreased in G2/M to increase again upon S phase in the following cycle (75 min). We conclude that a fraction of yeast Mcm4 is phosphorylated *in vivo* during S phase. To test whether this phosphorylation depends on CDK sites within the N-terminus, serines within all five SP or SPxK/R motifs (S7, 17, 32, 69, 145) were substituted to alanine. This allele (*mcm4-5A*) was subjected to PhosTag™ analysis as above. Mutation of these sites caused disappearance of all slower migrating bands (Fig. 2C), demonstrating that Mcm4 phosphorylation *in vivo* depends on one or more of these five CDK sites clustered Mcm4's N-terminus. However, the complex pattern of Mcm4 phosphorylation during the cell cycle precluded any simple assessment of the kinase phosphorylating these sites. Specific inhibition of CDK during an α–factor release using a small molecule (1-NMPP1) in a *cdc28-as1* strain led to a complete disappearance of slower migrating bands during the first 30 min of the time course (Fig. 2E). Although these cells never exited G1 (no budding, no DNA replication) slower migrating bands reappeared at later time points, indicating that Mcm4 phosphorylation can also occur in a CDK-independent manner. This suggests that several kinases can phosphorylate Mcm4 but that CDK inhibition prevents or slows down the initial phosphorylation of Mcm4 in late G1.

### ***mcm4-5A* is synthetic lethal with *clb5,6*Δ and *mcm5/bob1***

The biological importance of these five CDK phospho-acceptor sites (S7, S17, S32, S69, S145) within Mcm4 was assessed using flow cytometry, cell viability and minichromosome maintenance assays, which did not point toward obvious DNA replication defects in the *mcm4-5A* mutant strain (data not shown). Thus these sites cannot be essential CDK targets, but does not rule out that Mcm4 sites may act synergistically with other targets. Indeed, we found that the *mcm4-5A* allele is lethal in a strain lacking the S-phase cyclins Clb5 and 6, which on its own has normal viability despite delayed DNA replication (Fig. 3A) (Schwob and Nasmyth, 1993). Using a conditional allele of *CLB5* driven by the *GALs* promoter, we were able to test the consequences for DNA replication and cell division of depleting Clb5 in a *clb6 mcm4-5A* strain. Figure 3B shows that *GALs-CLB5 clb6 mcm4-5A* cells on glucose replicate DNA more slowly than *GALs-CLB5 clb6* control cells (compare the 45 and 150 min time points in both strains). The triple mutant cells underwent one or two additional divisions and then died without ever forming a colony (Fig. 3A). The additive effects (co-lethality) of *mcm4-5A* and *clb5,6*Δ mutations implies both that the N-terminal Mcm4 sites can be phosphorylated by other kinases, possibly M-phase CDKs, and that Clb5,6-CDK (obviously) targets other key proteins. The lethality of *mcm4-5A clb5,6*Δ cells would thus stem from the impossibility to phosphorylate Mcm4 combined to the absence (or delay) of

phosphorylation of replication factors normally targeted by Clb5,6-Cdk1, such as for example Sld3 (Zegerman and Diffley, 2007).

We also combined the *mcm4-5A* allele with various other mutations in replication initiation factors (including putative CDK sites within Mcm2 and Mcm10). One double mutant, *mcm4-5A bob1*, turned out to be cold-sensitive (cs) for growth (Fig. 4A). *bob1-1* is an allele of *MCM5* (changing Pro83 to Leu) that bypasses the requirement of *CDC7* and *DBF4* for DNA replication (Hardy *et al.*, 1997). The *mcm4-5A bob1* double mutant ceases cell proliferation at 22°C (and below) after 1-3 cell divisions. To see if these cells have problems to initiate DNA replication, *bob1* and *mcm4-5A bob1* cells were synchronized with α-factor at permissive temperature (32°C) to allow preRC formation and then released at 22°C (restrictive temperature). Flow cytometry analysis revealed that although S phase started on schedule (30 min after αF-release) in the double mutant, it progressed very slowly, reaching apparent completion only at 105 min (instead of 60 min in the *bob1* control; Fig4B). Figure 4C shows that the *mcm4-5A bob1* double mutant is also highly sensitive to hydroxyurea (HU) at permissive temperature. These cells did not die in the first cell cycle (unlike *rad53* mutants on HU) but formed micro-colonies composed of 10-20 cells, suggesting that they are capable of exiting mitosis under chronic HU exposure but suffer from gradual loss of viability.

***mcm4-5A bob1* double mutants have replication initiation defects**

The slow S phase in the *mcm4-5A bob1* mutant at 22°C could be due either to a decrease of the number of active origins or to elongation proceeding at a slower pace. To monitor origin firing efficiency, we analyzed BrdU-labeled DNA fibers straightened on a microscope glass slide using DNA combing (Lengronne *et al.*, 2001). Upon release from α-factor at 22°C into medium containing HU, BrdU gets incorporated into short 10-20 kb regions surrounding origins (Fig. 5A). The density of active origins was then calculated by measuring the distance separating the center of two successive BrdU tracks (inter-origin distance, IOD) in more than 200 fibers. Figure 5B shows that the mean IOD in the *mcm4-5A bob1* double mutant is more than twice that of *bob1* and wild type strains (90kb instead of 42kb), implying that the number of fired origins (at least for the subset of early origins) is significantly lower in the double mutant. The notion that *mcm4-5A bob1* cells have a slow S phase because of initiation, not elongation defects is supported by the absence of a significant delay in S-phase completion when *mcm4-5A bob1* cells are first arrested in HU at permissive temperature (allowing for early origins to fire) and then released at restrictive temperature (Fig. S1). We conclude that the lengthening of S phase in the *mcm4-5A bob1* double mutant is almost entirely due to a failure to activate origins.

**_mcm4-5A bob1_ replication initiation defects stem from DDK gain of function**

One trivial explanation for the synthetic effects between *mcm4-5A* and *mcm5/bob1* could be that each mutation causes a slight loss of function that together would affect the functionality of the MCM complex. A more attractive hypothesis is that the N-termini of Mcm4 and Mcm5 (where the *mcm4-5A* and *bob1* mutations reside) integrate regulatory signals conveyed by CDK and DDK. In this scenario, contradictory inputs brought about by the *mcm4-5A* (loss of CDK function) and *bob1* (DDK bypass) mutations would make the initiation mechanism inefficient. In keeping with the latter hypothesis (contradictory inputs) for the co-lethality of *mcm4-5A* and *bob1*, we found that ectopic DDK expression recapitulates the phenotypes obtained with *mcm5/bob1*. Plasmids bearing the *CDC7* and *DBF4* genes under control of a Tet-repressible promoter were introduced in the *mcm4-5A* strain. As shown in Figure 4D, the *mcm4-5A* mutant did not grow at 22°C in the absence of doxycyclin, when both *CDC7* and *DBF4* are co-expressed. This lethality was not observed when only one of the two DDK subunits was overexpressed, or at 32°C where cells grew equally well in the presence or absence of doxycyclin (not shown). These findings indicate that the lethality of the *mcm4-5A bob1* strain likely stems from precocious or constitutive DDK-dependent activation of the MCM complex, not from a loss of function of the $Mcm5^{Bob1}$ subunit. The cold-sensitive phenotype suggests that, in *mcm4-5A bob1* cells at 22°C, a fraction of preRCs might be locked in a conformation that is refractory to inducers of DNA replication.

**CDK and DDK actions need to be coordinated for efficient origin activation**

These observations prompted us to evaluate the possibility that the *mcm4-5A bob1* lethality was due to an imbalance between CDK and DDK activities. One possibility is that forced activation of the preRC by DDK without previous Mcm4 phosphorylation by CDK leaves pre-initiation complexes in a state that is unstable or refractory to origin firing. If this scenario were correct, delaying the activation of S-phase CDK in a *bob1* background should produce the same phenotype as the *mcm4-5A bob1* double mutant. Deletion of *CLB5* and *CLB6* leads to a 30-40 min delay in the initiation of DNA replication, which is then triggered by Clb1-4 cyclins (Schwob and Nasmyth, 1993). As predicted by the above hypothesis, the *clb5,6*Δ *bob1* triple mutant was also found cold-sensitive for growth (Fig. 6A), with DNA replication progressing very slowly at the non-permissive temperature (Fig. 6B). Moreover, the *clb5,6*Δ *bob1* triple mutant showed HU sensitivity similar to that of the original *mcm4-5A bob1* mutant (Fig. 6C), indicating that ablation of Clb5 and 6 recapitulates the effects caused by removing Mcm4 phospho-acceptor residues in a *bob1* context. We further explored whether, in this situation

of delayed CDK activation, hyperactivation of DDK could substitute for the *bob1* mutation. Indeed, Figure 6D shows that *clb5,6*Δ cells cannot grow when both *CDC7* and *DBF4* are overexpressed. The strong similarity of phenotypes obtained with *mcm4-5A* and *clb5,6*Δ in conditions of DDK bypass or up-mutations is consistent with residues S7, S17, S32, S69 and S145 of Mcm4 being *in vivo* targets of CDK. It also suggests that S-CDK and DDK must act in a balanced and coordinated manner to fire origins in an efficient way.

**Mitotic cyclins cannot substitute for Clb5,6 in *bob1* cells**

In the experiments described above (Fig. 6), the lethality of *bob1 clb5,6*Δ cells at low temperature could stem either from a delay in phosphorylating S-CDK substrates (if mitotic cyclins can substitute) or a complete lack thereof (if Clb1-4/Cdk1 cannot productively phosphorylate these targets). To distinguish between these possibilities, we introduced in the *bob1 clb5,6*Δ strain a version of *CLB5* under control of the G2/M-specific *SWI5* promoter, which causes Clb5-Cdk1 kinase activity to appear at the same time as that of Clb2-Cdk1 (Fig. S2). Figure 7A shows that the *SWI5pr-CLB5* allele completely suppressed the cold lethality of the *bob1 clb5,6*Δ mutant. Thus while Clb5 expressed in G2/M can rescue the replication defects of the *bob1 clb5,6*Δ triple mutant, mitotic Clb1-4 cyclins that are expressed and active at the same time cannot. We conclude that it is not the timing of CDK activation that is critical for the viability of *bob1* cells, rather the specificity of the cyclin-Cdk complex. Clearly Clb5,6/Cdk1 does something to *bob1* cells that Clb1-4/Cdk1 cannot. To strengthen this conclusion, we performed the reciprocal experiment by expressing mitotic cyclins at a time matching that of the Clb5,6 S-phase cyclins. To this aim, *CLB2*, *3* and *4* open reading frames were each introduced at the *CLB5* locus (under control of the *CLB5* promoter) in a *bob1 clb6*Δ *swe1*Δ strain. It was shown previously that *SWE1* deletion significantly increases the ability of *clb5::CLB2*, *3* or *4* to drive S phase to almost wild type kinetics in a *clb5,6*Δ strain (Hu and Aparicio, 2005). Furthermore, it is known that Clb2 protein made from the *CLB5* promoter is expressed at similar levels and timing as endogenous Clb5, while carrying stronger kinase activity (Cross *et al.*, 1999; Loog and Morgan, 2005). Despite this, we found that none of the mitotic cyclins expressed early could suppress the cold sensitivity of the *bob1 clb5,6*Δ *swe1*Δ strain (Fig. 7B). Thus while either S- or M-phase cyclins can trigger DNA replication in wild-type cells, only Clb5 and 6 can do so in a strain where DDK regulation has been bypassed, suggesting that Clb5,6/Cdk1 has the unique property to interface with DDK activity.

**Phosphorylation of the Mcm4 N-terminus improves origin firing efficiency**

What could be the role of Mcm4-Nter phosphorylation during the normal process of initiation? Although the *mcm4-5A* mutation did not confer obvious growth defects in otherwise wild-type cells, careful analysis of S-phase progression by flow cytometry and DNA combing revealed a significant and reproducible lengthening of S phase in the *mcm4-5A* mutant strain at 18°C. DNA replication was completed only 120 min after release from G1 compared to 90 min in wild type (Fig. 8). This lengthening was also present at 22°C, but less important (not shown). Using DNA combing we found that the mean distance between active origins was 61 kb in the *mcm4-5A* single mutant at 22°C, compared to 42 kb in wild type and *bob1* cells (Fig. 5 and S3), indicating that chromosomes are duplicated from fewer origins in *mcm4-5A* cells. These results demonstrate that, although not essential in laboratory conditions, the phosphorylation of the Mcm4 N-terminus contributes to the efficacy of origin firing.

## DISCUSSION

### Mcm4 N-ter phosphorylation and the initiation of DNA replication

Mcm4 is highly conserved in eukaryotes. In multiple alignments covering divergent species, the similarity is high throughout most of its length, reaching 61% identity over a 137 amino acids stretch. The similarity drops dramatically however within the first 170 residues of Mcm4, which are rich in potential CDK phosphorylation sites. Using phospho-specific antibodies Ishimi and co-workers showed that at least 7 out of the 11 N-terminal S/TP sites are phosphorylated *in vivo* in human cells (Komamura-Kohno *et al.*, 2006). *In vitro* studies revealed that these phosphorylations are CDK-dependent (Ishimi and Komamura-Kohno, 2001). Finally, phosphopeptide mapping showed that CycB-Cdk1 was responsible for most if not all Mcm4 phosphorylation in M phase *Xenopus* extracts (Hendrickson *et al.*, 1996; Pereverzeva *et al.*, 2000). Here we show that a fraction of Mcm4 molecules is phosphorylated during S phase in *S. cerevisiae* (Fig. 2). Although we could not provide a formal demonstration that CDK is indeed responsible for these phosphorylations, this idea is supported by the strong similarities between the phenotypes caused by the elimination of five CDK phospho-acceptor residues within ScMcm4's N-terminus and those obtained by deleting the S-phase cyclins, when these mutants are combined to DDK gain-of-function mutations.

We provide the first *in vivo* evidence that phosphorylation of Mcm4 contributes to an efficient initiation of DNA replication. This phosphorylation of Mcm4 is not essential for initiation since the *mcm4-5A* mutant strain, which lacks all five CDK phosphoacceptor sites, displays only subtle replication defects in laboratory conditions. Yet at temperatures of 22°C or below, which are common for yeast ecotypes, the *mcm4-5A* allele

causes a clear lengthening of S phase, which correlates with a reduced number of fired origins determined by DNA combing. Interestingly for our understanding of the mechanism of replication initiation, we show that more profound effects are observed when the *mcm4-5A* allele is combined to mutations that bypass (*mcm5*/*bob1)* or up-regulate (ectopic Cdc7-Dbf4 expression) DDK activity. In this context, *mcm4-5A* cells proceed exceedingly slowly through S phase at low temperatures, leading to cell death within the first three cell divisions. The terminal morphology consists of large dumbbell cells with DNA trapped in the neck or stretched between the mother and daughter cells (not shown). The decreased origin firing measured on single DNA molecules in the *mcm4-5A bob1* strain points to an activating, not inhibitory, role of Mcm4 phosphorylation by CDK in initiating DNA replication. BrdU tracks were not shorter, nor was S-phase significantly lengthened when *mcm4-5A bob1* cells were shifted to restrictive temperature after initiation, arguing against a role of Mcm4 N-ter phosphorylation in the elongation of DNA synthesis. In contrast to Mcm4 that we show here to only contribute to efficient origin firing, Sld2 and Sld3 are essential to promote DNA replication in a CDK-dependent manner (Tak *et al.*, 2006; Tanaka *et al.*, 2007; Zegerman and Diffley, 2007). We suggest that, in addition to promoting the formation of Sld2-Dpb11 and Sld3-Cdc45 complexes required for recruitment of GINS and replisome progression complexes (RPC), S-CDKs also perform a non-essential regulatory function on the MCM complex that interfaces with DDK function.

Our finding that Clb5 expressed late in the cell cycle (from the *SWI5* promoter), but not any of the mitotic cyclins Clb2, 3 or 4 expressed early (from the *CLB5* promoter in a *swe1*Δ background), can suppress the cold lethality of the *bob1 clb5,6*Δ mutant indicates that the defects of this strain stem from a lack, not simply a delay of phosphorylation of one or more Clb5,6-specific substrates. It is known that a number of CDK substrates, among which some key replication factors such as Orc6, Cdc6, Mcm3 and Sld2, are more efficiently phosphorylated *in vitro* by Clb5-Cdk1 than by Clb2-Cdk1 (Loog and Morgan, 2005). This specificity was shown to depend on the presence of a hydrophobic patch in Clb5 and, in some cases, of a Cy or RXL motif in the substrate (Wilmes *et al.*, 2004). Given the genetic evidence presented here, we propose that Mcm4 could be another member of this class of preferential Clb5-CDK targets. However Mcm4 is not phosphorylated exclusively by S-CDKs, as revealed by the synthetic lethal interaction between the *mcm4-5A* and *clb5,6Δ* mutations and by the residual phosphorylation after chemical inhibition of Cdc28. The kinases responsible for these phosphorylations are likely mitotic CDKs and DDK, which could modify Mcm4 but to a lower level than in the presence of S-CDK. These activities would be sufficient for viability of *clb5,6Δ* cells, but not for the same cells in the context of DDK gain-of-function mutations.

**Efficient origin firing entails coordinated CDK and DDK action**

It is thought that *bob1,* an allele of *MCM5,* bypasses the requirement of *CDC7* and *DBF4* for DNA replication by inducing a conformational change in the MCM complex that mimics activation by Dbf4-Cdc7 kinase. In keeping with this interpretation, it was shown that *bob1* cells exhibit abnormally high amounts of chromatin-bound Cdc45 in α-factor arrested G1 cells (Sclafani *et al.*, 2002). Cdc45 binding to chromatin depends on DDK and is therefore very low in wild type G1 cells. The notion that *mcm4-5A bob1* defects are linked to bypass of DDK regulation is corroborated by our finding that ectopic expression of *CDC7* and *DBF4* in the *mcm4-5A* mutant has the same effect as the *bob1* mutation. Thus it seems that MCM complexes pre-activated by DDK cannot stably promote origin firing, unless Mcm4 is also phosphorylated on its N-terminus by S-CDK. This suggests that Mcm4 phosphorylation by CDK compensates for a conformational defect caused by the *bob1* mutation or by DDK hyper-activation (Fletcher and Chen, 2006). Another way to think about these data is that there might be an order in the molecular events leading to preRC activation by CDK and DDK. In this view, forced pre-RC activation by DDK (either through *bob1* or ectopic DDK expression) without previous Mcm4 phosphorylation by S-CDK might render origins refractory to firing by affecting replisome assembly or stabilization.

**Intricacy of CDK and DDK actions may provide for directionality in replisome assembly**

Why should ectopic DDK activity in conditions where Mcm4 cannot be phosphorylated by CDK be deleterious for preRC activation? One hint could be that Cdc45 binds to chromatin much earlier in G1 in *bob1* than in *WT* cells (Sclafani *et al.*, 2002). Binding of Cdc45 prior to Mcm4 phosphorylation by Clb5,6-Cdk1 might lead to the assembly of a abnormal pre-initiation complex that might be locked, at least at low temperatures, in a conformation that cannot easily trigger initiation. Another hypothesis is suggested from recent studies on Mcm2 phosphorylation (Montagnoli *et al.*, 2006), where six *in vivo* phosphorylation sites on human Mcm2 have been identified, three dependent on DDK and three on CDK. Two of these modifications affect adjacent serines, with DDK phosphorylating S40 and CDK phosphorylating S41. Strikingly, it was found that while both kinases could phosphorylate their cognate serines (S40, S41) *in vitro* when the neighboring Ser was unphosphorylated, only DDK could do so when the other serine was already phosphorylated. That is, CDK is unable to phosphorylate S41 when S40 is already modified. Reciprocally, it was shown that Mcm2 phosphorylation by DDK is facilitated by prior phosphorylation by CDK (Masai *et al.*, 2000). This suggests that CDK and DDK must act sequentially (CDK first) to phosphorylate hMcm2 on Ser41 and 40, respectively, which also fits the notion that DDK targets S/T residues that are either embedded in acidic stretches or next to residues already carrying (negatively-

charged) phosphate moieties. Priming of a DDK substrate by prior CDK phosphorylation has been recently demonstrated for the yeast meiotic recombination protein Mer2, and could be a common mechanism for CDK-DDK co-regulation (Wan *et al.*, 2008). Mcm2 has an evolutionary divergent N-terminal domain that contains several such potential DDK-CDK bi-phosphorylation SSP (or STP) motifs. In fact, most of the potential CDK sites located in the Mcm4 N-terminal domains of many evolutionary distant species, as well as all five sites that we mutated in ScMcm4 belong to this category (Fig. 1). It was shown recently that Mcm4 is phosphorylated by DDK in *Xenopus* and budding yeast (Takahashi and Walter, 2005, Masai, 2006 #2058; Sheu and Stillman, 2006). Together with our data, these results suggest that one or more subunits of the MCM complex are phosphorylated both by CDK and DDK, whereby CDK might favor the action of DDK but, conversely, where precocious phosphorylation by DDK might forestall the action of CDK on the preRC. Such a dependency in kinase function might provide for directionality along the sophisticated path of origin firing, which entails the orderly addition of various sub-complexes to the preRC before DNA synthesis actually begins. We propose that the amino-terminal extensions of eukaryotic Mcm2,4 and 6 integrate regulatory signals conveyed by S-CDK and DDK. Failure to coordinate CDK and DDK activities on the MCM complex might destabilize the replisome and cause abortive firing of replication origins. We speculate that the regulation of MCM's N-terminal extensions by S-CDK and DDK may also account for the modulation of fork progression rates seen in eukaryotes (Raghuraman *et al.*, 2001) compared to Archaea (Lundgren *et al.*, 2004).

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TABLE 1. List of strains used in this study.

| Strain | Relevant genotype | Origin |
|---|---|---|
| E001 | *MATa, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100* | W303-1A |
| E718 | *MATa, bob1-1* | C. Hardy |
| E1000 | *MATa, ura3::URA3/GPD-TK(7x)* | Lengronne *et al*., 2001 |
| E1448 | *MATa, mcm4-5A* | This study |
| E1823 | *MATa, mcm4-5A, bob1-1, ura3::URA3/GPD-TK(7x)* | This study |
| E1825 | *MATa, bob1-1, ura3::URA3/GPD-TK(7x)* | This study |
| E1826 | *MATa, mcm4-5A, ura3::URA3/GPD-TK(7x)* | This study |
| E1968 | *MATa, clb5::HIS3, clb6::LEU2* | This study |
| E1971 | *MATa, clb5::URA3, clb6::LEU2* | This study |
| E2476 | *MATa, clb5::URA3, clb6::LEU2, bob1-1::TRP1* | This study |
| E2486 | *MATa, mcm4-5A, bob1-1* | This study |
| E2605 | *MATa, clb5::HIS3, clb6::LEU2, SWI5::SWI5pr-CLB5::URA3* | This study |
| E2606 | *MATa, clb5::HIS3, clb6::LEU2, bob1-1::TRP1, SWI5::SWI5pr-CLB5::URA3* | This study |
| E2726 | *MATa, MCM4::TEV-PrA-7His/ Sphis5*$^{+}$ | This study |
| E2729 | *MATα, clb6::LEU2, bob1-1::TRP1, swe1::KanMX, clb5::CLB3* | This study |
| E2731 | *MATα, clb5::URA3, clb6::LEU2, bob1-1::TRP1, swe1::KanMX* | This study |
| E2738 | *MATa, mcm4-5A::TEV-PrA-7His/Sphis5*$^{+}$ | This study |
| E2756 | *MATα, clb6::LEU2, bob1-1::TRP1, swe1::URA3, clb5::CLB4* | This study |
| E2781 | *MATα, clb6::LEU2, bob1-1::TRP1(Kl), swe1::HIS5, clb5::CLB2* | This study |
| E2799 | *MATa, clb6::LEU2, bob1-1::TRP1, swe1::KanMX* | This study |
| E3200 | *MCM4::TEV-PrA-7His/ Sp.his5*$^{+}$, *GALS-3HA-CLB5/nat, clb6::LEU2* | This study |
| E3215 | *mcm4-5A::TEV-PrA-7His/Sp.his5*$^{+}$, *GALS-3HA-CLB5/nat, clb6::LEU2* | This study |
| E3218 | *MATa, MCM4::TEV-PrA-7His/ Sphis5*$^{+}$, *cdc28-as1* | K. Shokat / this study |

All strains are congenic or at least backcrossed four times to W303-1a.

## FIGURE LEGENDS

**Figure 1.** Multiple alignment of the N-terminal domain of Mcm4 from different species. Sc: *S. cerevisiae*; Sp: *S. pombe*; Ca: *C.albicans*; Hs: *H. sapiens*; Mm: *M. musculus*; Xl: *X. laevis*; Dm: *D. melanogaster*; At: *A. thaliana*. Potential CDK phosphorylation sites are marked by black boxes.

**Figure 2**: The N-terminal extension of Mcm4 is phosphorylated *in vivo* during S phase in a CDK-dependent manner. **(A)** Specificity of western-blot detection of Mcm4 tagged with Protein A. Whole-cell extracts from untagged strain (lane 1), three MCM4-PrA transformants (lanes 2-4) and an unrelated strain expressing SmB1-TAP (lane 5) detected on western blots using PAP antibody and ECL. All signal derives from the PrA-tagged

protein. **(B-E)** Strains of the indicated genotype expressing a single copy of either *MCM4* or *mcm4-5A* tagged with Protein A were arrested with α-factor and released in YPD medium at 30°C (B,C) or in YPD containing 0.5 mM 1-NMPP1 (D,E). Samples were taken at the indicated time points and analyzed for DNA content by flow cytometry (left) and for Mcm4 phosphorylation using SDS-PAGE run with or without 25 µM Phos-Tag™ as indicated. The vertical bar and asterisk indicate slower-migrating phospho-Mcm4 species on western blots.

**Figure 3**: Synthetic lethality of *mcm4-5A* and *clb5,6*Δ. **(A)** Serial fivefold dilutions of strains E3013 (*GALs-CLB5 clb6*Δ), E3192 (*mcm4-5A*), E3205 (*GALs-CLB5 clb6*Δ *mcm4-5A*) and E3208 (*GALs-CLB5 clb6*Δ *mcm4-5A bob1-1*) were spotted on YPGal and YPD (Glu) plates and grown for 2 to 3 days at 30°C. **(B)** Strains E3200 (*GALs-CLB5 clb6*Δ) and E3215 (*GALs-CLB5 clb6*Δ *mcm4-5A*) were grown in YEPRafGal medium, arrested in G1 with α-factor, depleted for Clb5 by addition of 2% Glu to the medium 30min before release from G1 arrest. Samples were taken at the indicated times and analyzed for S-phase progression using flow cytometry.

**Figure 4**. Phenotypes of the *mcm4-5A bob1-1* mutant. **(A)** Serial fivefold dilutions of strains E001 (WT), E1448 (*mcm4-5A*), E718 (*bob1-1*) and E2486 (*mcm4-5A bob1-1*) were spotted on YPD plates and grown at 32° or 22°C. **(B)** *bob1-1* and *mcm4-5A bob1-1* strains grown in rich medium were arrested in G1 with α-factor at 32°C and released at 22°C by pronase addition. DNA content was analyzed by flow cytometry. BI: budding index. **(C)** Serial fivefold dilutions of WT (E001), *mcm4-5A* (E1448), *bob1-1* (E718) and *mcm4-5A bob1-1* (E2486) strains were spotted on YPD plates containing or not 66mM HU and grown at 31°C. **(D)** MCM4 (wild type, E001) and *mcm4-5A* (E1448) strains containing plasmids pCM189(*URA3*) (tet-U) and YCplac22(*TRP1*) (YCp-W), containing or not the *DBF4* or *CDC7* genes under control of a tetracycline-repressible promoter were spotted as serial fivefold dilutions on synthetic medium lacking uracil and tryptophan and containing (transcription OFF) or not (ON) doxycycline (2 µg/ml) at 22°C.

**Figure 5**. DNA combing analysis of origin firing. **(A)** WT, *bob1-1*, *mcm4-5A* and *bob1-1 mcm4-5A* strains containing seven copies of the thymidine kinase gene (respectively E1000, E1826, E1825, E1823) were grown in rich medium, arrested with α-factor at 32°C and released for 90 min at 22°C in medium containing 200 µg/ml BrdU and 0.2M HU. Genomic DNA was extracted from cells and DNA fibers combed on silanized glass slides. DNA fibers (red signals) and BrdU-substituted regions surrounding origins activated in early S phase (green signals) were visualized by fluorescence microscopy using anti-guanosine (Argene) and anti-BrdU (BU-75,

SeraLab) antibodies, respectively. **(B)** Distribution of interorigin distances (IOD), plotted as histograms. **(C)** The IOD distributions were compared between each strain using a Mann-Whitney statistical test, which determines if the medians of the distributions are significantly different.

**Figure 6.** *clb5,6*Δ *bob1-1* cells show defects very similar to those of *mcm4-5A bob1-1*. **(A)** Serial fivefold dilutions of strains E001 (WT), E718 (*bob1-1*), E1971 (*clb5,6*Δ) and E2476 (*clb5,6*Δ *bob1-1* ) were spotted on YPD plates and grown at 32° or 22°C. **(B)** *bob1-1* and *clb5,6*Δ *bob1-1* strains grown in rich medium were arrested in G1 with α-factor at 32°C and released at 18°C by pronase addition. DNA content was analyzed by flow cytometry. BI: budding index. **(C)** Serial fivefold dilutions of *clb5,6*Δ and *clb5,6*Δ *bob1-1* strains were spotted on YPD plates containing or not 0.2M HU and grown at 31°C. **(D)** E001 (*WT*) and E1448 (*mcm4-5A*) strains containing plasmids tet(U) (URA3), YCp(W) (TRP1), containing or not the *DBF4* or *CDC7* genes under control of a tetracycline-repressible promoter were spotted as serial fivefold dilutions on synthetic medium lacking uracil and tryptophan and containing (transcription OFF) or not (ON) doxycycline (2µg/ml) at 22°C.

**Figure 7.** *SWI5pr-CLB5* but not *clb5::CLB2,3* or *4* suppresses cold lethality of *clb5,6*Δ *bob1-1*. Serial fivefold dilutions of strains E1971 (*clb5,6*Δ), E2476 (*clb5,6*Δ *bob1-1*), E2605 (*clb5,6*Δ *SWI5pr-CLB5*) and E2606 (*clb5,6*Δ *SWI5pr-CLB5 bob1-1* ) (panel A), or *clb6*Δ *swe1*Δ *bob1-1* strains containing either wild type *CLB5* (E2799), *clb5*Δ (E2731) or *CLB2, CLB3* or *CLB4* under control of the *CLB5* promoter (E2781, E2729 and E2756, respectively) (panel B) were spotted on YPD plates and grown at 31° or 18°C.

**Figure 8.** S phase is slower in the *mcm4-5A* mutant. E001 (*WT*) and E1448 (*mcm4-5A*) strains grown in rich medium were arrested in G1 with α-factor at 32°C and released at 18°C by pronase addition. DNA content was analyzed by flow cytometry. BI: budding index.

## Supplementary figures

**Figure S1**. The completion of DNA replication after an HU block is not affected in *mcm4-5A bob1* cells. E718 (*bob1-1*) and E2486 (*mcm4-5A bob1-1*) strains grown in rich medium were arrested with α-factor, released in 0.2M HU at 32°C and then shifted to 22°C in fresh medium lacking HU. DNA content was analyzed by flow cytometry. BI: budding index.

**Figure S2**. Expression of *CLB5* from the *SWI5* promoter delays Clb5-Cdk1 kinase activation. (A) *CLB5* driven

by the *SWI5* promoter was integrated at the *SWI5* locus of a *clb5 clb6* strain. Clb5- and Clb2-associated H1 kinase activities were measured in cultures of wild type (E001; left) and *clb5 clb6 SWI5p-CLB5* (E447) strains synchronized in G1 by α-factor treatment. Cyc, cycling culture ; αF, a-factor arrested cells. (B). PhosphoImager quantitation of the kinase assays, showing that Clb5 kinase activity is delayed to G2/M and now overlaps with that of Clb2 in the *SWI5p-CLB5 clb5,6* strain (right).

**Figure S3.** Box-and-Whiskers plots of inter-origin distance distributions in the indicated strains. Inter-origin distance (IOD) histograms of Figure 5 were re-plotted as box-and-whiskers graphs. The beginning and end of the horizontal lines represent the minimal and maximal values of IODs, respectively. The three vertical lines delineating the two shaded boxes define, from left to right, the first, second (median) and third quartile values of the distribution.

```
Sc   ......MSQQSSSPTKEDNNSSSPVVPNPDSVPPQLSSPALFYSSSSSQGDIYGRNNSQN   54
Sp   ........MSSSQQSGRANELRTPGRANSSSREAVDSSPLFFPASSPGSTRLTTPRTTAR   52
Ca   MSSPPQSTNQNNSNPAQDSLERESGSISQSQQPPIVSSPLFFNSSNPGSDIGNFNSQSQS   60
Hs   ..........MSSPASTPSRRGSRRGRATPAQTPRSEDARSSPSQRRRGEDSTSTGELQP   50
Mm   ..........MSSPASTPSRRSSRRGRVTPTQSLRSEESRSSPNRRRRGED.SSTGELLP   49
Xl   ..........MSSPTSTPSRRRVNVGEAVTLQLLVERKCSLLRHRKDGQKIPHQLVSFCP   50
Dm   ..........MSSPARSPSVGGATPKQGARTPTRGIASQDVETPMRMGPGRAVRPSDNIS   50
At   ..........MASDSSLGNTNDGPPSPGENVSSPIENTYSSPAALHRRRRGRSSTPTQFA   50

Sc   LSQGEGNIRAAIGSSPLNFPSSSQRQNSDVFQSQGRQGRIRSSASASGRSRYHSDLRSDR  114
Sp   TPLASSPLLFESSSPGPNIPQSSRSHLLSQRNDLFLDSSSQRTPRSTRRGDIHSSVQMST  112
Ca   QSQSQ...RRNDISSPLHYTSSAQPTSDIGGFDSQRSARVQDVGRIMRRAQRSDVTDSVS  117
Hs   MPTSPGVDLQSTAAQDVLFSSPPQMHSSAIPLDFDVSSPLTYGTPSSR.VEGTPRSGVRG  109
Mm   MPTSPGADLQSPPAQNALFSSPPQMHSLAIPLDFDVSSPLTYGTPSSR.VEGTPRSGVRG  108
Xl   CQPHLQETFRVP.....CFLALAPSRHSAHQSELDLSSPLTYGTPSSR.VEGTPRSGIRG  104
Dm   LPPTSPGNISLP.......ATSPARGLGANMSEIDLSSPLNYGTPSSMGSIRTPRSGIRG  103
At   TPPPPPSRLASS.......NSTPPTSRPSAARSKGRNGHGGGGGGGGGDPGTPMSTDEP  103

Sc   ALPTSSSSLGRNGQNRVHMRRNDIHTSDLSSPRRIVDFDTRSGVNTLDTSSSSAPPSEAS  174
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Ca   SPQRSRRYFTQGRGNGPSNLN......................SSTSAQFSTDPAEPND  154
Hs   TPVRQRPDLGSAQKGLQVDLQS.....................DGAAAEDIVASEQSL  146
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At   LP.....................................SSDDGEEDGGDDTTPTFV  123
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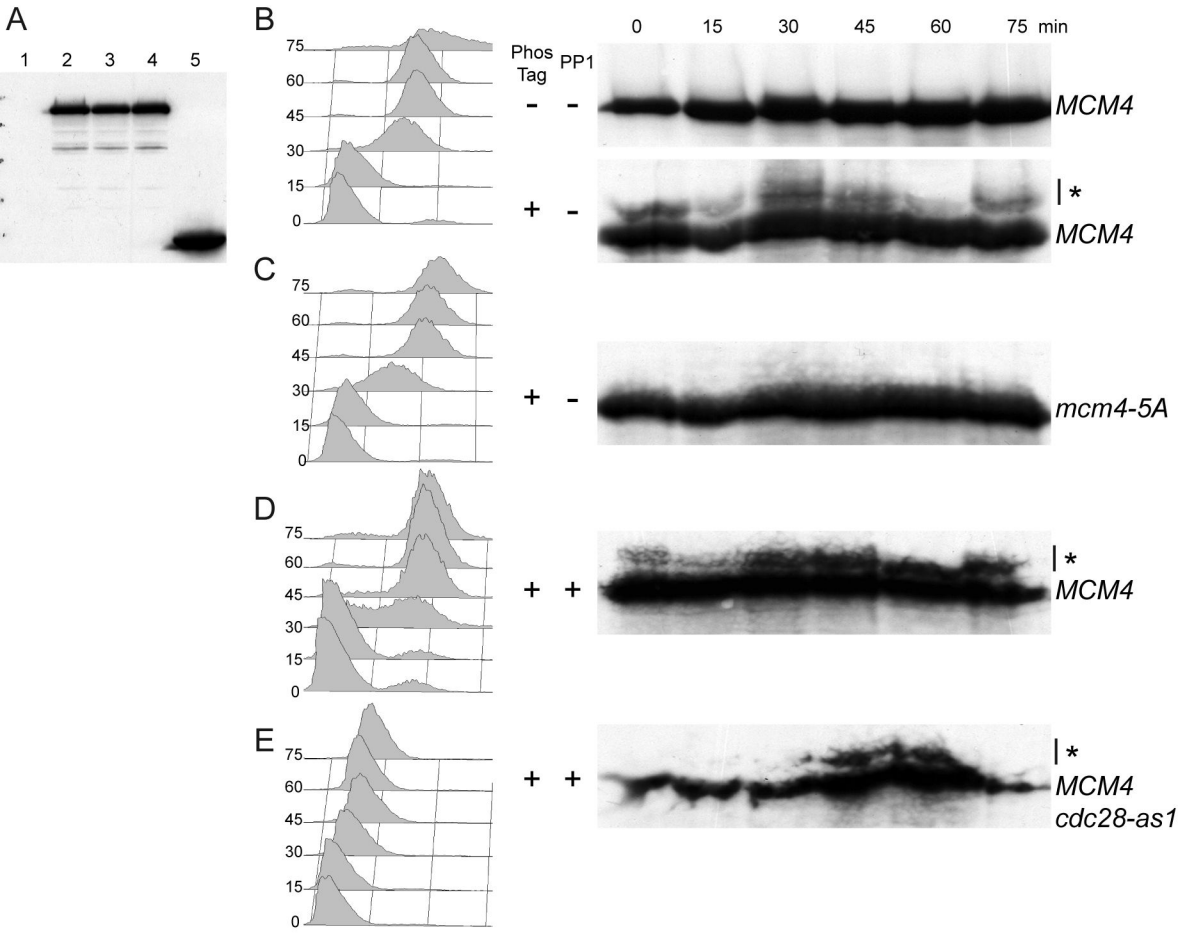
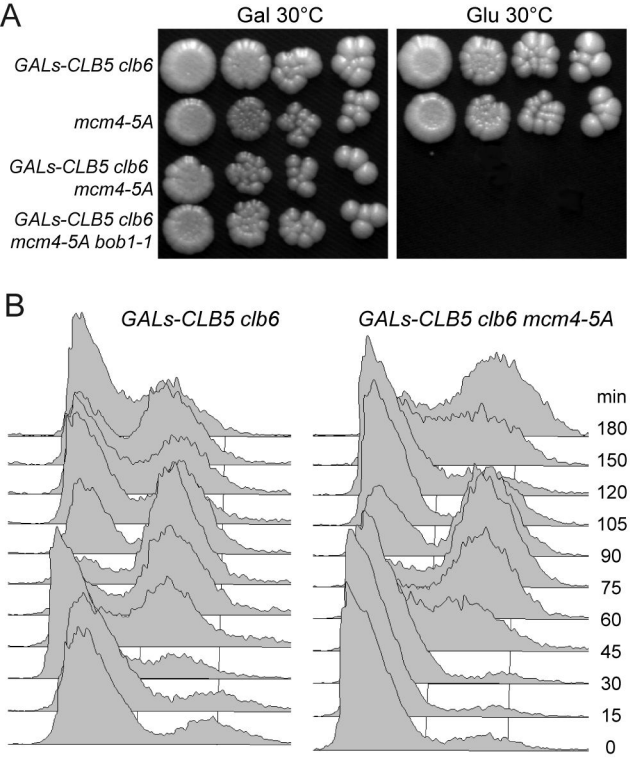
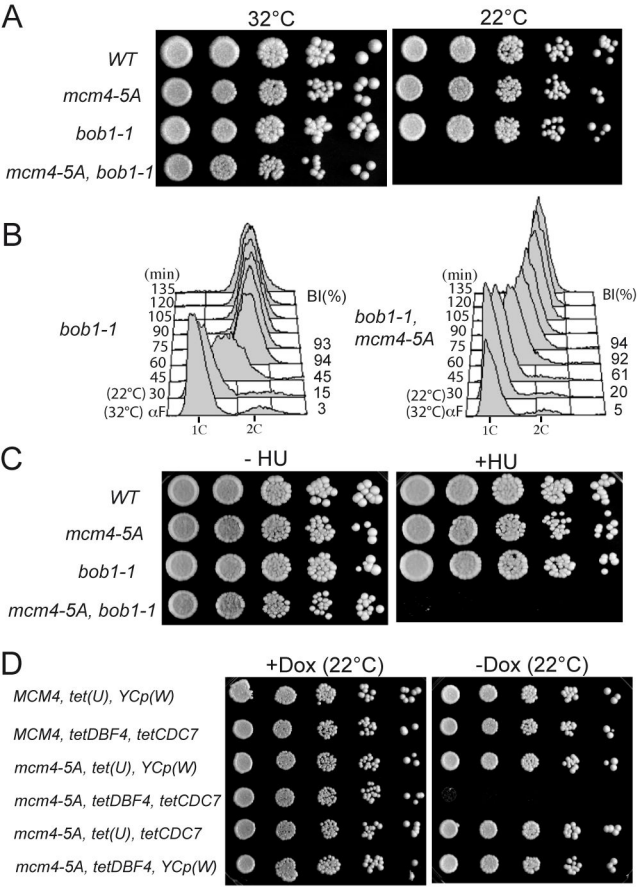



Devault et al - Figure 2

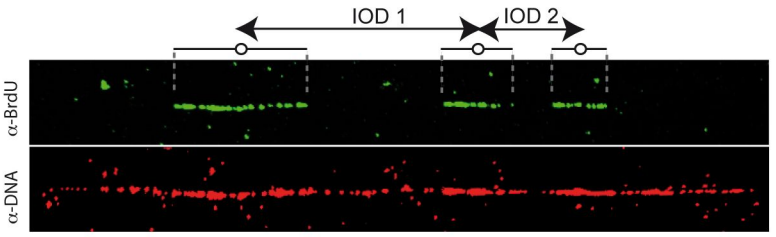



Devault et al - Figure 3




Devault et al - Figure 4

A

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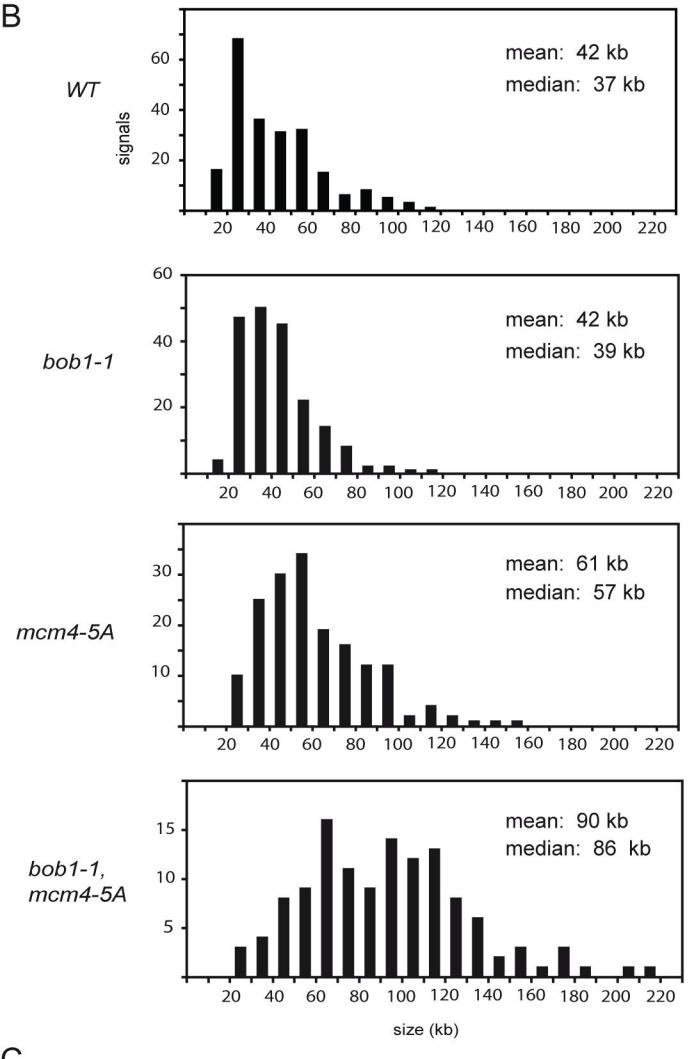
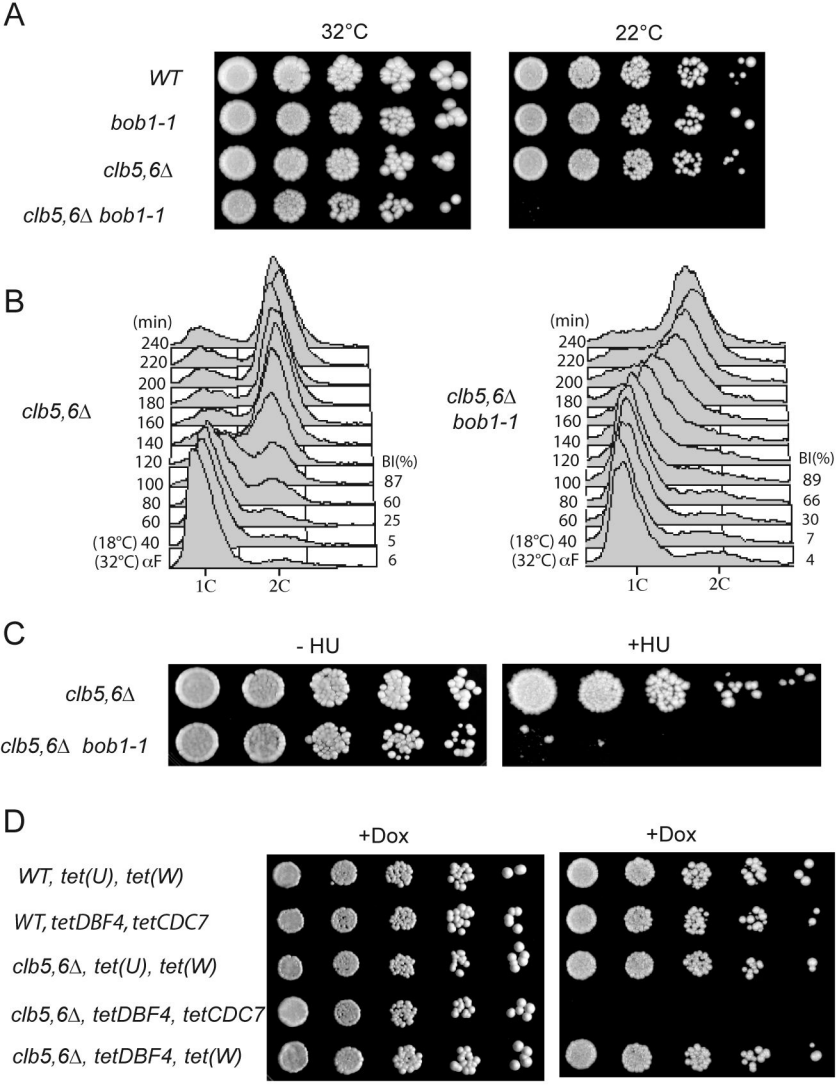


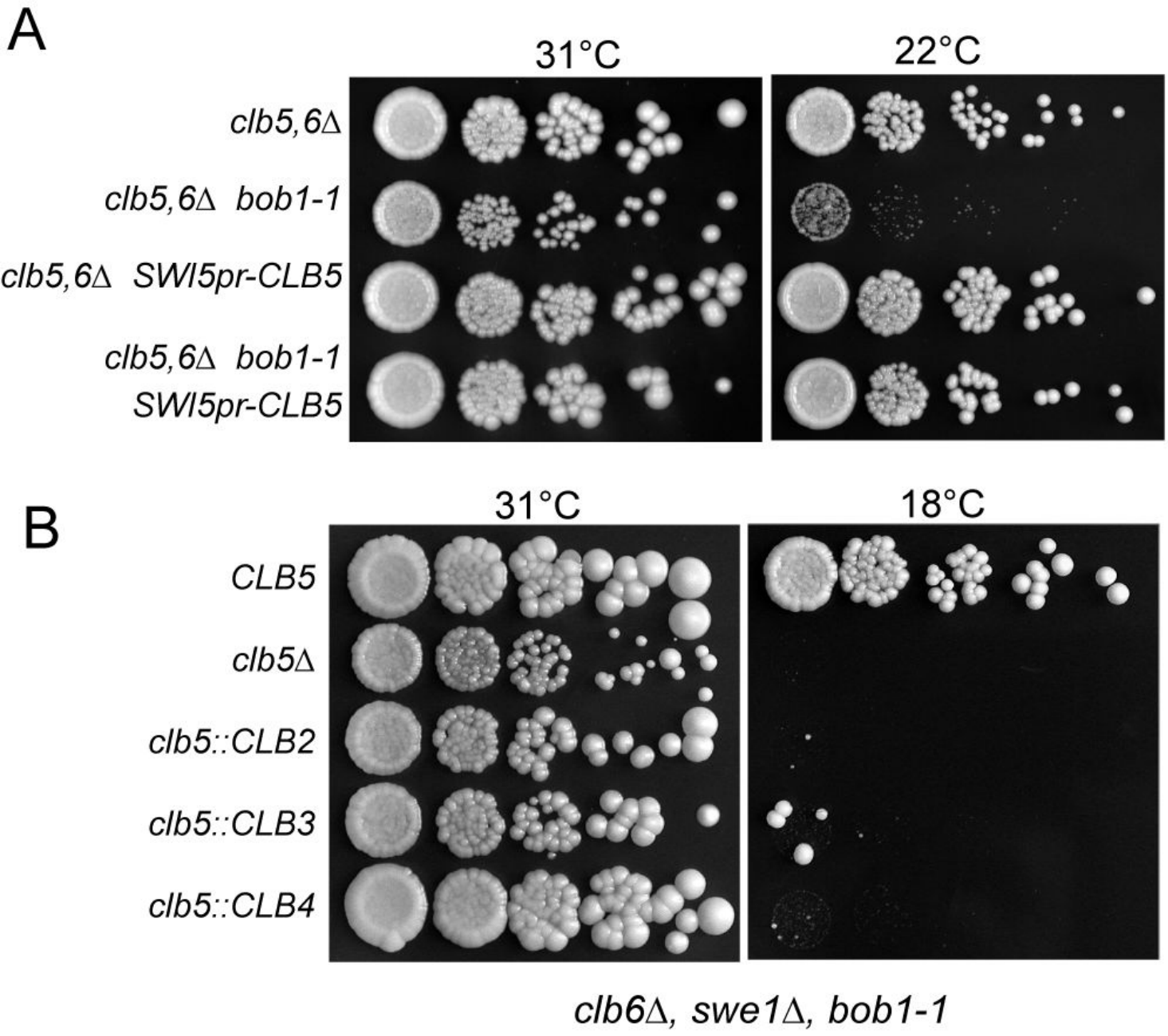

C

| Strains compared | $median_1 \neq median_2$ ? ($p < 0.05$) |
|---|---|
| *WT* vs *bob1-1* | No |
| *WT* vs *mcm4-5A* | Yes |
| *WT* vs *bob1-1, mcm4-5A* | Yes |
| *mcm4-5A* vs *bob1-1, mcm4-5A* | Yes |

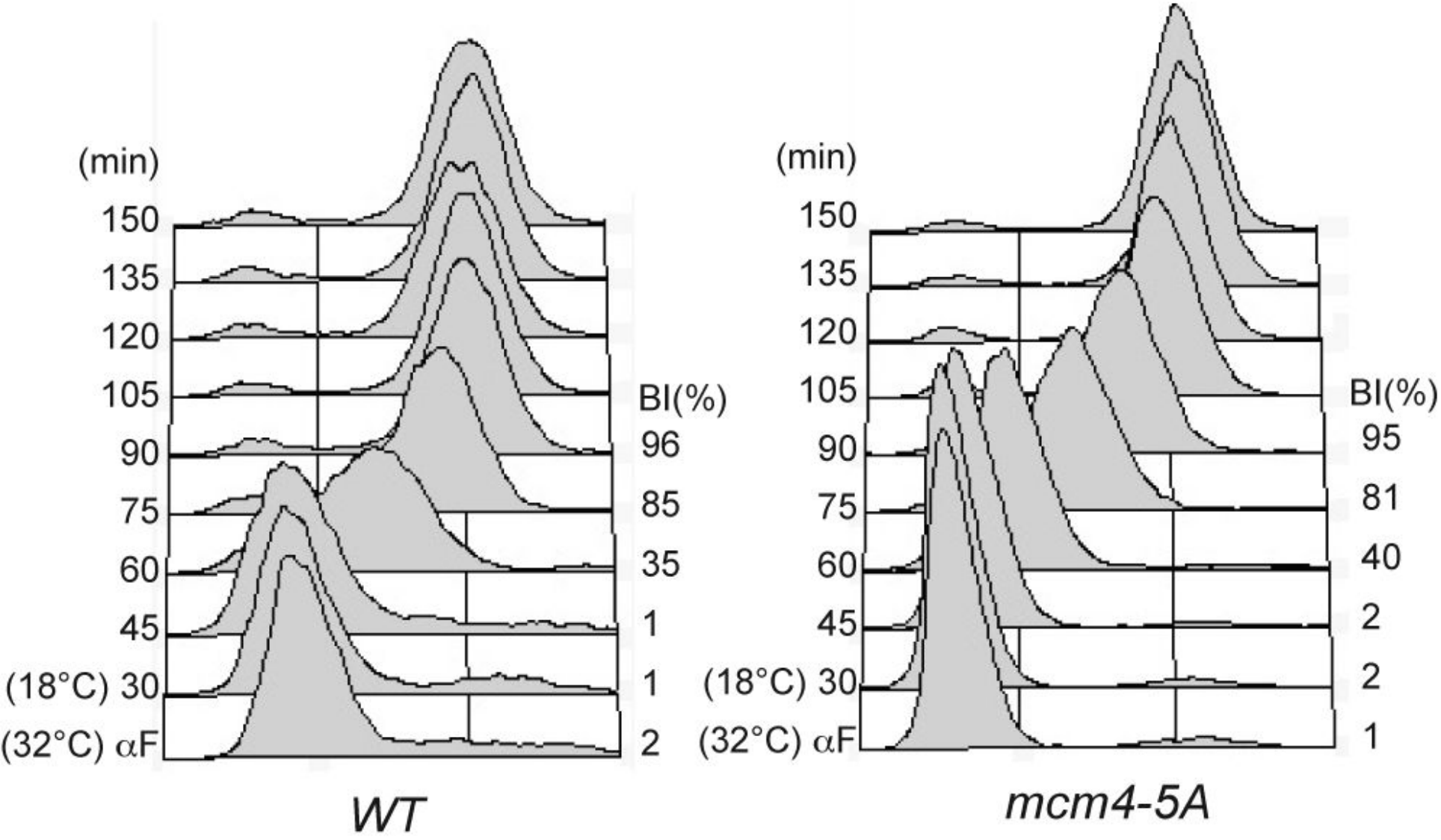
Devault et al - Figure 5




Devault et al - Figure 6




Devault et al - Figure 7




Devault et al - Figure 8