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Protein kinase CK2: a major regulator of the G1/S transition in *Saccharomyces cerevisiae*

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If we knew what it was we were doing,
it would not be called research, would it?

Science is a wonderful thing if one
does not have to earn one's living at it.

Albert Einstein

INDEX

Riassunto

La caseina chinasi 2 (CK2) è una chinasi essenziale, altamente conservata e distribuita in modo ubiquitario negli organismi eucarioti. Essa fosforila più di 300 substrati, ma il suo ruolo fisiologico ed il suo meccanismo di regolazione sono attualmente ancora poco chiari (Meggio e Pinna, 2003). CK2 è tradizionalmente considerato un enzima tetramerico, costituito da due subunità catalitiche e due subunità regolative, codificate in lievito rispettivamente dai geni *CKA1* e *CKA2*, *CKB1* e *CKB2*. La delezione dei geni che codificano per le due subunità regolative o per una delle due subunità catalitiche ha pochi effetti, ma la contemporanea delezione dei geni per le due subunità catalitiche è letale. In *Saccharomyces cerevisiae* è stata inoltre dimostrata una diversa specificità delle due subunità catalitiche (α e α'): la subunità α risulta coinvolta nella polarizzazione cellulare, mentre la subunità α' è legata alla regolazione del ciclo cellulare e sembra essere fondamentale sia in fase G1 che in mitosi. Infatti, mutanti *cka1Δcka2*^{ts} alla temperatura restrittiva arrestano la loro crescita e si bloccano con un fenotipo terminale costituito da 50% delle cellule non gemmate in fase G1 e 50% delle cellule arrestate in metafase ed anafase (Hanna et al., 1995).

Tra i substrati di CK2 sono inoltre note diverse proteine coinvolte nel ciclo cellulare sia in cellule di mammifero che in lievito (p21, p27, Cdc2, Cdk1, Cdc37) (Meggio e Pinna, 2003). Tra queste ci sono anche due proteine chiave della transizione G1/S in lievito: Sic1, inibitore dei complessi Cdk1-Clb5/6, fosforilato da CK2 sul residuo di Ser201 (Coccetti et al., 2004; Coccetti et al., 2006), e Cdc34, enzima E2 necessario per l'ubiquitinazione di molte proteine del ciclo cellulare (tra cui la stessa Sic1) che viene fosforilato da CK2 sui residui Ser207, Ser216 e Ser282 (Pyerin et al., 2005; Barz et al., 2006; Sadowski et al., 2007).

L'attività di ricerca del mio dottorato è stata principalmente volta a chiarire le relazioni tra CK2 e questi due importanti substrati (Sic1 e Cdc34), per comprendere i meccanismi con cui la chinasi CK2 interviene nella regolazione della transizione G1/S in lievito.

Precedenti analisi dimostravano che in ceppi *cka1Δcka2*^{ts} a 37°C i livelli dell'inibitore Sic1 aumentavano (Coccetti et al., 2006); abbiamo perciò voluto indagare se questo accumulo fosse responsabile del blocco in G1 che si osservava alla temperatura non permissiva in questi ceppi. Siamo così riusciti a dimostrare che all'aumento dei livelli di Sic1 corrispondeva una forte inibizione dell'attività chinasica del complesso Cdk1-Clb5. Inoltre, la delezione del gene *SIC1*, così come lo spegnimento della sua espressione, in un background *cka1Δcka2*^{ts}, portava al superamento dell'arresto in G1 a 37°C, determinando un blocco uniforme delle cellule con contenuto di DNA post-sintetico. Questi dati, pubblicati nel 2007 (Tripodi et al., 2007), hanno quindi per la prima volta chiarito gli eventi molecolari alla base del blocco in G1 dovuto all'inattivazione della CK2: l'accumulo di Sic1, inibendo il complesso Cdk1-Clb5, impediva l'inizio della replicazione del DNA.

Successivamente ci siamo concentrati su Cdc34, l'enzima E2 coinvolto nella degradazione di Sic1. Dati di letteratura e nostre analisi computazionali hanno rivelato che Cdc34 possiede numerosi siti consenso per CK2. Analisi di spettrometria di massa (MS) da noi condotte sulla proteina ricombinante fosforilata *in vitro* da CK2 hanno dimostrato una fosforilazione sui seguenti siti: S130, S167, S188, S195, S207, S282. Di questi, in particolare, S130 e S167, localizzati nel dominio catalitico della proteina, sono risultati siti altamente conservati tra le proteine omologhe a Cdc34 in diversi organismi, e sono stati effettivamente da noi individuati come siti fosforilati anche

in vivo in modo CK2-dipendente. Tramite un saggio tiolestere di legame tra l'ubiquitina e Cdc34 (wild-type e $Cdc34^{S130AS167A}$) *in vitro*, abbiamo osservato che la mancata fosforilazione dei siti S130 e S167 da parte di CK2 riduceva notevolmente la capacità di caricamento dell'ubiquitina da parte di Cdc34. Successive analisi *in vivo* ci hanno permesso di verificare il ruolo fisiologico di queste fosforilazioni. Abbiamo osservato che l'overespressione della proteina mutante $Cdc34^{S130AS167A}$ non era in grado di complementare la mutazione termosensibile *cdc34-2* (Schwob et al., 1994), e determinava un doppio blocco con contenuto di DNA sia pre-replicativo che post-replicativo; il blocco in fase G1 era caratterizzato dall'accumulo di Sic1 e veniva superato dalla sua delezione. Tuttavia, l'espressione di $Cdc34^{S130AS167A}$ ad un livello pari a quello della proteina endogena, portava ad un arresto uniforme delle cellule in fase G1 alla temperatura restrittiva, identico a quello del ceppo di controllo *cdc34-2^{ts}*. Questi dati, pubblicati nel 2008 (Coccetti et al., 2008), hanno quindi permesso di dimostrare che la fosforilazione del dominio catalitico di Cdc34 da parte di CK2 era necessaria per il funzionamento dell'enzima e quindi per l'ubiquitinazione dei suoi substrati *in vivo* (tra cui Sic1).

Parallelamente allo studio dei substrati di CK2 in fase G1, abbiamo indagato se le condizioni nutrizionali, fondamentali nella modulazione del ciclo cellulare, e della transizione G1/S in particolare, regolassero anche la stessa CK2. Utilizzando ceppi recanti le 4 subunità di CK2 marcate con un TAP-tag, abbiamo verificato che né i livelli totali, né la loro localizzazione subcellulare (che risulta prevalentemente nucleare sia in glucosio che in etanolo) variavano al variare della fonte di carbonio. Tuttavia, l'attività di CK2, misurata tramite un saggio tradizionale e tramite un nuovo saggio da noi sviluppato (in cui veniva

utilizzata la proteina ricombinante Sic1 come substrato di CK2), risultava nettamente inferiore in cellule cresciute in etanolo rispetto a quella di cellule cresciute in glucosio. Per comprendere se tale differenza di attività di CK2 fosse imputabile alla diversa velocità di crescita e/o al diverso metabolismo delle cellule cresciute in glucosio ed etanolo, abbiamo fatto crescere i ceppi di lievito tramite l'utilizzo del bireattore, in modo da poter considerare separatamente gli effetti della velocità di crescita e del metabolismo del carbonio. Tale sistema ha permesso di dimostrare che la velocità di crescita era il principale responsabile della modulazione dell'attività di CK2. Abbiamo inoltre dimostrato, tramite l'utilizzo di ceppi deleti nelle due singole subunità catalitiche di CK2, che entrambe le subunità (α e α') erano regolate dalle condizioni di crescita, e che la subunità α sembrava possedere una maggiore attività rispetto alla subunità α'.

Abbiamo quindi fornito la prima evidenza di una regolazione dell'attività di CK2 e di un suo coinvolgimento nel *sensing* dei nutrienti in lievito (articolo in preparazione).

Abstract

Casein kinase 2 (CK2) is a ubiquitous, essential and highly conserved eukaryotic kinase. It phosphorylates more than 300 substrates, but its physiological role and regulation mechanism are still poorly understood (Meggio and Pinna, 2003). CK2 is traditionally considered to be a tetrameric enzyme, composed of two catalytic subunits and two regulatory subunits, which are encoded in yeast by *CKA1* and *CKA2* genes, *CKB1* and *CKB2* genes respectively. Deletion of regulatory subunits, or of either catalytic subunit gene alone has few phenotypic effects, but simultaneous disruption of both *CKA1* and *CKA2* genes is lethal. In *Saccharomyces cerevisiae* a specialization of the two catalytic subunits (α e α') was shown: α subunit is involved in cell polarity, while α' subunit is linked to cell-cycle regulation and was shown to be fundamental both in G1 phase and in mitosis. In fact, at restrictive temperature *cka1Δcka2*^{ts} mutants arrest cell cycle with a terminal phenotype constituted of 50% of unbudded G1 cells and 50% of cells arrested in metaphase and anaphase (Hanna et al., 1995).

Among CK2 substrates, many cell-cycle proteins are known, both in mammalian cells and in yeast (p21, p27, Cdc2, Cdk1, Cdc37) (Meggio and Pinna, 2003). CK2 also phosphorylates two key regulators of the G1/S transition in yeast: Sic1, the Cdk1-Clb5/6 inhibitor, which is phosphorylated by CK2 on Ser201 (Coccetti et al., 2004; Coccetti et al., 2006), and Cdc34, the E2 enzyme required for the ubiquitination of many cell-cycle proteins (among which Sic1), which is phosphorylated by CK2 on Ser207, Ser216 e Ser282 (Pyerin et al., 2005; Barz et al., 2006; Sadowski et al., 2007).

My PhD research activity was focused on the relationship between CK2 and these two relevant substrates (Sic1 and Cdc34), in order to understand CK2-mediated regulation of the G1/S transition in yeast.

Previous analysis showed an increase of Sic1 level in *cka1Δcka2*^{ts} strain at 37°C (Coccetti et al., 2006); thus we investigated whether this increase was responsible for the G1 arrest of this strain at restrictive temperature. We showed that the observed increase of Sic1 level was responsible for an inhibition of Cdk1-Clb5 kinasic activity. Moreover, *SIC1* deletion, like the shutting-down of its expression, in a *cka1Δcka2*^{ts} background, bypassed the G1 arrest at 37°C, leading to a single cell-cycle arrest, in which cells showed a post-synthetic DNA content. These data, published in 2007 (Tripodi et al., 2007), explained for the first time the molecular mechanism of the G1 block due to CK2 inactivation: Sic1 accumulation inhibits Cdk1-Clb5 complex, thus preventing the onset of DNA replication.

We then worked on Cdc34, the E2 enzyme involved in Sic1 degradation. Literature data and our computational analysis revealed that Cdc34 protein present many consensus sites for CK2 phosphorylation. Mass spectrometry (MS) analysis on recombinant Cdc34 phosphorylated *in vitro* by CK2 showed phosphorylations on the following sites: S130, S167, S188, S195, S207, S282. In particular, among these, S130 and S167, within the catalytic domain of the protein, are highly conserved among Cdc34 homologues in various organisms, and were identified as phosphorylated sites *in vivo* in a CK2-dependent manner. Through a thiolester assay, we studied Cdc34 (wild-type and Cdc34^{S130AS167A}) binding to ubiquitin *in vitro*, and we observed that lack of CK2-mediated phosphorylation on S130 and S167 strongly reduced the ubiquitin-charging ability of Cdc34. Subsequent *in vivo* analysis allowed us to investigate the physiological role of these phosphorylations. We observed that Cdc34^{S130AS167A} overexpression is not able to complement the thermo-sensitive mutation *cdc34-2* (Schwob et al., 1994), and determined a

double arrest with both pre-replicative and post-replicative DNA content; the G1 block was characterized by Sic1 accumulation and was bypassed by *SIC1* deletion. Yet, $Cdc34^{S130AS167A}$ expression to a level comparable to the endogenous protein led to a uniform G1 arrest at restrictive temperature, like the arrest observed in the control strain *cdc34-2*^{ts}. Thus, these data, published in 2008 (Coccetti et al., 2008), showed that CK2 phosphorylation of the catalytic domain of Cdc34 was required for the function of the enzyme and for the *in vivo* ubiquitination of its substrates (among which Sic1).

A part from the study of CK2 substrate of G1 phase, we investigated if CK2 was regulated by nutritional conditions, which are important for the modulation of the cell-cycle and especially of the G1/S transition. We used yeast strains expressing TAP-tagged CK2 subunits, and we showed that neither total levels of the four subunits, nor their subcellular localization (which is mainly nuclear both in glucose and in ethanol) were modulated by carbon source. Then we measured CK2 activity, with a traditional assay and with a new assay that we developed using recombinant Sic1 as CK2 substrate; we showed that CK2 activity was clearly lower in ethanol growing cells than in glucose growing ones. We further investigated whether this difference in CK2 activity was due to the different growth rate or to the different carbon metabolism of cells growing in glucose or ethanol. To this purpose, we used bioreactor technology, to separately consider growth rate effects and metabolism effects. This system showed that growth rate was the main factor responsible for the modulation of CK2 activity. We also showed, by using mutant strains bearing a deletion in one of the two genes encoding for the catalytic subunits, that both subunits (α e α') were regulated by nutritional conditions; moreover, α subunit seems to have a higher activity than α' subunit.

Therefore we provided the first evidence of a regulation of CK2 activity and of CK2 involvement in the nutrient sensing in yeast (paper in preparation).

Introduction

1 Cell cycle in yeast

The cell cycle is the series of events that take place in a cell leading to its division and duplication. Yeast cell cycle is conventionally divided in four distinct phases: during G1 (Gap1) phase the cell grows and prepares for DNA replication; S (Synthesis) phase is the phase in which DNA is replicated; G2 (Gap2) is required to prepare for mitosis; and M (Mitosis) phase, when chromosome segregation, nuclear division and cytokinesis take place.

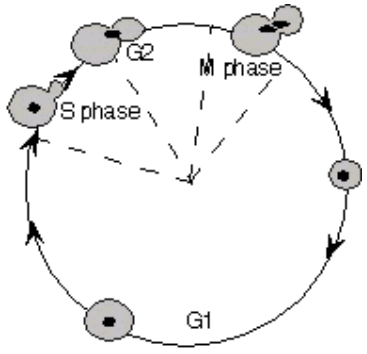


Fig. 1 Schematic rapresentation of the yeast cell-cycle.

During the cell cycle every step is thightly contolled, in order to avoid mistakes or loss of genetic information, that could be fatal for the cell. The main control area of the cell cycle in *Saccharomyces cerevisae* is called Start, a short interval in late G1 phase during which yeast cells commit to division. At the G1/S transition cells can coordinate environmental and internal signals to choose between different fates: mating, sporulation, quiescence or mitotic division. After Start, three semi-independent events occur (bud emergence, DNA synthesis and spindle pole body duplication) and cells are inevitably commetted to

cell-cycle progression and division. Start is also used to coordinate cell growth and cell division; in fact to pass Start cells should reach a critical cell size, in order to ensure enough resources to complete the cell cycle (Jorgensen and Tyers, 2004).

In yeast cell-cycle events are controlled by the Cyclin-Dependent Protein Kinase 1 (CDK1), encoded by the *CDC28* gene, which is a Ser/Thr protein kinase that phosphorylates residues in the S/T-P consensus motif. CDK1 activity is modulated by the association with nine different small regulatory subunits called cyclins. Cyclins can be grouped in three functional classes: G1 cyclins (Cln1,2,3), S phase cyclins (Clb5,6) and mitotic cyclins (Clb1,2,3,4), and are expressed at different times by a combination of transcriptional control and protein instability. Thus, different cyclins are present at different stages of the cell cycle and can regulate Cdk1 activity and its substrate specificity (Futcher et al., 1996; Mendenhall and Hodge, 1998).

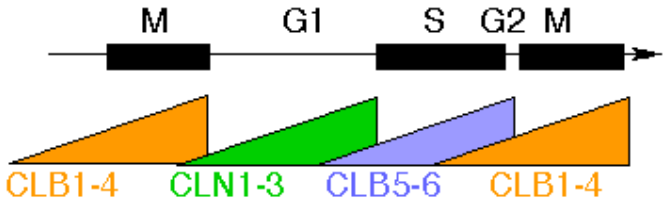


Fig. 2 Timely regulated transcription of cyclins in yeast.

Cln1,2, expressed in G1 phase, are responsible for bud emergence at Start, and their periodic expression depends on SCB sequences (Swi4/6-dependent cell-cycle box) on the promoter of *CLN1,2* genes, which are responsible for their transcription in G1 phase. SCB

sequences are recognized by the SBF complex (SCB binding factor), which is made of Swi4, a DNA binding protein, and Swi6.

The onset of DNA replication is instead due to Cdk1 association with Clb5,6, whose expression in late G1 phase is driven by the MBF complex, composed of Mbp1 and Swi6, which recognize MCB sequences (Mlu cell-cycle box) on *CLB5,6* promoters (Futcher et al., 1996; Mendenhall and Hodge, 1998; Breeden, 2003).

Cln3 significantly differs from all other cyclins, since it is present at lower levels and throughout the cell cycle, with only a modest peak of expression at the M/G1 border. Its role is fundamental for the cell cycle, because it works upstream to all other cyclins, by activating SBF and MBF transcription factors, which allow *CLN1,2* and *CLB5,6* to be transcribed (see below). Activation of SBF and MBF is due to the dissociation of the inhibitor Whi5 from SBF/MBF complexes (Costanzo et al., 2004; de Bruin et al., 2004). In fact, in late G1 phase, Cdk1-Cln dependent phosphorylation on multiple sites (de Bruin et al., 2004) dissociates Whi5 from SBF/MBF and triggers Whi5 nuclear export (Costanzo et al., 2004).

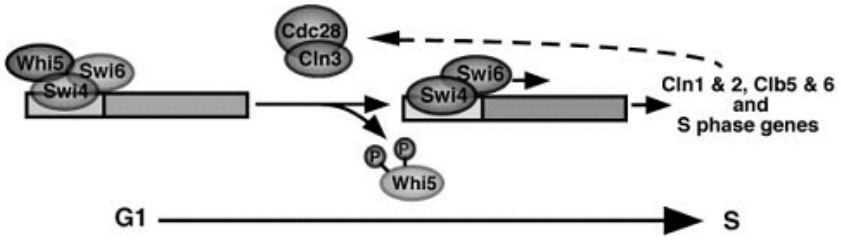


Fig. 3 Model of the regulation of G1-specific transcription in yeast (from de Bruin et al., 2004).

Starting form S phase, also M phase cyclins are synthetized. Clb3,4 are important for spindle formation, while later, Clb1,2 are required for spindle elongation and cell division (Futcher et al., 1996; Mendenhall and Hodge, 1998).

Regulation of cyclins expression is not only due to a periodic transcription, but also to a timely regulated degradation. In fact, all cyclins are polyubiquitinated and degraded through the proteasome complex in a strictly regulated fashion. Cyclins degradation is particularly important at two different cell-cycle stages. In late G1, degradation of cyclins and of the Cdk-inhibitor Sic1 (see paragraph 1.1) are due to the action of the SCF complex (Skp1, Cdc53, F-box protein). This complex is a E3 ubiquitin ligase complex, which recognizes proteins containing the typical PEST sequence (a sequence rich in Pro, Glu, Ser, Thr) that were phosphorylated by Cdk1. SCF ability to recognize different substrates is due to F-box proteins (Cdc4 or Grr1), that gives specificity to the polyubiquitination system. In anaphase, M phase cyclins, which contain the so called Destruction Box, are polyubiquitinated through a second E3 complex called APC (Anaphase Promoting Complex). In this case, target specificity is given by Cdc20 and Cdh1, which act on different substrates to allow the cell to exit from mitosis (Mendenhall and Hodge, 1998).

1.1 The inhibitor Sic1

Sic1 is a Cki (Cdk inhibitor), active on Cdk1-Clb complexes (Schwob et al., 1994). Its inhibitory activity, due to its C-terminal domain, is given by its ability to exclude substrates from Cdk1 active site. *SIC1* mRNA accumulates to high levels as cell exits from mitosis, thank to the action of two transcription factors: Swi5, which enters the nucleus at late anaphase and is the main responsible for *SIC1* transcription, and Ace2, that plays a minor role (Knapp et al., 1996). Thus, Sic1 starts accumulating in late mitosis, where it is involved in downregulation of Cdk1-Clb activity required for the exit form mitosis (Donovan et al., 1994). However, Sic1 most studied function is to prevent premature DNA replication in G1 phase (Lengronne and Schwob, 2002). In fact, despite Clb5,6, accumulation in G1 phase, DNA replication cannot start until Ckd1-Cln levels have risen sufficiently to complete bud initiation and spindle pole body duplication (Schwob et al., 1994). Thus, Sic1 main role in G1 phase is to inhibit Cdk1-Clb5,6, in order to avoid a premature DNA replication before Start. Although *SIC1* is a non-essential gene, *sic1Δ* cells show genomic instability, initiate DNA replication from fewer origins and have an extended S phase (Nugroho and Mendenhall, 1994; Lengronne and Schwob, 2002).

At the G1/S transition, Cdk1-Clb5,6 complexes becomes active because of Sic1 degradation. In fact during G1 phase, Cdk1-Cln1,2 complexes phosphorylate Sic1 on multiple sites, targeting the inhibitor to degradation via the ubiquitin-proteasome system (Verma et al., 1997b; Nash et al., 2001).

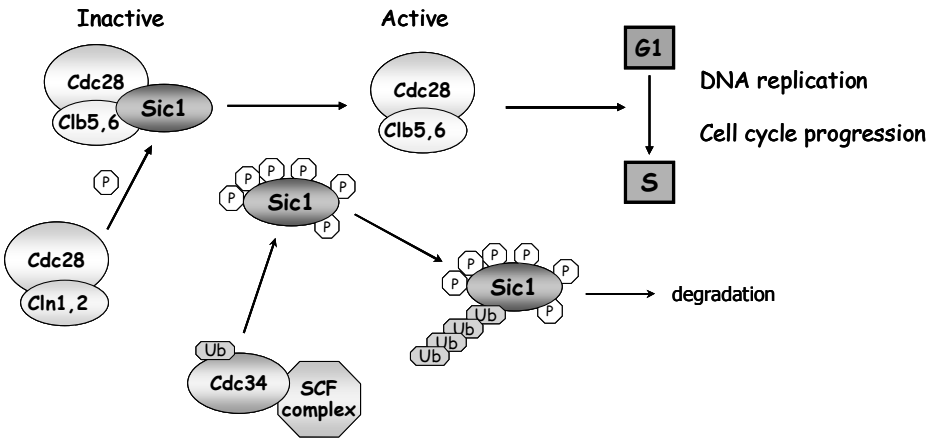


Fig. 4 Molecular events of the G1/S transition.

In particular, it was shown that a threshold of phosphorylation exists: Sic1 should be phosphorylate by Cdk1-Cln complexes on at least six of its nine sites (Nash et al., 2001), in order to be recognized by the Cdc34-SCF^{Cdc4} ubiquination complex (see paragraph 1.2) and thus degraded by the proteasome machinery.

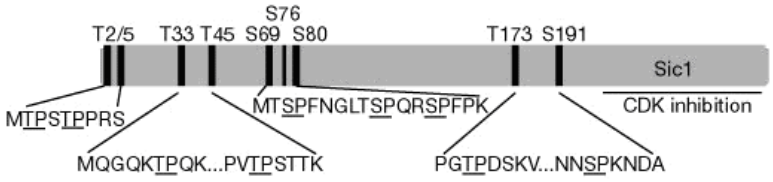


Fig. 5 Sic1 scheme reporting the nine Cdk1-Cln phosphorylation sites and the C-terminal Cdk inhibitory domain (from Nash et al., 2001).

However, recently, an interesting paper by Cross and coworkers showed that Sic1 degradation in not essential to pass Start, as it was previously suggested (Cross et al., 2007). In fact, they elegantly demonstrated that cells expressing an unphosphorylatable version of Sic1, able to inhibit Cdk1-Clb complexes, but which is not degraded at the end of G1 phase, are fully viable. However these cells show a lengthened G1 phase, variable cell-cycle delay and extreme sensitivity to Clb dosage, indicating that Sic1 phosphorylation and degradation is not essential, but is required for the correct timing of G1/S transition and especially for cell-cycle robustness (Cross et al., 2007).

A part form Cdk1, other kinases were shown to phosphorylate the inhibitor Sic1. Escotè and coworkers observed a Hog1-dependent phosphorylation on Thr173, which is responsible for the stabilization of Sic1 upon osmotic stress, leading to a cell-cycle arrest in G1 phase (Escotè et al., 2004). The same stabilizing phosphorylation at Ther173 was observed upon rapamycin exposure, which determines a G1 arrest with upregulation of the inhibitor Sic1 (Zinzalla et al., 2007). On the contrary, the stress-responsive Cdk Pho85 promotes Sic1 destabilization, through phosphorylation of Thr5 (Nishizawa et al., 1998). The only phosphorylation described so far that is not implied in Sic1 degradation is phosphorylation of Ser201 by protein kinase CK2 (Coccetti et al., 2004; Coccetti et al., 2006; see paragraph 2.6).

Sic1 is highly regulated by nutritional conditions. In fact, works from my laboratory previously showed that Sic1 level is modulated by carbon source: Sic1 level is around double in cells growing on ethanol

that in cells growing on glucose as carbon source (Rossi et al., 2005). Moreover, also Sic1 subcellular localization is regulated in a carbon-source dependent manner: Sic1 is exclusively nuclear in glucose growing cells in G1 phase, while it is partly delocalized in the cytoplasm in ethanol growing cells. This different Sic1 localization was found to be linked to a different Clb5 localization, showing for the first time a positive role for Sic1 in facilitating Clb5 nuclear localization (Rossi et al., 2005).

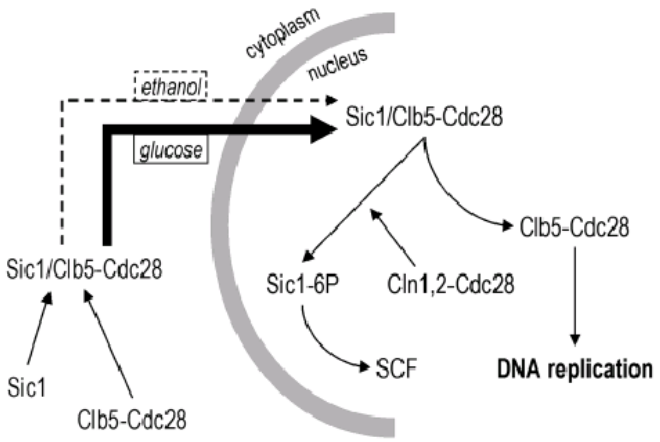


Fig. 6 Model for carbon-source dependent import of the Cdk1-Clb5-Sic1 complex (from Rossi et al., 2005).

1.2 The Cdc34-SCF^{Cdc4} complex

As described above, entry into S phase requires the activity of the S phase promoting complex Cdk1-Clb5, whose activation requires Sic1 degradation via the ubiquitin-proteasome machinery. Genetic and biochemical analysis showed that the E2 enzyme Cdc34 and the E3 complex SCF^{Cdc4} are required for Sic1 degradation at Start (Schwob et al., 1994; Verma et al., 1997a; Feldman et al., 1997).

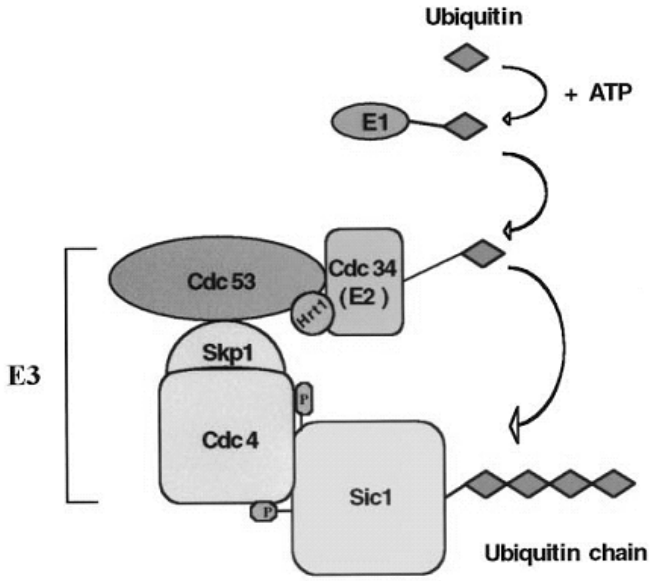


Fig. 7 Schematic representation of the yeast SCF complexes (from DeSalle and Pagano, 2001).

Substrate ubiquitination requires three sequential enzymatic steps: i) ATP-dependent activation of ubiquitin through the formation of a high-energy thiolester linkage with a ubiquitin-activating enzyme (E1); ii) transfer of the activated ubiquitin to a ubiquitin-conjugating enzyme

(E2); iii) transfer of activated ubiquitin to a specific lysine residue of the target protein, assisted by ubiquitin ligase complexes (E3). This sequence is repeated until multiple chains of ubiquitin are attached, thereby marking the protein for rapid degradation by the 26S proteasome (Hershko and Ciechanover, 1998). Yeast SCF complex is an E3 complex composed of multiple subunits: i) Skp1, an adaptor protein that function as a scaffold protein; ii) Cdc53, a cullin family member that recruits the ubiquitin conjugating enzyme Cdc34; iii) Hrt1 (also called Rbx1 or Roc1), a RING-finger protein that transfers ubiquitin molecules to substrates, without directly forming a thiolester bond with ubiquitin moieties; iv) one of several unique F-box proteins. F-box proteins are a large family of adaptor proteins (among which Cdc4, Grr1 and Met30), which share no homology outside of the F-box domain; they confer substrate specificity to the system, by recruiting a particular target to the core ubiquitination machinery (DeSalle and Pagano, 2001). Among these, at the G1/S transition SCF^{Cdc4} complex recognizes phosphorylated Sic1 through the F-box protein Cdc4, while SCF^{Grr1} binds phosphorylated Cln1,2 through the F-box protein Grr1 (DeSalle and Pagano, 2001).

The different SCF forms described can transfer ubiquitin molecules to target substrates by creating a complex with the E2 ubiquitin-conjugating enzyme Cdc34 (Mathias et al., 1998).
Cdc34 (or Ubc3) is an essential enzyme, involved in the ubiquitination of many cell-cycle proteins. Structurally, it consists of a highly conserved N-terminal catalytic domain (residues 1-170), and a 125 aa acidic C-terminal domain (residues 171-295). The catalytic domain is highly conserved among E2 enzymes and contains the ubiquitin-accepting Cys (Cys95), which is the only cysteine present in the

protein. The C-terminal extension contains the SCF-binding domain (residues 171-209), responsible for the interaction with the E3 complex (through Cdc53), for the regulation of cell cycle and for Cdc34 self-association (Ptak et al., 1994; Mathias et al., 1998). Finally, the function of the C-terminal tail is not clear; in fact it was shown that residues 210-295 are dispensable for viability, since a truncated version of Cdc34, $Cdc34_{1-209}$, is able to restore full viability of a *cdc34Δ* strain (Ptak et al., 1994).

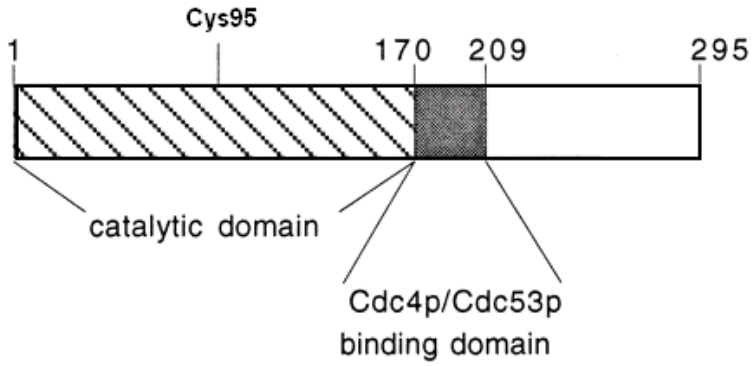


Fig. 8 Functional domains of Cdc34 (modified from Mathias et al., 1998).

Biochemical studies showed that Cdc34 is first activated by the ubiquitin transferred from the E1 enzyme. Then, the Cdc34-Ub thiolester complex self-associates, and, in combination with the SCF complex, the Cdc34-dimer directs the assembly of the multi-ubiquitin chain on the target substrate (Varelas et al., 2003).

1.3 The G1/S transition and the critical cell-size

To mantain a uniform size, cells must coordinate cell growth and cell division. In *Saccharomyces cerevisiae*, growth and division are coupled at Start; in fact, as mentioned before, cells have to reach a critical cell size (called Ps) to execute Start, in order to ensure that sufficient growth has occurred to traverse the cell cycle and to ensure cell size homeostasis (Tapon et al., 2001; Rupes, 2002). Critical cell size is modulated by nutrients: cells growing on glucose as carbon source (rich medium) show a bigger cell size than cells growing on ethanol (poor medium) (Alberghina et al., 2004).

Notably, due to the asymmetric cell division of budding yeast, newborn cells have to grow more than mother cells before being able to overcome the cell size checkpoint; conversely, larger mother cells can reach the critical cell size earlier. Since the duration of the budded phase is constant for all generations, what differs from mothers and daughters is the duration of the G1 phase, as represented in Fig. 9.

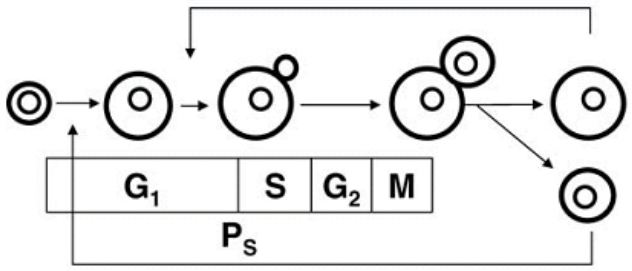


Fig. 9 Representation of the cell cycle and the asymmetrical division of the budding yeast *Saccharomyces cerevisiae* (from Barberis et al., 2007).

It has been proposed that the G1 cyclin Cln3 is a sensor of the translational rate, which reflects ribosome content and cell size (Futcher, 1996). Notably, the *CLN3* 5'UTR contains a small upstream ORF (uORF), which represents a translational control element, that is critical for the growth-dependent regulation of Cln3 synthesis (Polymenis and Schmidt, 1997). Given that during G1 phase the level of Cln3 is proportional to cell mass, and since Cln3 is localized in the nucleus (whose size is constant during G1), it has been proposed that cells pass Start upon reaching a critical Cln3 concentration in the nucleus (Futcher, 1996). However, several evidences indicated that Cln3 cannot be the only determinant of the cell sizer mechanism, and it was suggested that a threshold, involving an activator (Cln3) and an inhibitor, could control entry into S phase (Alberghina et al., 2001). This inhibitor was proposed to be Far1 (Alberghina et al., 2004), the Cdk1-Cln inhibitor, mainly known to mediate cell-cycle arrest in response to pheromone (Chang and Herskowitz, 1990).

Newborn cells are endowed with a certain level of Far1, which remains approximately constant during G1 phase; on the contrary, Cln3 level increases proportionally to cell mass. When the amount of Cln3 overcomes that of Far1, the first threshold is passed, free Cdk1-Cln3 complex phosphorylates Whi5, thus activating SBF and MBF dependent transcription of the *CLN1,2* and *CLB5,6* genes. As previously described, the newly formed Cdk1-Clb5,6 complexes are not immediately active, due to the presence of the inhibitor Sic1. Only after Sic1 degradation, this second threshold involving Clb5 and Sic1 can be overcome and cells can enter S phase (Alberghina et al., 2004).

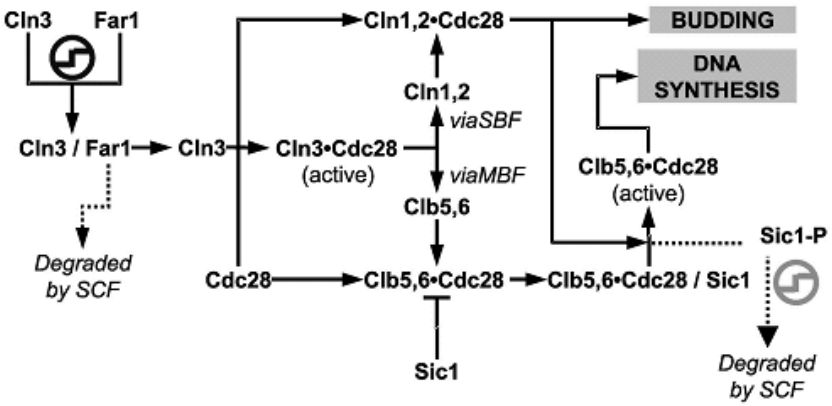


Fig. 10 Model of a two threshold network for the control of the G1/S transition (from Alberghina et al., 2004).

Taking together these data, a mathematical model of the G1/S transition in *S. cerevisiae* was constructed in our laboratory, showing that Ps is an emergent property of the G1/S network (Barberis et al., 2007).

Fig. 11 illustrates the "sizer plus timer" mechanism of the G1/S transition proposed from our group and confirmed form Di Talia and coworkers (Di Talia et al., 2007; Alberghina et al., 2009): the Cln3/Far1 threshold acts as a "sizer", while the "timer" is the period from the overcoming of the Cln3/Far1 threshold to the degradation of Sic1. Within the timer, the period from the previous cytokinesis to Whi5 exit from the nucleus (T1) is much longer for daughter cells than for mothers, while the period from Whi5 exit to budding and DNA replication (T2) is almost the same both in mother and daughter cells.

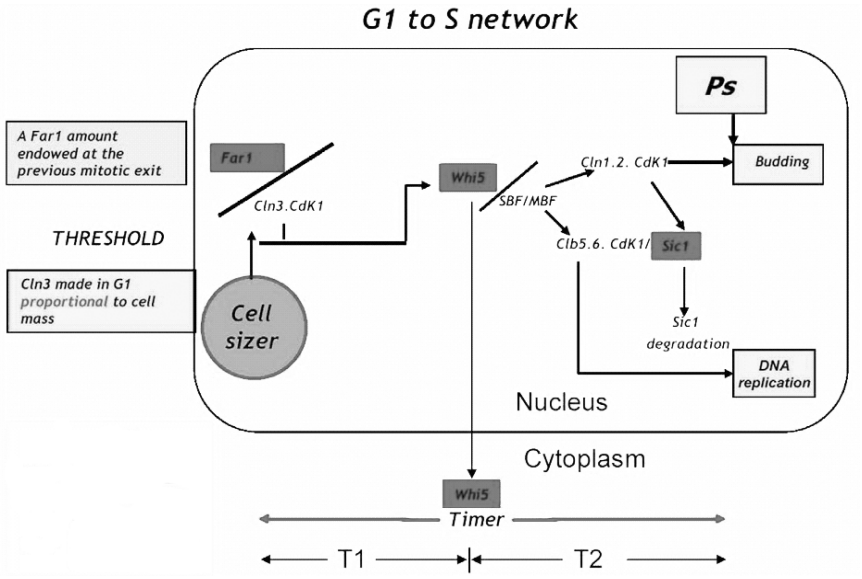


Fig. 11 Model of the G1/S transition in yeast (from Alberghina et al., 2009).

It should be noted however that other groups proposed different molecules as Cln3 inhibitors. For instance, Verges and coworkers showed that Cln3 is retained into the endoplasmic reticulum (ER) in early G1 cells, bound to the Ssa1,2 chaperones. In late G1, Cln3 is release form the ER by Ydj1 ATPase activation, thus leading to nuclear accumulation of Cln3. Since chaperone levels increase as cells grow in G1, they hypothesized that Ydj1 could transmit growth capacity information to the cell cycle for efficient setting of the cell size (Verges et al., 2007).

2. Protein kinase CK2: general features

CK2 (acronim for casein kinase 2) is one of the first protein kinases to be described, discovered togheter with CK1 by Burnett and Kennedy in 1954. Its activity was first detected in rat liver using casein as phosphorylatable substrate (from which the name of the kinase) and later it was ubiquitously found in a variety of tissues and organisms. Curiously, CK2 physiological role remained unclear for a long time and it is still not complitely understood (Meggio and Pinna, 2003).

CK2 is a highly conserved, essential protein kinase (Guerra and Issinger, 1999; Pinna, 2002), which phosphorylates more than 300 substrates, involved in transcription, translation, signal transduction, survival and cell cycle (Meggio and Pinna 2003). It is traditionally considered a tetrameric enzyme, composed of two cathalytic subunits (α) and two regulatory subunits (β), which enhance CK2 stability and activity and modulate substrate selectivity (Litchfield, 2003).

In many organism distinct isoforms of the cathalytic subunit have been identified. In humans two well carachterized isoforms exist (α and α'), and a third isoform (α'') was recently identified. On the contrary, only a single regulatory subunit (β) has been identified in most organisms, with few exceptions, such as *Saccharomyces cerevisae*, which posseses two regulatory subunits (β and β') (Guerra and Issinger, 1999).

CK2 phosphorylation sites always show acidic features, with the typical CK2 consensus site being the following: (E/D/X)-(S/T/y)-(D/E/X′)-(E/D/X)-(E/D)-(E/D/X), where X is any amino acid except basic ones and X′ is any amino acid except basic or proline residues (Pinna, 2002). Moreover, phosphorylated amino acids can efficiently replace Glu and Asp at any position. This feature includes CK2 into

that class of kinases whose targeting can be primed by another protein kinase and accounts for the occurrence of multiple phosphorylation sites among CK2 substates (Meggio and Pinna, 2003).

Although it was initially classified as a Ser/Thr protein kinase, CK2 can now be considered a dual specificity kinase, being able to phosphorylate also Tyr residues, although less efficiently (Marin et al., 1999), both in yeast (Wilson et al., 1997) and in mammalian cells (Vilk et al., 2008).

Another peculiarity of CK2 is its dual-cosubstrate specificity, that is the ability to efficiently use either ATP or GTP as a cosubstrate; generally in the presence of Mg^{2+} the Km values of CK2 for ATP are slightly lower than for GTP, while in the presence of Mn^{2+} the opposite has been reported (Niefind et al., 1999).

2.1 CK2 structure

The structure of CK2 is now well known, thank to the analysis of the crystallographic structures of the catalytic subunit CK2α, of the regulatory subunit CK2β and of the holoenzyme.

The structure of the CK2α monomer is a variant of the common bilobal architecture of protein kinases, with a β-rich N-terminal domain, an α-helical C-terminal domain, and the active site in the cleft between them (Niefind et al., 1998). A shown in Fig. 12, the N-terminal domain is the smaller one and it is mainly constituted of an anti-parallel β-sheet composed of five β-strands; the C-terminal domain has instead an α-helix fold, with most helices conserved among the eukaryotic protein kinase superfamily.

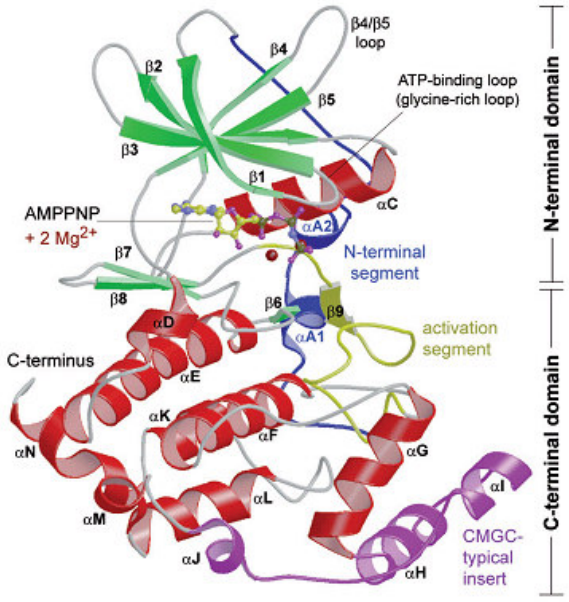


Fig. 12 Catalytic subunit CK2α from *Zea mays* (from Niefind et al., 2009).

On the contrary, the structure of CK2β subunit revealed a unique fold (Chantalat et al., 1999). The CK2β monomer, as CK2α, consist of two domains, but the main feature of this structure is represented by a zinc-finger motif, which can mediate the omodimerization of the protein.

In fact, in the holoenzyme the CK2β dimer constitutes the central building block, bridging the two CK2α subunits (Niefind et al., 2001). In this "butterfly" structure, each β monomer touches both α subunits, which in contrast make no contact with each other. Moreover, the structure of the tetrameric enzyme demonstrated that the non-hydrolysable ATP analogue AMPPNP (adenosine 5'-[β,γ-imido]triphosphate) is present in the ATP binding site of only one of the two catalytic CK2α subunits (see Fig. 13).

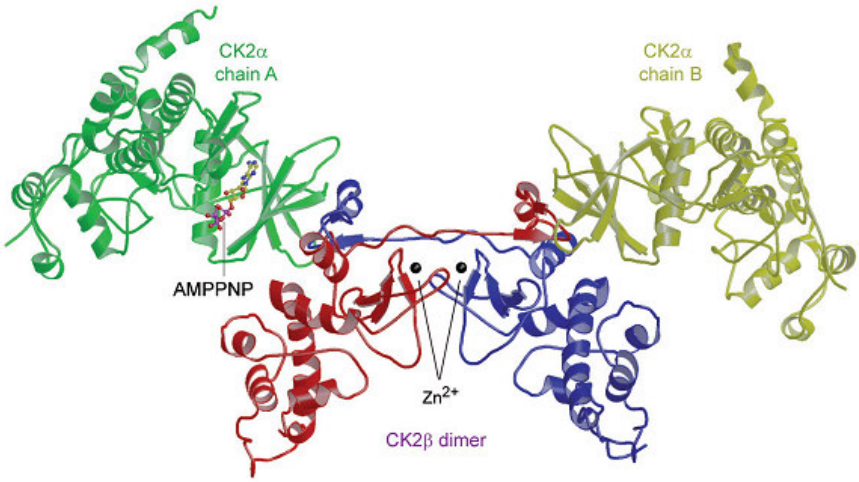


Fig. 13 Structure of the CK2 holoenzyme complex (from Niefind et al., 2009).

2.2 CK2 regulation

CK2 is a costitutively active enzyme and is indipendent of either secondary messengers or phosphorylation events (Meggio and Pinna 2003); yet, a number of distinct mechanisms contribute to its modulation, such as regulated expression and assembly, post-translational modifications, protein-protein interactions, regulation by various natural compounds, modulation of subcellular localization (Pinna, 2002; Litchfield, 2003; Bibby and Litchfield, 2005).

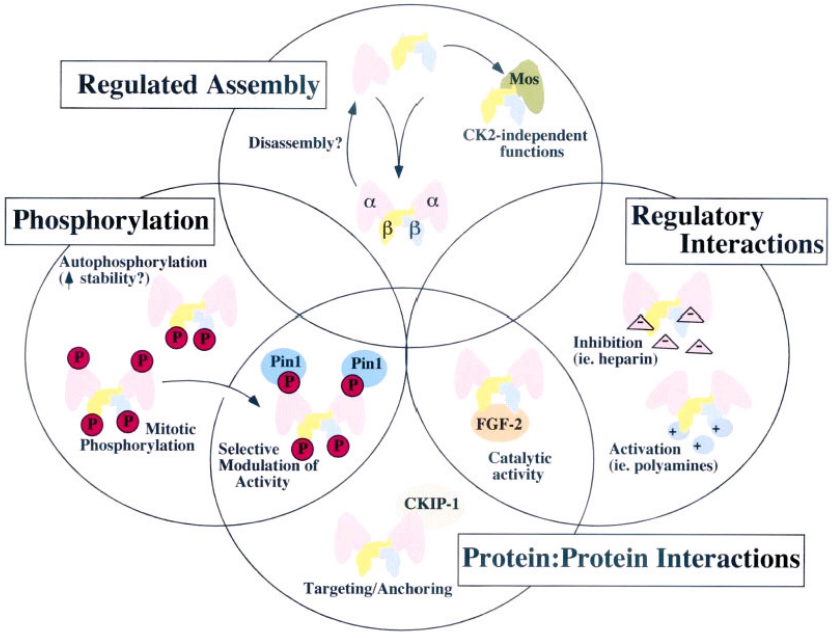


Fig. 14 Possible mechanisms of CK2 regulation (from Litchfield, 2003).

Regulated expression and assembly of CK2

Despite CK2 is expressed in all tissues and organs, and throughout the cell cycle, its levels often correspond to proliferation rate, and cells with higher proliferation rate exhibit higher CK2 activity (Munstermann et al., 1990). In fact, CK2 activity was found elevated in all analysed cancers (colorectal, melanomas, bladder, kidney, gastric and breats carcinomas, Munstermann et al., 1990) and in cancer cell lines (Prowald et al., 1984). CK2 activity is also increased by extracellular signals that promote cellular proliferation, such as insulin, epidermal growth factor (Sommercorn et al., 1987) and serum (Carrol and Marshak, 1989). The observed increase in CK2 activity is not always accompained by an increase of CK2 levels, thus reflecting other kind of regulation.

There are also evidences indicating an unbalanced expression of catalytic and regulatory subunits in a variety of tissues and in cancer cells (Bibby and Litchfield, 2005). Indeed, although CK2 was traditionally considered to be a tertrameric enzyme, both catalytic and regulatory subunits can exist outside the holoenzyme. For exemple, there are substrates, the prototype being calmodulin, that can be phosphorylated by free catalytic subunits, but not by the holoenzyme (Litchfield, 2003). Moreover, CK2β subunit was shown to have CK2-independent functions, since it was linked to DNA damage checkpoint both in yeast and in human (Bibby and Litchfield, 2005), and it was shown to be involved in the regulation of the enzymatic activity of other kinases that are distinct from CK2 (Olsen and Guerra, 2008).

Phosphorylation of CK2

Even if phosphorylation is not strictly required for CK2 activity, both α and β subunits show many physiological phosphorylation sites and it

cannot be excluded that they could partecipate to some degree in the regulation of CK2 activity *in vivo*. For instance, CK2 is phosphorylated at Ser209 of CK2β subunit (Litchfield et al., 1995), as well as Thr344, Thr360, Ser362, and Ser370 in the C-terminal domain of CK2α, in a cell-cycle dependent manner (Bosc et al., 1995). CK2 was also shown to be a substrate of Tyr-kinase Abl and of the oncogenic form Bcr-Abl (Hériché and Chambaz, 1998).

Moreover CK2 holoenzyme undergoes autophosphorylation at serine and threonine residues of the catalytic subunit, and at two serine residues (Ser2 and Ser3) close to the N-terminus of its β subunit, which enhance CK2β stability (Zhang et al., 2002). Finally, free catalytic subunits can undergo intermolecular tyrosine-autophosphorylation on Tyr182 in the activation loop, which, however, seems to have no effect on CK2 activity *in vitro* (Donella-Deana et al., 2001).

Regulation by natural compounds

CK2 has typically been classified as a messenger-independent kinase. However, different small molecules were described to partecipate in some aspects of CK2 regulation. For instance, it has long been known that CK2 is inhibited by negatively charged compounds, such as heparin, and activated by positively charged compounds, like polyamines (Litchfield, 2003). Yet, physiological effects of these compounds are not completely clear.

Protein-protein interactions

Protein-protein interactions represent a major mechanism for regulation of protein-kinases and the identification of several proteins interacting with CK2 is consistent with this concept. A part from

revealing new CK2 substrates, the analysis of CK2 interactome will include proteins that regulate functional properties of the enzyme. Among these, of great interest are those proteins which interact only with specific subunits, like the peptide prolyl isomerase Pin1 and CKIP-1, which interact with CK2α but not with CK2α' (Litchfielad et al., 2001). On this basis, recently Gyenis and Litchfield examined the emerging CK2 interactomes both in yeast and in humans. This analysis revealed new insights regarding CK2 cellular functions as well as functional specialization of individual subunits (Gyenis and Litchfield, 2008).

CK2 subcellular localization

CK2 is also regulated by a thight modulation of its subcellular localization, since it exerts different functions in the various compartments where it is found (Faust and Montenarh, 2000). In particular, protein kinase CK2 mainly shows a nuclear localization (Krek et al., 1992; Penner et al., 1997; Martel et al., 2001; Filhol et al., 2003), which has been reported to increase after serum treatment and in tumor cells (Sommercorn et al., 1987; Filhol et al., 1990; Faust et al., 1999; Guo et al., 1999; Wang et al., 2003; Homma and Homma, 2008), thus seeming to be linked to high cellular proliferation.

Anyway, CK2 pools were also found in the cytoplasm, associated to the endoplasmic reticulum, to the membrane and to the cytoskeleton (Faust and Montenarh, 2000). In general, CK2 subcellular localization is considered a dynamic process, that changes with the requirement of CK2 activity at a particular cellular district in response to various stimuli and stress signals (Faust and Montenarh, 2000; Filhol and Cochet, 2009).

As concerns the nuclear pool of CK2, Filhol and coworkers showed that each CK2 subunit is separately imported into the nucleus through specific mechanisms. In CK2α subunit, nuclear localization is due to a unique cluster of predominantly basic amino acids, at position 74-77. On the contrary, CK2β subunit does not contain any recognizable nuclear localization sequence (NLS), but it is also imported to the nucleus, maybe bound to other proteins; its nuclear import requires dimerization but does not require interaction with CK2α. Likewise, CK2 subunits may also form the holoenzyme in the cytoplas, which can bind ligands (such as FGF-2) able to target the nuclear translocation of the holoenzyme (see Fig. 15, Filhol et al., 2003).

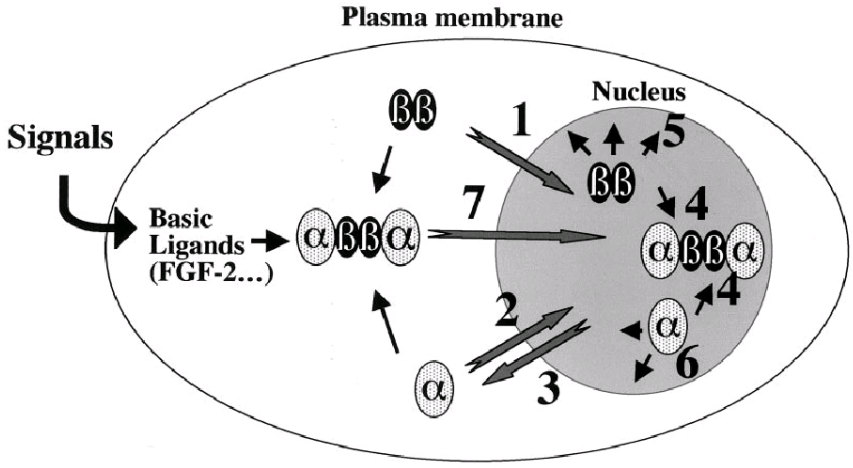


Fig. 15 Model for intracellular dynamics of CK2 subunits (Filhol et al., 2003).

2.3 CK2 and cancer

CK2 activity is strongly correlated to proliferation rate, being higher in highly proliferating cells; in particular, as previously mentioned, CK2 activity was shown to be higher in cancer cell lines if compared with normal tissues (Prowald et al., 1984; Munstermann et al., 1990; Guerra and Issinger, 1999; Tawfic et al., 2001) and, more outstanding, CK2 was found upregulated in all cancers that have been examined (Trembley et al., 2009). Thus, CK2 dysregulation has often been associated with dysregulated proliferation and cell growth. However, it should be noted that elevated levels of CK2 alone are not indicative of dysregulation, since high levels of CK2 are intrinsically present in certain organs (such as brain and testes), and are associated to certain developemental stages. Instead, CK2 elevation in neoplasia reflects a complex state of dysplasia, not simply a higher proliferation rate (Trembley et al., 2009).

CK2 cannot be considered an oncogene by itself and there is no evidence of mutations of CK2 giving rise to tumors. Yet, modest alterations in the activity of CK2 are sufficient to induce dramatic effects; for exemple, in transgenic mice, targeted expression of CK2α in T cells and in mammary glands leads to lymphomagenesis and mammary tumorigenesis respectively (Xu et al., 1999). More importantly, CK2 exhibits oncogenic co-operativity when combined with several oncogenes, like c-myc, tal-1, Ha-Ras oncogenes, or in p53-deficient mice (Orlandini et al., 1998; Litchfield, 2003; Trembley et al., 2009). Also the inclusion of many oncogenes and viral proteins among CK2 targets strongly suggests a role for CK2 in cancer. Thus, even if it is not an oncogene, CK2 is universally considered a contributing and sustaining factor in cancer. But CK2 oncogenic

potential is not only due to its association to high proliferation rate, but mainly to the fact that CK2 is a potent suppressor of apoptosis, acting on different cellular functions, signaling pathways and biochemical reactions involved in cell death (Ahmad et al., 2008; Ruzzene and Pinna, 2009). In some cancers, decreased apoptotic activity is an even more important factor in cancer progression than increased proliferation. It was shown that phosphorylation by CK2 may protect proteins (like Max, Bid, connexin 45.6, HS1, and presenilin-2) from caspase-mediated degradation (Meggio and Pinna, 2003); besides, CK2 has a strong impact on IAPs (inhibitors of apoptosis proteins) and CK2 inhibition leads to a potent induction of apoptosis (Wang et al., 2008).

Angiogenesis and formation of metastasis are two more processes important for cancer progression, in which CK2 seems to be involved, even if there are no systematic studies on these subjects (Trembley et al., 2009). Finally, CK2 was shown to counteract the efficacy of antitumor drugs and to contribute to the multi-drug resistance phenotype (Ruzzene and Pinna, 2009). Thus, CK2 contributes to potentiate the tumor phenotype, by creating favorable conditions for tumorigenesis whenever its activity reaches a critical threshold, and this makes CK2 a potential prognostic marker and an attractive target in a variety of neoplastic diseases (Sarno and Pinna, 2008).

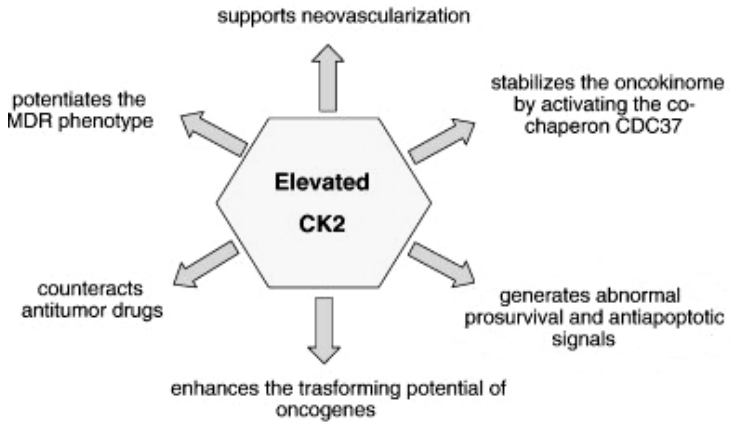


Fig. 16 Effects promoted by abnormally high CK2 levels (form Ruzzene and Pinna, 2009).

Nowadays, a number of potent and selective inhibitors of CK2 are available, and great efforts are made to find new and more potent ones. Yet, while there is a strong rationale for considering CK2 as a target for cancer therapy, it is important to note that CK2 is a ubiquitous and essential enzyme, rising concerns about potential tossicity of CK2-targeting drugs. By the way, recently, Perea and coworkers developed a novel peptide that abrogates CK2 phosphorylation by blocking the substrate (Perea et al., 2004), which is currently under clinical trials and seems to be safe and well tolerated, providing an early proof-of-principle of possible clinical benefits (Solares et al., 2009).

2.4 CK2 and cell-cycle progression

Protein kinase CK2 is involved in cell cycle progression, both in yeast (Hanna et al., 1995) and in mammalian cells (Pepperkok et al., 1994; Homma and Homma, 2005; Homma et al., 2005).

In mammalian cells some ambiguous data come from the measurement of CK2 activity during cell cycle. In fact, while some groups failed to observe any difference in CK2 avtivity in different cell cycle phases (Schmidt-Spaniol et al., 1993; Bosc et al., 1999), many others showed significant differences (Pepperkok et al., 1994; Marshak and Russo, 1994; Wang et al., 2003; Homma et al., 2005; Homma and Homma, 2008). In particular, CK2 activity was shown to be required for specific cell-cycle phases in mammalian cells, mainly G0/G1 and G1/S transitions, while it is not necessary for S phase (Pepperkok et al., 1994). Moreover, many key cell-cycle regulators are CK2 substrate (Meggio and Pinna, 2003), like p21, p27, p53, but the effect of these phosphorylations on the regulation of cell cycle is often not so clear. Homma and Homma showed that CK2 phosphorylation of the tumor suppressor protein APC (adenomatous polyposis coli), mutated in most colorectal tumors, is involved in the regulation of cell-cycle progression (Homma and Homma, 2005). In the same year, they showed that phosphorylation of the translation initiation factor eIF5 by CK2 is important for cell-cycle progression at the S/G2 boundary (Homma et al., 2005).
Of great interest was also the finding of the phosphorylation of cyclin H by CK2, which was shown to be critical for a full activation of the CAK complex (Schneider et al., 2002). The CAK (Cdk Activating Kinase) complex, which is composed of the regulatory subunit cyclin

H, the catalytic subunit Cdk7 and the assembly factor Mat1, is responsible for the phosphotylation of Thr160 in the activation loop of Cdks, thus being fundamental for cell-cycle progression.

In *Saccharomyces cerevisiae* genetic studies suggested two major requirements of CK2 activity during the cell cycle, since CK2 inactivation leads to an arrest either in G1 and in M phase (Hanna et al., 1995). Detailed description of cell-cycle involvement of CK2 in yeast can be found in paragraph 2.5.

2.5 CK2 in *Saccharomyces cerevisiae*

In yeast, CK2 is composed of two catalytic subunits (α and α') encoded by *CKA1* and *CKA2* genes, and two different regulatory subunits (β and β'), encoded by *CKB1* and *CKB2* respectively. Deletion of either catalytic subunit gene alone has few if any phenotypic effect, but simultaneous disruption of both *CKA1* and *CKA2* genes is lethal (Padmanabha et al., 1990). However, despite this partial redundancy of the two catalytic subunits, analysis of yeast temperature-sensitive mutants for CK2 revealed a specialization of α and α' subunits. In fact, CK2α is required for maintenance of cell polarity (Rethinaswamy et al., 1998), while CK2α' is mainly involved in cell-cycle control (Hanna et al., 1995, see below). On the contrary, both single and double deletion of *CKB1* and *CKB2* genes shows only modest phenotypic defects under standard growth conditions (Li^+ and Na^+ enanched sensitivity; Glover, 1998); besides, CK2β subunits were linked to the adaptation to DNA damage checkpoint (Toczyzki et al., 1997).

Recently, a biochemical characterization of CK2 from budding yeast was carried out by the group of R. Szyszka. They interestingly showed that *S. cerevisiae* holoenzyme requires both β and β' regulatory subunits for its activity (Kubinski et al., 2007). Moreover, they were able to discern between the activity of the holoenzyme and of the α' monomer by using two different specific CK2 inhibitors (TBBt and TBBz, Zien et al., 2003), thus providing a useful instrument for further biochemical characterization of different CK2 molecular forms.

Anyway, the more studied process in which CK2 was shown to be involved in yeast is cell-cycle progression. As mentioned earlier, CK2 is mainly involved in G1 and in M phase (Hanna et al., 1995). In fact,

following a shift to the non permissive temperature, CK2α' *ts* mutants block cell cycle and exhibit a dual arrest phenotype, with 50% of the population being arrested as unbudded G1 cells and 50% as large-budded cells in G2/M. Moreover, comparative genome-wide expression analysis of CK2 subunit deletion mutants indicated that expression levels of several genes linked to cell-cycle progression and cell division in yeast are affected by CK2 (Barz et al., 2003). For instance, *CLN3* and *CLN2* are repressed in *ckaΔ* and *ckbΔ* strains respectively and many CK2-affected genes encode proteins involved in chromatin remodeling and modification.

Recent works also showed that CK2 phosphorylates key cell-cycle regulatory proteins, such as the cyclin-dependent kinase Cdk1 (Russo et al., 2000), the molecular chaperone Cdc37 (Bandhakavi et al., 2003), the inhibitor Sic1 (Coccetti et al., 2004; Coccetti et al., 2006) and the ubiquitin-conjugating enzyme Cdc34 (Barz et al., 2006; Sadowski et al., 2007).

Notably, most of the known CK2 substrates are involved in the G1 phase of the cell cycle, thus accounting for the role of CK2 in G1 phase (Hanna et al., 1995). However, a recent review focused on potential CK2 substrates controlling M phase (Alberghina et al., 2009), showing that more than 30 key mitotic proteins contain CK2 consentus sites. A deepening of this interesting observation could be crucial to understand the involvement of CK2 in mitosis at a molecular level.

The cyclin-dependent kinase Cdk1

One of the first CK2 cell-cycle substrates discovered in yeast was Cdk1, the main cyclin-dependent kinase of *S. cerevisiae*, that is phosphorylated on Ser46 by CK2 both *in vitro* and *in vivo* (Russo et

al., 2000). Notably, Ser46 corresponds to Ser39 of the human homologous Cdc2, which is also CK2 substrate (Russo et al., 1992), thus indicating a conserved mechanism between yeast and human cells. Mutational analysis on Cdk1-S46 showed that phosphorylation of Ser46 does not influence Cdk1 kinase activity; rather, it is involved in the regulation of cell size. In fact, a strain expressing Cdk1-S46A showed a decrease of cell size, that was modest in exponential phase and much more pronounced in stationary phase (Russo et al., 2000).

The molecular chaperone Cdc37

The molecular chaperone Cdc37 was first identified as a temperature-sensitive cell-division cycle mutation that arrests cells in G1 at the non-permissive temperature (Reed, 1980). Cdc37 was soon linked to Hsp90 because of its identification in Hsp90-dependent signaling pathways and its physical association with Hsp90 clients; however, recent evidences demonstrated that Cdc37 can function as a molecular chaperone by itself (MacLean and Picard, 2003).

Cdc37 is also the only known multicopy suppressor of temperature-sensitive alleles of CK2 in *Saccharomyces cerevisiae*. Cdc37 is phosphorylated by CK2 on Ser14 and Ser17 (Bandhakavi et al., 2003), and a strong conservation of this phosphorylation is observed, since also in mammalian cells Cdc37 is phosphorylated by CK2 on Ser13 (corresponding to Ser14, Miyata and Nishida, 2005). Yeast cells bearing mutations on these two phosphorylation sites showed growth defects and a reduced CK2 activity. Thus, it was proposed that Cdc37 and CK2 constitute a positive feedback loop, that promotes the activity of multiple kinases (Bandhakavi et al., 2003). Notably, the same feedback loop model was proposed for mammalian Cdc37 (Miyata and Nishida, 2005).

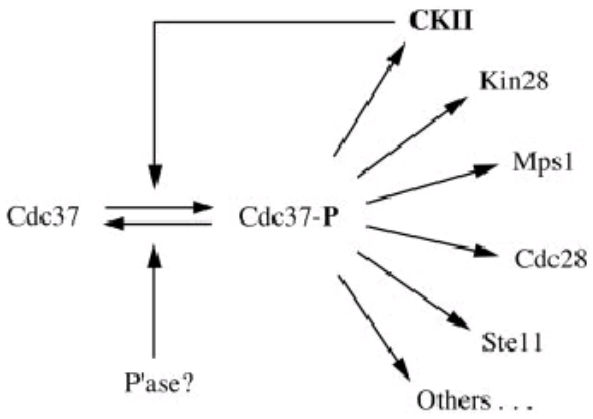


Fig. 17 A positive feedback loop between CK2 and Cdc37 positively regulates multiple protein kinases (from Bandhakavi et al., 2003).

The Ckd-Clb inhibitor Sic1

Coccetti and coworkers showed that the Cdk inhibitor Sic1 is a physiological CK2 substrate, both *in vitro* (Coccetti et al., 2004) and *in vivo* (Coccetti et al., 2006). As mentioned above, Ser201 phosphorylation by CK2 was the first and only phosphorylation described by now, that is not involved in the process of degradation of Sic1. In fact, Ser201 phosphorylation does not alter Sic1 ability to be degraded *in vivo*, but is important for the coordination between cell growth and cell-cycle progression. Indeed, a strain expressing Sic1-S201A showed a decrease in Ps; on the contrary, a strong reduction of Ps was observed in a strain expressing Sic1-S201E. Moreover, in Sic1-S201A cells, Ckd1-Clb5 kinase activity is much lower than in wild-type cells, and this is reflected by a slower G1/S progression of this strain. Conversely, in Sic1-S201E cells, Ckd1-Clb5 activity is condensed in a shorter time window, and G1/S transition execution showed a strong lack of coordination between budding and DNA

synthesis (Coccetti et al., 2004). However, although *in vivo* data suggested that Sic1-S201 phosphorylation promotes G1/S transition (Coccetti et al., 2004), Sic1 phosphorylated by CK2 was observed to be a stronger *in vitro* inhibitor of Cdk-cyclin complexes than the unphosphorylated one (Barberis et al., 2005a). This apparent discrepancy is currently under investigation in our laboratory, to shed light on the role of Sic1-Ser201 phosphorylation.

The ubiquitin-conjugating enzyme Cdc34

Yeast Cdc34 was described as CK2 substrate by Pyerin and coworkers (Pyerin at al., 2005; Barz et al., 2006), who observed a role of Cdc34 in the methionine biosynthesis pathway. They showed that CK2 phosphorylates Cdc34 on at least two sites, Ser207 and Ser282; phosphorylation at one of these sites, Ser282, has a significant impact on MET gene expression (Barz et al., 2006). Later, Cdc34 was shown to be phosphorylated by CK2 at Ser207 and Ser216 (Sadowski et al., 2007), in the acidic tail of Cdc34. The authors noticed a higher ubiquitination activity *in vitro* when Cdc34 was phosphorylated by CK2, and linked this difference in activity to a slight difference in cell-cycle progression in strains bearing mutation at these two phosphorylation sites (Sadowski et al., 2007). However, as mentioned above, the acidic tail of Cdc34 is dispensable for its activity *in vivo* (Ptak et al., 1994). Interestingly, CK2 phosphorylates also human Cdc34 in its C-terminal tail on multiple residues, regulating its nuclear localization (Block et al., 2001), and affecting β-catenin degradation (Semplici et al., 2002).

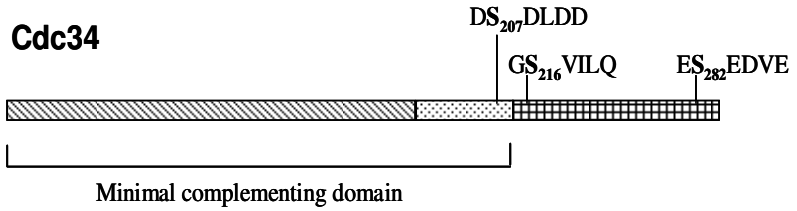


Fig. 18 CK2-mediated phosphorylations of yeast Cdc34 described so far in literature.

Results

3 In CK2 inactivated cells the cyclin dependent kinase inhibitor Sic1 is involved in cell cycle arrest before the onset of S phase

CK2 plays an important role in cell-cycle progression and several cell-cycle proteins have been reported to be CK2 substrates. In particular, literature data showed that *cka1Δcka2^{ts}* mutants arrest cell cycle in both G1 and M phase at 37°C (Hanna et al., 1995). Despite this interesting discovery, no detailed analysis was carried out to define the molecular mechanism underling this double arrest.

Work from my laboratory previously showed that Sic1, the inhibitor of Clb5-Cdc28 complexes required for the G1/S transition, is a physiologically relevant CK2 substrate (Coccetti et al., 2004; Coccetti et al., 2006).

Here we show that CK2 inactivation up-regulates Sic1 level resulting in severe down-regulation of Clb5-Cdc28 kinase activity. Concurrent inactivation of Sic1 and CK2 leads to a single cell-cycle arrest, in which cells show a post-synthetic DNA content and short/elongated spindles, typical of cells arrested in mitosis. These findings clearly explain that the G1 arrest of *cka1Δcka2^{ts}* mutants is due to Sic1 accumulation, which prevents S phase entry at restrictive temperature.

The result of this analysis has been published in 2007 by Tripodi et al.

3.1 Results

Sic1 protein level increases in *cka1Δcka2^{ts}* strains at restrictive temperature

Until now, no CK2 target responsible for either G1 or G2/M cell cycle arrest has been identified (Canton and Litchfield, 2006). We previously reported that Sic1 level increased in *cka1Δcka2-8^{ts}* arrested cells at 37°C (Coccetti et al., 2006). Since Sic1 is largely a G1-specific protein, this increased level may be the passive result of the accumulation of cells in the G1 phase or, on the contrary, a fraction of CK2-inactivated cells may arrest in G1 due to Sic1 hyper-accumulation. To answer this point, we analyzed the time course of Sic1, Clb5 and Cln2 accumulation in *cka1Δcka2-8^{ts}* strains expressing one of the following tagged proteins, Cln2-4HA, Clb5-9myc and Sic1-4HA shifted at the restrictive temperature (37°C). At the indicated time points after the shift, samples were taken for monitoring cell density, DNA content by FACS analysis and specific protein level by western blot analysis. As shown in Fig. 1A, after 1 h of incubation at 37°C, cells started accumulating with a 1N DNA content and, as previously reported, within 6 h after the temperature shift, the increase in cell number was completely arrested with about 50% of the cell population in G1 phase (Hanna et al., 1995). A strong Sic1 accumulation was detected as early as 1 h after CK2 inactivation. Sic1 level steadily increased at later time points reaching a maximum between 4 and 6 h after the temperature shift (Fig.1B) corresponding to complete cell cycle arrest (Fig. 1C). The levels of cyclins Cln2 and Clb5 also remained high throughout the duration of the experiment (Fig. 1B).

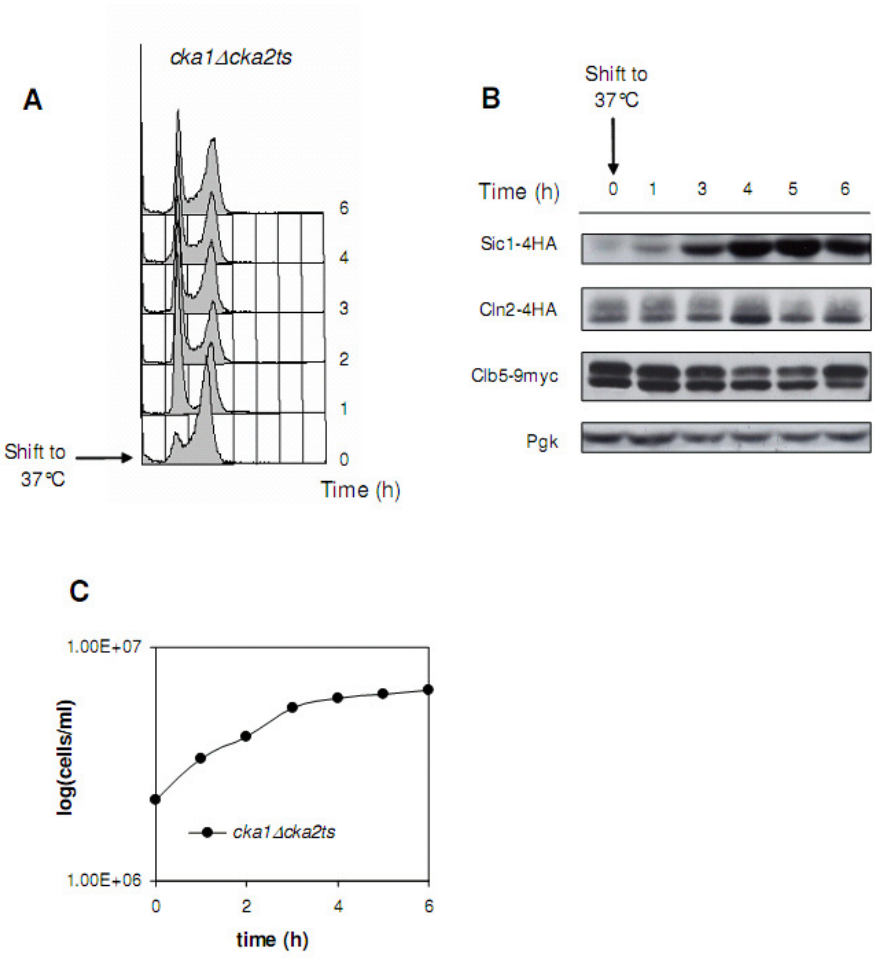


Fig. 1 Cell cycle arrest due to CK2 inactivation involves Sic1 up-regulation. *cka1Δcka2^{ts}* strains expressing Sic1-4HA, Cln2-4HA and Clb5-9myc were grown in synthetic minimal medium at 25°C and at time 0 shifted to 37°C. Samples were taken at the indicated time points to assay (A) DNA content by FACS analysis; (B) protein levels by western blot analysis using anti-HA 12CA5 antibody (dilution 1:1000) and anti-myc 9E10 antibody (dilution 1:1000) (Pgk was used as a loading control); (C) cell density.

Clb5-Cdc28 kinase activity is severely reduced in a *cka1Δcka2-8^{ts}* strain at restrictive temperature

Results reported above indicate that Sic1 accumulates and cells do not start DNA replication, despite the high content of Clb5, possibly because the high Sic1 level keeps the Clb5-Cdc28 complex in a catalytically inactive state. Clb5-Cdc28 activity was thus assayed. Exponentially growing *cka1Δcka2-8^{ts}* cells, endogenously expressing a Clb5-myc protein, were treated with α-factor for two hours at 25°C, shifted to 37°C for one additional hour and then released into fresh medium at 37°C. As a control we analysed the same strain arrested with α-factor and released at permissive temperature (the scheme of the experiment is reported in Fig. 2A). Samples were taken at the indicated time points to analyse Clb5-associated kinase activity, Sic1 and Clb5 protein levels and DNA content by FACS analysis. As expected, at 25°C cells started to enter S phase 30 min after the release, coincidently with an increase in Clb5-Cdc28 kinase activity that reached its maximum between 40 and 65 min, coincident with Sic1 degradation (Fig. 2B-C). In contrast, at restrictive temperature, only half of the population escaped from the G1 block and arrested within one cell cycle as large budded cells with a 2N DNA content (Fig. 2B). Furthermore, at 37°C the level of Sic1 remained quite stable and high (Fig 2C). Consistently with our hypothesis, little Clb5 associated kinase activity was observed at 37°C during the time course of the experiment, due to Sic1 inhibition (Fig 2C).

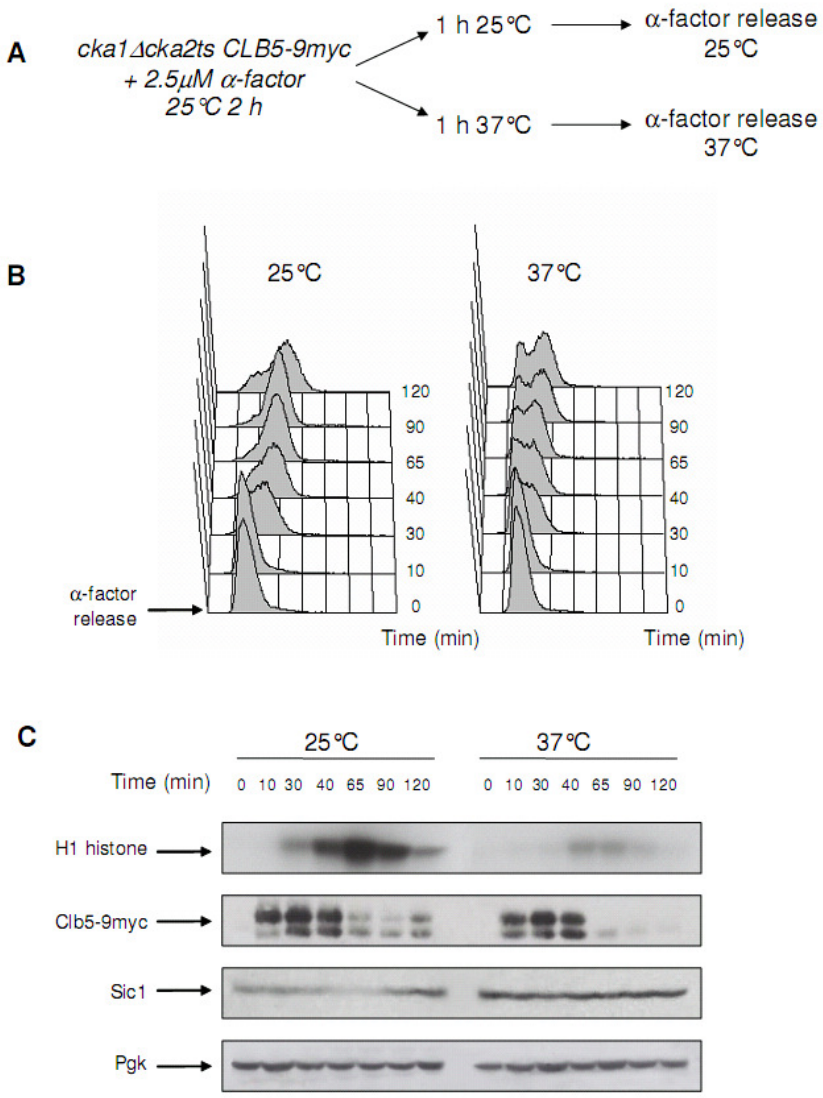


Fig. 2 Clb5/Cdc28 associated kinase activity is altered in *cka1Δcka2^{ts}* mutant. *cka1Δcka2^{ts}* yeast strain was synchronized in G1 by α-factor treatment and released in fresh medium at time 0 both at 25°C and at 37°C (in the latter case, the culture was shifted to the non-permissive temperature 1 h prior to the removal of α-factor, as shown in A). Samples were taken at

the indicated time points to assay (B) DNA content by FACS analysis; (C) Clb5-associated H1 kinase activity and protein levels by western blot analysis using anti-Sic1 antibody (dilution 1:1000) and anti-myc 9E10 antibody (dilution 1:1000) (Pgk was used as a loading control).

Increase in G1 cells upon CK2 depletion requires Sic1

Our data support the notion that up-regulation of Sic1 contributes to the failure to enter S phase of CK2-depleted cells by inhibiting the activity of the Clb5-Cdc28 complex. If this is the case, then *SIC1* deletion in a *cka1Δcka2-8^{ts}* background should result in a uniform arrest of cells with a post-synthetic DNA content. The *cka1Δcka2-8^{ts}* mutant and the isogenic *cka1Δcka2-8^{ts}sic1Δ* strain were grown in synthetic minimal medium at 25°C until early exponential phase and then incubated at 37°C. Both strains exhibited a transient increase in the proportion of G1 cells during the first 1 h following the shift to restrictive temperature as a consequence of the heat shock, but they diverged afterwards. In fact, FACS analysis confirmed that cells expressing Sic1 arrested growth with both 1N and 2N DNA content, as reported in Fig. 3A (left panel) and in Hanna et al., 1995. Instead *sic1Δ* strain started accumulating cells with a 2N DNA content after 3 h of incubation at 37°C (Fig. 3A, right panel), ultimately leading to a complete cell cycle arrest after 6 h at 37°C with all cells harbouring a postreplicative DNA content (Fig. 3A-B). The analysis of the profile of mitotic spindles of *sic1Δ* strain indicated that cells accumulated both with short and elongated spindles, typical of cells arrested in mitosis (Fig. 3C). Thus, *cka1Δcka2-8^{ts}sic1Δ* cells are unable to accumulate in G1 phase after CK2 inactivation. The same results were obtained using the strain *cka1Δcka2-11^{ts}* (Hanna et al., 1995), indicating that the results are not dependent on a specific CK2 allele (data not shown).

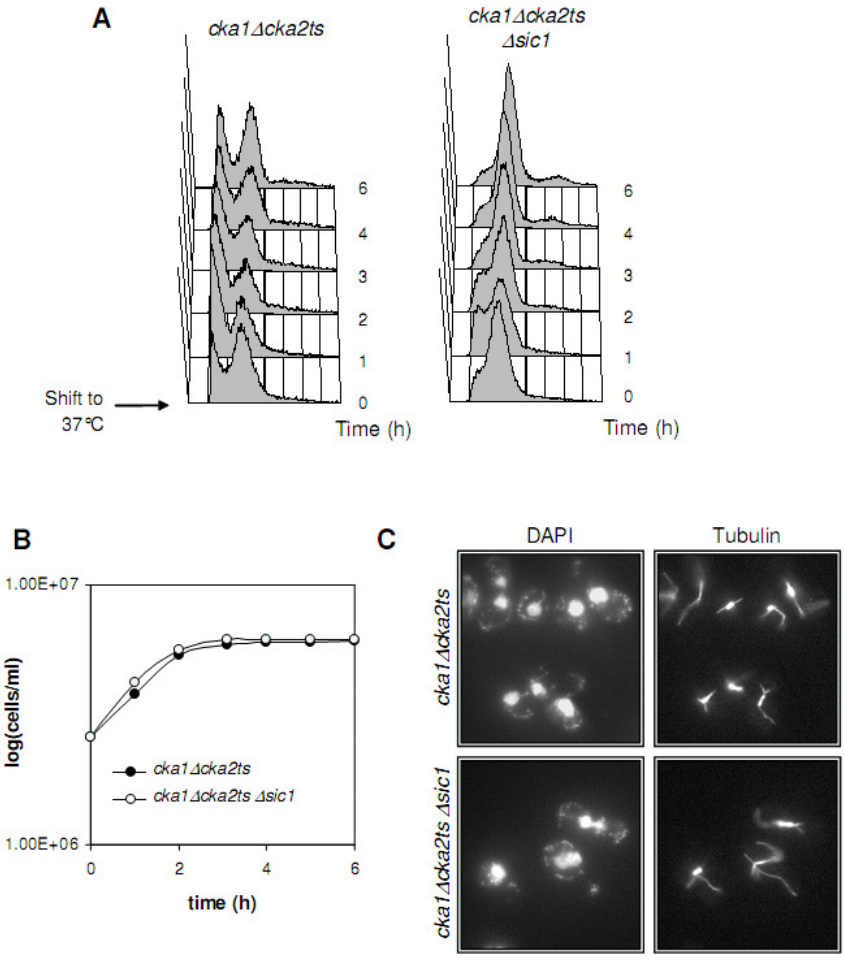


Fig. 3 Deletion of *SIC1* suppresses the G1 arrest due to CK2 inactivation. *cka1Δcka2-8^{ts}* and *cka1Δcka2-8^{ts} sic1Δ* strains were grown in synthetic minimal medium at 25°C and at time 0 shifted to 37°C. Samples were taken at the indicated time points to assay (A) DNA content by FACS analysis; (B) cell density; (C) nuclear and spindle morphology of the arrested strains at 37°C.

The role of Sic1 in the G1 arrest following CK2 inactivation, was further confirmed by shutting-down Sic1 expression in a *cka1Δcka2-8^{ts}sic1Δ* strain, expressing the Sic1-4HA protein under the control of a tetracycline-repressible promoter (pTet-*SIC1*) (Rossi et al., 2005). The strain was grown in minimal synthetic medium to early exponential phase at permissive temperature. Cells expressing Sic1 were treated or untreated for 4 h with doxycycline (2 µg/ml final concentration) in order to shut off *SIC1* expression and maintained at 25°C or shifted to 37°C. Samples were taken to analyse DNA content by FACS analysis and Sic1 level by western blot. As expected, doxycycline treatment caused Sic1 depletion within 0.5 h at 25°C and determined a concomitant increase of the budding index (data not shown) and of the percentage of cells with a 2N DNA content (Fig. 4A-B, left panels), as observed in a *sic1Δ* strain (Lengronne and Schwob, 2002). On the contrary, Sic1 stabilization could be observed as early as 1 h in untreated cells shifted to 37°C, reaching a maximum between 2 and 4 h after the temperature shift (Fig. 4A, middle panel). As previously shown, the arrested population consisted of a mixture of cells with either 1N and 2N DNA content (Fig. 4B, middle panel). At 37°C, as at the permissive temperature, doxycycline treatment depleted Sic1 in less than 0.5 h after the temperature shift and the cells did not progress through the M phase and accumulated with a 2N DNA content (Fig. 4A-B, right panels). The arrested cells exhibited the same terminal phenotype, regarding budding index and mitotic spindle morphology, to that described in the previous paragraph. The majority of cells arrested with a large bud and with either short and elongated spindles (data not shown).

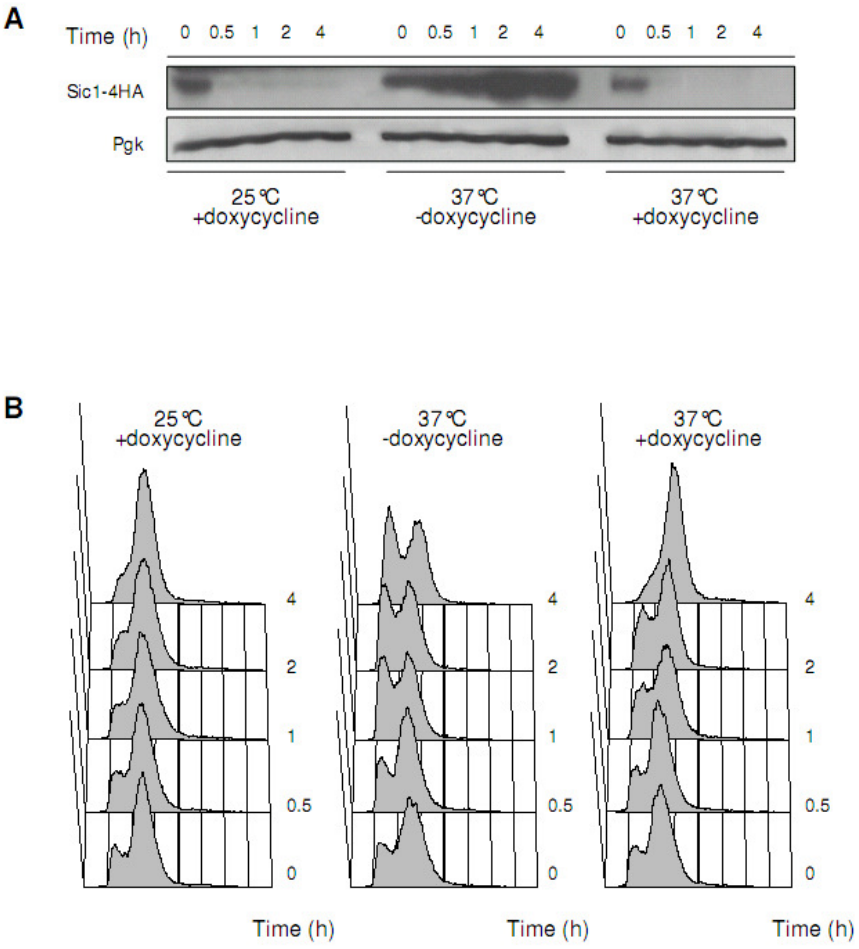


Fig. 4 Shutting off Sic1 expression suppresses the G1 arrest due to CK2 inactivation. *cka1Δcka2-8^{ts} sic1Δ pTet-SIC1-4HA* strain was grown at 25°C, at time 0 cells were either maintained at 25°C or shifted to 37°C, in the absence or in the presence of doxycycline (2 µg/ml) to switch off *SIC1* expression. Samples were taken at the indicated time points to assay (A) protein level by western blot analysis using anti-HA 12CA5 antibodies (dilution 1:1000) (Pgk was used as a loading control); (B) DNA content by FACS analysis.

In summary, these data show that *SIC1* depletion, obtained either by ablation of the *SIC1* gene or by shutting off its transcription, severely interferes with the G1 arrest of a *cka1Δcka2-8^{ts}* mutant, confirming that Sic1 plays a major role in arresting cell cycle before the onset of S phase in CK2-inactivated cells.

3.2 Conclusions

CK2 activity is required both in the G1 phase and at the G2/M transition (Hanna et al., 1995). Recent work from our group showed that Sic1, a major inhibitor of the G1/S transition, is phosphorylated by CK2 on Ser201 both *in vitro* (Coccetti et al., 2004) and *in vivo* (Coccetti et al., 2006) and that Ser201 mutations alter cell growth-cell cycle coordination at the G1/S transition (Coccetti et al., 2004). Results reported in this paper newly indicate a major involvement of the inhibitor Sic1 in G1 arrest due to CK2 inactivation since: (i) Sic1 accumulation is an early event following CK2 inactivation; (ii) in synchronous cells released from an α-factor-induced G1 block, the accumulated Sic1 inhibits the Clb5-Cdk1 complex, effectively blocking the G1/S transition; (iii) *SIC1* deletion does not rescue the overall cell growth defect of a *cka1Δcka2*^{ts} strain, but almost completely abolishes the G1 block, so that a *cka1Δcka2*^{ts} *sic1Δ* strain arrests in metaphase and in anaphase, *i.e.* at a time point when in wild type cells Sic1 is not present (metaphase) or just starts to be synthesized (anaphase).

Up-regulation of Sic1 accumulation has been reported to play a major role in rapamycin-induced G1 arrest in yeast (Zinzalla et al., 2007), as it is rapamycin up-regulation of $p27^{Kip1}$, the mammalian functional equivalent of Sic1 (Barberis et al., 2005a), in primary mouse embryo fibroblasts (Cheng et al., 1999). Thus, data presented in this paper reinforce the notion that the control of Ckis level, sub-cellular localization (Rossi et al., 2005) and post-translational modification (Coccetti et al., 2006) play a major role in fine tuning the G1/S transition in eukaryotes as a function of intracellular and extracellular signals.

3.3 Materials and Methods

Yeast strains and growth conditions. *S. cerevisiae* strains used in this study are listed in Table 1.

Table 1 **Yeast strains used in this study**

Strain	Relevant genotype	Origin
cka1Δcka2-8^{ts}	MATa, cka1-Δ1::HIS3, cka2-Δ1::TRP1, [cka2-8]	Hanna *et al*., 1995
cka1Δcka2-8^{ts} sic1Δ	MATa, cka1-Δ1::HIS3, cka2-Δ1::TRP1, [cka2-8], sic1::KanR	This study
cka1Δcka2-8^{ts} SIC1-4HA	MATa, cka1-Δ1::HIS3, cka2-Δ1::TRP1, [cka2-8], SIC1-4HA (KanR)	This study
cka1Δcka2-8^{ts} CLN2-4HA	MATa, cka1-Δ1::HIS3, cka2-Δ1::TRP1, [cka2-8], CLN2-4HA (KanR)	This study
cka1Δcka2-8^{ts} CLB5-9myc	MATa, cka1-Δ1::HIS3, cka2-Δ1::TRP1, [cka2-8], CLB5-9myc (URA3)	This study
cka1Δcka2-8^{ts} pTet-SIC1-4HA	MATa, cka1-Δ1::HIS3, cka2-Δ1::TRP1, [cka2-8], sic1::KanR, [pCM189-Sic1-4HA]	This study

Yeast strains were grown in synthetic medium containing 2% glucose, 6.7 g/l YNB (Difco), 100 mg/l of Ade, and 50 mg/l of Ura and required amino acids. Cell density of liquid culture grown at 25°C and 37°C was determined with a Coulter counter on mildly sonicated and diluted samples. Percentage of budded cells was determined by direct microscopic count of at least 300 cells after mild sonication. For G1 synchronization cells were grown to exponential phase, then α-factor was added to a final concentration of 2.5 µM; cells were allowed to arrest for approximately 2 h at 25°C and, where indicated, shifted at 37°C for 1 additional hour; synchronization was monitored by microscopic analysis and confirmed by DNA distribution profile obtained with FACS analysis.

Recombinant and genetic techniques. DNA manipulation and yeast transformations were carried out according to standard techniques. To obtain the tagged strains, proteins were tagged with a

C-terminal 4HA or 9myc epitope by an in locus 3' in-frame insertion. The 4HA-Kan^R fragment used to tag Sic1 and Cln2 was PCR amplified from the pDHA plasmid. It was constructed by replacing the *SalI-BglII* fragment (containing the *CFP* gene) of the pDH3 plasmid (kindly provided by Trish N. Davis) with a PCR amplified 4HA fragment. The 9myc-URA3 fragment used to tag Clb5 was amplified from pST-9myc-URA3 plasmid. It was obtained by inserting a PCR amplified 9myc fragment and the *URA3* marker in the pSTBlue-1 plasmid digested with *EcoRI* and *BamHI*. To obtain the *cka1Δcka2-8^{ts}sic1Δ* strain, we disrupted *SIC1* with the cassette *SIC1::Kan^R* in the *cka1Δcka2-8^{ts}* strain kindly supplied by C.V.C. Glover (Hanna et al., 1995). All primers used are listed in Table 2.

Table 2	**Primers used in this study**
Name	**Sequence (5' --> 3')**
5'-SIC1-HA-Kan	AAGGTTAACGGATGAAGAAAAGAGAAGATTCAAGCCAAAGGCATTGTTT CAATCTAGGGATCAAGAGCATCGGCCGCATGGATCCTATCC
3'-SIC1-HA-Kan	TTGCAAATAAATGTAGAATAAGTAAGTAAATAAAATATAATCGTTCCAGA AACTTTTTTTTTTCATTTCTGGATGGCGGCGTTAGTATCG
5'-CLN2-HA-Kan	ATAAATAGCGGTAAATCTAGCAGTGCCTCATCTTTAATTTCTTTTGGTAT GGGCAATACCCAAGTAATACGGCCGCATGGATCCTATCC
3'-CLN2-HA-Kan	CTCTCTTTTCCCGCAGAATATGAAAGCTTTTCTTTTATAAATCTTATAAT ATTGGTCTCTTTTTGGTACGGATGGCGGCGTTAGTATCG
5'-CLB5-myc-URA3	TTATTTCCAAACTTTCAAGTGGTGTACATCCGAAATGCATAGCAACTTTC AAAATCTATTTAATCTTAAGAGATCTCTTAGCGGCCGCTCT
3'-CLB5-myc-URA3	CCTTTTAGTTCAGCAAAAAGAAAAGAAAATGTAAAGAGTATGCGAATTC ATGAGCATTACTAGTACTAATGCATGCTGCAGACGCGTTACG
5'-SIC1delta-Kan	AACAACTAAGTCTTTTAAAAATGCGCCATTATTAGCACCTCAGCTGAAG CTTCGTACGCT
3'-SIC1delta-Kan	TTCATTTCTTCAATGCTCTTGATCCCTAGATTGAAACAATATAGGCCACT AGTGGATCTG

To obtain the Sic1 overexpressing strain the *cka1Δcka2^{ts}sic1Δ* strain was transformed with the vector pTet-*SIC1*-4HA bearing *SIC1* gene under the control of tetracycline-repressible promoter. Silencing of *SIC1* was obtained by adding 2 µg/ml doxycycline to cultures of cells carrying the *SIC1* gene on plasmid with tetracycline-repressible promoter exponentially growing in synthetic media (Rossi et al., 2005).

Protein extraction, immunoblotting, immunoprecipitation and kinase assays. Samples of cells were harvested and lysed using ice-cold NP40 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$ and 1% Nonidet P-40) plus 1 mM PMSF, proteases inhibitor mix (Complete EDTA free Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitor mix (Sigma). Total protein extraction was performed as previously described (Coccetti et al., 2006). Protein concentration was determined using the Bio-Rad protein assay. Western blot analysis was performed using anti-Sic1 polyclonal antibody, anti-HA monoclonal antibody (12CA5, Roche), anti-myc monoclonal antibody (9E10, Santa Cruz Biotechnology) and anti-Pgk monoclonal antibody (Molecular Probes) (1:1000 dilution for each antibody). Enhanced chemioluminescence system (ECL, Amersham Biosciences) was used for immunoblot detection according to the manufacturer's instructions. Samples of cells for Clb5 kinase assay were collected by filtration, resuspended in ice-cold NP40 buffer plus inhibitor and broken as previously described (Coccetti et al., 2006). *In vitro* kinase assay to detect Clb5/Cdc28 kinasic activity on histone H1 was performed as previously described (Coccetti et al., 2004).

Flow cytofluorimetric analysis. A total of 1 x 10^7 cells was collected by filtration for each sample, fixed in 70% ethanol and subsequently processed for FACS analysis. Cells were washed three times with PBS (3.3 mM NaH_2PO_4, 6.7 mM Na_2HPO_4, 127 mM NaCl, 0.2 mM EDTA, pH 7.2) and incubated over night in PBS plus 1 mg/ml RNAse (Roche).The day after, cells were washed once with 50 mM Tris pH 7.5 and resuspended in 50 mM Tris plus 1 μM Sytox Green (Molecular Probes). Analysis was performed with a BD Biosciences FACScan.

4 The CK2 phosphorylation of catalytic domain of Cdc34 modulates its activity at the G1 to S transition in *Saccharomyces cerevisiae*

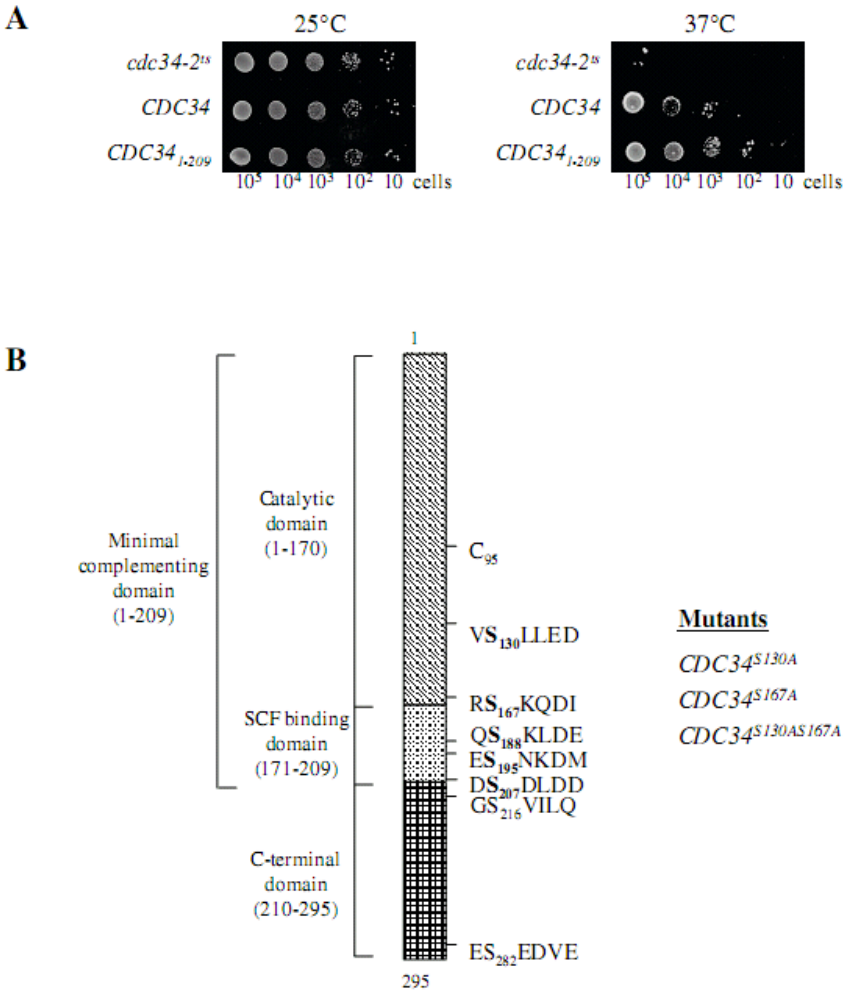
The ubiquitin-conjugating enzyme Cdc34 was recently shown to be phosphorylated by CK2 on the C-terminal tail in *Saccharomyces cerevisiae* (Barz et al., 2006; Sadowski et al., 2007). Here we present novel findings indicating that in budding yeast CK2 phosphorylates Cdc34 within the N-terminal catalytic domain. Specifically, we show, by direct mass spectrometry analysis, that Cdc34 is phosphorylated *in vitro* and *in vivo* by CK2 on Ser130 and Ser167, and that the phosphoserines 130 and 167 are not present after CK2 inactivation in a *cka1Δcka2-8^{ts}* strain. CK2 phosphorylation of Ser130 and Ser167 strongly stimulates Cdc34 ubiquitin charging *in vitro*. The Cdc34^{S130AS167A} mutant shows a basal ubiquitin charging activity which is indistinguishable from that of wild type but is not activated by CK2 phosphorylation and its expression fails to complement a *cdc34-2^{ts}* yeast strain, supporting a model in which activation of Cdc34 involves CK2-mediated phosphorylation of its catalytic domain.

The result of this analysis has been published in 2008 by Coccetti et al.

4.1 Results

CK2 phosphorylates Cdc34 on Ser130 and Ser167 both *in vitro* and *in vivo*

By consensus sequence analysis, five putative CK2-phosphorylation sites were identified within the complementing domain of Cdc34 ($Cdc34_{1-209}$, Fig. 1A and Ptak et al., 1994): Ser130 and Ser167 inside the catalytic domain, and Ser188, Ser195 and Ser207, clustered in the SCF-binding domain (Fig. 1B). Amino acid sequence alignment indicates that CK2 consensus sites centered on Ser130 and Ser167 are evolutionarily conserved in other yeasts and in metazoa, suggesting that they are functionally relevant (Fig. 1C). Fig. 1D shows that both the full length His_6-Cdc34, as previously reported (Pyerin et al., 2005; Barz et al., 2006; Sadowski et al., 2007), and His_6-$Cdc34_{1-209}$ were phosphorylated *in vitro* by CK2, confirming that sites phosphorylatable by CK2 are present within the complementing domain of Cdc34.


A
25°C
37°C
cdc34-2ts
CDC34
CDC34 1-209
10⁵ 10⁴ 10³ 10² 10 cells
B
1
Minimal complementing domain (1-209)
Catalytic domain (1-170)
SCF binding domain (171-209)
C-terminal domain (210-295)
C95
VS130LLED
RS167KQDI
QS188KLDE
ES195NKDM
DS207DLDD
GS216VILQ
ES282EDVE
295
Mutants
CDC34S130A
CDC34S167A
CDC34S130AS167A

C

```
Cdc34_S.cerevisiae   -MSSRKSTASSLLLRQYRELTDPKKAIPSFHIELEDDSNIFTWNIGVMVLNEDSIYHGGF 59
Q6CRG7_K.lactis      -MSG-KNTAASLLLRQYRELTDPKKAIPSFHIALEDDSNIFLWNIGVMVLNEESIYHGGY 58
Q5AH09_C.albicans    -MSS--KSAAAILQRQFKDLTDPKKGIPSFHIELDDD-NIFLWNVGVMVLNPESLYHGGY 56
Q7SZU1_X.laevis      MAHQQMTSSQKALMLELKSLQE--EPVEGFRITLVDESDLYNWEVAIFGP-PNTLYEGGY 57
Cdc34B_human         MAQQQMTSSQKALMLELKSLQE--EPVEGFRITLVDESDLYNWEVAIFGP-PNTLYEGGY 57
CDC34_human          MARPLVPSSQKALLLELKGLQE--EPVEGFRVTLVDEGDLYNWEVAIFGP-PNTYYEGGY 57
                           ::   *  : : * :  : : .*:: * *: ::: *::.::    :: *.**:

Cdc34_S.cerevisiae   FKAQMRFPEDFPFSPPQFRFTPAIYHPNVYRDGRLCISILHQ-SGDPMTDEPDAETWSPV 118
Q6CRG7_K.lactis      FKAQMKFPDDFPFSPPTFRFTPAIYHPNVYRDGRLCISILHQ-SGNPTSDEPDEETWSPV 117
Q5AH09_C.albicans    FKGQMRFPQDFPFSPPSFRFTPALYHPNVYRDGRLCISILHQ-GGDPTSDEPESETWSPA 115
Q7SZU1_X.laevis      FKAHIKFPIDYPYSPPTFRFLTKMWHPNIYENGDVCISILHPPVDDPQSGELPSERWNPT 117
Cdc34B_human         FKAHIKFPIDYPYSPPTFRFLTKMWHPNIYENGDVCISILHPPVDDPQSGELPSERWNPT 117
CDC34_human          FKARLKFPIDYPYSPPAFRFLTKMWHPNIYETGDVCISILHPPVDDPQSGELPSERWNPT 117
                     **.:::** *:*:*** *** . ::***:*. * :******   .:* :.*   * *.*.

                             130                                   167
Cdc34_ S.cerevisiae  QTVESVLISIVSLLEDPNINSPANVDAAVDYRKNPE------QYKQRVKMEVERSKQDIP 172
Q6CRG7_K.lactis      QTVESVLISIVSLLEDPNISSPANVDAAVDYKKNPD------QYKQKVKLEVERSKQDIP 171
Q5AH09_C.albicans    QTVESVLISIISLLDDPNGNSPANIDASVEFRKNYN------VYKKKVLQEVERSKKDIP 169
Q7SZU1_X.laevis      QNVRTILLSVISLLNEPNTFSPANVDASVMFRKWRDSKGKDKEYAEIIRKQVSATKAEAE 177
Cdc34B_human         QNVRTILLSVISLLNEPNTFSPANVDASVMFRKWRDSKGKDKEYAEIIRKQVSATKAEAE 177
CDC34_human          QNVRTILLSVISLLNEPNTFSPANVDASVMYRKWKESKGKDREYTDIIRKQVLGTKVDAE 177
                     *.*.::*:*::***::**  ****:**:* ::*  :       * . :  :*  :* :

                                188        195        207
Cdc34_ S.cerevisiae  KGFIMPTSESAYISQSKLDE--PESNKDMADNFWYDSDLDDDENGSVILQDDDYDDGNNH 230
Q6CRG7_K.lactis      QGFVMPESTKAYVSQRKANEGIDTIKETVADNFWYDSDEGDEDVDIYAYEDSEDEEDNDD 231
Q5AH09_C.albicans    EDFIMPETSDNAYSNYNNSKNKEGAEEPVDEDFWYESEEEES------FDEESLMDENMD 223
Q7SZU1_X.laevis      KDGVKVPTTLAEYCIKTKVPSSDNSSDLLYDDLYDDDIDDEDEE-----EEDADCYDDDD 232
Cdc34B_human         KDGVKVPTTLAEYCIKTKVPSNDNSSDLLYDDLYDDDIDDEDEE-----EEDADCYDDDD 232
CDC34_human          RDGVKVPTTLAEYCVKTKAPAPDEGSDLFYDDYYED--GEVEEE-----ADSCFGDDEDD 230
                     .. :   :     .  .        .. . :: : :     .         :.     : .

Cdc34_ S.cerevisiae  IPFEDDDVYNYNDNDDDDERIEFEDDD----DDDDSIDNDSVMDRKQPHKAEDESEDVE 286
Q6CRG7_K.lactis       VFSKDSSNLNFSKKANKND-IDSDEDEDLVPEEAEDSLD-ESVMDRRS--KLMEESEELE 287
Q5AH09_C.albicans    DQFEDDD--------------EEEEDD----EDED--------MGGK------------- 244
Q7SZU1_X.laevis      SGNEES---------------------------------------------------- 238
Cdc34B_human         SGNEES---------------------------------------------------- 238
CDC34_human          SGTEES---------------------------------------------------- 236
                        ::.

Cdc34_ S.cerevisiae  DVERVSKKI 295
Q6CRG7_K.lactis      DAENK---- 292
Q5AH09_C.albicans    ---------
Q7SZU1_X.laevis      ---------
Cdc34B_human         ---------
CDC34_human          ---------
```

D

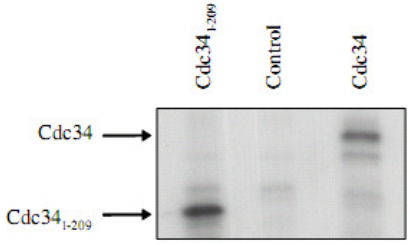


Fig. 1 *In vitro* CK2 phosphorylation of His$_6$-Cdc34$_{1-209}$. (A) Strains of the indicated genotype (*cdc34-2^{ts}*, *CDC34* and *CDC34$_{1-209}$*) were equalized, spotted at 10-fold serial dilutions and grown at 25°C and 37°C. (B) A

schematic representation of Cdc34 functional domains. The CK2 phosphorylation sites in the complementing domain (aa. 1-209) are indicated in bold. Mutants analysed in this study are indicated on the right. (C) Multiple sequence alignment of E2 enzymes using ClustalW. Conserved CK2 phosphorylation sites are marked by arrows. (D) *In vitro* phosphorylation of recombinant His_6-$Cdc34_{1-209}$ (lane 1) and His_6-Cdc34 (lane 3) with purified human CK2 (10 U for 30 min. at 30°C); as a negative control, a crude *E. coli* extract was used (lane 2). The result was confirmed from three separate experiments and one was reported as an example.

In order to identify residues within the complementing domain of Cdc34 phosphorylated by CK2, recombinant full length His_6-Cdc34, His_6-$Cdc34^{S130A}$ and His_6-$Cdc34_{1-209}$ were phosphorylated *in vitro* by CK2, digested with trypsin or Asp-N and analyzed by mass spectrometry (Fig. 2A). Operating in the linear mode, a peak at 2840.06 *m/z* corresponding to a phosphorylated 108-133 peptide (^{108}DEPDAETWSPVQTVESVLISIVS$_{130}$LLE^{133}, calculated monoisotopic mass 2840.16) and a peak at 1668.89 *m/z* corresponding to a phosphorylated 160-173 peptide (^{160}VKMEVERS$_{167}$KQDIPK^{173}, calculated average mass 1668.92) were detected in the wild type protein (Fig. 2B). Phosphatase treatment allowed the detection of the corresponding non-phosphorylated peaks (data not shown). The phosphorylation site within the 160-173 peptide was unequivocally identified as Ser167, since this is the only phosphorylable residue present within the peptide (Fig. 2A). To identify Ser130 as the phosphorylated residue, we performed MS/MS experiments using a nanoLC-ESI-MS/MS on His_6-Cdc34 and His_6-$Cdc34^{S130A}$ phosphorylated *in vitro* by CK2 and digested with Glu-C. $_{123}$SVLISIVpS$_{130}$LLE$_{133}$ peptide showed very low

ionization with ESI source, as previously reported for other phosphorylated peptides.[29,30] Instead, MS/MS analysis on His_6-$Cdc34^{S130A}$ mutant protein revealed a not phosphorylated peptide containing S130A ($_{123}$SVLISIVA$_{130}$LLE$_{133,}$ $[M+2H]^{2+}$ 578.6 *m/z* Mass=1155.2), confirming the absence of other phosphorylated serines within the 123-133 peptide (Fig. 2C).

Therefore, Ser130 was identified as the phosphorylated residue (i) because other serines/threonines present within the peptide have a poor CK2 consensus [14] and, more strikingly, (ii) because MS/MS analysis on mutant $_{123}$SVLISIVA$_{130}$LLE$_{133}$ peptide confirmed the absence of any phosphorylated residue (Fig. 2C).

In order to identify whether phosphorylation on Ser130 and on Ser167 occurs *in vivo*, $Cdc34_{1-209}$ and Cdc34 proteins were immunopurified with anti Cdc34-antibody from glucose-grown yeast cells and resolved by SDS-PAGE followed by Silver staining for MS analysis *in vivo*. Bands corresponding to Cdc34 or $Cdc34_{1-209}$ from six individual lanes were pooled, digested with trypsin or Glu-C and analyzed by MALDI-TOF. Identity of Cdc34 was assessed by database search. The spectra revealed the presence of phosphorylated peptides spanning Ser130 as well as Ser167 (Fig. 2D). In particular, two peaks were detected at 2181.68 *m/z* and at 1155.05 *m/z* corresponding to Cdc34 114-133 and 123-133 peptides, respectively, phosphorylated at Ser130. A peak corresponding to a Cdc34 160-168 peptide with a decrease of 18 units was detected at 1087.67 *m/z*. This result was further confirmed by MS of the peptide mixture obtained by *in situ* digestion of $Cdc34_{1-209}$ with endoproteinase Glu-C (peptide 164-181, Fig. 2D).

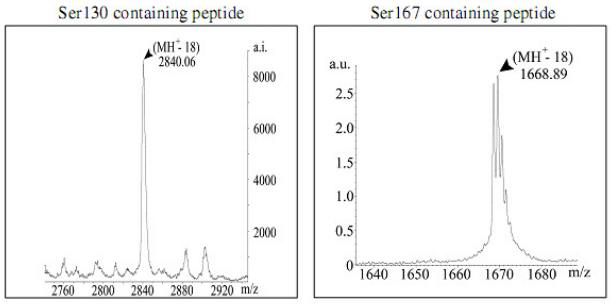
Additional confirmation that the kinase involved in Cdc34 phosphorylation on Ser130 and Ser167 is CK2 came from the

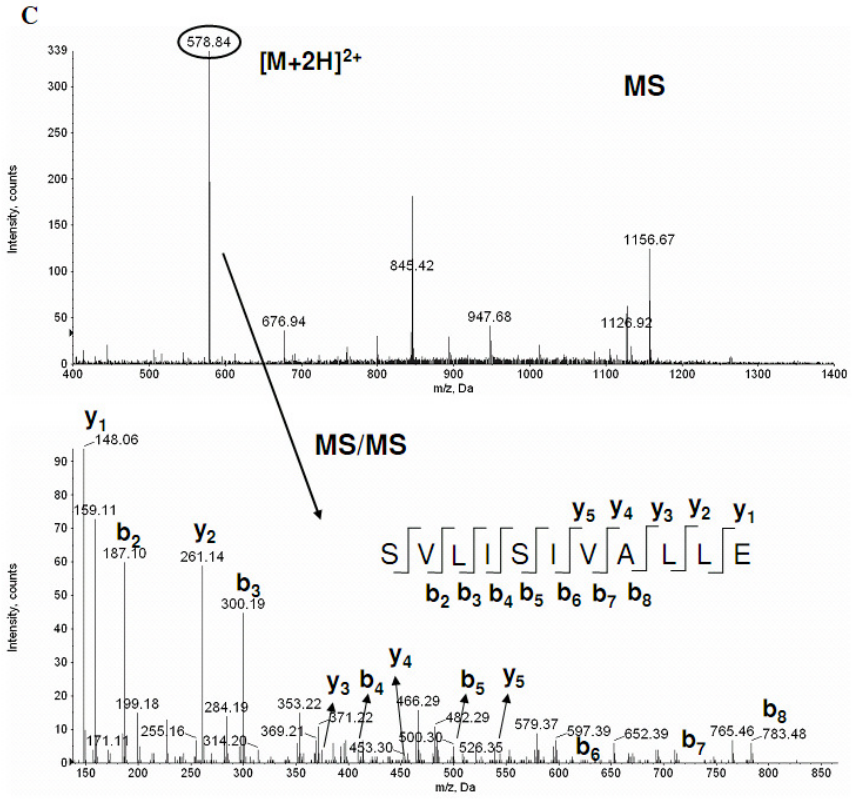
phosphorylation status of Cdc34 immunopurified from the *cka1Δcka2-8^{ts}* strain shifted to 37°C for 6 h (Hanna et al., 1995). In fact, after CK2 inactivation, two peaks (spanning Ser130 and Ser167) were detected at 2199.88 *m/z* and at 3281.58 *m/z* corresponding to Cdc34 114-133 and 166-194 peptides, respectively, containing Ser and Thr residues not phosphorylated (Fig. 2E).

A

Cdc34 peptides phosphorylated by CK2 *in vitro*				
Peptide	**Species**	**Predicted mass**	**Identified mass**	**Protease**
Ser130				
^{108}DEPDAETWSPVQTVESVLISIVS$_{130}$LLE^{133}	P-SER -18	2840.16 (average)	2840.06	Asp-N
^{108}DEPDAETWSPVQTVESVLISIVS$_{130}$LLEDPNINSPANV^{143}	P-SER -18	3862.25 (average)	3861.52	Asp-N
^{108}DEPDAETWSPVQTVESVLISIVS$_{130}$LLE^{133}	P-SER +80	2938.17 (average)	2938.47	Asp-N
Ser167				
^{160}VKMEVERS$_{167}$KQDIPK^{173}	P-SER -18	1668.92 (monoisotopic)	1668.89	Trypsin
^{162}MEVERS$_{167}$K^{168}	P-SER -18	860.44 (monoisotopic)	860.45	Trypsin

B




C
578.84
[M+2H]2+
MS
1156.67
845.42
676.94
947.68
1126.92
Intensity, counts
m/z, Da
MS/MS
y1
148.06
159.11
b2
187.10
y2
261.14
b3
300.19
y5 y4 y3 y2 y1
S V L I S I V A L L E
b2 b3 b4 b5 b6 b7 b8
y4
y3 b4
b5 y5
b8
199.18
284.19
353.22
466.29
371.22
482.29
579.37
597.39
652.39
765.46
783.48
171.11
255.16
314.20
369.21
453.30
500.30
526.35
b6
b7

D

Cdc34 peptides phosphorylated *in vivo*				
Peptide	Species	Predicted mass	Identified mass	Protease
Ser130				
^{114}TWSPVQTVESVLISIVS$_{130}$LLE^{133}	P-SER -18	2182.21 (monoisotopic)	2181.68	Glu-C
^{123}SVLISIVS$_{130}$LLE^{133}	P-SER -18	1154.71 (monoisotopic)	1555.05	Glu-C
Ser167				
^{160}VKMEVERS$_{167}$K^{168}	P-SER -18	1087.6 (monoisotopic)	1087.67	Trypsin
^{164}VERS$_{167}$KQDIPKGFIMPTSE^{181}	P-SER +80	2142.06 (monoisotopic)	2142.62	Glu-C

E

Cdc34 peptides identified *in vivo* after CK2 inactivation				
Peptide	Species	Predicted mass	Identified mass	Protease
Ser130				
^{114}TWSPVQTVESVLISIVS$_{130}$LLE^{133}	Unmodified	2200.21 (monoisotopic)	2199.88	Glu-C
Ser167				
^{166}RS$_{167}$KQDIPKGFIMPTSESAYISQSKLDEPE^{194}	Unmodified	3281.63 (monoisotopic)	3281.58	Glu-C

Fig. 2 Cdc34 is phosphorylated on Ser130 and Ser167 by CK2. *In vitro* detection of Cdc34 phosphorylated on Ser130 and Ser167. (A) 8 µg of His$_6$-Cdc34, His$_6$-Cdc34^{S130A} and His$_6$-Cdc34$_{1-209}$ were phosphorylated in vitro by purified human CK2 (10 U for 30 minutes at 30°C). Then, samples were denatured in 8 M urea and subjected to reduction and alkylation in solution by iodoacetamide. After dilution and incubation with either trypsin, Asp-N or Glu-C endoproteinases (1:25 enzyme/protein, w/w) over night at 37°C, samples were analysed by Mass analysis (as described in the experimental section). The presence of the phosphate group results in an increase in mass of 80 units (peptide 108-133 containing Ser130) or in a decrease of 18 units due to the loss of the phosphate and a water molecule (all the other peptides in Fig. 2A). (B) MALDI-TOF spectra of two phosphorylated peptides reported in Fig. 2A: the peak at 2840.06 m/z, corresponding to peptide 108-133 (on the left), and the peak at 1668.89 m/z, corresponding to peptide 160-173 (on the right), are indicated. Data are representative of one of three experiments performed. (C) MS/MS spectrum of $_{123}$SVLISIVA$_{130}$LLE$_{133}$ peptide (578.6 m/z) from recombinant His$_6$-Cdc34^{S130A} protein

phosphorylated by CK2; identified fragment ions are shown. *In vivo* detection of Cdc34 phosphorylated on Ser130 and Ser167. (D) Cdc34 and $Cdc34_{1-209}$ were immunopurified from 10 mg of total yeast extract with anti-Cdc34 antibody and resolved by SDS-PAGE followed by silver staining for MS analysis *in vivo*. Bands corresponding to Cdc34 and $Cdc34_{1-209}$ were trypsin-digested and analyzed by MALDI-TOF. Peptide 164-181 shows an increase in mass of 80 units, all the other peptides show a decrease of 18 units. (E) Cdc34 was immunopurified with anti-Cdc34 antibody from the *cka1Δcka2-8^{ts}* yeast strain shifted to 37°C for 6 h, resolved and analyzed as previously described. The identified mass of Cdc34 114-133 and 166-194 peptides are indicated. Data are representative of one of two experiments performed.

Taken together our results provide evidence that Cdc34 is phosphorylated *in vitro* and *in vivo* both on Ser130 and on Ser167 and support a direct role of CK2 in phosphorylating Cdc34 within the catalytic domain.

In vitro CK2 phosphorylation on Ser130 and Ser167 activates ubiquitin-binding activity of Cdc34

As noted above, Ser130 and Ser167 lie within relatively well conserved regions. Thus, we decided to investigate the effect of CK2 phosphorylation of these residues on the efficiency of ubiquitin charging by E1 of His_6-Cdc34 and His_6-$Cdc34^{S130AS167A}$ proteins, in a thiolester assay (Block et al., 2005). Phosphorylation *in vitro* by CK2 strongly enhanced the ubiquitin charging of His_6-Cdc34 (Fig. 3A, compare lanes 1-4 to lanes 5-8). The double mutant His_6-$Cdc34^{S130AS167A}$ was unaffected in basal ubiquitin charging by E1 compared to wild type (lanes 9-12 *vs*. 1-4), but such a binding could not be further stimulated by CK2 phosphorylation, despite the fact that comparable amounts of proteins were used (Fig. 3B) and that,

under our assay conditions, other residues (Ser188, Ser195 and Ser282) were phosphorylated by CK2 (Fig. S1, supplementary material).

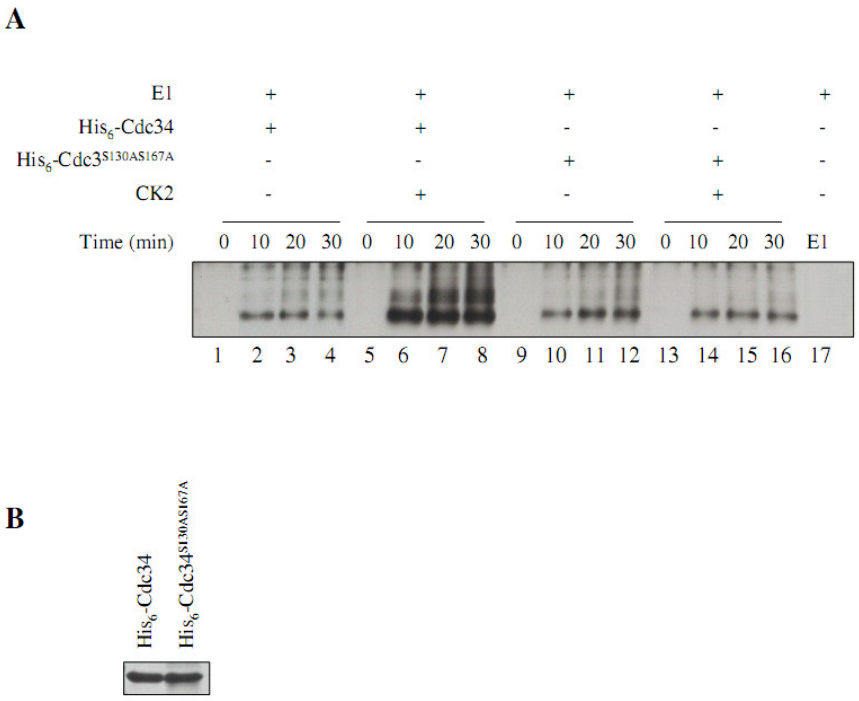


Fig. 3 CK2 phosphorylation of Ser130 and Ser167 promotes Cdc34-ubiquitin binding. (A) Recombinant His_6-Cdc34 and His6-$Cdc34^{S130AS167A}$, either phosphorylated *in vitro* by CK2 or not, were incubated with E1, ATP and biotinylated ubiquitin. Samples were taken at the indicated time points. As a control, Cdc34 was omitted from the reaction (last lane). (B) Coomassie blue staining of His_6-Cdc34 and His_6-$Cdc34^{S130AS167A}$ proteins used in (A). Data are representative of one of three experiments performed.

CDC34^{S130AS167A} expression is unable to complement the growth defect of a *cdc34-2*^{ts} strain

Results reported above suggest that inability to phosphorylate Ser130 and Ser167 could seriously hamper Cdc34 activity and its ability to support cell cycle progression, thus predicting that expression of a *CDC34*^{S130AS167A} gene should be unable to complement a *cdc34-2*^{ts} mutation. To test this hypothesis, wild type and several *CDC34* mutants, cloned under the control of a tetracycline-repressible promoter (*CDC34*^{S130A}, *CDC34*^{S167A} and *CDC34*^{S130AS167A}; Fig. 1B), were tested for their ability to complement the *cdc34-2*^{ts} strain. Log-phase cultures expressing the appropriate *CDC34* gene, were grown at 25°C. At time 0, each culture was split: one-half remained at 25°C while the other half was shifted to 37°C. In keeping with our prediction, the double mutant *CDC34*^{S130AS167A} was fully inactive (Fig. 4A), with its terminal phenotype after 6 h at 37°C characterized by 50% of the cells with elongated buds (Fig. 4B). The mutant *CDC34*^{S167A} complemented the *cdc34-2*^{ts} strain to a similar extent as wild type *CDC34*, on the basis of an increase in cell number (panel A) and cell morphology (panel B). Assay of the same parameters showed that expression of *CDC34*^{S130A} gave full complementation of the *cdc34-2*^{ts} strain in terms of cell division, although more than 40% of the population displayed some morphological defects in the mother cell, suggesting that the cell wall integrity defects associated with the *cdc34-2*^{ts} mutant (Varelas et al., 2006) could not be fully suppressed (panel B). Inability of *CDC34*^{S130AS167A} to complement the *cdc34-2*^{ts} strain was not merely due to an instability of the mutated protein, as shown by western blotting with anti-Cdc34 antibody (Fig. 4C). On the contrary, the mutant *CDC34*^{S188AS195AS207A} was able to fully complement the *cdc34-2*^{ts} strain (Fig. S2, supplementary material).

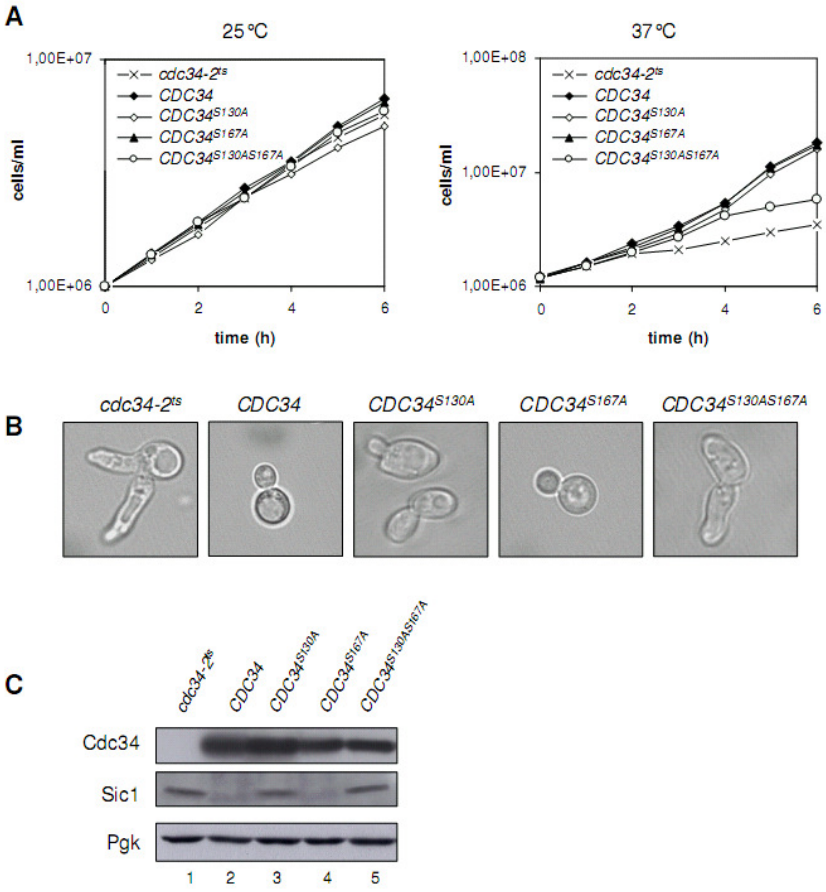


Fig. 4 *CDC34*^{S130AS167A} mutant fails to complement the *cdc34-2*^{ts} strain. (A) *cdc34-2*^{ts}, *CDC34*, *CDC34*^{S130A}, *CDC34*^{S167A} and *CDC34*^{S130AS167A} strains were grown to exponential phase at 25°C and either maintained at 25°C (left panel) or shifted to 37°C (right panel); samples were taken at the indicated time points to assay cell density. Shown are representative curves of three independent experiments. (B) Cellular morphology of the strains after 6 h at 37°C. (C) Crude extracts of the strains shifted to 37°C for 6 h were analyzed by western blot with anti-Cdc34 antibody (1:1000 v/v final dilution) and with anti-Sic1 antibody (1:1000 v/v final dilution). Crude extracts of *cdc34-*

2^{ts}strain (lane 1) was loaded as a negative control for the expression of the protein Cdc34 at restrictive temperature. Pgk was used as a loading control. One representative immunoblot is shown.

SIC1 deletion bypasses the G1 arrest caused by *CDC34*^{S130AS167A}

A sizable Sic1 accumulation was observed when the mutant *CDC34*^{S130AS167A} was arrested for 6 h at restrictive temperature (Fig. 4C). Sic1 up-regulation was also observed in the *CDC34*^{S130A} strain, which, however, did not arrest at 37°C (Fig. 4C). Yet, increased Sic1 level is not enough *per se* to arrest cell cycle, but rather determines the correct timing for entering into S phase (Cross et al., 2007).

SIC1 deletion in a *cdc34-2*^{ts} or *cka1Δcka2*^{ts} background bypasses G1 arrest at 37°C, resulting in a block of growth with a post-synthetic DNA content (Schwob et al., 1994; Tripodi et al., 2007). Results reported above suggest that the Cdc34^{S130AS167A} protein is catalytically inactive: hence, it is predicted that *SIC1* deletion in a *CDC34*^{S130AS167A} background should result in arrest of cells with a post-synthetic DNA content. Hence, *CDC34*^{S130AS167A}, *CDC34*^{S130AS167A} *sic1Δ* strains and the single point mutants (*CDC34*^{S130A}, *CDC34*^{S130A} *sic1Δ, CDC34*^{S167A}, *CDC34*^{S167A} *sic1Δ*) were grown at 25°C to early exponential phase and then shifted to 37°C. *CDC34*^{S130AS167A} showed a complete cell-cycle arrest after 6 h at 37°C with 50% of the population with a presynthetic DNA content (Fig. 5). *CDC34*^{S130AS167A} *sic1Δ* mutant exhibited a transient increase in the proportion of G1 cells during the first 1 h following the temperature shift, then started to accumulate cells with a 2N DNA content after 2 h at 37°C, ultimately leading to a complete arrest with cells endowed with a postreplicative DNA content (as the control strain *cdc34-2*^{ts} *sic1Δ*) (Fig. 5). The majority of

cells arrested with a large bud and with a nuclear morphology indicative of segregated chromosomes (data not shown).

On the contrary, the single point mutants *CDC34*^{S130A} and *CDC34*^{S167A} did not arrest cell growth in a *sic1Δ* background as well, although the strain *CDC34*^{S130A} *sic1Δ* showed a reduction of cells in G1 phase at restrictive temperature compared to its *SIC1* isogenic counterpart (Fig. 5), consistently with its Sic1 up-regulation.

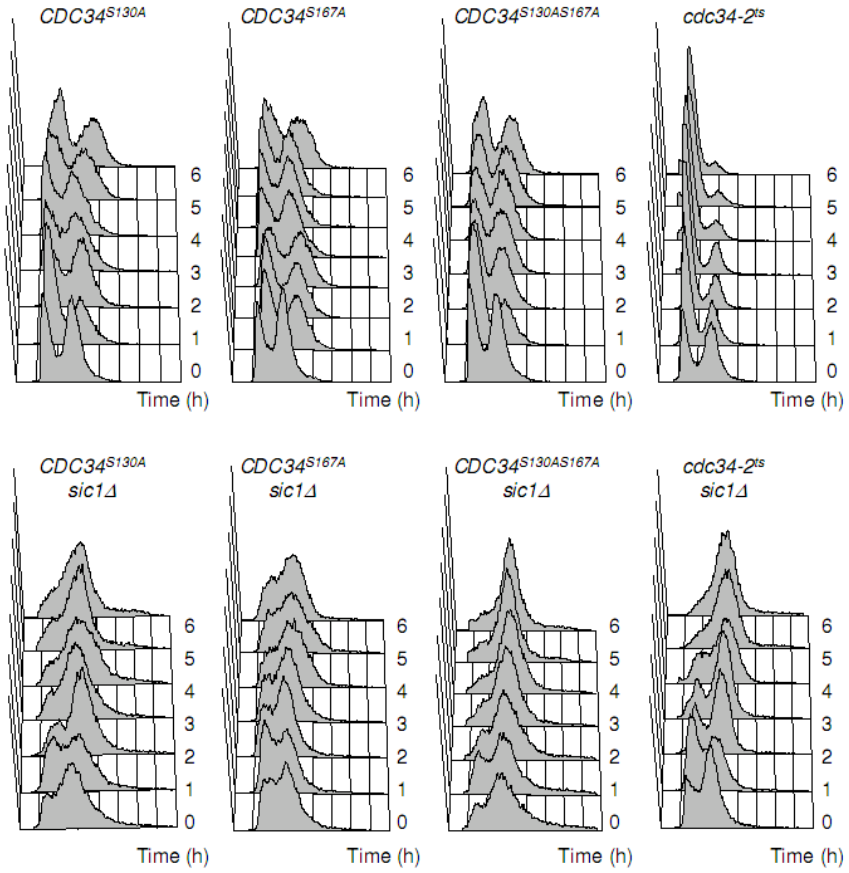


Fig. 5 Deletion of *SIC1* suppresses the G1 arrest of the *CDC34*^{S130AS167A} strain at 37°C. Strains of the indicated genotype (*cdc34-2*^{ts}, *CDC34*^{S130A}, *CDC34*^{S167A}, *CDC34*^{S130AS167A}) and the correspondent *sic1Δ* strains (*cdc34-*

2^{ts} sic1Δ, CDC34^{S130A} sic1Δ,.*CDC34^{S167A} sic1Δ, CDC34^{S130AS167A}* sic1Δ) were grown in synthetic minimal medium at 25°C and at time 0 were shifted to 37°C. Samples were taken at the indicated time points to assay DNA content by FACS analysis. Data are representative of one of two experiments performed.

Downregulation of *CDC34^{S130AS167A}* expression induces a complete G1 arrest

We next determined the physiological effect of downregulating expression of ectopically expressed wild type and mutant *CDC34* genes by adding doxycycline (0.5 µg/ml and 1 µg/ml) to the growing cultures in order to obtain an expression comparable to the endogenous *CDC34*. The scheme of the experiment is shown in Fig. 6A. Samples were taken 6 h after the shift to 37°C to analyze cell growth, DNA content by FACS analysis and Cdc34 level. At restrictive temperature Cdc34 disappeared in a *cdc34-2^{ts}* background (Fig. 4C, lane 1), thus expressed Cdc34 proteins were only the ones encoded by the genes under the control of the *tet*-promoter.

First, we confirmed that doxycycline did not cause any perturbation to yeast cell growth as reported (Garì et al., 1997); in fact, *CDC34* fully complemented the *cdc34-2^{ts}* mutant also in the presence of doxycycline (Fig. 6B-D). Expression of *CDC34^{S130A}* and *CDC34^{S167A}* to a similar level of the endogenous *CDC34* (Fig. 6B, compare lane 5 to lanes 7-8 and lane 9 to lanes 11-12) did not lead to substantial changes in cell growth and DNA content (Fig. 6C-D). On the contrary, downregulation of *CDC34^{S130AS167A}* (Fig. 6B, compare lane 13 to lanes 15-16) confirmed the inability of the mutant to complement the *cdc34-2^{ts}* strain (Fig. 6C) with its terminal phenotype resembling that of a *cdc34-2^{ts}* strain characterized by multiple and elongated buds (inset

Fig. 6D). Remarkably, the whole population arrested with a presynthetic DNA content (Fig. 6D).

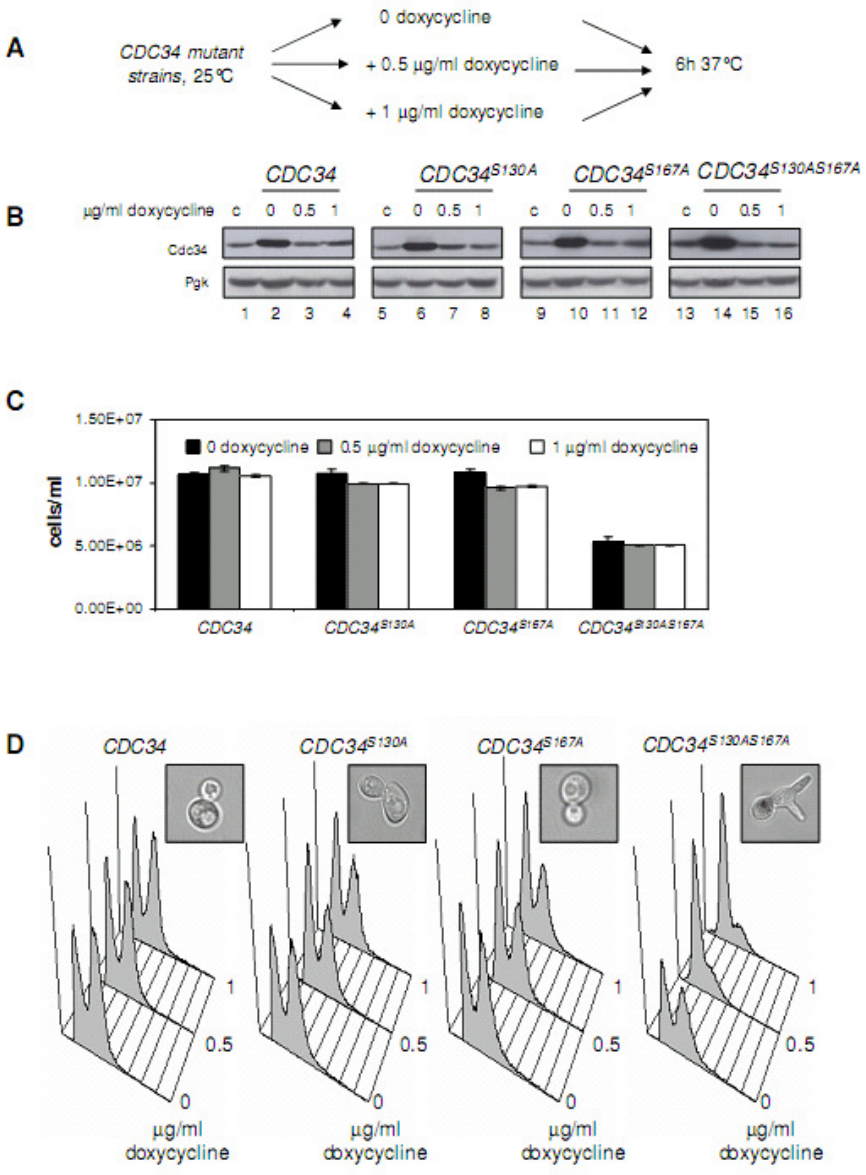


Fig. 6 Downregulation of *CDC34*^{S130AS167A} expression induces a complete G1 arrest. (A) *CDC34*, *CDC34*^{S130A}, *CDC34*^{S167A} and *CDC34*^{S130AS167A} strains were grown to exponential phase at 25°C and shifted to 37°C for 6 h, in the absence or in the presence of doxycycline (0.5 µg/ml and 1 µg/ml). (B) Crude extracts were analysed by western blot with anti-Cdc34 antibody (1:1000 v/v final dilution); protein extracts from exponentially growing wild type cells were used as a control for the expression of the endogenous Cdc34 protein (c, lanes 1, 5, 9 and 13). Pgk was used as a loading control. Samples were taken to assay (C) cell density and (D) DNA content by FACS analysis. Insets show cellular morphology after 6 h at 37°C in the presence of doxycycline (1 µg/ml). Data are representative of one of two experiments performed.

4.2 Supplemental Data

To test the functional significance of CK2-mediated phosphorylations within the SCF-binding domain of Cdc34, the mutant *CDC34*^{S188AS195AS207A} was tested for its ability to complement a *cdc34-2*^{ts} mutation. Log-phase cultures grown at 25°C were divided in two and one-half was grown at 25°C while the other half was shifted to 37°C. As shown in Fig S2, the mutant *CDC34*^{S188AS195AS207A} complemented the *cdc34-2*^{ts} strain to a similar extent as wild type.

Cdc34 peptides phosphorylated by CK2 *in vitro*			
Peptide	**Species**	**Predicted mass**	**Identified mass**
Ser188			
^{169}QDIPKGFIMPTSESAYISQ**S$_{188}$**K^{189}	P-SER -18	2309.16 (monoisotopic)	2309
^{170}DIPKGFIMPTSESAYISQ**S$_{188}$**KLDEPE**S$_{195}$**NK^{197}	P-SER +80	3193.46 (average)	3193.62
Ser 167 and Ser 188			
^{162}MEVER**S$_{167}$**KQDIPKGFIMPTSESAYISQ**S$_{188}$**K^{189}	2P-SER -36	3152.63 (average)	3152.43
Ser 195			
^{191}DEPE**S$_{195}$**NKDMADNFWY^{205}	P-SER -18	1843.93 (average)	1844.09
Ser 282			
^{271}DRKQPHKAEDE**S$_{282}$**E^{283}	P-SER +80	1648.73 (average)	1649.32
^{274}QPHKAEDE**S$_{282}$**EDVEDVERVSK^{293}	P-SER -18	2308.08 (monoisotopic)	2308.38

Fig. S1 Cdc34 is phosphorylated *in vitro* on Ser188, Ser195 and Ser282 by CK2. His$_6$-Cdc34 and His$_6$-Cdc34$_{1-209}$ were phosphorylated *in vitro* by CK2 and analysed by MS.

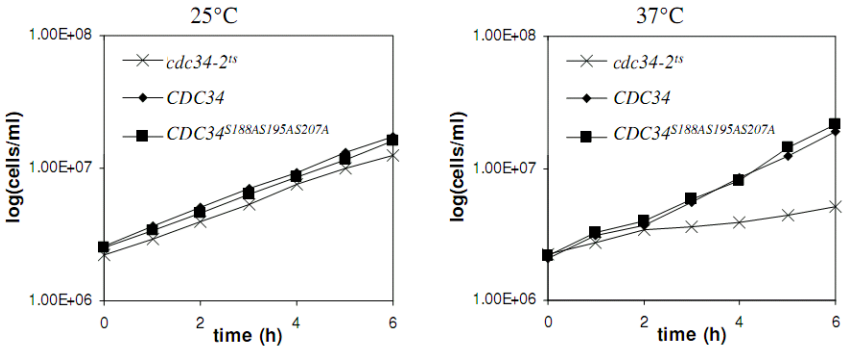


Fig. S2 S188A, S195A, S207A mutations do not alter cell-cycle progression. *cdc34-2*^{ts}, *CDC34, CDC34*^{S188AS195AS207A} strains were grown at 25°C and either maintained at 25°C (left panel) or shifted to37°C (right panel); samples were taken at the indicated time points to assay cell density.

4.3 Discussion

It is known that many proteins are substrates of CK2, but mutation of the respective phosphorylation sites does not originate a lethal phenotype (Canton and Litchfield, 2005). In several instances a modulatory effect has been shown on the process at hand (Wang et al., 2008). An exception is constituted by the molecular chaperone Cdc37 which is phosphorylated by CK2 on Ser14 and Ser17. A *CDC37*^{S14AS17A} strain, lacking these phosphorylation sites, exhibits severe growth and morphological defects, presumably because of the negative effect on the activity of multiple protein kinases, including CK2 itself (Bandhakavi et al., 2003). These results have been interpreted as evidence that Cdc37 is the major CK2 target in yeast (Bandhakavi et al., 2003). Consistently, it has been reported that CK2 failure to phosphorylate Cdc37 could explain the cell cycle arrest of a *cka1Δcka2*^{ts} strain (Hanna et al., 1995).

Cdc34 has also long been recognized as a CK2 substrate (Pyerin et al., 2005). More recent findings identified at least three CK2-mediated phosphorylation sites within its C-terminal domain (Ser207, Ser216 and Ser282) (Barz et al., 2006; Sadowski et al., 2007). The major observed phenotypic defect was connected with methionine biosynthesis and was identified in a strain expressing a Cdc34^{S282A} mutant (Barz et al., 2006), despite the fact that Ser282 (as well as Ser216) are located within a region that is dispensable for *in vivo* Cdc34 activity (Ptak et al., 1994). Physiological function of Ser207 phosphorylation has not yet been determined (Barz et al., 2006). Other results reported by Sadowski et al. indicate that Cdc34 mutants, carrying substitutions at positions Ser207 and Ser216, do not affect

growth rate but show only a moderate increase in the rate of Sic1 degradation (Sadowski et al., 2007).

Here we report the first evidence that CK2 phosphorylates Cdc34 on Ser130 and Ser167, *i.e.* within the catalytic domain, both *in vitro* and *in vivo*. Our studies also show that CK2-mediated phosphorylation at positions 130 and 167 is required to stimulate its ubiquitin charging activity *in vitro* above a basal level that is the same for both wild type and Cdc34^{S130AS167A} proteins. Interestingly, expression to a level comparable to endogenous *CDC34* lead to a pure G1 arrest, while overexpression of *CDC34*^{S130AS167A} determines a dual cell cycle arrest (Fig. 6D); thus defects brought about by a Cdc34 protein with unaltered basal activity, but faulty activation by CK2, can be partially overcome by increased expression.

The cell-cycle arrest of the *cdc34-2*^{ts} strain expressing *CDC34*^{S130AS167A} is accompanied by an increase of the level of Sic1 at restrictive temperature and can be bypassed by deletion of the *SIC1* gene, consistently with the notion that Sic1 is the major Cdc34 substrate in the G1/S transition. A G1 arrest coupled to Sic1 up-regulation is also observed in a *cka1Δcka2-8*^{ts} strain shifted to 37°C (Tripodi et al., 2007). Also in this case, *SIC1* deletion suppresses the G1 arrest (Tripodi et al., 2007).

Experimental data suggest that CK2 failure to phosphorylate Cdc37 (Bandhakavi et al., 2003) or Cdc34 (this paper) could explain the cell cycle arrest of a *cka1Δcka2*^{ts} strain. They indicate that neither Cdc37 nor Cdc34 is the only relevant CK2 substrate in yeast. As known, the distribution of control over different steps in a pathway (or nodes in a network) is a general property of biological networks, first discovered in metabolic analysis and then reported in signal transduction pathways (Thomas et al., 1998) and cell cycle control as well (Russo

et al., 2000; Barberis et al., 2007). Thus, Cdc37 and Cdc34 emerge as crucial CK2 substrates because of their determinant role in cell cycle progression: Cdc37 maintains the activity of several important protein kinases and Cdc34 catalyses the major step required for the correct timing for the G1/S transition.

As summarized in Fig. 7 (that highlights novel results obtained in this paper and puts them into relation with available literature data) yeast CK2 appears to play a relevant role in regulating the G1/S transition by conveying information to a large number of components of the network.

Basal ubiquitin ligation of Cdc34 by E1 (not shown) is inefficient (dotted line), becoming stronger (solid line) upon Ser130 and Ser167 phosphorylation by CK2. Then, ubiquitin-bound Cdc34 polyubiquitinates Sic1 (phosphorylated by Cln1-2/Cdc28, not shown) leading to its degradation and activation of the Clb5-6/Cdc28 complex. In the absence of CK2 phosphorylation of Cdc34, as a result of either CK2 inactivation (Hanna et al., 1994; Tripodi et al., 2008) or of the presence of the *CDC34*^{S130AS167A} allele (this paper), cells arrest in G1, largely because of inability to destroy Sic1. Consistently, the G1 arrest due to the above described mutation or by inactivation of Cdc34 (Schwob et al., 1994) can be bypassed by depletion of Sic1 that is itself a physiologically relevant CK2 substrate (Coccetti et al., 2004; Coccetti et al., 2006), as are the cyclin dependent kinase Cdc28 (Russo et al., 2000) and the chaperone Cdc37, whose phosphorylation by CK2 positively regulates Cdc28 (Bandhakavi et al., 2003). Thus, by phosphorylating multiple substrates within the same complex, CK2 can effectively integrate signals required to modulate execution of the G1/S transition.

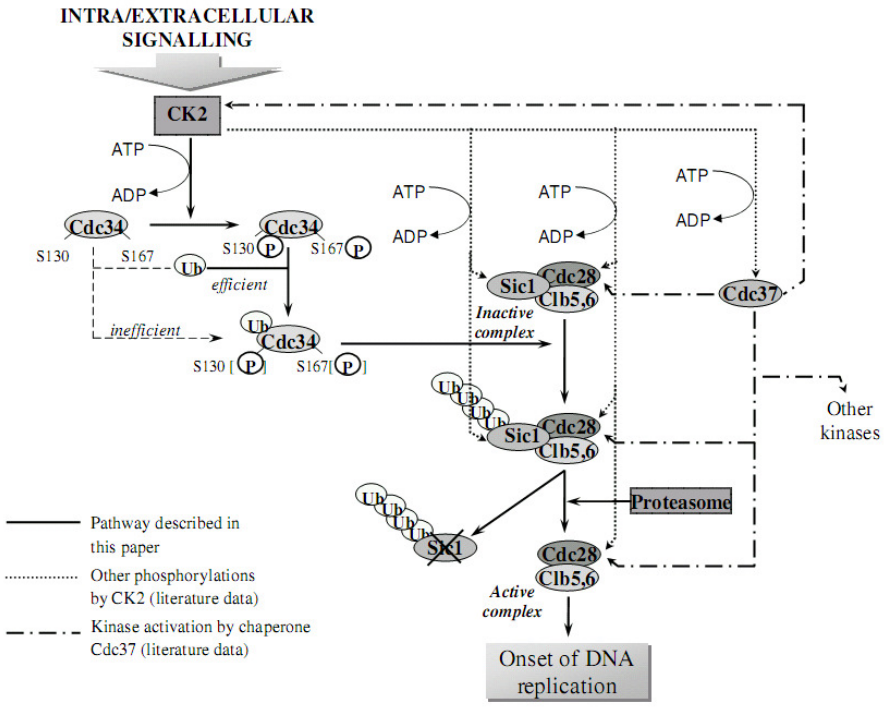


Fig. 7 A model of the regulatory role of CK2 phosphorylation in the G1/S transition. See text for details.

4.4 Materials and Methods

Yeast strains and growth conditions. *S. cerevisiae* strains used in this study are listed in Table 1.

Table 1 **Yeast strains used in this work**

Strain	Genotype	Origin
cdc34-2^{ts}	*Mata, cdc34-2, ade2-11, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi, [pCM189]*	This work
CDC34	*Mata, cdc34-2, ade2-11, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi, [pCM189-CDC34]*	This work
CDC34^{S130A}	*Mata, cdc34-2, ade2-11, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi, [pCM189-CDC34^{S130A}]*	This work
CDC34^{S167A}	*Mata, cdc34-2, ade2-11, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi, [pCM189-CDC34^{S167A}]*	This work
CDC34^{S130AS167A}	*Mata, cdc34-2, ade2-11, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi, [pCM189-CDC34^{S130AS167A}]*	This work
CDC34$_{1-209}$	*Mata, cdc34-2, ade2-11, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi, [pCM189-CDC34$_{1-209}$]*	This work
cdc34-2^{ts} sic1Δ	*Mata, cdc34-2, ade2-11, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi, [pCM189], sic1::HIS3*	This work
CDC34^{S130AS167A} sic1Δ	*Mata, cdc34-2, ade2-11, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi, [pCM189-CDC34^{S130AS167A}], sic1::HIS3*	This work
cka1Δcka2-8^{ts}	*MATa, cka1-Δ1::HIS3, cka2-Δ1::TRP1, [cka2-8]*	Hanna *et al*., 1995
cka1Δcka2-8^{ts} CDC34	*MATa, cka1-Δ1::HIS3, cka2-Δ1::TRP1, [cka2-8], [pCM189-CDC34]*	This work

Synthetic media contained 2% glucose, 6.7 g/L YNB (Difco) and 50 mg/L of required nutrient. Cell density of liquid cultures grown at 25°C or at 37°C was determined with a Coulter counter on mildly sonicated and diluted samples.

FACS analysis. Flow cytometric DNA analysis was performed as previously described on a BD Biosciences FACScan (Tripodi et al., 2007).

Recombinant DNA techniques and genetic manipulations. DNA manipulations and yeast transformations were carried out according to standard techniques. *E. coli DH5α* and *BL21(DE3)[pLysE]* were used in cloning experiments and for expression of recombinant proteins, respectively. *CDC34* was cloned in pIVEX2.4a plasmid and mutant *CDC34* genes were obtained by site-directed mutagenesis, using customly designed primers. Then, the *NotI-PstI* fragments containing *CDC34* and *CDC34* mutant genes were cloned into the *MCS* of the yeast centromeric vector pCM189, under the control of *tetO-CYC1* promoter (Garì et al., 1997). *SIC1* gene was disrupted as reported (Schwob et al., 1994).

Generation and purification of His_6-tag fusion proteins of Cdc34. Recombinant proteins used in this study were purified as previously reported (Coccetti et al., 2004). Then, the proteins were loaded on a DEAE Sepharose™ and eluted at 250-350 mM NaCl using a linear NaCl gradient (50-450 mM) in 20 mM Na_2HPO_4 pH 6. The purest fractions were dialysed against 50 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM $MgCl_2$, 10% glycerol and stored at -80°C.

Protein extraction, immunoblotting and immunoprecipitation. Total protein extraction and immunoprecipitation were performed as previously described (Coccetti et al., 2006). Cdc34 and $Cdc34_{1-209}$ were immunopurified from 10 mg of total protein extract using anti-Cdc34 antibody and resolved by SDS-PAGE followed by silver staining for MS analysis *in vivo*, according to manufacturer's instruction (GE Healthcare). Western blot analysis was performed using anti-Cdc34 polyclonal antibody, anti-Sic1 polyclonal antibody

(produced in our laboratory) and anti-Pgk monoclonal antibody (Molecular Probes) (1:1000 dilution for each antibody).

In vitro phosphorylation and thiolester assay. *In vitro* phosphorylation of recombinant proteins (8 µg of each purified protein) was performed as previously described (Coccetti et al., 2004), using 10 U of purified human CK2 (BIOMOL) in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 100 µM ATP for 30 min at 30°C. CK2 phosphorylation was controlled using [γ-^{32}P]ATP, as reported (Coccetti et al., 2004). Both phosphorylated and not phosphorylated His_6-Cdc34 and His_6-Cdc34^{S130AS167A} were used in a thiolester assay using biotinylated-ubiquitin and then analyzed by non-reducing SDS-PAGE and blotting with Streptavidin-HRP (Block et al., 2005).

MALDI-TOF analysis, sample preparation and mass spectrometry techniques. To reveal phosphorylated peptides His_6-Cdc34, His_6-Cdc34^{S130A}, His_6-Cdc34^{S167A} and His_6-Cdc34$_{1-209}$ were phosphorylated *in vitro* by CK2 (as previously reported) and subjected to reduction and alkylation in solution by iodoacetamide. After dilution, the sample was incubated with either trypsin, Asp-N or Glu-C endoproteinases (1:25 enzyme/protein, w/w) overnight at 37°C. In gel digestion and peptide extraction were performed as previously reported (Coccetti et al., 2006). Mass spectrometry analysis was carried out on a Bruker Daltonics Reflex IV instrument (Bruker Daltonics, Milano, Italy) equipped with a nitrogen laser and with a matrix of α-ciano-4-hydroxy-cinnamic acid. Each peptide mixture was loaded on an AnchorChip plate (Bruker Daltonics) and Bruker peptide

calibration standards were used for calibration. Each spectrum was accumulated for at least 200 laser shots.

nanoLC Mass Spectrometry analysis. The digested peptide mixtures by Glu-C were analysed by LC-MS/MS analysis using a Q-Star Elite coupled to the nanoHPLC system Tempo (Applied Biosystems). The mixtures were loaded on an Agilent reverse-phase pre-column cartridge at 10 µl/min (A solvent 0.1% formic acid, loading time 5 min). Peptides were separated on the Agilent reverse-phase column, at a flow rate of 0.25 µl/min with a 0% to 65% linear gradient in 60 min (A solvent 0.1% formic acid, 2% ACN in MQ water; B solvent 0.1% formic acid, 2% MQ water in ACN). Nanospray source was used at 1.8 kV with liquid coupling, with a declustering potential of 60 V, using an uncoated silica tip from NewObjectives (O.D. 360 µm, I.D. 50 µm, T.D. 8 µm). Data were acquired in information-dependent acquisition (IDA) mode, in which a full scan mass spectrum was followed by MS/MS of the 3 most abundant ions (2 s each). In particular, spectra acquisition of MS/MS analysis was based on a survey MS scan (TOF MS) from 400 m/z to 2000 m/z at scan time range of 0.2-1.5 s. This scan mode was followed by MS/MS spectra (product ion) of three most intense ions that were acquired, using the best collision energy calculated on the bases of m/z values and charge state, from 100 m/z to 1400 m/z at scan time range of 0.2-1.5 s. Data were acquired and processed using Analyst software (Applied Biosystems).

5 CK2 activity is modulated by growth rate in budding yeast

Protein kinase CK2 activity and proliferation rate were often suggested to be correlated in mammalian cells (Prowald et al., 1984; Munstermann et al., 1990; Guerra and Issinger, 1999; Tawfic et al. 2001); however, a clear demonstration of this assumption has never been provided, due to difficulties in modulating growth rate in mammalian cell cultures.

Besides, nutritional conditions strongly modulate growth rate and cell cycle in yeast (Tapon et al., 2001; Rupes, 2002; Rossi et al., 2005). Thus, it was of great interest to analyze the correlation between CK2 and growth rate, both major regulators of the yeast cell cycle.

Here we used budding yeast as a model to investigate the correlation between CK2 activity and growth rate, which can be modulated by changing nutritional conditions. We found that although levels and localizations of either catalytic and regulatory subunits are not affected by carbon sources, CK2 activity is significantly higher in glucose growing cells than in ethanol growing ones. Remarkably, using chemostat cultures we show that the different activity is mainly correlated to growth rate since CK2 activity is higher in populations growing with high dilution rate. These findings reveal a previously unrecognized link between proliferation rate and CK2 activity in budding yeast.

These data are going to be submitted.

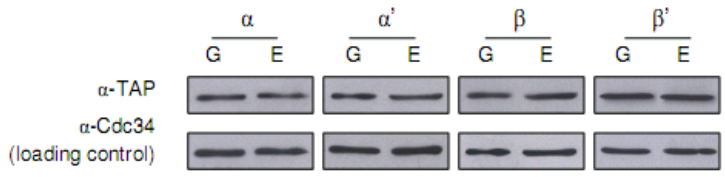
5.1 Results

CK2 levels and localization are not modulated by carbon source

To investigate whether CK2 was modulated by growth conditions in yeast cells, we started analyzing levels and localizations of the catalytic and regulatory subunits in cells growing in different nutritional conditions. Strains expressing TAP-tagged CK2 subunits (α, α', β, β') were grown in glucose or ethanol containing medium, harvested in mid-exponential phase, and levels and localizations of the four CK2 subunits were tested by western analysis. The levels of all subunits were not altered in ethanol growing cells in comparison with those of glucose growing ones and catalytic and regulatory subunits were detectable in the nuclear fractions in both conditions (Fig. 1A-B). Our results then indicated that CK2 is a nuclear enzyme, in agreement with data from Huh and coworkers, showing that the four GFP-fused CK2 subunits are mostly localized in the nucleus of glucose growing cells (Huh et al., 2003); in addition, they also indicate that CK2 nuclear localization is unaffected by carbon source.

uh et al., 2003) ddition, they also indicate that CK2 nuclear localization is unaffected by carbon source.

(A)



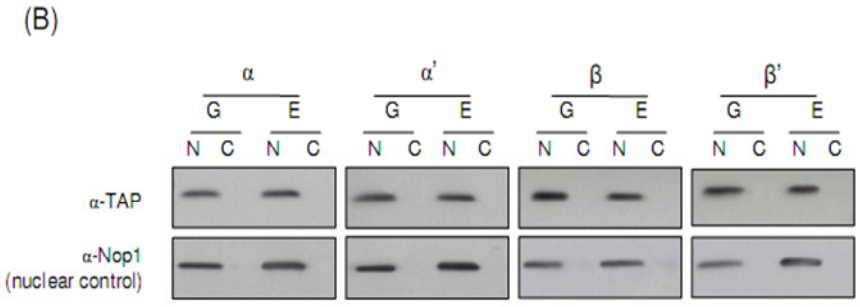


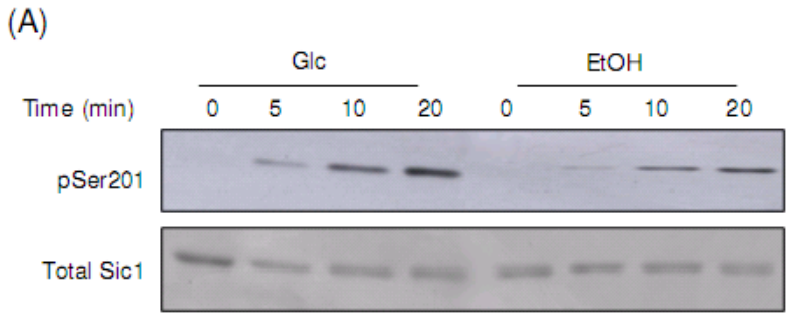
Fig. 1 CK2 Levels and localization are not modulated by carbon source. Strains expressing TAP-tagged CK2 subunits (α, α', β, β') were grown in medium containing glucose or ethanol as carbon source. Samples were taken in mid exponential phase to compare (A) the level of CK2 subunits and (B) their subcellular localization by western analysis using anti-TAP antibody. (anti-Cdc34 antibody and anti-Nop1 antibody were used as loading control and nuclear marker respectively). One of three independent experiments is reported.

CK2 activity is modulated by carbon source

Since previously reported data showed that CK2 activity can be affected without any significant alteration of the level of its subunits (Sommercorn et al., 1987; Homma et al., 2005), we analysed CK2 activity in lysates of cells growing on glucose and ethanol. To this purpose, we developed a new *in vitro* kinase assay using His_6-Sic1 purified form *E. coli* as a CK2 substrate and anti-pSer201-Sic1 antibody to detect phosphorylation, since we previously reported that Sic1, the cyclin-dependent kinase inhibitor, is phosphorylated on Ser201 by CK2 (Coccetti et al., 2004; Coccetti et al., 2006). His_6-Sic1 was more phosphorylated on Ser 201 using cells growing in glucose as the CK2 source (Fig. 2A).

These experiments were complemented with determinations of CK2 activity directly on the cellular lysates using the peptide substrate (RRRADDSDDDDD) which is specific for the CK2 kinase (Pinna et al., 1984). Remarkably, CK2 activity was found higher in glucose growing cells than in ethanol growing ones (Fig. 2B) and using the selective CK2 inhibitor TBB we also confirmed the specificity of the measured activity, as previously reported (Sarno et al., 2001).

Taken together these data suggest that CK2 activity is higher in cell populations growing on glucose with a high proliferation rate not only on the synthetic peptide but also on the physiological target His_6-Sic1 and that the anti-pSer201-Sic1 antibody can effectively be used for investigating CK2 activity.



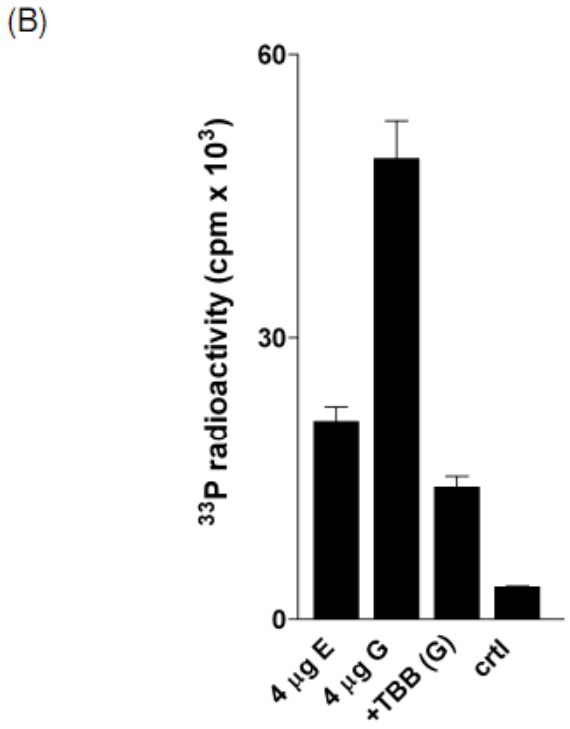


Fig. 2 CK2 activity is modulated by carbon source. (A) CK2 activity in protein extracts from cells exponential growing in glucose or ethanol containing media were assayed towards the recombinant protein His_6-Sic1. Samples were taken at the indicated time points during the *in vitro* reaction. (B) 4 µg of total protein extracts from cells growing in glucose or ethanol containing media were used to assay CK2 activity towards the specific peptide RRRADDSDDDDD. Controls in the absence of the peptide and in the presence on 10 µM TBB are shown. Means ± standard deviations are indicated.

CK2 activity is detectable only in nuclear fractions

Literature data (Huh et al., 2003) and our reported results demonstrate that CK2 is a nuclear enzyme (Fig. 1B). In order to avoid interferences due to the presence of cytoplasmic components, nuclear fractions were isolated from cells growing on glucose and ethanol media and CK2 activity was then tested on His_6-Sic1 and on the synthetic peptide RRRADDSDDDDD. In keeping with results obtained using total protein extracts (Fig. 1A-B), a significant enhancement of Sic1 phosphorylation on Ser201 and of the synthetic peptide was observed using nuclear extract from glucose growing cells (Fig. 3A-B). Moreover, CK2 activity was undetectable in cytoplasmic extracts (data not shown), consistently with the nuclear localization of CK2.

Similar results were obtained by measuring the activity from yeast strains expressing only one single catalytic subunit. Cells bearing a deletion in one of the two genes encoding for the two catalytic subunits (*cka1Δ* or *cka2Δ*) were grown in glucose or ethanol supplemented media, and nuclear CK2 activity was tested using His_6-Sic1 as a substrate. Both strains exhibited a higher CK2 activity in glucose than in ethanol growing cells, indicating that both subunits are modulated by growth rate (Fig. 3C). Notably, the activity of α subunit was significantly higher than the activity of α' subunit; however, despite being lower, α' activity can be crucial for the phosphorylation of its targets, especially considering the specialization of the two catalytic subunits.

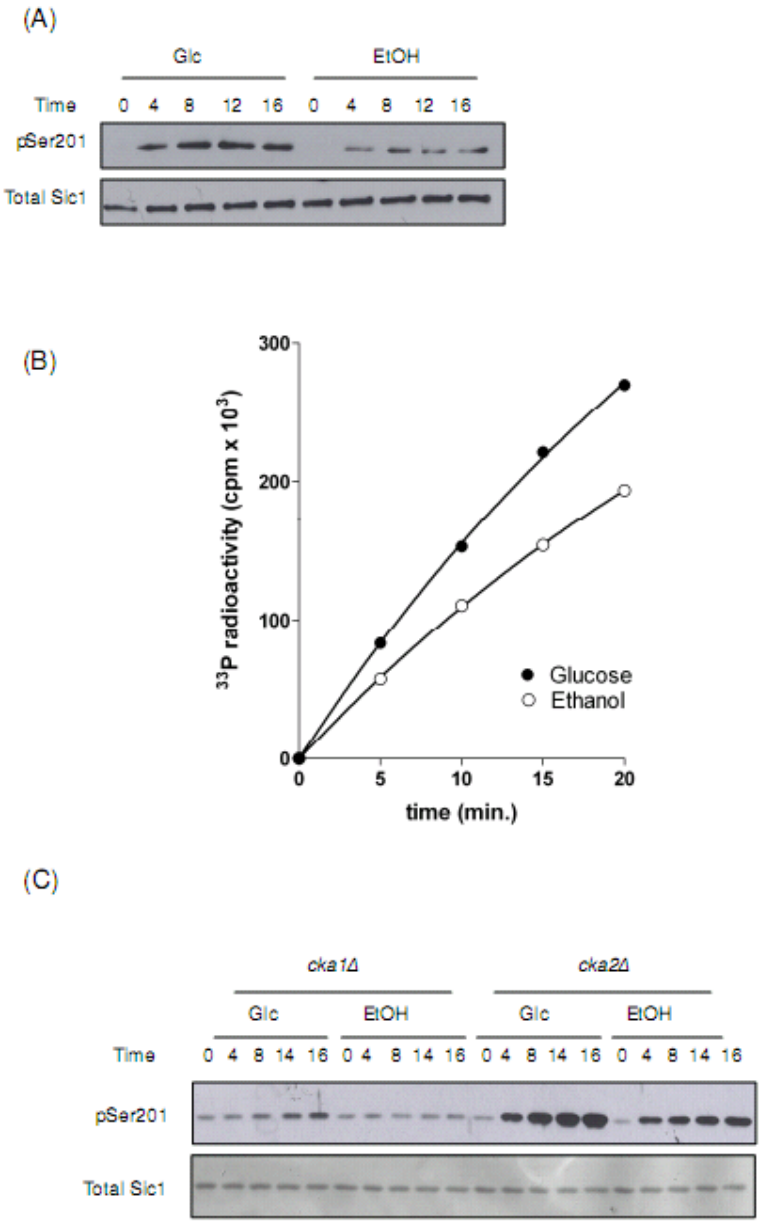


Fig. 3 CK2 activity is detectable only in nuclear fractions (A) CK2 activity in nuclear extracts from cells growing in glucose or ethanol containing media were assayed towards the recombinant protein His_6-Sic1. Samples were

taken at the indicated time points during the *in vitro* reaction. (B) 1.5 µg of nuclear protein extracts from cells growing in glucose or ethanol containing media were used to assay CK2 activity towards the specific peptide RRRADDSDDDDD. Samples were taken at the indicated time points during the *in vitro* reaction. (C) *cka1Δ* or *cka2Δ* strains were grown in glucose or ethanol containing media, and CK2 nuclear activity was assayed towards the recombinant protein His_6-Sic1 during a time course (0, 4, 8, 12, 16 min).

CK2 activity is modulated by growth rate

Our results suggested that CK2 activity is higher in cells with a higher growth rate (*i.e.* glucose growing cells, Td=1.5 h) than in cells with a lower one (*i.e.* ethanol growing cells, Td=2.75 h), in accordance with preoviuos indication of correlation between CK2 activity and proliferation rate obtained in mammalian (Prowald et al., 1984; Munstermann et al., 1990). Nevertheless, our experimental setup cannot exclude any contribution of the different metabolisms on CK2 activity. Cells growing on glucose are in fact characterised by a high glycolysis and a respiro-fermentative metabolism, while growth on ethanol requires the activation of gluconeogenetic and glyoxylate pathways, and is a full respiratory process (Gancedo and Serrano, 1989).

In order to discriminate the contribution of the carbon source and of the growth rate to CK2 activity, we used the chemostat technique. It is well known that by varying the dilution rate (i.e. the flow feed rate) in a continuous culture, different specific growth rates can be obtained (Herbert, 1959). In a steady state condition the dilution rate equals to the growth rate. To enucleate the contribution of growth rate CEN.PK113.7D, a strain suitable for chemostat growth, was grown in glucose limited chemostat cultures at D=0.23 h^{-1} and D=0.1 h^{-1}. In these cultural conditions the metabolism is fully respiratory, as

indicated by a high biomass yield and by the absence of by-products (Fig. 4A), since for CEN.PK113.7D strain respiro-fermentative metabolism sets in above a critical dilution rate of 0.28 h^{-1} (Porro et al., 2003). The contribution of the carbon source has been evaluated with an ethanol-limited chemostat culture at D=0.1 h^{-1} (Fig. 4A). Nuclear extracts from the three chemostat cultures were assayed for CK2 activity using His_6-Sic1 as a substrate (Fig. 4B). This assay clearly showed that cells population with higher growth rate (D=0.23 h^{-1}) exhibited a higher CK2 activity than cells with lower one (D=0.1 h^{-1}) (Fig. 4B). A slight reduction of CK2 activity was also observed between cells grown with the same dilution rate (D=0.1 h^{-1}) in glucose and ethanol, suggesting that carbon metabolism could influence, although to a lesser extent, CK2 activity using His_6-Sic1 as a substrate (Fig. 4B).

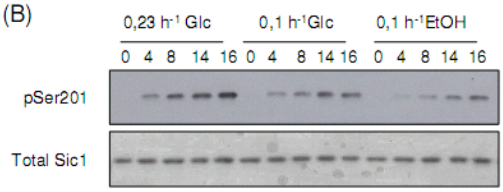
The effect of growth rate on CK2 activity has been further characterize by the determination of CK2 kinetic parameters (i.e. Km_{app} and Vmax) on nuclear extracts from the three steady-state chemostat cultures. Varying the concentrations of the specific peptide, we built the Michaelis-Menten plot (upper panel) and the Lineweaver–Burk plot (lower panel) for CK2 in all the conditions tested (Fig. 4C). Cells with the higher dilution rate (D=0.23 h^{-1}) exhibited a significantly higher CK2 activity toward the synthetic peptide RRRADDSDDDDD than cells grown with the lower dilution rate (D=0.1 h^{-1}). No significant differences were observed for chemostat cultures grown in glucose and ethanol with the same dilution rates (D=0.1 h^{-1}) (Fig. 4C). In particular, Km_{app} showed a similar value in all the conditions tested ranging from 79 to 83 µM; on the contrary Vmax values derived from cultures with the same dilution

rates were similar, while Vmax was about 45% higher in cells with 0.23 h^{-1} dilution rate.

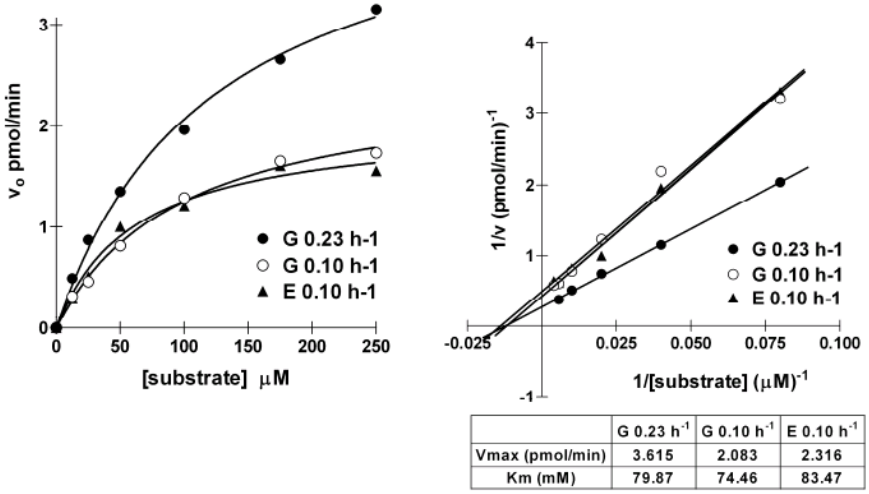
(A)

Dilution Rate D (h^{-1})	Carbon Source
0,23	Glucose
0,1	Glucose
0,1	Ethanol

(B)



(C)



	G 0.23 h^{-1}	G 0.10 h^{-1}	E 0.10 h^{-1}
Vmax (pmol/min)	3.615	2.083	2.316
Km (mM)	79.87	74.46	83.47

Fig. 4 CK2 activity is modulated by growth rate. (A) Growth parameters of *S. cerevisiae* CEN.PK113-7D strain in chemostat cultures. Data are the average of at least two experiments. Values for biomass Yield ($Y_{S/X}$, gram of

cells per gram of carbon source) measured are very close to the theoretic ones (0.51 and 0.60 for glucose and ethanol, respectively) and are consistent with a fully respiratory metabolism. Carbon recovery is the percentage of the fed carbon found in the products assayed. (B) CK2 activity of the cells in (A) was assayed using His_6-Sic1 or (C) the specific peptide RRRADDSDDDDD as substrate. (C) 1.5 µg of nuclear protein extracts from cells in (A) were used to estimate Michaelis-Menten kinetic parameters towards the specific peptide RRRADDSDDDDD. CK2 activity was assayed with varying concentrations of peptide. Michaelis-Menten plot (left panel) and Lineweaver–Burk plot (right panel) are reported. One of two independent experiments is presented. Kinetic results obtained in this study are listed.

5.2 Conclusions

Using budding yeast as a model organism, we gave the first demonstration of a correlation between CK2 activity and growth rate, an assumption that was often suggested in the past, but was never unequivocally demonstrated in budding yeast. In fact, we showed that CK2 activity is higher in cells with higher growth rate, while carbon metabolism seems to exert a lesser influence, and only towards certain substrates.

In conclusion, this data provide a clear evidence of an *in vivo* modulation of CK2 activity in budding yeast and suggest a previously uncharacterized role for CK2 in nutrient response.

5.3 Materials and Methods

Yeast strains and growth conditions. *S. cerevisiae* strains used in this study are listed in Table 1.

Yeast strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open BioSystem
CKA1-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CKA1-TAP*	Open BioSystem
CKA2-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CKA2-TAP*	Open BioSystem
CKB1-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CKB1-TAP*	Open BioSystem
CKB2-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CKB2-TAP*	Open BioSystem
CKA1Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cka1::kanMX4*	Euroscarf
CKA2Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cka2::kanMX4*	Euroscarf
CEN.PK113-7D	*MATa MAL2-8^{c} SUC2*	L. Brambilla collection

Batch culture were grown in synthetic complete media, prepared by assembling 2% carbon source (glucose or ethanol), 6.7 g/L yeast nitrogen base and complete supplemented mixture (CSM, MP Biomedials).

Protein extraction and western blotting. Crude protein extracts were obtained by standard glass beads method using lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% NP-40, 10% glycerol) plus 1 mM PMSF, protease inhibitor mix (Complete EDTA free protease inhibitor cocktail tablets, Roche) and phosphatase inhibitor mix (Sigma). Nuclear and cytoplasmic extracts were obtained using NE-PER Extraction kit (Pierce Biotechnology) on spheroplasts, as

reported in Rossi et al., 2005. Western blot analysis was performed using anti-TAP monoclonal antibody (1:2500 dilution, Open Biosystems), anti-Nop1 antiboby as nuclear control (1:5000 dilution, EnCor Biotechnology) and anti-Cdc34 policlonal antibody as loading control (1:1000 dilution).

CK2 activity towards a peptide substrate. Crude protein extracts from yeast cells were obtained as reported above, using lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% NP-40, 10% glycerol) plus 1 mM DTT, protease inhibitor mix (Complete EDTA free protease inhibitor cocktail tablets, Roche) and phosphatase inhibitor mix (Sigma). CK2 activity was tested on 4 µg of crude protein extracts or 1.5 µg of nuclear protein extracts in a medium containing 50 mM Tris/HCl buffer (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl and 100 µM [γ -^{33}P]ATP (specific radioactivity 1,000-2,000 cpm/pmol) in the presence of the specific peptide substrate RRRADDSDDDDD (Marin et al., 1994). TBB (4,5,6,7- tetrabromobenzotriazole) (10 µM) was used as selective CK2 inhibitor in control reactions. Assays were carried out in a 30 µl volume at 30°C and stopped by spotting on to phosphocellulose filters and cooling in ice. Filters were washed in 75 mM phosphoric acid four times and dried before counting. Initial rate data were fitted to the Michaelis-Menten equation and Vmax and Km_{app} values was determined from Lineweaver–Burk plots.

CK2 activity toward recombinant His_6-Sic1. His_6-Sic1 was expressed and purified from *E. coli* as previously reported (Coccetti et al., 2004). CK2 activity in crude protein extracts (0.75 µg) towards the recombinant His_6-Sic1 (6 µg) was tested in a reaction mix containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP at

30°C. CK2 phosphorylation was analyzed by SDS-PAGE and blotting with anti-Sic1-pSer201 antibody (dilution 1:2000), and with anti-His probe antibody (dilution 1:1000) as control.

Chemostat cultivation. Chemostat cultivations were performed as reported (Porro et al., 2003). For all the experiments glucose concentration in the reservoir was 5 g l^{-1}, while ethanol concentration was 2.5 g l^{-1}. Steady state was achieved after at least six volume changes had passed through and no oscillations had occurred. Each experiment has been run at least in double with a carbon recovery >95%. In each condition cells and carbon dioxide were the only products.

Discussion

Protein kinase CK2 plays an important role in cell-cycle regulation, both in yeast (Hanna et al., 1995) and in mammalian cells (Pepperkok et al., 1994; Homma and Homma, 2005; Homma et al., 2005), and several cell-cycle proteins have been reported to be CK2 substrates (Meggio and Pinna, 2003). In particular, literature data showed that *cka1Δcka2^{ts}* mutants arrest cell cycle at 37°C, showing a double block: 50% of the population arrests as unbudded G1 cells with pre-replicative DNA content, while 50% of the population attests in M phase, with post-replicative DNA content and spindles typical of metaphasic and anaphasic cells (Hanna et al., 1995). Despite this interesting discovery, no detailed analysis had been carried out to define the molecular mechanism underling this double arrest. During my PhD, we investigated the cause of the G1 arrest observed in half of the population after CK2 inactivation. Results reported in Tripodi et al., 2007 indicated a major involvement of the inhibitor Sic1 in this G1 arrest. In fact, we showed that Sic1 levels strongly increase upon CK2 inactivation, and this accumulated Sic1 inhibits the Clb5-Cdk1 complex, effectively blocking the G1/S transition. Besides, we observed that *SIC1* deletion does not rescue the overall cell growth defect of a *cka1Δcka2^{ts}* strain, but almost completely abolishes the G1 block, thus leading to a unique arrest in mitosis.

Thus, we identified Sic1 as the molecular reason of the G1 arrest observed upon CK2 depletion. Sic1 itself was shown to be a physiological CK2 substrate, which is phophorylated on Ser201 (Coccetti et al., 2004; Coccetti et al., 2006). Yet, Sic1 accumulation cannot be a direct consequence of CK2 depletion in cka1Δcka2ts mutants, since our group showed that Ser201 phosphorylation does not affect Sic1 stability (Coccetti et al., 2004). Instead, we believe that Sic1 accumulation is an indirect consequence of CK2 depletion, due

to the lack of phosphorylation of the ubiquitin-conjugating enzyme Cdc34. Indeed, in Coccetti et al., 2008 we reported that CK2 phosphorylates Cdc34 on Ser130 and Ser167, i.e. within the catalytic domain of the protein, both *in vitro* and *in vivo*. Our studies showed that CK2-mediated phosphorylation is required to stimulate Cdc34 ubiquitin charging activity *in vitro*. We also observed that overexpression of the partially active protein $Cdc34^{S130AS167A}$ in a $cdc34\text{-}2^{ts}$ background determines a dual cell-cycle arrest, but its expression to a level comparable to endogenous Cdc34 leads only to a G1 arrest. Interestingly, the cell-cycle arrest of a $CDC34^{S130AS167A}$ strain is accompanied by an increase of Sic1 level at restrictive temperature and can be bypassed by *SIC1* deletion, as in $cka1\Delta cka2^{ts}$ mutant cells.

Therefore, data published during this thesis (Tripodi et al., 2007; Coccetti et al., 2008) suggest that upon CK2 depletion, Cdc34 phosphorylation on Ser130 and Ser167 is reduced, thus impairing the ubiquitin-charging activity of Cdc34. In this condition, Cdc34-SCF complex is unable to poly-ubiquitinate its substrates, among which Sic1, that accumulates and inhibits Cdk1-Clb5 complex, ultimately leading to the observed block in G1 phase (Hanna et al., 1995). Taken together, these data indicate that CK2 is a major regulator of the G1/S transition in yeast, which can effectively integrate signals required to modulate Start execution, by phosphorylating multiple substrates involved in S phase entrance (Cdc28, Sic1, Cdc34, Cdc37).

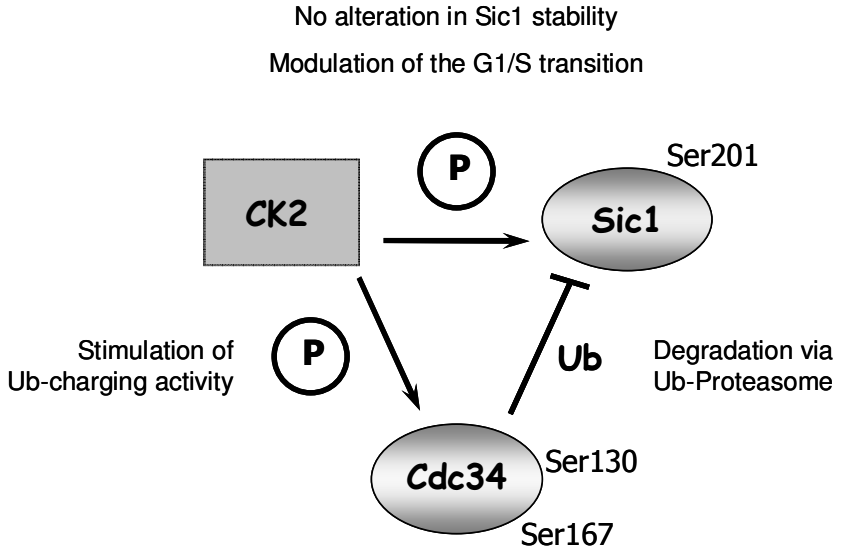


Fig. 1 CK2-mediated phosphorylation of Sic1 and Cdc34 modulates G1/S transition in yeast.

Nutritional conditions highly regulate yeast cell cycle, especially at the G1/S transition. In fact, cells growing in glucose containing medium show a faster growth rate and a bigger cell size than cells growing in ethanol containing medium (Tapon et al., 2001; Alberghina et al., 2004). Notably, many key cell-cycle regulators are modulated by nutritional conditions: Cln3, Far1, Clb5 and Sic1 levels are higher in glucose than in ethanol growing cells; moreover Sic1 subcellular localization is influenced by growth conditions (Alberghina et al., 2004; Rossi et al., 2005).

Since CK2 is a major regulator of the cell cycle, and since nutritional conditions are strongly involved in cell cycle regulation , we decided to investigate whether there is a correlation between CK2 and growth conditions in yeast.

Literature data from studies in mammalian cells suggested a correlation between CK2 activity and proliferation rate, since cells

with higher proliferation rate exhibit higher CK2 activity (Munstermann et al., 1990). However this correlation has never been directly proved, and we think that yeast could be used as a model system also to this purpose. In fact growth rate in *Saccharomyces cerevisiae* can be easily modulated changing nutritional conditions, both in batch and using chemostat technology. In this way, we presented evidences that CK2 activity is strongly connected to growth rate also in yeast cells. We found that CK2 levels and localization do not vary in glucose *vs* ethanol growing cells; however, CK2 activity is significantly higher in glucose growing cells than in ethanol growing ones. We could correlate this difference in activity with different growth rates; in fact, through chemostat cultures, for the first time we showed that CK2 activity was higher in yeast cells with higher growth rate. During these studies, we also developed a new *in vitro* assay to highlight qualitative differences in CK2 activity from yeast protein extracts. The bigger advantage of this system is to use a physiological CK2 substrate (Sic1) instead of a synthetic peptide (that is commonly used to estimate CK2 activity); moreover, it avoids the use of radioactive phosphate, preserving researchers and the environment.

Thus, we provided the first evidence of a regulation of CK2 activity in yeast, and the first clear demonstration of a correlation between CK2 activity and growth rate. This modulation of CK2 activity by nutritional condition suggests that nutrients could exert their influence on cell cycle, at least in part, through CK2.

Therefore, it would be of great interest to analyse CK2-mediated phosphorylations in different growing conditions. We are currently starting a comparative study of CK2-mediated phosphorylations on Sic1 and Cdc34 in glucose *vs* ethanol growing cells. In keeping with

data presented in this thesis, since CK2 is less active in ethanol than in glucose growing cells, Cdc34 should be less phosphorylated on CK2-specific sites, thus accounting, at least in part, for the higher stability of Sic1 in ethanol. Yet, we should remember that subcellular compartimentalization has a strong influence on phosphorylation events. Thus, further analysis will be necessary to elucidate the role of protein kinase CK2 in nutritional modulation of the yeast cell cycle.

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