Sic1 as a timer of Clb cyclin waves in the yeast cell cycle – design principle of not just an inhibitor

Matteo Barberis¹,²

¹ Institute for Biology, Theoretical Biophysics, Humboldt University Berlin, Germany
² Max Planck Institute for Molecular Genetics, Berlin, Germany

Keywords
budding yeast; cell cycle; Clb cyclins; Clb module; cyclin waves; design principle; homology modelling; oscillations; Sic1; systems biology

Correspondence
M. Barberis, Institute for Biology, Theoretical Biophysics, Humboldt University Berlin (HUB), Invalidenstraße 42, 10115 Berlin and Max Planck Institute for Molecular Genetics (MPIMG), Ihnestraße 63-73, 14195 Berlin, Germany
Fax: +49 3020 938813
Tel: + 49 3020 938383
E-mail: matteo.barberis@biologie.hu-berlin.de (HUB), barberis@molgen.mpg.de (MPIMG)

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Design principles of molecular networks

The physiological properties of cellular organisms are carried out by functional modules formed by complex sets of interacting molecules [1]. Biological systems are governed by general design principles (i.e. the characteristic network topology and regulatory interactions between molecules that are responsible for a certain physiological function) [2]. In this context, comprehension of an interacting network of genes, proteins and metabolites that are responsible for normal and perturbed cellular functions is crucial for rationalizing design principles. The discipline that tackles this challenge is called cellular systems biology [3,4], which aims at a mechanistic understanding of cellular functions through the dynamic interactions of constituent molecules in space and time [5].

Abbreviations
APC, anaphase promoting complex; Cdk, cyclin-dependent kinase; CK2, casein kinase 2; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonance energy transfer; KID, kinase inhibitory domain; SCF, Skp1/Cdc53/F-box protein.
To investigate the design principles of regulatory networks, it is essential to analyze the dynamic output generated by network motifs that are assumed to repeat over and over again in biological systems. These motifs include, amongst others, autoregulation and positive- and negative-feedback loops, as well as feedforward loops [6–10], which strongly constrain the possible choices of network architecture and network parameters (the specific terminology used through the text is defined in Table 1). The mode of regulation in which network motifs are built to generate a particular physiological behaviour is assembled in functional modules, discrete entities that involve a small number of components accomplishing a certain autonomous function [1]. The understanding of the molecular mechanisms and timing in which each functional module occurs has increasingly attained new levels of comprehension, with systems biology incorporating inter-disciplinary fields such as structural biology, biophysics, biochemistry, theoretical frameworks and single-molecule techniques.

In this review, we describe a systems biology approach that addresses the role of the cyclin-dependent kinase (Cdk) inhibitor Sic1, a crucial regulator of the Cdk1/Clb kinase activities necessary to set the timing of cell cycle progression in budding yeast (the proteins considered with respect to their functions in the budding yeast cell cycle are listed in Table 2). We summarize all of the existing literature addressing Sic1 regulation and show how combined experimental and theoretical approaches reveal new functions of the inhibitor. In detail, we provide evidence of how a property of yeast cells (i.e. temporal oscillations of Cdk1/Clb activities) is driven by a functional module (i.e. the ‘Clb module’), with different Cdk1/Clb complexes regulated in accordance with a specific design principle (i.e. interaction of all Cdk1/Clb complexes with their stoichiometric inhibitor Sic1). Furthermore, we provide indications of the mechanisms that can potentially regulate Sic1 availability to control the precise timing of waves of Clb cyclins during cell cycle progression.

### Table 1. Specific terminology used through the present study.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Allovalency</td>
<td>Kinetic principle based on the interaction between a polyvalent disordered ligand and a single receptor site</td>
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<tr>
<td>Bi-stable system</td>
<td>System that exists in two distinct stable states and can switch from one state to the other in response to a specific external input</td>
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<tr>
<td>Conjugate gradient</td>
<td>Iterative algorithm used for the numerical solution of particular systems of linear equations; used to solve unconstrained optimization problems such as energy minimization</td>
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<tr>
<td>Docking</td>
<td>Method of molecular modelling that predicts the preferred orientation and strength of association (binding affinity) of two molecules when bound to each other in a stable complex</td>
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<tr>
<td>Feedback loop</td>
<td>Mechanism that is looped back to control a system within itself; a positive-feedback increases the event that caused it, whereas a negative-feedback reduces the input signal that caused it</td>
</tr>
<tr>
<td>Feed-forward loop</td>
<td>Three-component pattern composed of two inputs (e.g. transcription factors) where one regulates the other and both jointly regulating a target (e.g. gene); each interaction can be activating or repressing (eight possible structural types)</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>Measure of the extent of cooperativity in ligand binding by an allosteric protein; a Hill coefficient of 1 indicates independent binding, whereas a value &gt; 1 shows positive cooperative binding (one ligand facilitates the binding of subsequent ligands at other sites on the allosteric protein)</td>
</tr>
<tr>
<td>Homology modelling</td>
<td>Procedure to build an atomic-resolution model of a target protein from its amino acid sequence and an experimental 3D structure of a related homologous protein that functions as a template; relies on (a) identification of one or more known protein structures that resemble the structure of the query sequence and (b) the production of an alignment that maps residues in the query sequence to residues in the template sequence</td>
</tr>
<tr>
<td>Kinetic model</td>
<td>Representation of a system with differential equations describing the involved reactions by definite rate laws</td>
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<tr>
<td>Molecular dynamics</td>
<td>Computer simulation method that mimics the motion of atoms and molecules that are allowed to interact for a period of time</td>
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<tr>
<td>Monte Carlo simulation</td>
<td>Algorithm that relies on repeated random sampling to compute the results</td>
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<tr>
<td>Response coefficient</td>
<td>Measure of the dependence of a system component on an external parameter (e.g. concentration of the component, numerical value of a kinetic reaction)</td>
</tr>
<tr>
<td>Stochastic simulation</td>
<td>Procedure where the rates of all possible changes to the state of a model are computed</td>
</tr>
<tr>
<td>Ultrasensitivity</td>
<td>Biochemical switch characterized by a small response to low input signal (stimulus) and a progressively higher response to increased input signal; the output response is more sensitive to stimulus change compared to the hyperbolic Michaelis–Menten response</td>
</tr>
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</table>
Table 2. Proteins considered in the present study and their functions in the budding yeast cell cycle.

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
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<tbody>
<tr>
<td>Ace2</td>
<td>Transcription factor required for septum destruction after cytokinesis; its phosphorylation prevents nuclear exit during the M/G1 transition, promoting localization to daughter cell nuclei, and also prevents nuclear import during cell cycle phases other than cytokinesis</td>
</tr>
<tr>
<td>Ash1</td>
<td>Transcription factor that specifies daughter cell fate in mating-type switching; translated in the distal tip of anaphase cells and accumulates in daughter cell nuclei</td>
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<tr>
<td>Cdc14</td>
<td>Protein phosphatase located in the nucleolus and released upon entry into anaphase to promote mitotic exit; reduces Cdk1/Cib activity</td>
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<tr>
<td>Cdc20</td>
<td>Cell cycle-regulated activator of APC; required for metaphase/anaphase transition; directs ubiquitination of mitotic cyclins</td>
</tr>
<tr>
<td>Cdc34</td>
<td>Ubiquitin-conjugating enzyme (E2) and catalytic subunit of SCF; regulates cell cycle progression by targeting substrates for degradation</td>
</tr>
<tr>
<td>Cdc4</td>
<td>F-box protein that associates with Skp1 and Cdc53 to form SCF; promotes G1/S transition by targeting G1 (Cln) cyclins and Sic1 for degradation</td>
</tr>
<tr>
<td>Cdc53</td>
<td>Cullin that associates with Cdc4 and Cdc53 to form SCF; promotes G1/S transition by targeting G1 (Cln) cyclins and Sic1 for degradation</td>
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<tr>
<td>Cdh1</td>
<td>Cell cycle-regulated activator of APC; promotes mitotic exit by directing the ubiquitination of cyclins</td>
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<tr>
<td>Cdk1</td>
<td>Catalytic subunit of the main cell cycle cyclin-dependent kinase; associates with G1 (Cln) and mitotic (Cib) cyclins</td>
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<tr>
<td>Clb1, Clb2</td>
<td>B-type cyclins that activate Cdk1 to promote G2/M transition</td>
</tr>
<tr>
<td>Clb3, Clb4</td>
<td>B-type cyclins that activate Cdk1 to promote G2/M transition; involved in spindle assembly</td>
</tr>
<tr>
<td>Clb5, Clb6</td>
<td>B-type cyclins that activate Cdk1 to promote DNA replication initiation during the S phase; involved in spindle assembly</td>
</tr>
<tr>
<td>Cin1, Cin2</td>
<td>G1 cyclins that activate Cdk1 to promote G1/S transition</td>
</tr>
<tr>
<td>Cin3</td>
<td>G1 cyclin that activates Cdk1 to promote G1/S transition; regulates the transcription of Cin1 and Cin2</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein kinase 2; serine/threonine kinase with roles in cell growth and proliferation; phosphorylates many substrates including transcription factors and RNA polymerases</td>
</tr>
<tr>
<td>Dbr2</td>
<td>Serine/threonine kinase involved in transcription and stress response; activated during exit from mitosis</td>
</tr>
<tr>
<td>Dcr2</td>
<td>Phosphatase; dosage-dependent positive regulator of G1/S transition</td>
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<tr>
<td>Fkh2</td>
<td>Forkhead transcription factor required for the expression of genes at the G2/M transition; positively regulates transcriptional elongation and negatively regulates chromatin silencing</td>
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<tr>
<td>Gis2</td>
<td>Translational activator for mRNAs with internal ribosome entry sites; associates with polysomes and binds to a specific subset of mRNAs</td>
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<tr>
<td>Hek2</td>
<td>RNA binding protein that represses translation of ASH1 mRNA and regulates its asymmetric localization</td>
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<tr>
<td>Hog1</td>
<td>Mitogen-activated protein kinase involved in osmoregulation; mediates recruitment and activation of RNA polymerase II</td>
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<tr>
<td>Ime2</td>
<td>Serine/threonine kinase involved in the timely destruction of Sic1 during sporulation and in activation of meiosis</td>
</tr>
<tr>
<td>Nab2</td>
<td>Nuclear polyadenylated RNA-binding protein required for nuclear mRNA export and poly(A) tail length control; autoregulates mRNA levels</td>
</tr>
<tr>
<td>Pcl1, Pcl2</td>
<td>Cyclins that interact with the cyclin-dependent kinase Pho85; regulate polarized growth, morphogenesis and cell cycle progression; localize to sites of polarized cell growth</td>
</tr>
<tr>
<td>Pcl9</td>
<td>Cyclin that interacts with the cyclin-dependent kinase Pho85; activated by Swi5</td>
</tr>
<tr>
<td>Pho4</td>
<td>Basic helix-loop-helix transcription factor that activates transcription in response to phosphate limitation; regulated by phosphorylation at multiple sites and by phosphate availability</td>
</tr>
<tr>
<td>Pho80</td>
<td>Cyclin that interacts with the cyclin-dependent kinase Pho85; regulates the cellular response to nutrient levels such as phosphate limitation</td>
</tr>
<tr>
<td>Pho85</td>
<td>Cyclin-dependent kinase that regulates the cellular response to nutrient levels and cell cycle progression</td>
</tr>
<tr>
<td>Rad23</td>
<td>Polyubiquitin-binding protein involved in nucleotide excision repair</td>
</tr>
<tr>
<td>Rpn10</td>
<td>Polyubiquitin-binding proteins; non-ATPase base subunit of the 19S regulatory particle of the 26S proteasome</td>
</tr>
<tr>
<td>Rub1</td>
<td>Ubiquitin-like protein; conjugation substrates include Cdc53 cullin</td>
</tr>
<tr>
<td>Sic1</td>
<td>Inhibitor of Cdk1/Cib complexes that control G1/S and M/G1 transitions; prevents a premature S phase and ensures genomic integrity</td>
</tr>
<tr>
<td>Skp1</td>
<td>Kinetochore protein and subunit of SCF ubiquitin ligase complex (E3 enzyme)</td>
</tr>
<tr>
<td>Swi5</td>
<td>Transcription factor that activates the transcription of genes expressed at the M/G1 transition and in the G1 phase; its nuclear localization in the G1 phase is regulated by Cdk1-mediated phosphorylation</td>
</tr>
<tr>
<td>Yrb1</td>
<td>Ran GTPase binding protein involved in nuclear protein import, RNA export and ubiquitin-mediated protein degradation during cell cycle progression</td>
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Sic1: the key regulator of entrance into the S phase

In the budding yeast *Saccharomyces cerevisiae*, the coordination between cell growth and DNA replication is guaranteed by the fact that: (a) the S phase does not take place until cells reach a critical cell size and (b) multiple mechanisms ensure genome duplication once per cell cycle. This coordination occurs during a narrow interval in the late G1 phase known as Start [11] and it is connected to two crucial events. On the one hand, growth-dependent accumulation of waves of G1 (Cln) and B-type (Clb) cyclins regulates the order and the timing of cell cycle phases by binding to the Cdk1 kinase [12–18]. On the other hand, the presence of Sic1, a potent inhibitor of Cdk1/cyclin complexes containing Clb but not Cln cyclins [19–21], prevents premature DNA replication onset by anchoring to both cyclin and kinase subunits, as reported previously [22,23].

Originally discovered as a high-affinity *in vivo* substrate of Cdk1, Sic1 was identified as a 40-kDa polypeptide (also called p40) co-precipitating with, and a substrate of Cdk1, which binds tightly to Cdk1 inhibiting its kinase activity [19,24]. The *SIC1* gene was subsequently cloned by screening a library of yeast genomic DNAs with a degenerate mixture of oligonucleotide probes capable of encoding a peptide derived from Sic1 [25] and as a high copy suppressor of temperature-sensitive mutants of Dbf2, a kinase involved in mitotic exit [26]. *SIC1* is not an essential gene and its deletion is viable, although *sic1Δ* mutants have an extended S phase, a high frequency of broken and lost chromosomes, and a 575-fold increase in gross chromosomal rearrangements [25,27]. Moreover, chromosome combing showed that the distance between replicons is 1.5 times longer in *sic1Δ* cells compared to wild-type [28]. The deletion strain shows an altered morphology and a significant percentage of large-budded cells arrested at the anaphase/telophase transition, with sister chromatids that are inefficiently separated during anaphase [25,27,29]. Further evidence demonstrated that DNA replication and budding are uncoupled in these cells [30]. In *sic1Δ* mutants, DNA replication initiates prematurely as a result of activation of Cdk1/Clb5,6 kinase complexes in the early G1 phase, which trigger genome duplication by firing DNA replication origins during the S phase [27]. The precocious Cdk1/Clb5,6 activation causes severe genome instability that alters S phase dynamics by inhibiting the formation of the pre-replication complex on replication origins, an event that occurs normally in the late G1 phase [31]. In these cells, some origins never fire and, consequently, DNA replication is not completed in a timely manner, with cells segregating incompletely replicated chromosomes. Cell cycle defects are only rescued by delaying Cdk1/Clb5,6 activation [27]. Interestingly, Sic1 promotes nuclear import of Cdk1/Clb5 as cytoplasmic Clb5 accumulation is observed upon inactivation of the *SIC1* gene, thus revealing its positive role in promoting the G1/S transition to initiate DNA replication [32].

In contrast to this scenario, stabilization of Sic1 arrests the cell cycle between Start and the initiation of DNA replication, resulting in cells with elongated buds [20,25,33]. However, a nondegradable form of Sic1 lengthens the G1 phase by transiently blocking Cdk1/Clb activation and exhibits an extreme sensitivity to Clb dosage, rather than a lethal cell cycle block [34]. This suggests that, ultimately, the total level of Cdk1/Clb could increase above the Sic1 level to drive cell cycle progression. Our recently obtained results support the validity of this interpretation, showing that a stable Sic1 delays the onset of DNA replication as a result of the retarded activation of Clb cyclin waves, therefore delaying cell cycle progression because of the altered timing of Cdk1/Clb activities [35]. Taken together, these data demonstrate that Sic1 promotes origin licensing in the late G1 phase and sets the correct timing of S phase onset by preventing Cdk1/Clb kinase activity and thus genome instability [27,36].

Cell cycle-dependent mechanisms regulating Sic1

Sic1 protein expression is limited to the M/G1 transition [26,37] and has been shown to inhibit kinase activities associated with Cdk1/Clb5, which triggers DNA replication, and Cdk1/Clb2, which triggers mitotic events [20,38]. These studies highlight the fact that Sic1 strongly binds to Cdk1 in correspondence to its catalytic site, therefore preventing the access of substrates. Thus, Sic1 achieves two major functions in cell cycle regulation. On the one hand, it prevents premature S phase onset by inhibiting Cdk1/Clb5,6 until Sic1 is phosphorylated by Cdk1/Cln complexes and targeted for degradation via the ubiquitin-mediated proteolysis pathway [20,39,40]. In this scenario, Cdk1 is able to phosphorylate Sic1 when in complex with Cln cyclins, thus relieving its own inhibition [30,33,37,41] and the essential role of Cln cyclins in this process is confirmed by the fact that deletion of *SIC1* gene recovers the *cln1Δ cln2Δ cln3Δ* lethal phenotype [30,42]. On the other hand, Sic1 contributes to the abolishment of Cdk1/Clb2 activity required for mitotic exit [26,29,43], although its binding to the
kinase does not target Clb2 for proteolysis [44]. The mechanisms that are adopted at the critical cell cycle transitions (M/G1 and G1/S) to regulate Sic1 availability are described below.

**Sic1 activation at the M/G1 transition**

Cell division is guaranteed upon removal of the kinase activity associated with Cdk1/Clb2 via anaphase promoting complex (APC)-dependent proteolysis [45] and the appearance of Sic1 in late anaphase is timed to mediate a reduction in this kinase activity [29,46,47]. Consistent with this role, sic1Δ mutants show a delay from anaphase to telophase [29].

*SICl* transcription is regulated by the activity of Swi5 and Ace2 transcription factors, with their levels partially determined in the G2 phase and their activity stimulated by a reduction of Cdk1/Clb2 in anaphase [29,46]. The timing of *SICl* transcriptional bursts depends mainly on Swi5 activity, which maintains *SICl* transcription at a basal level throughout the cell cycle, and is responsible for 50% of the total Sic1 expression and the majority of its periodicity [29,46], whereas Ace2 is responsible for the residual periodicity. Therefore, Swi5-driven *SICl* transcription initiates earlier than the Ace2-driven transcription. Swi5 has a cytoplasmic localization when Cdk1/Clb2 activity is high and it is nuclear when Cdk1/Clb2 activity is low, indicating that phosphorylation of Swi5 by Cdk1/Clb2 prevents the entry of Swi5 into the nucleus [48,49] in telophase, therefore explaining the discrepancy between *SICl* transcription levels driven by Swi5 and Ace2 [29]. The first burst of nuclear Swi5 generates a positive-feedback loop as a result of the Sic1 produced inhibiting any residual Cdk1/Clb2 not destroyed in anaphase, further reducing Swi5 phosphorylation and increasing its nuclear localization [50]. This, in turn, allows Ace2 to enter into the nucleus. Interestingly, evidence has been provided showing that the Skp1/Cdc53/F-box protein Cdc4 (SCF) ubiquitin ligase complex regulates S phase entry not only through degradation of Sic1, but also by regulating Swi5 availability [47]. Swi5 has been shown to be ubiquitinated and degraded through SCF(Cdc4) leading to termination of *SICl* transcription in the early G1 phase, therefore ensuring efficient entry into the S phase [47].

The cellular localization of Swi5 depends on the phosphorylation state of a nuclear localization sequence, which retains the transcription factor in the cytoplasm after Cdk1/Clb2-mediated phosphorylation and promotes its nuclear translocation after dephosphorylation by the Cdc14 phosphatase [51]. The requirement of Cdc14 for the mitotic exit is indicated by the arrest of cdc14Δ mutants in late anaphase under restrictive conditions with an elongated mitotic spindle. In these mutants, Sic1 does not accumulate, in agreement with the impairment of *SICl* expression. On the one hand, Cdc14 triggers Sic1 accumulation and stabilization by dephosphorylating Swi5 and Sic1, respectively [52,53]. On the other hand, Cdc14 activates the degradation of Clb cyclins by dephosphorylating Cdh1, a factor of the APC [53,54]. The highly regulated network that controls Sic1 availability is shown in detail in Fig. 1.

![Fig. 1. Mechanisms of Sic1 activation and inactivation. During the G1 and S phases, Cdk1/Cln and Cdk1/Clb activities prevent Sic1 accumulation by phosphorylating Sic1 and its transcription factor Swi5, therefore promoting Sic1 degradation via SCF(Cdc4) and inhibiting *SICl* transcription, respectively. The phosphatase Cdc14 is raised in anaphase and activates APC(Cdh1), which in turn inhibits its Cdk1/Clb activity by promoting Clb degradation. Moreover, Cdc14 dephosphorylates both Sic1 and Swi5, therefore stimulating Sic1 stabilization and *SICl* transcription, respectively. Active Sic1 inhibits Cdk1/Clb activity until Cdk1/Cln is raised in the G1 phase to drive Sic1 phosphorylation. Activating interactions are indicated by a normal arrow and repressing interactions are indicated by a barred arrow.](image-url)
Sic1 inactivation at the G1/S transition

SIC1 transcription occurs in late anaphase [29,46,47] and its protein levels initially increase from anaphase to the G1 phase, followed by a rapid turnover at the G1/S transition when Sic1 is directed to ubiquitin-dependent degradation mediated by Cdk1/Cln complexes [26,37,39,40]. Sic1 is an unstable protein with a half-life of approximately 1–5 min [55] and its N-terminal region was recognized as being necessary and sufficient for its ubiquitination in vitro [56]. Ubiquitinated intermediates of Sic1 have been observed in vivo [57] and its ubiquitination has been demonstrated in vitro [33,57,58]. Moreover, the inhibition of enzymes needed for the proteolysis-mediated ubiquitination stabilizes Sic1 [20,30,55].

Cdk1 inactivation leads to Sic1 accumulation and the inhibition of the mechanism responsible of its proteolysis at Start, resulting in a nonphosphorylatable Sic1 [20,26]. Sic1 has nine consensus phosphorylation sites for Cdk1, which are targets of in vitro and in vivo Cdk1/Cln- and Cdk1/Cln5-mediated phosphorylations [19,20,24,30,33,42,57], and amino acids Thr5, Thr33 and Ser 76 appear to be critical because mutations of these sites with nonphosphorylatable ones increase the half-life of Sic1 in vivo, resulting in a stable Sic1 that is poorly ubiquitinated in vitro [33]. It has been reported that Sic1 undergoes degradation after phosphorylation on a minimum of six of its nine potential sites [33,59–61]; however, binding studies showed that specific sequences acting as starting place of degradation, called degrons, might be sufficient to drive Sic1 degradation (i.e. pairs of phosphorylation sites: Thr5/Thr9, Thr45/Thr48, Ser76/Ser80) [62]. Although Sic1 phosphorylation is primarily dependent on Cdk1/Cln1,2 activity [20,33], Cdk1/Cln2, Cdk1/Cln5 and Cdk1/Cln2 complexes are capable of phosphorylating Sic1 in vitro [26,33,57]. Interestingly, recent evidence has provided novel insights into the multisite phosphorylation mechanism of Sic1, which creates a network of docking connections to achieve proper tuning of the timing of the G1/S transition [63]. This study highlights a sophisticated mechanism involving phosphorylations mediated by Cln2 and Cln5 for the timing of Sic1 degradation driven: (a) by the interaction between RXL motifs in Sic1 and the hydrophobic patch docking site of Cln5 and (b) by a specific stretch of 10 amino acids in Sic1 that confer Cln2 specificity [63,64].

Phosphorylated Sic1 is recognized by Cdc4, a subunit of the E3 ligase SCF [57,59–61,65–68] that polyubiquitinates Sic1 on the N-terminal residues together with the E2 enzyme Cdc34 [33,56,58,69–74], and the process can occur when Sic1 is either free or bound to Cdk1/Cln5 [26,57]. Sic1 directly recognizes and co-precipitates with Cdc4 but not with the other components of the SCF(Cdc4) ubiquitin ligase complex [26,57]. Moreover, Sic1 interaction with Cdc4 is enhanced by Skp1 [57,58,75] and Rub1 appears to assist Cdc53 in Sic1 ubiquitination by modifying Cdc53 via covalent binding [76]. However, mutations of SCF components showed low Sic1 degradation [20,30,55] and lead to a G1 phase arrest with multi-budded cells [55,77–79], a phenotype that can be partially restored by deleting Sic1 [20,55]. Cdc34 is the only essential ubiquitin-conjugating enzyme [80] and cdc34 mutants show multi-budded cells with DNA not replicated and spindle pole bodies not separated, a phenotype that is also observed in cells deficient for Cln function [20] or expressing a stable Sic1 [33]. This phenotype is a result of the failure of degradation of Sic1, which accumulates in this mutant [20,30]. When Sic1 is phosphorylated, the polyubiquitin-binding proteins Rpn10 and Rad23 and the Ran-binding protein Yrb1 target the inhibitor to the proteasome for its efficient degradation [81–84].

Sic1 multisite phosphorylation provides a switch-like cell cycle transition

Cyclin-dependent kinase inhibitors ensure the correct timing of cell cycle phases and define thresholds for Cdk/cyclin activity [85,86]. Therefore, cell cycle progression depends on the relative concentration of Cdk inhibitors and cyclins (and thus Cdk/cyclin activity), which represents an effective biochemical threshold mechanism that can be irreversible when the inhibitor is degraded [87]. This threshold mechanism has been proposed to control Start, where a cell sizer controls entrance into the S phase by activating waves of cyclins that set the timing for the onset of mitosis and cell division [88], with Sic1 as a central regulator.

The molecular mechanism in which Sic1 controls cell cycle progression has been the subject of combined mathematical modelling and experimental efforts, with a special focus on the Cdk1/cyclin threshold necessary to coordinate the timing of Sic1 destruction with cell cycle transitions [59,63,89–91]. The advantage of a multisite phosphorylation mechanism based on kinetic considerations was suggested to provide a switch-like response for Sic1 degradation to rationalize the timing of DNA replication dynamics [59]. Based on the evidence that successive elimination of Cdk1 phosphorylation sites progressively diminishes the recruitment of Sic1 by Cdc4, its ubiquitination and degradation [33], the nine consensus Cdk1 sites on Sic1 were converted to alanine and restored one by one in the order of
their importance (as measured by the degree to which elimination of a single site affects the Sic1 turnover). The mutants were then tested for their ability to bind Cdc4 in vitro after phosphorylation by Cdk1/Cln1,2 or to arrest cell proliferation [59]. Re-addition of five phosphorylation sites did not restore Sic1 binding to Cdc4, however the presence of a sixth Cdk1 site restored the ability of Sic1 to bind Cdc4 and abrogated the toxicity as a result of Sic1 overexpression. This finding demonstrates that there is a threshold number of phosphorylated sites required for the Sic1–Cdc4 binding, with the Sic1 turnover creating an ultrasensitive switch-like response with a maximum theoretical Hill coefficient of 6 [59]. The behaviour of Sic1 reveals the anatomy of a biochemical switch that is responsible for the G1/S transition: a requirement for six equivalent phosphorylations sets a threshold for Cdk1/Cln1,2 activity, such that: (a) Sic1 is stable at low levels of Cdk1/Cln1,2 and (b) a temporal threshold is generated to eliminate Sic1 at progressively increasing levels of Cdk1/Cln1,2, promptly inactivating the inhibitor in a switch-like manner [59,65,92]. Positive-feedbacks are likely to participate in the Sic1 ubiquitination switch because Cdk1/Clb5,6 can phosphorylate Sic1 when they become active, allowing its ubiquitination by SCF(Cdc4) [57,58]. This could further steepen the ultrasensitivity profile, making it conceivable that Sic1 degradation approaches a bi-stable system, as observed for the Mos–mitogen-activated protein kinase pathway in Xenopus oocytes, where a bi-stable system is generated when an ultrasensitive system is combined with a positive-feedback loop [93]. Interestingly, recent findings show that both Cdk1/Cln2 and Cdk1/Clb5 realize a multi-phosphorylation cascade of Sic1 by using a precise interaction mediated by cyclin-specific docking motifs in Sic1. In late G1, Cdk1/Clb5 is inhibited by Sic1 and Cln2 carries out the initial phosphorylation of paired degrons, which serves as a docking platform for phosphorylation mediated by Clb5 released from Sic1 inhibition [63]. Cdk1/Clb5 generates a positive-feedback loop that is required for a switch-like Sic1 destruction, providing evidence that Cdk1/Clb complexes also can contribute to maintain Sic1 at low levels, as suggested previously [14,63]. The Cdk1-mediated phosphorylation of Sic1 induces ubiquitination and the subsequent degradation of the inhibitor, which ensures prompt Cdk1/Clb5,6 activation after Cdk1/Cln activity is established [94].

To provide a possible explanation for the cooperative phosphorylations that drive Sic1 recognition by Cdc4, a mathematical model based on the interaction between a polyvalent disordered ligand and a single receptor site (a kinetic principle called allovalency) has been developed. The model accounts for the experimentally observed cooperativity of the phosphorylated sites on the polyvalent ligand Sic1 in its interaction with a single phosphoepitope binding site on Cdc4, predicting a multiplicative increase in affinity to explain the phosphorylation threshold [89]. Furthermore, cumulative electrostatic forces derived from phosphate groups have been indicated as the physical basis for the Sic1–Cdc4 interaction [60] and NMR studies showed that multiple phosphorylated sites of Sic1 interact with the single receptor site of Cdc4 in dynamic equilibrium [61]. An increasing number of phosphorylation sites creates an efficient phosphorylation threshold; however, above the threshold, the response may not always generate an efficient switch but, instead, may increase in an hyperbolic fashion, as reported previously [90]. This issue between thresholding and switching was recently tackled, aiming to investigate the timing of a substrate decreasing as the kinase level increases for critical cell cycle transitions (i.e. how multisite phosphorylation leads to Sic1 switch-like protein degradation at the G1/S transition) [91]. In that study, a temporal response coefficient was defined to characterize the steepness of the response curve for phosphorylation-triggered protein degradation over time, and to quantify to what extent the response is switch-like by combining temporal thresholding and a rapid decrease after the threshold. The degradation is influenced by the type of kinase stimulus (step-function, linear, nonlinear) that drives the increase of a kinase and by the strength and duration of the stimulus (when the stimulus strength is low, its duration is important and affects the extent to which Sic1 is degraded; when the stimulus strength overcome a critical level, Sic1 is largely eliminated by the time the kinase reaches its maximum level and, thus, the duration of the kinase signal is not relevant). Strikingly, the extent of the switch-like degradation was found to be affected by the number of sites phosphorylated by the kinase, and this design enables Sic1 to respond to the kinase signal in a highly switch-like manner, generating both an observable temporal threshold and a rapid decrease of the phosphorylation threshold, enhancing the robustness of the degradation machinery [91]. Taken together, these studies shed new light on the general design principles at the basis of protein degradation switches for regulating the timing of cell cycle transitions.

**Dual function of Sic1 in regulating S phase dynamics**

The activation of Sic1 in anaphase is an important event for a faithful cell division, as well as its deactivation at
the G1/S transition. The inhibitor is a critical regulator that maintains a temporal window free from Cdk1/Clb activity, permitting the pre-replication complex to be assembled on the replication origins during the G1 phase [31]. It has been shown that sic1Δ mutants initiate DNA replication prematurely as a result of early activation of Cdk1/Clb5 activity [27]. Considering that, in this strain, only a few origins of replication are fired (sparse origin firing), a working hypothesis is that this could be a result of the different availability of Cdk1/Clb5,6 in the nucleus for their timely activation. In sic1Δ cells, DNA replication is not completed on schedule with cells segregating incompletely-replicated chromosomes, a clear indication that origin density is crucial for genome integrity. Thus, by inhibiting Cdk1/Clb activity in the G1 phase, Sic1 promotes efficient formation of the pre-replication complex, potentially on sites that serve as dormant origins [95]. To investigate whether genomic instability in budding yeast is potentially regulated by Sic1, a mathematical model of the G1/S transition has been developed [96]. The implications of the balance between Sic1 and Cdk1/Clb5,6 for the timely cell cycle progression have been investigated, and the efficiency of this threshold mechanism to activate origins of replication at the S phase entrance was simulated [96,97]. Importantly, considering the double role of Sic1 as a stoichiometric inhibitor of Cdk1/Clb complexes [20,22] and as a promoter of Cdk1/Clb5 transport from the cytoplasm into the nucleus [32,96], a possible explanation for the sparse origin firing phenotype observed in sic1Δ cells has been rationalized (Fig. 2). In a stochastic simulation of replication origin activation that considers the rate of firing dependent on the amount of nuclear Cdk1/Clb5,6, an early firing of the replication origins was observed in the sic1Δ mutant compared to the wild-type [97]. This is a result of Cdk1/Clb5 being transported into the nucleus by diffusion because no Sic1 is available for active transport via its nuclear localization sequence [32]. The Cdk1/Clb5 activity immediately available in the nucleus initiates origin firing but, considering that the amount of complex accumulated in the nucleus via diffusion is lower compared to that titrated by Sic1, the firing process proceeds slowly and does not activate the whole set of replication origins [97,98], as observed experimentally [27]. This result strongly suggests that the fine tuning of Cdk1/Clb5,6 activity by Sic1 controls the precise temporal activation of DNA replication dynamics for timely G1/S transition, as recently noted in computational analyses [99,100].

Sic1 prevents genomic instability coordinating the timing of Cdk1/Clb activation

The timely activation of Cdk1/Clb5,6, as well as the other waves of kinase activity associated with Cdk1/Clb3,4 and Cdk1/Clb1,2 complexes between the S and M phases, is strictly dependent on a reduction in Sic1 levels [87,88]. However, whether the mechanism of the regulation of cell cycle timing involves Sic1 or
not is not well understood. To investigate whether Sic1 may function as a timer in coordinating the appearance of the waves of phase-specific Cdk1/Clb activities throughout cell cycle progression, a combined computational and experimental approach has been employed. Kinetic models based on ordinary differential equations and describing Cdk1/Clb dynamics over time have been carried out, implementing interactions of Sic1 with one or more Cdk1/Clb complexes [35], with the aim of reproducing the characteristic pattern known as waves of cyclins (i.e. various Cdk1/Clb complexes are activated and inactivated in a fixed, temporally regulated sequence) [12–18]. These modular networks are sufficiently small for an accurate modeling; generally, kinetic models have been successfully used to predict signalling properties when a sufficient small network is considered and kinetic parameters are available, or when parameters are unknown but components and reactions are known [101].

Cdk1/Clb activation has been considered as a series of events in which each kinase complex activates the next one in a progressive cascade. In detail, after CLB5,6 transcription by the MBF (Swi6-Mbp1) transcriptional complex [102], Cdk1/Clb5,6 activates CLB3,4 transcription via a yet unknown transcription factor, and the formed Cdk1/Clb3,4 activates CLB1,2 transcription via the Fkh2 transcription factor [103,104] (Fig. 3A, red lines). This cascade is inferred from evidence provided in literature and incorporating the well-known mechanisms of transcriptional regulation of Clb cyclins [35]. We refer to this small network as the ‘Clb module’, which has been extended by encompassing the different regulatory interactions that Sic1 can establish with the Cdk1/Clb complexes: (a) Sic1 binds only to Cdk1/Clb5,6; (b) Sic1 binds to both Cdk1/Clb5,6 and Cdk1/Clb1,2; and (c) Sic1 binds to Cdk1/Clb5,6, Cdk1/Clb1,2 and Cdk1/Clb3,4. Computational simulations revealed that the temporal appearance of waves of Clb cyclins with an oscillation-like behaviour is observed only when Sic1 binds to all Cdk1/Clb complexes [35] (Fig. 3B). This result has been tested for internal consistency to investigate

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**Fig. 3.** Signalling network describing Cdk1/Clb regulation. (A) After production of Cdk1/Clb5,6 (re1), Sic1 binds, forming the Sic1-Cdk1/Clb5,6 complex (re2, solid line). Sic1 is degraded primarily by Cdk1/Cln1,2 (not shown) and by Cdk1/Clb activities (re5), and Clb5,6 is degraded in both Cdk1/Clb5,6 (re6) and Sic1-Cdk1/Clb5,6 (re4) complexes. Cdk1/Clb5,6 activates Cdk1/Clb3,4, in addition to its basal production (re7), and Sic1 binds to Cdk1/Clb3,4 forming the Sic1-Cdk1/Clb3,4 complex (re15, dashed line). Sic1 is degraded by Cdk1/Clb activities (re18) and Clb3,4 is degraded in both Cdk1/Clb3,4 (re8) and Sic1-Cdk1/Clb3,4 (re17) complexes. Cdk1/Clb3,4 activates Cdk1/Clb1,2 together with Cdk1/Clb5,6, in addition to its basal production (re9). Sic1 binds to Cdk1/Clb1,2 forming the Sic1-Cdk1/Clb1,2 complex (re11, dotted line). Sic1 is degraded by Cdk1/Clb activities (re14) and Clb1,2 is degraded in both Cdk1/Clb1,2 (re10) and Sic1-Cdk1/Clb1,2 (re13) complexes. The sequential transcriptional activation of Cdk1/Clb complexes is shown in red: Cdk1/Clb5,6 activates both Cdk1/Clb3,4 (arrow A) and Cdk1/Clb1,2 (arrow Cl), Cdk1/Clb3,4 activates Cdk1/Clb1,2 (arrow B) and Cdk1/Clb1,2 autoactivates itself (arrow D). Clb-regulated degradation by Cdk1/Clb complexes is shown in blue: Clb5,6 degradation is driven by both Cdk1/Clb3,4, Cdk1/Clb1,2 (arrow F), whereas Clb3,4 (arrow G) and Clb1,2 (arrow H) degradations are driven by Cdk1/Clb1,2. C1, C2 and C3 indicate Cdk1/Clb5,6, Cdk1/Clb3,4 and Cdk1/Clb1,2, respectively. (B) Computational time course of total Clb cyclin levels of the network shown in (A) considering only the sequential transcriptional activation of Cdk1/Clb complexes (red arrows). (C) Computational time course of total Clb cyclin levels of the network shown in (A) considering the sequential transcriptional activation of Cdk1/Clb complexes (red arrows) and the Clb-regulated degradation by Cdk1/Clb complexes (blue arrows). The curves represent the total amount of each specific Clb cyclin, given by the contribution of Clb free and Clb bound to Sic1. Figure reproduced with permission from Barberis et al. [35].
whether specific values of kinetic parameters assigned to each biochemical reaction can influence the time delay between Clb cyclins. This analysis, called global sensitivity analysis, is based on a Monte Carlo simulation and employs the random sampling of 10,000 kinetic parameter sets that are varied between 0.1- and 10-fold of their initial values. The analysis revealed that any change of parameters affects the delay of Clb cyclin appearance only when Sic1 binds to all Cdk1/Clb complexes [35]. Supporting these findings, association of Sic1 with various Clb cyclins has been detected using in vitro assays and in vivo methods by high-throughput genome-wide screenings for complexes [56,105–112]. In addition, independent validation of the interactions of Sic1 with all Clb cyclins has been carried out using in vitro analyses (e.g. yeast-two-hybrid and glutathione S-transferase pull-down) [35] and in living yeast cells with Clb2, Clb3 and Clb5 by Förster resonance energy transfer (FRET) via fluorescence lifetime imaging microscopy (FLIM) [113]. This evidence, together with data indicating that Sic1 is a substrate for Clb2-, Clb5- [57,58,114] and Clb3-(M. Barberis, A. González-Novo and F. Posas, unpublished data) associated kinase activities and that Sic1 levels are observed throughout the cell cycle [115,116], supports the hypothesis that Sic1 might be involved in the regulation of waves of Clb cyclins.

Computational simulations carried out perturbing the structure of the mathematical model and testing Cdk1/Clb dynamics in the absence of Sic1 - mimicking a sic1Δ mutant - predicted an abolishment of Clb cyclin waves, with their levels reaching a different plateau over the simulation time as compared to the characteristic periodicity of Clb levels of a wild-type cell. Strikingly, G1-elutriated sic1Δ cells completely loose the timing and regulated periodicity of Clb cyclins, revealing that not only Clb5, but also Clb3 and Clb2 arise at the beginning of the G1 phase with levels that progressively increase to reach a different plateau, as predicted previously [35]. This finding is in agreement with early data showing that a sic1Δ strain accumulates Clb5 in the early G1 phase and thus precocious Cdk1/Clb5 activity, which promotes genome duplication from a few replication origins [27]. Consequently, an uncontrolled temporal pattern of Clb cyclins may lead to cell division with chromosomes not being completely replicated and therefore genomic instability [25]. Taken together, these findings, as well as the fact that Sic1 coexists in time with all Clb cyclins [35], suggest that Sic1 triggers the timing and oscillatory pattern of the waves of Clb cyclins, and therefore Clb-associated kinase activities, through a feed-forward regulation. The different abundance of Clb cyclins in a cell [117,118] could also be relevant for the localization of Sic1/Clb interactions, as well as their temporal window of activity.

Considering that Sic1 degradation is crucial for the proper regulation of cell cycle progression, we investigated the temporal dynamics of Clb cyclin waves upon the abolishment of Sic1 degradation. We showed, by computational and experimental analyses, that the constitutive expression of a nondegradable form of Sic1, Sic1-0P, does not impair the accumulation of waves of Clb2, Clb3 and Clb5, which can still be observed although temporally delayed [35,96], as inferred recently [34]. This is in agreement with the fact that oscillations in Sic1 level are sufficient to trigger the feed-forward loop that is necessary to switch oscillations of Cdk1/Clb activities [119]. Computational simulations impairing the degradation of Sic1 by any of the Cdk1/Clb complexes reproduced recent data showing that cells overexpressing Sic1-0P are lethal in the absence of either Clb2, Clb3 or Clb5 [34] as a result of an improper timing of the accumulation of the remaining active Clb cyclins [120]. This result clearly reflects the specificity that Clb cyclins acquire at various cell cycle stages [117,121] and, indeed, indicates that the computational predictions are valid, supporting a role for Sic1 in the regulation of Cdk1/Clb complexes to prevent genomic instability.

**Sic1, and not Clb degradation, promotes waves of Clb cyclins**

The sequential pattern of accumulation and disappearance of Clb cyclins gives directionality to cell cycle events. Cdk1/Clb complexes are activated by positive-feed-forward loops depending on the regulated transcription of CLB genes [12,122] and by negative-feedback loops via down-regulation of Clb levels by the SCF ubiquitin ligase complex and the APC [50,123–126]. This mechanism of degradation occurs through the mutual regulation of two forms of APC, APC(Cdc20) and APC(Cdh1), which are differentially regulated by Cdk1/Clb-mediated phosphorylation: S phase Cdk1/Clb complexes inactivate Cdh1, mitotic Cdk1/Clb complexes activate Cdc20, and their subsequent inactivation promotes Cdh1 preventing the destruction of Sic1, which starts to accumulate [45,127–132].

To investigate the influence of Sic1 regulation and mechanisms of Clb degradation on the timing of Clb cyclin waves, the network presented in Fig. 3A was modified to incorporate the role of specific Cdk1/Clb complexes in the APC-driven Clb degradation, without including explicitly APC complexes and their
interactions with Clb cyclins. Specifically, we introduced Clb5,6 degradation stimulated by both Cdk1/Clb3,4 and Cdk1/Clb1,2 complexes, and degradation of Clb3,4 and Clb1,2 stimulated by Cdk1/Clb1,2 [45, 127–130] (Fig. 3C, blue arrows). As observed in the previous computational analysis, only the binding of Sic1 to all Cdk1/Clb complexes generates a time delay between Clb cyclins. Interestingly, the simulation indicated that the Cdk1/Clb-regulated degradation of Clb cyclins is needed to correctly shape Clb waves and reduce Clb levels at the end of the cell cycle in a compact way. However, this result suggests that waves of Clb cyclins derive from the binding of Sic1 to all three Clb pairs rather than as a result of Clb degradation. In agreement with this finding, it has been reported that regulated Clb proteolysis is not inherently essential because oscillations in Sic1 levels can substitute for APC as regulator of the Cdk1/Clb activity [119,133]. Moreover, waves of Cdk1/Clb activity are still observed under conditions of constitutive Clb3 expression, providing further evidence that Sic1 levels are sufficient to trigger the feed-forward loop necessary to switch Cdk1/Clb complexes between states of high and low concentrations [119].

Furthermore, it has been shown that the irreversibility of mitotic exit is not driven by Clb proteolysis but that a feedback loop involving Sic1 is required to maintain low Cdk1/Clb2 activity and to prevent cyclin resynthesis [134]. Taken together, these data demonstrate that the unidirectionality of mitotic exit is not a consequence of Clb2 proteolysis and that Clb degradation is not an absolute requirement for a viable cell cycle, highlighting an additional role for Sic1 in regulating Cdk1/Clb waves and coordinating the timing of their appearance.

**Molecular mechanism of the Sic1-Cdk1/Clb interaction**

The occurrence of Clb cyclin waves is fundamental for the timely activation of phase-specific substrates throughout cell cycle progression, and the data reported above indicate that a potential mechanism to regulate them is realized by the interaction of Sic1 with each Clb subtype. At present, the mode and the strength of these interactions remain unknown and, although atomic details of molecular interactions derive only from 3D structures, attempts to crystallize Sic1 have failed until now [135]. Despite the lack of precise molecular details, NMR studies showed that Sic1 is a disordered protein both in its free state and when bound to the Cdc4 ubiquitin-protein ligase [68] but, when phosphorylated before degradation, it maintains a compact structure that keeps phosphate groups close together to form an electrostatic field between Sic1 and Cdc4 [61]. Biophysical methods have also shown that the C-terminal sequence of Sic1 cor responding to the kinase inhibitory domain (KID) [136] possess a propensity for structural order [137–139], which retains a dynamic helical structure and populates collapsed states of different compactness [140]. This observation suggests that this region is prone to assume a 3D folding upon binding with Cdk1/Clb complexes, as it has been shown for the structure of its mammalian counterpart p27Kip1 [141,142]. Indeed, p27Kip1-KID binds to the Cdk2/ cyclin A kinase complex via a sequential mechanism by first occupying a conserved hydrophobic pocket on cyclin A [143] and then by blocking the ATP binding pocket at the active site on Cdk2 [144].

Structural studies for the interaction between Sic1 and Clb cyclins are lacking as a result of limitations concerning the analysis of large protein complexes; therefore, homology modelling has been employed to investigate the interactions on the basis of the X-ray structure of p27Kip1. Despite a very low sequence identity between the KIDs of Sic1 and p27Kip1, the sequences reveal common elements of secondary structure and also the conservation of hydrophobic residues recognizing the cyclin subunit and the catalytic site on the kinase [22,23,120]. A 3D model of the Sic1-KID-Cdk1/Clb5 ternary complex has been generated using the mammalian counterpart p27Kip1-KID-Cdk2/cyclin A as a template, and was optimized by the conjugate gradient method of energy minimization and molecular dynamics. The interactions between the LF (Leu32-Phe33) domain of p27Kip1-KID that recognizes a docking site for substrate identification on cyclin A [141,145] are conserved in the yeast complex, where the LV (Leu224-Val225) domain of Sic1-KID interacts...
Regulation of timely Clb cyclin waves by Sic1

M. Barberis

with Clb5 (Fig. 4A,B) [120], supporting the observation that p27^Kip1 interacts with Clb5 in vivo [106]. In addition, a conserved long amphipathic α-helix present in both KIDs of Sic1 and p27^Kip1 appears to function as a bridge keeping the Cdk/cyclin complex tightly assembled (Fig. 4C) [22,23,120,141]. Interestingly, amino acid Arg233 of Sic1-KID, although not hydrophobic, is threaded within Clb5 structure, making use of the alkylmoieties to effect specific hydrophobic interactions. Furthermore, amino acid Leu276 of Sic1-KID occupies a hydrophobic pocket on Cdk1 resembling the steric features that amino acid Tyr88 of p27^Kip1-KID establishes with the catalytic site on Cdk1 (Fig. 4D) [120]. These findings reveal that Sic1 and p27^Kip1 are structural homologues in their KIDs, as supported by experimental evidence. Indeed, binding kinetics between Sic1 and Cdk2/cyclin A through surface plasmon resonance indicate that Sic1 binds to the cyclin subunit with high affinity, followed by a low-affinity binding to the kinase [22], therefore realizing its inhibitory function via a two-step mechanism, by interacting first with the docking site on the cyclin and subsequently lengthening on the kinase to reach its catalytic site. Intriguingly, both Sic1 and p27^Kip1 protect Cdk1/Clb5 and Cdk2/cyclin A, respectively, from tyrosine phosphorylation, allowing the accumulation of nonphosphorylated kinase complexes that are immediately active when released from inhibition to promote DNA replication [146]. Altogether, Sic1 is functional homologous of p27^Kip1 and potent inhibitor of both yeast and mammalian Cdk/cyclin activities [22,23]; moreover, it plays a similar role in the cell cycle regulation of rum1 in fission yeast [147] but structural similarities remain to be investigated. At present, the atomic details of various Sic1/Clb interactions are not known, however it is evident that addressing the precise molecular mechanism that Sic1 engages to realize its inhibitory function on Cdk1/Clb complexes is undoubtedly challenging for understanding how the timing of cell cycle regulation is accomplished.

**Sic1: more than just promoting Cdk1/Clb inactivation?**

Despite the molecular details of Cdk1/Clb inhibition by Sic1 are not completely available yet, we would like to pose a fundamental question: why should events necessary for the precise timing of the appearance of Clb be positively regulated by Sic1, whereas apparently Sic1 should be fully degraded to permit the onset into the S phase? We hypothesize that the gradual inactivation of Sic1 persists until the next bulk of *SIC1* transcript in anaphase [29,46]. Accordingly, previous data showed that both *SIC1* transcript levels [46,148] and Sic1 protein levels [115,116] are detectable throughout the cell cycle, although their precise quantification is still missing. Early mitotic events depend on an increasing Cdk1/Clb activity, with entry into mitosis requiring less Cdk1/Clb activity than progression through the prophase/metaphase transition, and it was proposed that this might establish the order of mitotic events [149]. Perhaps a gradual inactivation of Sic1 and hence a gradual increase of Cdk1/Clb activity after entry into the S phase follows a similar mechanism. A slow transient inactivation of Sic1 by the accumulation of diverse Cdk1/Clb complexes could coordinate cell cycle events by their timely release from inhibition to guarantee the activation of specific substrates. Subsequent activation of Sic1 by Swi5 reduces Cdk1/Clb levels and hence promotes exit from mitosis countering Cdk1/Clb2 activity. This hypothesis is compatible with recent findings showing that Cdk1 activity on cell cycle substrates progressively increases from the G1 phase to mitosis [64] and suggests that the fine-tuning of Cdk1/Clb activities could be regulated in a temporal way by variable Sic1 levels and, potentially, by molecular mechanisms that could lead to a different Sic1/Clb binding affinity.

How does Sic1 regulate the timing of Clb waves? Sic1 degradation by Cdk1/Cln1,2 and the release of Cdk1/Clb5,6 lead to the activation of the mitotic Cln cyclin cascade [130], which is started by a sophisticated phosphorylation mechanism [63]. Cdk1/Clb activities in turn phosphorylate Sic1 and these progressive phosphorylation events could function as thresholds, a prerequisite for Cdk1/Clb complexes to promote their dissociation from the inhibitor in sequential waves, thus triggering phase-specific substrates in the S phase and early mitosis. Therefore, Sic1 remains spread throughout the cell cycle, giving the inhibitor time to set events that lead to Cdk1/Clb activation; for example, it could progressively destabilize the interaction with Cdk1/Clb complexes from the S phase to the M phase. However, although the mechanism is still not understood, it is expected that Sic1 interacts in vivo with all Clb cyclins pairs. FLIM-FRET has been employed to detect the close co-localization of fluorescent-tagged proteins in living yeast cells, providing high spatial and temporal resolution (nanoseconds) [150,151]. The occurrence of FRET was measured by monitoring the change in Sic1 lifetime in the presence and absence of Clb cyclins, based on the fact that the reduction of fluorescence lifetime as a result of FRET occurs only when molecules are in very tight proximity. The interaction of Sic1 with each Clb cyclin...
subtype in the nucleus of yeast cells has been observed, with variable FRET efficiencies measured for Sic1/Clb2, Sic1/Clb3 and Sic1/Clb5 pairs [113]. Sic1 and Clb cyclins localize in both the nucleus and cytoplasm [118]; however, our findings suggest that, ultimately, the functional inhibition of Cdk1/Clb complexes occurs in the nucleus, as it has been shown for Clb5 [32].

Interestingly, the interaction between Sic1 and Clb4 was not revealed, although recent high-throughput studies reported an association between both proteins [111,112]. A specific interaction between these partners cannot be ruled out at this stage, despite not being detected by FRET, potentially as a result of a structural organization of the Sic1/Clb4 complex that does not allow an interaction between the fluorescent tags associated with Sic1 and Clb4. However, this result is in agreement with the fact that Cdk/Clb3 constitutes the majority (67%) of Clb3,4-associated Cdk1 activity in asynchronous log-phase cells [152]. Similarly, it was shown that Cdk1/Clb2 constitutes the majority (85%) of Clb1,2-associated Cdk1 activity in mitosis-arrested cells [152] and it is expected to interact more strongly than Clb1 with Sic1. The fact that each Clb subtype interacts in vivo with Sic1 is consistent with the evidence that: (a) all Clb subtypes and Sic1 coexist during the whole cell cycle progression despite variable Sic1 levels and (b) sic1Δ cells lose the regulated periodicity of Clb cyclins [35]. Taken together, these data provide evidence that Sic1 can directly coordinate the appearance of Cdk1/Clb complexes by temporally regulating waves of Clb cyclins, and suggest that, despite the high functional homology between the cyclins [153,154], only some of them might target Sic1 preferentially to enable its function, supporting their divergence of function [155,156]. Further investigations are needed to unravel the temporal dynamics and specific localization of the Sic1/Clb interactions during different cell cycle stages.

**Have we discovered all regulatory functions of Sic1?**

During the last 15 years, improvements towards an understanding of how Sic1 controls the G1/S transition and is involved in mitotic exit, as well as how the inhibitor itself is regulated, have been made. Precise experimental settings and focused computational analyses created the basis for addressing these mechanisms, and further investigations are required to reveal the molecular details. However, several questions remain still unresolved. How is the translocation of Sic1 from the cytoplasm to the nucleus regulated at the molecular level? How are different Cdk1/Clb complexes induced timely to phosphorylate the inhibitor? How do the Cdk1/Clb kinases and Cdc14 phosphatase implicated in the regulation of the inhibitor function together to set Sic1 levels over time? Cdc14 antagonizes oscillations of the Cdk1/Clb activity [52,157]. It has been shown that the balance between Cdk1/Clb2 and Cdc14 is critical for proper regulation at mitotic exit [158] and that the balance between oscillating Cdk1/Clb activity and the execution of Cdc14 functions is central in the regulation of cell cycle progression at mitotic exit [159]. Supporting this vision, recent analyses reveal a different function for Clb1, Clb2 and Clb3 at mitotic exit, and are consistent with the hypothesis that Clb-associated functions are antagonized by Cdc14 to couple cell cycle progression with cytokinesis at mitotic exit [160]. The specific functions of Clb cyclins during cell cycle progression and cytokinesis have been investigated, revealing that: (a) Clb1 inactivation mainly inhibits cell cycle progression, with less influence on the inactivation of Clb2 or Clb3, (b) Clb2 inactivation is involved in the formation of the actin ring structure and (c) inactivation of both Clb1 and Clb3 is sufficient to complete cytokinesis and therefore cell division, with an additional effect of Clb3 in promoting polarized growth [160]. These data are in agreement with early evidence showing that Clb2 and Clb5 differ strikingly in the cell cycle events that they can drive efficiently, even when present at similar levels and at similar times [155]. This distinction suggests that a progressive activation of Cdc14 antagonizes Clb cyclins in a different manner, aiming to ensure that bud emergence and DNA replication are executed after the completion of cytokinesis. Early mitotic events depend on an increasing Cdk1/Clb activity [149] and it has been recently reported that increasing Cdc14 concentrations are required for the sequential dephosphorylation of mitotic substrates, with a greater catalytic efficiency for the targets that are active at early stages of mitosis [161]. This scenario shows how quantitative changes of the kinase/phosphatase ratio during mitotic exit activate substrate dephosphorylation in sequential thresholds for the temporal order of mitotic exit. The gradual activation and inactivation of Sic1 are therefore tightly regulated from this ratio, where different Clb-associated inactivation of Sic1 are progressively antagonized by Cdc14.

**Kinases and phosphatases: diverse mechanisms of Sic1 regulation**

Based on the presented data, we argue that Sic1 may act as a timer to coordinate oscillations of Clb waves.
An additional function of Sic1 might be to synchronize waves of Clb levels with other cell cycle processes. Stabilization and destabilization of Sic1 in late stages of the cell cycle remain to be proven, although there is evidence for a possible involvement of kinases and phosphatases, in addition to Cdk1 and Cdc14, for its fine-tuned regulation. Under osmotic stress conditions, Sic1 is phosphorylated by the stress-activated protein kinase (SAPK) Hog1, which stabilizes the inhibitor and prevents its degradation at the G1/S transition, thereby halting Cdk1/Clb5,6 activity and preventing premature entry into the S phase until the cell has adapted [162–165]. Cells deleted in the SIC1 gene or containing a Sic1 allele mutated in the Hog1 phosphorylation site do not arrest in the G1 phase upon Hog1 activation and enter into the S phase without being properly adapted [162,163].

The Pho85 kinase functions in the control of phosphate metabolism, interacting with the Pho80 cyclin to regulate the transcription factor Pho4 [166]. Pho85 associates with G1 cyclin homologues, including Pcl1, Pcl2 and Pcl9, whose expression is periodic during cell cycle progression [167]. Although the precise function of Pho85 remains unclear, its role on Sic1 stability has been shown. Pho85 in complex with Pcl1 phosphorylates Sic1 at specific Cdk consensus sites, leading to its prompt degradation and onset into the S phase, and Sic1 levels increase in pho85Δ mutants [167,168]. A novel role for Pho85 has been proposed in late mitosis as a result of its association with the Pcl9 cyclin, which is unstable with peak levels occurring in the late M phase. Interestingly, the expression of PCL9 and SIC1 genes occurs in the same temporal window [148]. Although it is still unclear which cyclin associated with Pho85 is required in vivo for the phosphorylation and destabilization of Sic1 [169], recent evidence shows that Pho85 associated with Pcl1 and Pho80 cyclins positively regulates autophagy by promoting the degradation of Sic1, which has been proposed to function as a negative regulator of autophagy [170]. Rapamycin-sensitive TOR kinase complex 1 (TORC1) is a major regulator of autophagy [171] and, under nutrient starvation conditions, it is inhibited and autophagy is induced. Interestingly, rapamycin-mediated G1 phase arrest is dependent on Sic1 up-regulation, with Sic1 accumulating in the nucleus and impairing Cdk1/Clb5,6 activity, therefore avoiding improper initiation of DNA replication under conditions of poor nutrient availability [172]. Cells deleted in the SIC1 gene do not arrest upon rapamycin treatment, making cells sensitive to a sublethal dose of rapamycin and nutrient starvation.

The casein kinase 2 (CK2) is a ubiquitous, highly pleiotropic and constitutively active serine/threonine kinase conserved in all eukaryotes [173] and an essential regulator of cell cycle progression. CK2 phosphorylates the E2 enzyme Cdc34 that, together with SCF(Cdc4), polyubiquitinates Sic1 [174], therefore potentially influencing the dynamics of its degradation. Moreover, CK2-mediated phosphorylation of Sic1 alters the timing of the G1/S transition by affecting Sic1 affinity for Cdk1/Clb5 [23,116,175] and mutations that impair (Ser201/Ala) or mimic (Ser201/Glu) phosphorylation by CK2 affect the coordination between cell growth and cell cycle progression [175]. However, analyses of the mutants did not reveal any appreciable effects on the structural conformation of isolated Sic1 [137]. Biochemical, structural and computational studies have been undertaken aiming to study the potential role of CK2 phosphorylation in regulating Sic1 activity, with the prediction that Sic1 might have a lower binding affinity for Cdk1/Clb5,6 in cells grown on a poor medium compared to cells grown on a rich medium [22] and that the phosphorylation in the latter might be dependent on CK2 [23]. Interestingly, support for this prediction was found in a subsequent study showing that inactive CK2 leads to high Sic1 levels and the abolishment of Cdk1/Clb5 activity [176]. This condition is comparable to the growth in a poor medium, where high Sic1 levels observed experimentally [32] might be the result of a decrease in CK2 activity on Sic1. This scenario suggests that the phosphorylated state of Sic1 could influence its localization, and thus the timing of S phase onset, ensuring that no premature origin licensing takes place until Sic1 proteolysis is activated, when higher Cdk1/Clb activity necessary to initiate DNA replication is then provided [120].

Ime2 is a kinase necessary for the timely destruction of Sic1 during sporulation [177]. Meiosis differs from mitosis with respect to the mechanism that regulates Sic1 stability. Indeed, the destruction of Sic1 and activation of a Clb5-dependent kinase in meiotic cells requires the meiosis-specific protein Ime2 and not Cdk1 activity because Cdk1/Cln complexes are not active during meiosis [177–179]. Despite the fact that Ime2 phosphorylates Sic1 at multiple PXS/T sites in vitro, the kinase is not sufficient to trigger Sic1 degradation [180]. However, Sic1 destruction is an important event in meiosis. Indeed, over-expression of Sic1 mutated at multiple Cdk1-targeted phosphorylation sites during the mitotic cell cycle stabilizes Sic1 and prevents premeiotic DNA replication [154,180], leading to DNA re-replication [181].
Although several kinases are known to regulate Sic1, the only phosphatase previously found to be associated with Sic1 is Cdc14, which counteracts the Cdk-dependent phosphorylation of Sic1, and its over-expression strongly stabilizes Sic1 [52]. In addition to kinases and Cdc14 phosphatase, the phosphorylation state of Sic1 is also regulated by other phosphatases, such as Dcr2 [182]. Dcr2 interacts functionally and physically with Sic1, and its over-expression destabilizes the inhibitor, leading to genome instability [182].

The incorporation of multiple kinases and phosphatases in an extended model of Sic1 regulation would potentially lead to a greater understanding of protein degradation switches. To support this argument, we tested the effect of Sic1 stabilization in late stages of cell cycle progression. Taking advantage of the network presented in Fig. 3A, we stopped the degradation of Sic1 (\(k_5\), \(k_{14}\) and \(k_{18}\)) for 30 min and at different time points. Fig. 5 shows that the formation of each Clb wave starts at the same point, although the amplitude of the maximum peak increases over time and the response is also prolonged. As a result of retardation of Sic1 degradation, Clb levels appear to accumulate and overcome the blockage. This effect is pronounced for Clb3,4 (Fig. 5B) and even more evident for Clb1,2 (Fig. 5C) compared to Clb5,6 (Fig. 5A), which is possibly explained by the lack of detail in the upper part of the network where the steps required for Cdk1-Clb5,6 formation are not taken into account. The prediction that Sic1 could be regulated throughout the cell cycle requires further experimental investigations and could be verified, on the one hand, by testing the involvement in late stages of cell cycle progression of kinases and phosphatases that are already known to play a role in Sic1 regulation and, on the other hand, by searching for novel regulators of Sic1 activity.

**Additional mechanisms to regulate Sic1 function?**

Sic1 accomplishes its cell cycle functions using a double mechanism of activation/inhibition, on the one hand by transporting Cdk1/Clb5,6 complexes from the cytoplasm to the nucleus to timely activate origins of replication [32,96] and, on the other hand, by being a potent inhibitor of the Cdk1/Clb activity [19–21]. An interesting question that remains to be addressed is whether Sic1 is active as an inhibitor in the cytoplasm. Biochemical analyses indicate that Sic1 is a high specific inhibitor of Cdk/cyclin complexes of yeast and mammalian cells [22], which would argue that this is the case. However, this model has a possible, although yet to be proven, analogy with the mammalian coun-
rationalize cell cycle dynamics in mouse fibroblasts [187]. The model recapitulates events from growth factor stimulation to S phase onset following phosphorylation states associated with the activation or deactivation of p27^Kip1 in the nucleus and cytoplasm. Interestingly, variability in the entrance into the S phase has been monitored against the relative concentration of the inhibitor depending on the translocation of binary and ternary Cdk/cyclin complexes, and restriction point dynamics are comparable with the corresponding experimental ones [187].

Sic1 was found to bind to Gis2, an activator for mRNAs [188], as well as to the RNA-binding proteins Hek2 [189] and Nab2 [190]. Both Gis2 and Hek2 are cytoplasmic proteins, whereas Gis2 has a nuclear localization [118]. Gis2 is a translational activator for mRNAs with internal ribosome entry sites; therefore, it is tempting to speculate that SIC1 mRNA could be stabilized during its translation and its copy number tightly regulated as a result of the critical role that the inhibitor exploits at the G1/S transition. Recent evidence indicating that a small number of SIC1 mRNA molecules generates a low level of noise in the translation process to regulate the entrance into the S phase [191] suggests that this could be the case. A stochastic model that addresses Sic1 transcription and the resulting noise at the G1/S transition has been developed, revealing that an increased amount of SIC1 mRNA leads to an amplified dispersion of Sic1 protein levels and suggesting that both SIC1 mRNA and Sic1 protein levels are critical for setting the timing of S phase onset [191]. Therefore, low SIC1 transcription could comprise a mechanism for ensuring the robust timing of Sic1 down-regulation. Accordingly, it has been reported that, under conditions of nutritional limitation, cell cycle progression is delayed and SIC1 mRNA levels increase [192], with Sic1 levels remaining high compared to wild-type [96].

Hek2 is a RNA binding protein involved in asymmetric localization of the mRNA of ASH1, a transcription factor that acts to specify daughter cell fate in mating-type switching [193,194]. Translation of ASH1 mRNA is coordinated with its transport to the bud tip, with translation being delayed when Ash1 is localized [195,196]. The fact that Sic1 binds with Hek2 suggests that both Sic1 and Ash1 accumulate in new-born daughter cell nuclei to provide an adequate amount of the transcripts necessary to control the early stages of cell cycle progression.

**Conclusions**

Identification of the structure of a functional module requires hypothesis-driven experiments and theoretical approaches to elucidate the underlying design principles. Each module is constituted by defined molecules connected with a particular topology [197,198] to generate a similar physiological behaviour, as suggested from the dynamically organized modularity in the budding yeast interactome network [199,200].

The biochemical regulation that controls cell cycle progression has been recognized to be the oscillation of kinase activities, where phase-specific Clb cyclins bind to the Cdk1 kinase with a characteristic staggered behaviour known as ‘waves of cyclins’ [12–18]. However, the molecular mechanisms of this coordinated regulation remain elusive. Here, we have recapitulated the available evidence showing how waves of Cdk1/Clb activities are generated, and investigated the role of the cyclin-dependent kinase inhibitor Sic1 in their regulation by reconstituting the functional ‘Clb module’ from their constituent components, the Cdk1/Clb kinase complexes and Sic1, following a bottom-up systems biology strategy [201]. We have shown that Sic1, rather than Clb proteolysis, acts as a timer in cell cycle regulation by coordinating the temporal oscillations of waves of Clb activity, therefore uncovering the design principle driving waves of Clb activity and rationalizing a mechanism that leads to abnormal replication dynamics and genome instability in the absence of the inhibitor. The model presented can be reduced to a variation of the classical biochemical switch between an inhibitor I (Sic1) and an activator A (Clb) (Fig. 6A). In this view, the scheme follows the typical hybrid feedback loop

![Fig. 6. Negative-feedbacks loops of the signalling network. (A) Biochemical switch between an inhibitor I (Sic1) and an activator A (Clb). (B) Hybrid feedback loop motif of natural oscillations. The mutual inhibition between I and A results in a positive-feedback loop on A characteristic of double negative-feedback loops. Activating interactions are indicated by a normal arrow and repressing interactions are indicated by a barred arrow. Figure reproduced with permission from Barberis et al. [35].](image-url)
motif of oscillations found in nature [202], where double inhibition between $I$ and $A_i$ results in a positive-feedback loop on $A_i$ (Fig. 6B).

The final goal of systems biology is to obtain a quantitative description of cellular functions to elucidate complex human diseases such as cancer and neurodegenerative disorders. Small functional modules, such as the one we have described, are able to explain physiological behaviours and predict biochemical activities and new functional interactions by incorporating both experimental and theoretical approaches ranging from mathematical (deterministic and stochastic) modelling and computational simulation to protein–protein interactions (PPIs), biochemical assays, biophysical methods, structural modelling, time-resolved dynamics and live cell imaging (Fig. 7). The approach presented here provides a useful view of how synergy can be developed with the aim of improving our comprehension of specific network-driven physiological properties and thus the detailed mechanisms that control cell cycle progression.

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