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Structure based Functional Distinction between Cln1 and Cln2 Depends on the Ubiquitin-Proteasome Pathway

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Abstract

Cln1 and Cln2, G1/S cyclins of the ascomycetous budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), oscillate during the cell cycle, rising in late G1 and falling in early S phase. We have been tried to elucidate the structure basis of the functional distinction between Cln1 and Cln2. Here we performed *in silico* simulations: construction and evaluation of three dimensional structures of Cln1-Cdc28 and Cln2-Cdc28 complexes. Our *in silico* simulations suggested that the interaction of Cln1 and Cln2 with Cdc28 were in the two distinct situations, designated as flip and flop conformation, at the extra amino acid region in the cyclin box of Cln1 and Cln2. We speculated the trigger of this flip-flop conversion of the extra amino acid region in the cyclin box of Cln1-Cdc28 and Cln2-Cdc28 might be regulated by the ubiquitination of the sequences rich in Pro (P), Glu (E), Ser (S) and Thr (T), so-called PEST motifs, in Cln1 and Cln2. Furthermore, we presumed that the functional superiority between Cln1 and Cln2 in the G1/S phase of *S. cerevisiae* might be controlled by flip-flop conversion and ubiquitin-proteasome pathway.

Keywords: Cln1; Cln2; Cdc28; Ubiquitination; PEST; *In silico* simulation

Abbreviations: *S. cerevisiae*: *Saccharomyces cerevisiae*; Cdk: Cyclin-Dependent Kinase; Cdc28: Cyclin-dependent kinase of *Saccharomyces cerevisiae*; Cln3: G1 cyclin of *Saccharomyces cerevisiae*; Cln1, Cln2: G1/S cyclin of *Saccharomyces cerevisiae*; SBF: Swi4/6-dependent cell cycle Box Binding Factor; MBF: MluI-binding factor; PEST motif: Sequences rich in Pro (P), Glu (E), Ser (S) and Thr (T); 3D: Three Dimension; MD: Molecular Dyanamics; IE: Interaction Energy; EC: Electrostatic Complementarity

Introduction

The control system of cell cycle is the regulatory network that governs the order and timing of cell cycle events. The key components of the cell cycle regulation system are the cyclin-dependent kinase (Cdk) enzymes and their regulators of cyclins, which are assembled into a robust and versatile regulatory network that is responsive to a variety of intracellular and extracellular information [1-3]. Cyclins are highly conserved proteins that activate CDKs to regulate the cell cycle, transcription and other cellular processes. These different cyclin types are produced at different cell cycle stage to accumulate at specific times during the cell cycle, leading to waves of activation of distinct cyclin/Cdk complexes.

In the ascomycetous budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), a single Cdk (Cdc28), forms complexes with nine cyclins: G1 cyclin Cln3, G1/S cyclins Cln1 and Cln2, S cyclins Clb5 and Clb6, G2/M cyclins Clb1, Clb2, Clb3, and Clb4 [4,5].

As summarized in Figure 1, after *S. cerevisiae* cell is born, Cdc28-kinase complexes promote cell-cycle commitment once the cell reaches a critical size during late G1 phase; this commitment phase of the cell cycle has been termed 'START'. The timing of START, a major control point at the G1/S transition, in *S. cerevisiae* cells are controlled principally by G1 cyclin Cln3 and G1/S cyclins Cln1 and Cln2 [6]. Each of these three cyclins contains a sequence motif conserved among all of them, which is thought to mediate their association with the cell cycle regulatory kinase Cdc28 [7]. However, Cln3 is distantly related to Cln1 and Cln2, it differs markedly from them in its transcriptional regulation and biochemical activity [8]. Moreover, Cln3, its protein and kinase activity levels are no oscillation throughout the cell cycle, activates a transcription program mediated by the transcriptional factors SBF and MBF that regulate the rate of G1 progression in *S. cerevisiae* (Figure 1) [9-12].

On the other hands, Cln1 and Cln2 oscillate during the cell cycle, rising in late G1 and falling in early S phase [6,13-17]. Cln1 and Cln2, which are synthesized as a consequence of SBF activation wave, control the trigger of post-START processes such as budding, spindle pole body duplication, and the initiation of DNA replication (Figure 1) [18]. Finally, degradation of Cln1 and Cln2 is performed by the ubiquitination of the sequences rich in Pro (P), Glu (E), Ser (S) and Thr (T), so-called PEST motifs [19]. Thus, the extensive work carried out on G1/S cyclins has revealed an extraordinary similarity between Cln1 and Cln2. In addition to the numerous reports outlining the similarity between Cln1 and Cln2, some differences have been previously described [20,21].

In this study, we investigated the cell cycle control mechanism between Cln1 and Cln2 from a point of view of their structure-function relationship. We focused on the role of the extra amino acid region in the cyclin box of Cln1 and Cln2, which is typically observed in G1/S cyclins (Cln1 and Cln2) but not in G1 cyclin (Cln3). Moreover, we investigated the allosterically regulation of the extra amino acid region in the cyclin box of Cln1 and Cln2 by the ubiquitin-proteasome pathway. We also provide a structural mechanisms contributing to the functional distinction between Cln1 and Cln2.

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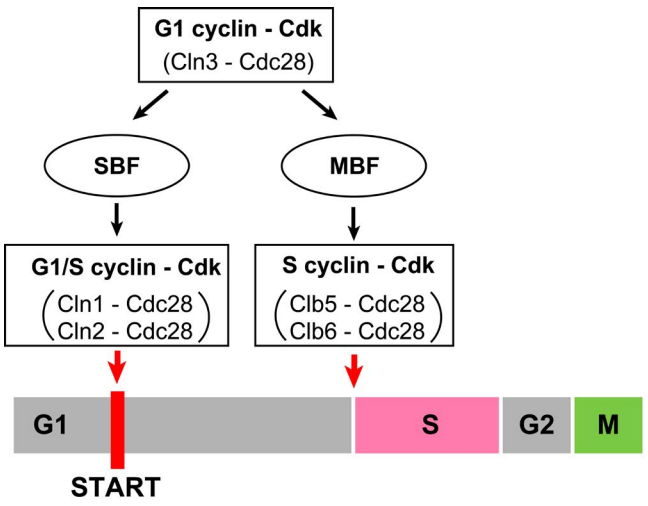

Figure 1: Overview of the cell cycle control system of *S. cerevisiae* G1/S phase.

Materials and Methods

Calculation of sequence identity

Protein sequence of Cln 1, Cln2, and Cln3 were obtained from the National Center for Biotechnology Information advances science and health by providing access to biomedical and genomic information (NCBI). Accession number of Cln1, Cln 2, and Cln3 are P20437.2: Full=G1/S-specific cyclin CLN1 (*Saccharomyces cerevisiae* S288c); P20438.2: Full=G1/S-specific cyclin CLN2 (*Saccharomyces cerevisiae* S288c), and P13365.2: Full=G1/S-specific cyclin CLN3 (*Saccharomyces cerevisiae* S288c), respectively. The amino acid sequence identity of the cyclin box among Cln1 (101-189), Cln2 (103-191), and Cln3 (137-202) were calculated with GENETYX (version 15, GENETYX CORPORATION, Tokyo, Japan)

Prediction of secondary structures

Protein secondary structure of the cyclin box of Cln1 (101-189), Cln2 (103-191) and Cln3 (137-202) were predicted with the protein secondary structure prediction server (Jpred3: http://www.compbio.dundee.ac.uk/www-jpred/).

Prediction of PEST motif

PEST motif of Cln1 and Cln2 were predicted by the calculation with GENETYX Software for PEST sequnce (version 15, GENETYX CORPORATION, Tokyo, Japan) with the consideration of the report by Hadwiger et al. [6].

Molecular modeling and evaluations

The three dimensional structures of Cln1-Cdc28, Cln2-Cdc28, and Cln3-Cdc28 were constructed with homology model in MOE (version 2009, CCG Inc., Montreal, Canada) according to the Brookhaven Protein Databank 1E9H. The molecular mechanics calculations were performed to obtain the local minimum structure using amber99 force field in MOE.

Molecular dynamics (MD) simulations were performed using the AMBER9 package with the modified TIP3P water potential (CONFLEX, Tokyo, Japan). The starting structures, the two distinct conformations (flip state and flop state) at the extra amino acid region in the cyclin box of Cln1 and Cln2, were placed in a pre-equilibrated solvent box consisting of water and sodium ions, where the number of sodium ions was adjusted to neutralize the systems. The solvent box was extended 10 Å beyond the Cln1-cdc28 and Cln2-cdc28 atoms. MD simulations were performed on the resulting systems for 1000 ps at 1 atm and 300 K using the isobaric-isothermal (NPT) ensemble. The integration time step was 2 fs, and the SHAKE algorithm was used to constrain all covalent bonds involving hydrogen atoms. The long-range electrostatic interactions beyond the cutoff distance were calculated using the particle mesh Ewald method.

To calculate interaction energy and electrostatic complementarities, we used MOE and MolFeat-EC (FiatLux, Tokyo, Japan) as previously described with some modifications [22-25]. The three-dimensional structures of these complexes were displayed using MolFeat (Ver. 4, FiatLux, Tokyo, Japan).

All figures from the MD simulations of Cln1-cdc28, Cln2-cdc28, and Cln3-cdc28 complexes were produced using VMD (http://www.ks.uiuc.edu/Research/vmd/).

Ligand Interaction of PEST motifs of Cln1-Cdc28 and Cln2-Cdc28 were calculated and displayed with MOE.

Results and Discussion

The cell cycle is controlled by a series of biochemical switches that trigger the events of the cycle in the correct order [1]. Cln1 and Cln2, G1/S cyclins of the ascomycetous budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), contribute to the control of new cell-cycle entry in response to growth or extracellular factors [6]. To clarify the structure-function relationship of G1/S cyclins of *S. cerevisiae*, we performed *in silico* simulations [26]: construction of three dimensional (3D) structure by homology modeling [27,28], optimization of 3D structure by molecular mechanics calculations and molecular dyanamics (MD) simulations [29], evaluation of interaction energy (IE) [30] and electrostatic complementarity (EC) [22] of Cln1-Cdc28 and Cln2-Cdc28 complexes. Additionally, we also performed *in silico* simulations for Cln3-Cdc28 to elucidate the structural differences between G1 and G1/S cyclins (Table 1).

Construction of 3D structures of Cln1-Cdc28 and Cln2-Cdc28

To sufficiently analyze the structural properties of G1/S cyclins of *S. cerevisiae*, we constructed the 3D structure of Cln1-Cdc28 and Cln2-Cdc28.

Prior to perform the construction of the 3D structure of them, we checked the sequence alignment and the secondary structure of G1/S cyclins Cln1 and Cln2. They have an identity of 57% at sequence level. However, the N-terminal regions of them, which contain the cyclin box (about 100 a.a.), are retained high similarity (74% identity). Furthermore, as shown in Figure 2a, Cln1 and Cln2 characteristically possessed the extra amino acid region (21 a.a.) in the cyclin box region among the G1 and G1/S cyclins of fungi, such as *A. fumigatus* Cln1, *C. neoformans* Cln1, *S. pombe* Puc1, etc. [31]. On the other hand, conservation is relaxed in their C-terminal regions (45% identity). This

	Interaction Energy (kcal/mol)	EC
Cln1 (flip) - Cdc28	- 745.2	0.50
Cln1 (flop) - Cdc28	- 836.1	0.54
Cln2 (flip) - Cdc28	- 672.8	0.51
Cln2 (flop) - Cdc28	- 875.7	0.54

Table 1: Interaction Energy and Electrostatistic Complementarity of Cln1-Cdc28, and Cln2-Cdc28.

would suggest that the elements responsible for the distinction between both cyclins (for instance, specific protein-interacting domains) could be located in the C-terminal portions of the proteins.

Subsequently, we predicted the secondary structure of the cyclin box of these cyclins. Figure 2b shows that the extra amino acid region in the cyclin box of Cln1 and Cln2 are proposed to be a linear structure, partially beta-sheet was included in Cln2 [32].

Taken together these information, we have constructed the 3D structure of Cln1-Cdc28 and Cln2-Cdc28 by using protein homology modeling applications in MOE: 1) homology sequence analysis, 2) multiple-structure alignment and superposition, 3) structural homologue identification, and 4) 3D model building.

Notably, the resulted 3D structure of Cln1-Cdc28 and Cln2-Cdc28 were demonstrating the two distinct conformations at the extra amino acid region in the cyclin box of Cln1 and Cln2.

As shown in Figure 3a-1 and 3b-1, the one conformation represented the extra amino acid region in the cyclin box of Cln1 and Cln2 did not bind with Cdc28, designated as the flip state. On the other hand, the other conformation represented the extra amino acid region of them bind with Cdc28, designated as the flop state (Figure 3a-2 and 3b-2). We intuited that these two distinct conformations might be playing an important role to carry out the function of G1/S cyclins function of *S. cerevisiae*. Therefore, we performed MD simulations for the flip and flop conformation of Cln1-Cdc28 and Cln2-Cdc28 to observe the state of the structural change, respectively.

In fact, prior to MD simulations, we presumed that the extra amino acid region in the cyclin box were in the equilibrium between flip and flop conformation. However, the results of MD simulations were deviated from our expectation. We did not observe any typical structural change, such as the flip-flop conversion, during the course of the 1,000 ps MD simulations (Supplementary Figure 1).

In addition, we also performed *in silico* simulation of Cln3-Cdc28 and summarized in Supplementary Figure 2.

Evaluation of 3D structures of Cln1-Cdc28 and Cln2-Cdc28

To better understand the flip-flop conversion of the extra amino acid region in the cyclin box of Cln1 and Cln2, we checked the interaction energy (IE) and electrostatic complementarity (EC) of the most stable complex structure of Cln1-Cdc28 and Cln2-Cdc28 during MD simulations.

The IE values of Cln1-Cdc28 in flip and flop conformation was -745.2 kcal/mol and -836.1 kcal/mol, respectively. On the other hand, that of Cln2-Cdc28 was -672.8 kcal/mol and -875.7 kcal/mol respectively. Moreover, the energy gap in IE values between flip and flop conformation of Cln1-Cdc28, ΔE(Cln1-Cdc28), and Cln2-Cdcx28, ΔE(Cln2-Cdc28) were 90.9 kcal/mol and 202.9 kcal/mol, respectively. Therefore, we suspected that the flip-flop conversion is easily induced on Cln1-Cdc28, ΔE(Cln1-Cdc28): 90.9 kcal/mol, rather than Cln2-Cdcx28, ΔE(Cln2-Cdc28): 202.9 kcal/mol. These results presumably indicated that the role of flop conformation is to stabilize Cln1-Cdc28 and Cln2-Cdc28 complexes for carrying out G1/S cyclins function. Furthermore, it is consistent with *in vitro* results that Cln2-Cdc28 plays a major role as G1/S cyclins of *S. cerevisiae* than Cln1-Cdc28 [6].

In addition to calculation of IE, we investigated EC for quantitative analysis of the protein-protein interaction domain of G1/S cyclins of *S. cerevisiae*. Figure 4 depicted the electrostatic potential on the molecular surfaces buried in the interface between the two proteins of the complex as a set of folding doors presentation (image of open book). In accordance with our observation, the EC value of Cln1-Cdc28 in flip and flop state was 0.50 and 0.54, respectively. On the other hand, that of Cln2-Cdc28 was 0.51 and 0.54, respectively. Indeed, the symmetricalness of the electrostatic potential (blue; positive charge, red; negative charge) on the protein-protein interface domain of flop state of Cln1-Cdc28 and Cln2-Cdc28 were higher than that of flip state. We presumed that these gap of the electrostatic potential between flip and flop conformation were induced by the high EC of the extra amino acid region in the cyclin box of Cln1 (0.68) and Cln2 (0.63). Taken together these results, it seems difficult to induce the flip-flop conversion on Cln1-Cdc28 and Cln2-Cdc28 without any driving forces.

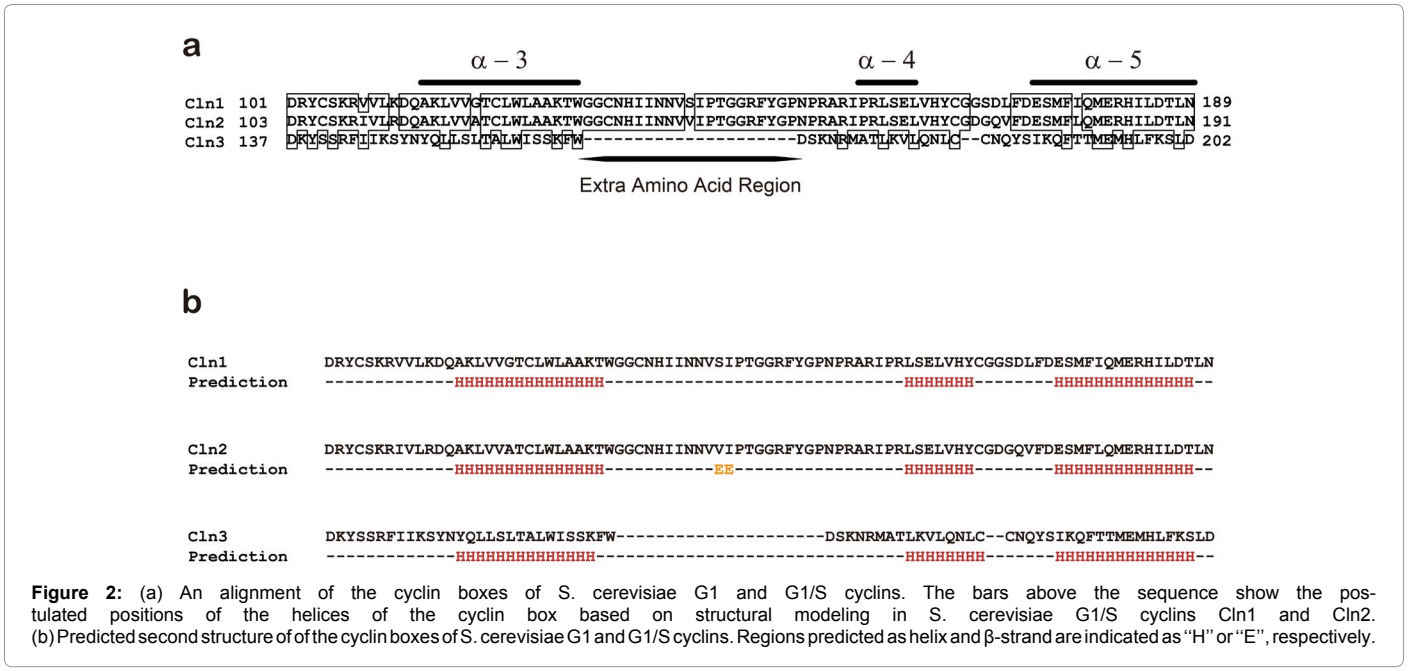

Figure 2: (a) An alignment of the cyclin boxes of S. cerevisiae G1 and G1/S cyclins. The bars above the sequence show the postulated positions of the helices of the cyclin box based on structural modeling in S. cerevisiae G1/S cyclins Cln1 and Cln2. (b) Predicted second structure of of the cyclin boxes of S. cerevisiae G1 and G1/S cyclins. Regions predicted as helix and β-strand are indicated as "H" or "E", respectively.

Additionally, the IE and EC value of Cln3-Cdc28 are -653.6 kcal/mol and 0.59, respectively (Supplementary Figure 2). IE value of Cln3-Cdc28 (-653.6 kcal/mol) is lower than that of Cln1(flop)-Cdc28 (-836.1 kcal/mol) and Cln2(flop)-Cdc28 (-875.7 kcal/mol). On the other hand, EC value of of Cln3-Cdc28 (0.59) is also lower than that of Cln1-Cdc28 (0.68: the extra amino acid region) and Cln2-Cdc28 (0.63: the extra amino acid region). The difference of these values among them is presumably due to the existence of the extra amino acid region in the cyclin box which is possessed in Cln1 and Cln2. Moreover, these results showed coincidence with the functional difference of among them that Cln3 is a very-low-abundance protein with a much weaker associated kinase activity relative to Cln1 and Cln2 [9].

Conversion and Degradation of Cln1-Cdc28 and Cln2-Cdc28

It has been proposed that the oscillation of G1/S cyclins of *S. cerevisiae* Cln1 and Cln2 during the cell cycle is controlled by the rapid degradation of them through the ubiquitination of the sequences rich in Pro (P), Glu (E), Ser (S) and Thr (T), so-called PEST motifs [6]. Here, we hypothesized that the flip-flop conversion of Cln1-Cdc28 and Cln2-Cdc28 might be regulated allosterically through the ubiquitination of G1/S cyclins of *S. cerevisiae* Cln1 and Cln2 [19,33-35].

To sufficiently characterize the structure-function relationship of the PEST motifs of G1/S cyclins of *S. cerevisiae* Cln1 and Cln2, we predicted the location of them on the 3D structure of Cln1 (35-546, flop conformation)-Cdc28 and Cln2 (37-534, flop conformation)-Cdc28. As shown in Figure 5a, two PEST motifs were predicted in Cln1. One is located near the Cdc28 binding domain in the cyclin box that is close to the N-terminal domain (233-274) and the other is located on the C-terminal domain (398-425). On the other hand, as shown in Figure

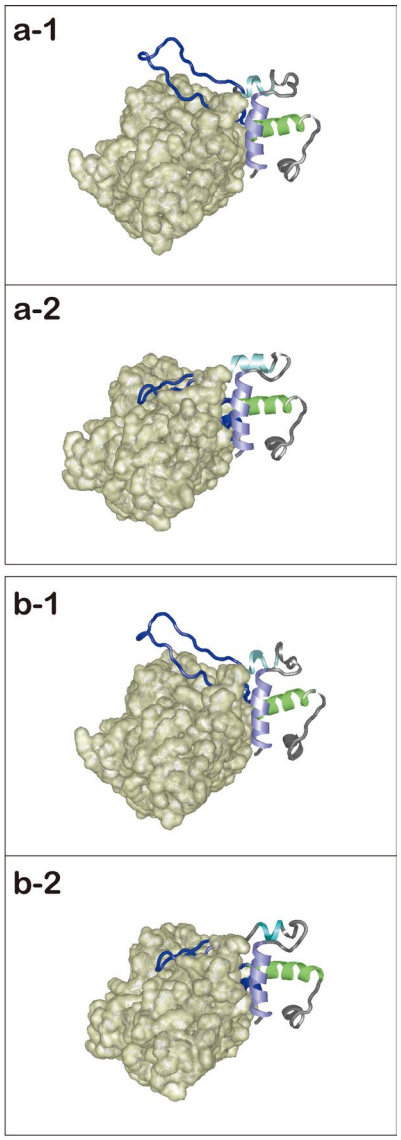

Figure 3: Molecular interaction models of (a-1) the flip state, (a-2) the flop state of Cln1(101-189)-Cdc28(5-298), (b-1) the flip state, (b-2) the flop state of Cln2(103-191)-Cdc28(5-298). Spacefilling model with brown is Cdc28. Ribbons are Cln1 and Cln2. Regions as the extra amino acid region, α-3, α-4, and α-5 helix are indicated as blue, green, cyan and purple, respectively.

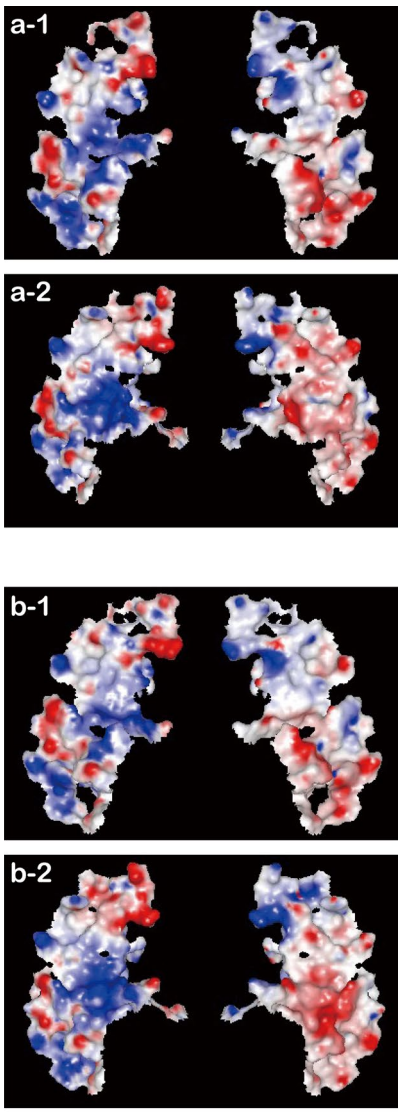

Figure 4: Electrostatic potential on the molecular surfaces buried in the interface between Cln1/2 and Cdc28. Electrostatic Complementarities on the buried molecular surface of (a-1) the flip state of Cdc28 (left) and Cln1 (right), (a-2) the flop state of Cdc28 (left) and Cln1 (right), (b-1) the flip state of Cdc28 (left) and Cln2 (right), (b-2) the flop state of Cdc28 (left) and Cln2 (right) generated by its own charges. Blue is positive, white is neutral and red is negative charged region.

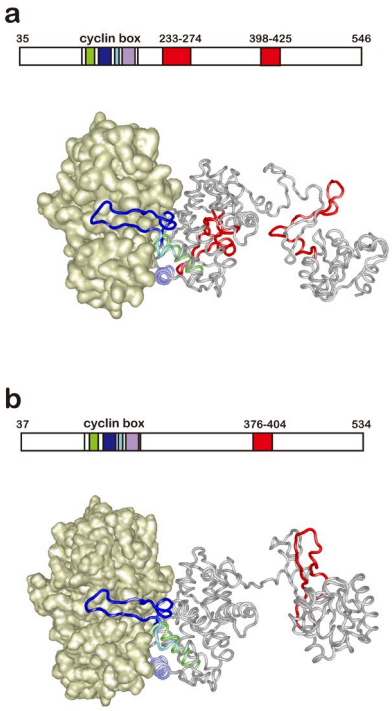

Figure 5: Schematic models and the flop state molecular interaction models of (a) Cln1 (35-546)-Cdc28 (5-298) and (b) Cln2 (37-534)-Cdc28 (5-298). Ribbon; Cln1/2, Spacefilling; Cdc28. Gray region in schematic models is the cyclin box (Cln1; 101-189, Cln2; 103-191), blue and red in both schematic models and the flip state molecular interaction models is the extra amino acid region and the PEST motif, respectively.

5b, only one PEST motif was predicted in the C-terminal domain (376-404) of Cln2.

Then, to examine the ligand interaction circumstances of the PEST motifs of G1/S cyclins of *S. cerevisiae* Cln1 and Cln2, we predicted the hydrophilicity and ligand interaction networks of the PEST motifs. As a consequence every PEST motifs possessed hydrophilic region where expose to solvent (Supplementary Figure 3). Therefore, we presumed that E3 ubiquitin ligase can recognize and access each PEST motifs equally. From this result, it was assumed that flip-flop conversion, a drastic structural change of the extra amino acid region in the cyclin box, could be easily proceed by the ubiquitination of the PEST motif in the N-terminal domain of Cln1. Taken together, according to the location and the structural features of the PEST motifs of G1/S cyclins of *S. cerevisiae* Cln1 and Cln2, multi PEST motifs of Cln1 might become predominant on degradation by the ubiquitin-proteasome pathway compared with a single PEST motif of Cln2 [35].

As summarized in Figure 6, we speculate that Cln1 will be rapidly shifted from flop to flip by ubiquitination of the PEST motif and will be destabilized subsequently. On the other hand, the PEST motif of Cln2 is located in C-terminal domain. Therefore, the extra amino acid region in the cyclin box of Cln2 might be less affected by ubiquitination and produce the modest degradation [33]. These results might be consistent with *in vitro* results that Cln2-Cdc28 plays a major role as G1/S cyclins of *S. cerevisiae* than Cln1-Cdc28 [6]. It is inferred from these observations that the flip-flop conversion by the ubiquitination of the PEST motif plays a dominant role in oscillation of Cln1-Cdc28 and Cln2-Cdc28 function during the cell cycle.

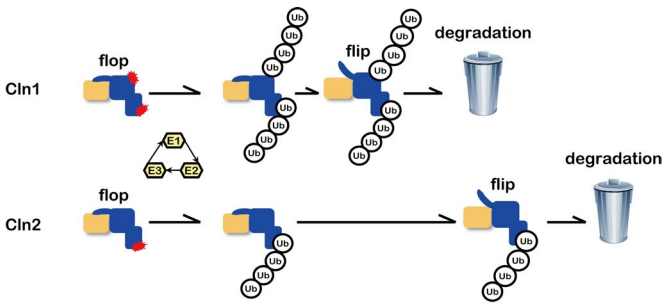

Figure 6: Model of the flip-flop regulation mechanism of Cln1 and Cln2 during late G1/S phase Cln1/2 in blue, Cdc28 in brown, and the PEST motif in red.

Conclusion

In conclusion, our research may be deciphering the role of the extra amino region in the cyclin box of G1/S cyclins of *S. cerevisiae*, which, until now, to our knowledge, has not been assigned significance in cyclins functions and structural analysis.

We insinuated that the flip-flop conversion of the extra amino region might be one of the key characteristics functions of the G1/S cyclins of *S. cerevisiae*, which controls the ocillation during cell cycle in G1/S cyclins (Cln1 and Cln2) but not in G1 cyclin (Cln3).

Furthermore, we speculate that the functional superiority between Cln1 and Cln2 in the G1/S phase of *S. cerevisiae* might be regulated by the flip-flop conversion by the ubiquitination of PEST motifs. However, Cln1 and Cln2 share an overlapping role in execution of G1/S function, Cln2 could be a major regulatory element of G1/S phase control because of the higher flip-flop conversion energy and the modest degradation through the ubiquitin-proteasome pathway [6,21].

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