Integrating Cdk signaling in *Candida albicans* environmental sensing networks

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Abstract

Cyclin-dependent protein kinases (Cdks) control cell cycle progression and morphological switches in eukaryotic cells. Based on recent findings concerning the evolution of Cdk phosphorylation sites in the Ascomycete linage, we shall analyze the density of Cdk motifs in the *Candida* proteome using the S_{LR} algorithm, focusing on protein sequences of regulatory modules that play important roles in the environmental sensing of *C. albicans*. Since Cdks are also involved in morphogenesis and environmental signaling, this search could help us to speculate about how Cdk signaling might be integrated in these regulatory networks that control *C. albicans* morphopathogenic determinants.

Table of contents

1. Introduction

In eukaryotic cells, global regulatory networks control cell physiology in response to external and internal cues. Many of these signaling networks are highly modular, making them more evolvable and providing increased fitness in competitive and changing environments (Bhattacharyya et al. 2006). Fungi are able to colonize and occupy highly divergent niches, ranging from high osmotic environments to plants and mammalian hosts. Their ability to adapt to these hostile environments depends on their capability to sense a variety of external cues, transduce the signals to specific cytoplasmic targets, and activate the appropriate responses.

Unlike the majority of fungi, the Ascomycete *Candida albicans* sis normally found as a commensal in the gastrointestinal tract of humans and warm-blooded animals. Although generally asymptomatic, *C. albicans* can cause mucosal infections in healthy people. In patients with a deficient immune system, such as HIV-compromised individuals or patients treated with immunosuppressive drugs after organ transplantation, this yeast can produce systemic infections in which the fungus can spread to all major organs of the body, leading to death in around 50% of bloodstream infections (Eggimann et al., 2003; Gudlaugsson et al., 2003).

C. albicans has several attributes that allow it to adapt rapidly to changing environmental signals that contribute to host colonization. One of those best studied is the ability to switch between different morphologies, such as yeast, pseudohyphae and hyphae (Sudbery et al. 2004). The yeast-to-hypha transition, triggered by a wide range of environmental cues, is regulated by multiple signaling pathways that control the transcription of a set of hypha-specific genes (HSGs), many of which encode known virulence factors (Biswas et al. 2007; Calderone and Fonzi 2001; Whiteway and Bachewich 2007). The promoter regions of HSGs integrate the signals of multiple activators and repressors, although the interplay between them remains largely unknown. The two major transcriptional activators are Cph1 and Efg1 (Liu et al. 1994; Stoldt et al. 1997), which act downstream of the MAPK and the cAMP-PKA pathways, respectively. The double mutant *cph1*Δ *egf1*Δ blocks hyphal transitions under most conditions tested and shows reduced virulence (Lo et al. 1997). Negative regulation of hyphal growth is achieved through the combinatorial association of the Tup1 repressor with the DNA-binding proteins (DBPs) Nrg1, Rfg1 and Mig1 (Braun et al. 2000; Braun and Johnson 1997; Kadosh and Johnson 2005; Murad et al. 2001a; Murad et al. 2001b). Cells depleted of any of these repressors are able to activate hyphal growth under yeast-growth conditions. Since Mig1-Tup1 regulate a set of genes other than those regulated by Nrg1-Tup1, it has been suggested that DBPs target Tup1 to specific subsets of genes. However, Mig1 and Nrg1 can also repress the expression of other genes in a Tup1-independent manner (Murad et al. 2001a), suggesting that the DBPs associated with Tup1, (rather than Tup1 itself) are likely to be regulated

during the yeast-to-hypha transition (Braun et al. 2000; Braun and Johnson 1997; Kadosh and Johnson 2005; Murad et al. 2001a; Murad et al. 2001b).

Another example of the enormous plasticity of *C. albicans* cells is white-opaque (W/O) switching. This fungus undergoes an epigenetic switch between two cell types, known as white and opaque (Slutsky et al. 1987). These cell types differ in their cell morphology, metabolic state and mating behavior, and in their ability to form biofilms, their preferred niches in the host and interactions with the immune system. In recent years, new insights into the mechanisms that control this transition have allowed researchers to uncover complex relationships that relate switching, mating and pathogenesis (Lohse and Johnson 2009; Soll 2009).

Although *Saccharomyces cerevisiae* and *C. albicans* share some common features, they also exhibit many significant differences since they diverged from a common ancestor more than 500 million years ago. The shared evolutionary fate of *C. albicans* and its hosts might have allowed it to evolve several developmental programs that would be activated by environmental cues within the host and that could aid in the colonization of different niches. These colonization sites represent different environments in terms of cohabitant microbiota, pH, nutrients and O_2 or CO_2 levels. Thus, this distinct life style might have been a driving force in rewiring *C. albicans* signaling networks (Li and Johnson 2010).

2. Evolution of phosphoregulation

Protein phosphorylation is a ubiquitous and reversible modification that is crucial for the regulation of cellular events (Seet et al. 2006). Comparative studies of the phosphoproteome of three yeast species (*C. albicans*, *S. cerevisiae* and *Schizosaccharomyces pombe*) suggest that protein kinases probably contribute to a substantial extent to the evolution and generation of phenotypic diversity (Beltrao et al. 2009).

An important feature of *C. albicans* is that the CUG codon is decoded as Ser instead of Leu (Santos and Tuite 1995), with an average frequency of 1-6 CUGs per gene (Butler et al. 2009; Massey et al. 2003). Since Ser is a substrate of protein kinases, this change in codon usage might increase the number of potential phosphorylation sites per protein, adding new possible layers of phosphoregulation to protein networks. In fact, the ratio of phosphoserines/total proteins determined by mass spectrometry (MS) analysis in *C. albicans* is higher (0.54) than in *S. cerevisiae* (0.39) and *S. pombe* (0.32) (Beltrao et al. 2009).

Protein kinases regulate the function of their target proteins by adding a phosphate group to specific sites, which can change the activity of the protein through two different mechanisms (Holt et al. 2009). First, phosphorylation could drive a precise conformational change in the structure of the protein because the phosphate modifies the network of hydrogen bonds of several neighboring amino acids. This type of regulation, common in metabolic enzymes, is highly context-

dependent and exhibits strong evolutionary conservation. Alternatively, the addition of phosphates to disordered regions (either the N- or C-termini or internal loops) of substrates can modify their interaction with other proteins (Serber and Ferrell 2007; Strickfaden et al. 2007) or can create new interactions through the phosphopeptide-binding modules present in other molecules, such as the SH2, 14-3-3, or WW domains (Bhattacharyya et al. 2006; Morrison 2009; Seet et al. 2006). In these cases, the position of the phosphoacceptor residue(s) is less context-dependent and therefore it can undergo a higher rate of change and a greater potential to generate functional diversity (Beltrao and Serrano 2007).

Cyclin-dependent protein kinases (Cdks) control progression along the eukaryotic cell cycle. These proteins are proline-directed kinases that preferentially phosphorylate substrates with the full consensus sequence S/T-P-X-K/R (where X is any amino acid), although they can also phosphorylate the minimal consensus sequence S/T-P (Echalier et al. 2010; Songyang et al. 1994). In this chapter, based on recent findings concerning the evolution of Cdk phosphorylation sites in the Ascomycete linage (Holt et al. 2009), we analyze the density of Cdk motifs in the *C. albicans* proteome using the S_{LR} algorithm (Moses et al. 2007a) and focusing on proteins that are components of regulatory modules that play important roles in environmental sensing. Since Cdks are also involved in morphogenesis and environmental signaling (Huang et al. 2007; Moseley and Nurse 2009; Wang 2009), this search could help us to speculate about how Cdk signaling might be integrated in the regulatory networks that control morphopathogenic determinants in *C. albicans.*

3. Cyclin-dependent kinases in C. albicans

In yeast, cell cycle progression is driven by a single Cdk1 (Cdc28 in *S. cerevisiae* and *C. albicans*, Cdc2 in *S. pombe*). The combinatorial association of Cdk1 with G1 or G2 cyclins is thought to generate Cdk complexes with different substrate specificities that regulate different cell cycle transitions (Bloom and Cross 2007; Loog and Morgan 2005). However, phylogenetic studies of yeast B-type cyclins and experimental yeast models are consistent with the idea of an ancestral eukaryote with a single Cdk/cyclin module driving the cell cycle (Archambault et al. 2005; Coudreuse and Nurse 2010; Fisher and Nurse 1996). It is likely that the appearance of multiple cyclins in most eukaryotic lineages would have introduced new regulatory layers to fine-tune the single core Cdk module, providing more flexibility in the control of the cell cycle in response to different inputs (Bloom and Cross 2007; Coudreuse and Nurse 2010; Loog and Morgan 2005).

In addition to driving the cell cycle, Cdks coordinate cell morphology switches (Moseley and Nurse 2009; Wang 2009). In *C. albicans*, modifications of cyclin levels produce dramatic morphological changes. This fungus contains three G1 (Ccn1, Hgc1 and Cln3) and two G2 (Clb2 and Clb4) cyclins. Depletion of Cln3,

Clb2 or Clb4 in yeast cells results in hyperpolarized growth in the absence of hypha-inducing conditions (Bachewich and Whiteway 2005; Bensen et al. 2005; Chapa y Lazo et al. 2005). Ccn1 is a non-essential G1 cyclin that is expressed during the G1/S transition and is required for the maintenance, but not the initiation, of hyphal growth under certain conditions (Loeb et al. 1999; Sinha et al. 2007). Hgc1 is a hypha-specific G1 cyclin-like protein that preferentially localizes to the dividing apical cell of the hyphae (Wang et al. 2007; Zheng and Wang 2004). Transcription from the *HGC1* promoter is essential for this asymmetric cell localization, since Hgc1 no longer exhibits the preferential apical accumulation when expressed under the control of the *MAL2* promoter (Wang et al. 2007). Deletion of *HGC1* prevents hyphal growth under all hypha-inducing conditions and results in reduced virulence in mouse models (Zheng and Wang 2004). Unlike the cell-cycle regulated transcription of other cyclin genes, *HGC1* expression is activated by hypha-inducing signals through the cAMP/PKA signaling pathway. The evolution of such control would probably have been crucial for ensuring the cell-cycle independent polarized growth of hyphae (Wang 2009; Zheng and Wang 2004). In agreement with this hypothesis, the expression of one allele of *CCN1* under the control of the *HGC1* promoter rescues the *hgc1*Δ mutant (P. Gutiérrez-Escribano and J. Correa-Bordes, unpublished results).

In recent years, insight into the links between Cdks and cell polarity proteins has been obtained in *C. albicans*, highlighting the importance of Cdk1 complexes in the control of cell morphogenesis during yeast and hyphal growth (González-Novo et al. 2008; Sinha et al. 2007; Wang et al. 2009; Zheng and Wang 2004; Zheng et al. 2007). An excellent summary of the role of Cdks in the yeast-hyphal transition of *C. albicans* has been published recently (Wang 2009).

4. Predicting Cdk targets in C. albicans proteins

Past efforts aimed at the identification of HSGs have underlined the importance of the cAMP-PKA and MAPK pathways in the transcriptional activation required for hyphal growth. However, recent findings have suggested that post-translational modifications mediated by Cdks are also important mechanisms in the regulation of polarized growth immediately after hyphal induction, independently of the cAMP-PKA and MAPK pathways (Sinha et al. 2007). These results suggest the existence of an additional signaling pathway(s) that plays a major role in the control of hyphal development, which is mediated by the Cdk-phosphorylation of key regulatory proteins in response to hypha-inducing signals.

4.1. Lessons from *S. cerevisiae*

Global analysis of the Cdk1-dependent *S. cerevisiae* phosphoproteome identified the position of 547 phosphorylation sites on 308 proteins, based on the specific chemical inhibition of Cdk1 and quantitative MS (Holt et al. 2009). Study of the structural context of the Cdk1 sites revealed some interesting features. First, more than 90% of the sites were located in loops and disordered regions. Second, Cdk1 substrates tended to be phosphorylated at multiple sites. Finally, Cdk1 phosphorylation sites tended to cluster in the primary sequence, suggesting that multiple phosphorylation events would modulate the same protein surface. Notably, comparisons of the substrates with their orthologs in another 32 Ascomycetes showed that the position of most of the phosphorylation sites was not highly conserved; instead, they shifted position inside the rapidly evolving disordered regions (Holt et al. 2009). In sum, although the minimal Cdk1 phosphorylation motif (S/T-P) is conserved over long evolutionary timescales, the regions containing them show rapid evolution. Thus, these features allow Cdk1 control mechanisms to adapt rapidly to developmental challenges that have arisen or may arise during the course of evolution. This flexibility in phosphorylation site positioning might have important biological implications, since the appearance of a linage-specific Cdk cluster in a protein could give rise to new regulatory controls. For example, it has been suggested that the Cdk-driven regulation of nuclear localization of the pre-Replicative Complex component Mcm3 could have appeared in the *S. cerevisiae* linage after its divergence from *C. albicans* through the acquisition of Cdk sites clustered at the ScMcm3 C-terminus (Moses et al. 2007b).

A new computational strategy aimed at identifying proteins that contain high densities of strong (S/T-P-X-R/K) and weak (S/T-P) Cdk consensus sites closely spaced in the amino acid sequence has recently been developed (Moses et al. 2007a). This method allows the identification of proteins in which Cdk clusters deviate from random expectation by calculating the likelihood ratio statistic (S_{LR}). This cluster-based method measures the enrichment of motifs in a sequence and their spatial clustering. In order to define a S_{LR} cut-off value to use in the prediction of Cdk substrates, a comparison of the distribution of S_{LR} scores using either the real Cdk consensus motifs or scrambled versions (P-R/K-X-S/T and P-S/T) was performed and a score threshold of 3.5 was defined. Therefore, cluster-based methods used in combination with other evidence, such as structural properties (Iakoucheva et al. 2004) or evolutionary conservation (Budovskaya et al. 2005), could be exploited to predict Cdk targets (Moses et al. 2007a).

4.2. Prediction of Cdk targets in the *C. albicans* proteome

Given the rapid evolution of Cdk phosphorylation site positioning in the disordered regions of proteins, we propose a speculative model whereby the commensal life style of *C. albicans* might have led Cdk evolution to be connected to a much broader range of signaling pathways than in *S. cerevisiae*. This would have allowed the integration of Cdk-control mechanisms with the different develop-

mental programs triggered by environmental cues found in the host. This hypothesis would imply the existence of *Candida* linage-specific Cdk clusters in proteins involved in the response to environment signals, such as cell signaling proteins and transcriptional regulators. To test this hypothesis, we searched the *C. albicans* proteome for proteins containing putative regulatory Cdk clusters using the S_{LR} algorithm. In order to reduce the number of false positives, a second criterion was used; this was that Cdk clusters had to be located in disordered regions, as determined by the PONDR algorithm (www.pondr.com). Finally, to test whether the identified putative regulatory clusters were linage-specific, we compared such regions with their ortholog proteins of other Hemiascomycota species

To identify putative Cdk targets in *C. albicans*, we analyzed the proteome using the S_{LR} algorithm for the presence of clusters of strong and weak Cdk motifs and used a threshold S_{LR} score of 3.5 (named S_{LRF} analysis). This analysis identified 91 proteins with an S_{LR} value above the cut-off, which represents the 1.46% of the total proteins (Fig. 1, inset). Of these 91 predictions, 52 of them had orthologs in *S. cerevisiae*. Gene Ontology (GO) analysis of the putative substrates revealed a strong enrichment for cell cycle-related functional categories (20/52; 38%). In addition, 34 of them (63%) showed Cdk1-dependent phosphorylation in *S. cerevisiae* or *C. albicans* (Beltrao et al. 2009; Holt et al. 2009; Ubersax et al. 2003). Accordingly, the value of 3.5 seemed to be a good threshold when strong and weak Cdk motifs were used. However, given the existence of Cdk substrates lacking strong consensus sites regulated by Cdk phosphorylation at weak sites (Nash et al. 2001; Strickfaden et al. 2007), we performed a second analysis searching for clustering of weak motifs only (named S_{LRW} analysis). 267 proteins (4.3% of the proteome) with a score above 3.5 were identified. To reduce the number of false positives, the threshold was increased to 4.8, a score at which the enrichment for cell cycle-related proteins was 24%; similar to that obtained in the identification of Cdk1 targets in *S. cerevisiae* (Holt et al. 2009). This reduced the set to 175 positive proteins (2.68%), which included 42% of the proteins identified in the S_{LRF} analysis (Fig. 1). In sum, the combination of S_{LRF} and S_{LRW} allowed the identification of 228 proteins, representing the 3.6% of the *C. albicans* proteome. GO analysis revealed that in addition to cell cycle-related processes there was an enrichment in proteins belonging to other cellular processes, such as filamentous growth (33/228, 14.5%) or transcription regulation (27/228 11.8%). A selection of putative Cdk1 substrates grouped by GO cellular component is shown in Fig. 1. Notably, we found that proteins involved in environmental sensing were also present in the predicted Cdk substrates. Environmental sensing is a complex process that includes several steps, and putative regulatory targets were found in proteins involved in cell wall regulation (CWR), transcriptional control and cell signaling. In the following sections, we shall describe some examples to illustrate how Cdk-control mechanisms could have been integrated with other signaling pathways in *Candida* and that are different from those seen in *S. cerevisiae*.

4.2.1. Transcriptional regulation

Transcription factors constitute one of the gene families enriched in pathogenic *Candida* species (Butler et al. 2009). In our analysis, we found several transcriptional regulators involved in environmental responses (Fig. 2A), suggesting that Cdk signaling could regulate their activity. These factors are: Sfu1 and Hap43 (iron response); Dal81 and Hsf1 (heat response); Mrr1 and Ndt80 (drug resistance); Ace2 (biofilm); Sfl1 (caspofungin response) and Mig1 and Nrg1 (yeast-to-hypha transition). Comparison with their *S. cerevisiae* orthologs showed that five of them -Hap43, Ndt80, Hsf1, Ace2 and Nrg1- have at least one cluster with five or more Cdk sites in regions that are not present in *S. cerevisiae*. This suggests that their regulation could have additional layers of complexity in *Candida*. As an example, an alignment of the N-terminal region of *C. albicans* Hap43 with *S. cerevisiae* Hap4 is shown in Fig. 2B. This region contains the CBC domain that is required for the interaction with the Hap2/3/5 complex (Bourgarel et al. 1999), which is conserved in both proteins. Interestingly, in *C. albicans* the CBC domain is located in a disordered region predicted by PONDR (1 to 128), which also contains 6 weak Cdk sites not present in *S. cerevisiae*, suggesting that Cdk1 might modulate the interaction of Hap43 with the complex. Iron homeostasis is essential for microorganisms, such as *C. albicans*, that compete for iron in a mammalian host. Hap43 is essential for iron-responsive transcriptional regulation and virulence in *C. albicans* (Hsu et al. 2011). Iron regulatory networks have undergone a differential evolution since *S. cerevisiae* and *C. albicans* diverged, regulation in each yeast species being adapted to their specific growth conditions.

4.2.2. Cell wall regulation

In addition to its protective role, the cell wall plays an important function in interaction with the environment, both in sensing external cues and in interacting with other cells. There is a significant expansion of cell wall gene families in pathogenic species, suggesting that cell wall regulation could be important for virulence (Butler et al. 2009). Recently, phenotypic analysis regarding sensitivity to cell wall stresses of a collection of protein kinase mutants has shown that cell wall signaling networks in *C. albicans* are expanded in comparison to those of *S. cerevisiae*, and that some signaling pathways have been rewired and integrated in the cell wall integrity response in *Candida* (Blankenship et al. 2010). Five kinases related to the CWR were identified in our analysis (Fig. 2A): three proteins (Bck1, Gin4, and Mob2, this latter being the regulatory subunit of the Cbk1-Mob2 complex) with conserved roles in CWR in *C. albicans* and *S. cerevisiae*, and two (Swe1 and Hst7) with apparent *C. albicans*-CWR specific functions (Blankenship et al. 2010).

These kinases have high S_{LR} scores, suggesting that Cdks might modulate their kinase activity under specific circumstances. All of them contain clusters of Cdk

sites of different lengths located at disordered regions that are either absent in their *S. cerevisiae* orthologs or that are at different regions of the protein. The most striking example is Gin4 (S_{LRF} 18.34), which has an amino acid sequence with 6 contiguous strong Cdk sites (S^{443}; S^{447}, S^{451}, S^{455}, S^{459} and S^{463}) in a disordered region. The alignment of Gin4 with its orthologs shows some interesting aspects. First, this cluster is absent in the *Saccharomyces* clade (Fig. 3), although other flanking Cdk sites are more or less conserved in position. Second, in the *Candida* clade, an increase in the number of Cdk sites is observed from *C. lusitaniae* to *C. albicans.* Thus, this progressive accumulation of strong Cdk sites at the same disordered region suggests a linage-specific regulation of Gin4 by Cdks. The Gin4 kinase is involved in septin organization in both yeasts (Longtine et al. 1998; Wightman et al. 2004). In *S. cerevisiae*, Gin4 is activated during mitosis in a Cdc28/Clb2-dependent manner (Altman and Kellogg 1997). This regulation is also probably conserved in *C. albicans*, since Gin4 phosphorylates the septin Cdc11 at Ser395 at the end of the cell cycle (Sinha et al. 2007). It is likely that in both yeasts cell cycle regulation could be exerted through the conserved Cdk sites, while the cluster of Cdk sites in CaGin4 might create new interactions through other phosphopeptide-binding proteins. Indeed, it has been shown that a *gin4*Δ mutant is hypersensitive to oxidative or osmotic stresses in *C. albicans* but not in *S. cerevisiae* (Blankenship et al. 2010).

4.2.3. Cell signaling

MAP kinase-mediated pathways are important stress-signaling modules essential for the development of appropriate acute and adaptative responses to environmental cues. In pathogenic fungi, these pathways are important for virulence (Román et al. 2007). Our analysis suggests that the Cek1-mediated pathway could receive inputs from Cdk signaling at different levels, since several components of the network were identified (Fig. 4). In *C. albicans*, this pathway is involved in mating and hyphal growth (Chen et al. 2002; Cote et al. 2011; Kohler and Fink 1996; Leberer et al. 1996; Liu et al. 1994; Yi et al. 2011). This network is composed of Cst20, the Ste11-Hst7-Cek1 module, the scaffolding protein Cst5, and the transcription factor Cph1. Finally, the Cpp1 phosphatase inhibits the pathway, probably by dephosphorylating Cek1 (Csank et al. 1997). During mating, this MAPK pathway activates different transcription factors depending on the cell type (Soll 2011)(Fig. 4). Recently, it has been shown that the exposure of *C. albicans* cells to β-glucan is controlled by the Cek1-mediated pathway (Galán-Díez et al. 2010), suggesting that this pathway might modulate innate immunoresponses triggered through dectin-1.

Our computational analysis also suggests that Cdks could regulate the RAM signaling pathway (Nelson et al. 2003) through the phosphorylation of Mob2 (Fig. 2 and 3). In *S. cerevisiae*, this pathway controls cell separation and polarized

growth through the activity of the NDR kinase Cbk1 (Weiss et al. 2002), which requires interaction with Mob2 for its function. Whereas CaMob2 showed a S_{LRF} value of 6.75 (the top 38), its *S. cerevisiae* ortholog had a S_{LRF} value of 0.77 (the top 887 from the *S. cerevisiae* proteome). This divergence suggests a new role for Cdks in the regulation of the Cbk1/Mob2 complex in *C. albicans*, which has been experimentally demonstrated (Gutiérrez-Escribano et al. 2011). In agreement with this idea, the cluster of four full Cdk sites (S^{44}; S^{51}, S^{67} and S^{97}) present in the amino terminal region of CaMob2 is absent in the *Saccharomyces* clade (Fig. 3).

Hyphal growth is characterized by robust polarized growth at cell tips and by the inhibition of cell separation after cytokinesis. Therefore, polarized growth and cell separation, the two major outputs of the RAM pathway, are differentially regulated in yeast and hyphae. We found that hyphal-inducing cues modulate the function of Cbk1/Mob2 through Cdk-dependent phosphorylation of Mob2 in a growth-dependent manner (Gutiérrez-Escribano et al. 2011). Phenotypic analysis of cell expressing a phosphodeficient Cdk Mob2 mutant suggests a role for these types of phosphorylation in promoting maintained polarized growth and inhibiting cell separation specifically in the hyphal form but not in yeast cells.

5. Conclusions

Several studies have shown the existence of rewiring in *C. albicans* transcriptional regulatory pathways (Li and Johnson 2010) and protein kinases (Blankenship et al. 2010). Recently, we have shown that Cdk is essential to for the differential modification of the outputs of a core signaling system, the Cbk1/Mob2 complex, depending on the environmental signals that activate different cell fate programs (yeast or hypha) (Gutiérrez-Escribano et al. 2011). In this chapter we have speculated that Cdks might be connected to broader range of signaling pathways than in *S. cerevisiae* through the acquisition of *Candida* linage-specific Cdk sites clustered in proteins involved in environmental responses. Our Cdk cluster-based search in combination with other evidences, such as localization in disordered regions and the evolution of such clusters in other Hemiascomycetes, could be used to predict new layers of Cdk regulation in *C. albicans* environmental sensing networks.

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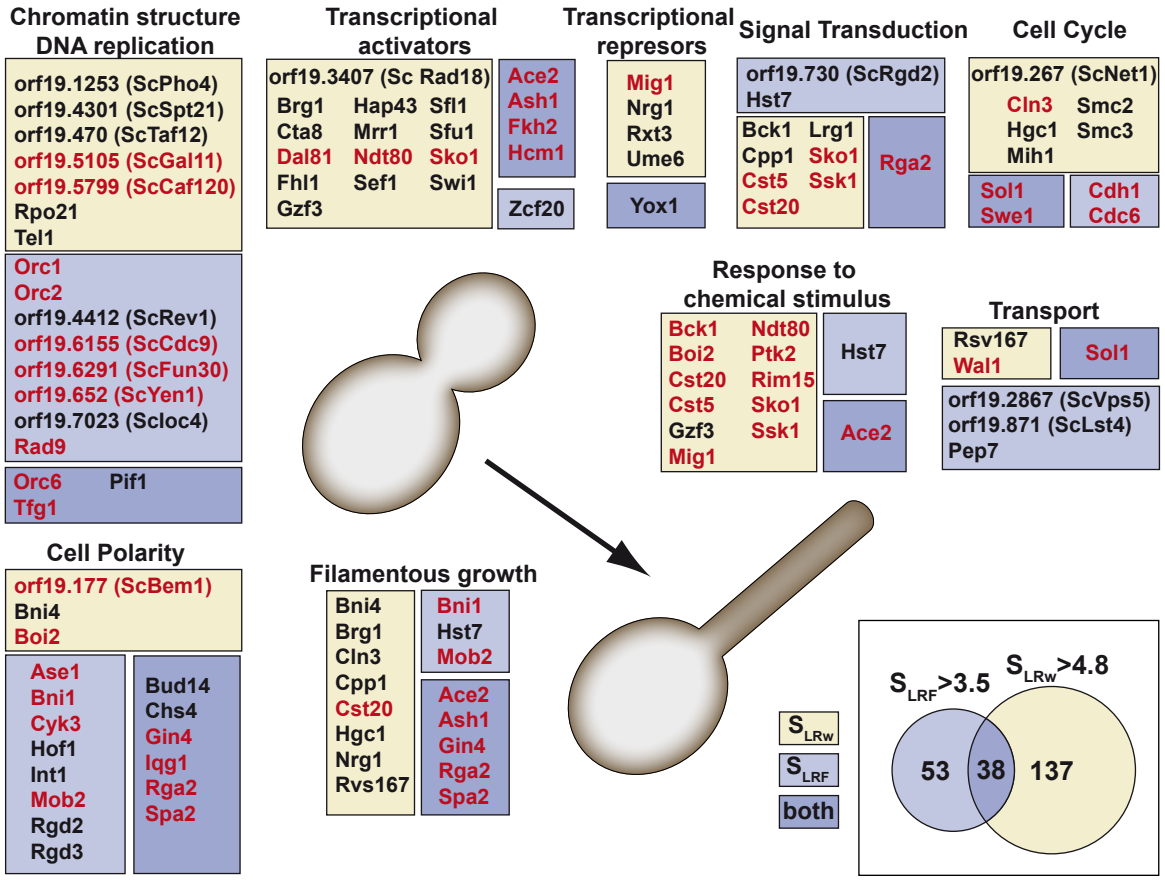
FIGURE LEGENDS

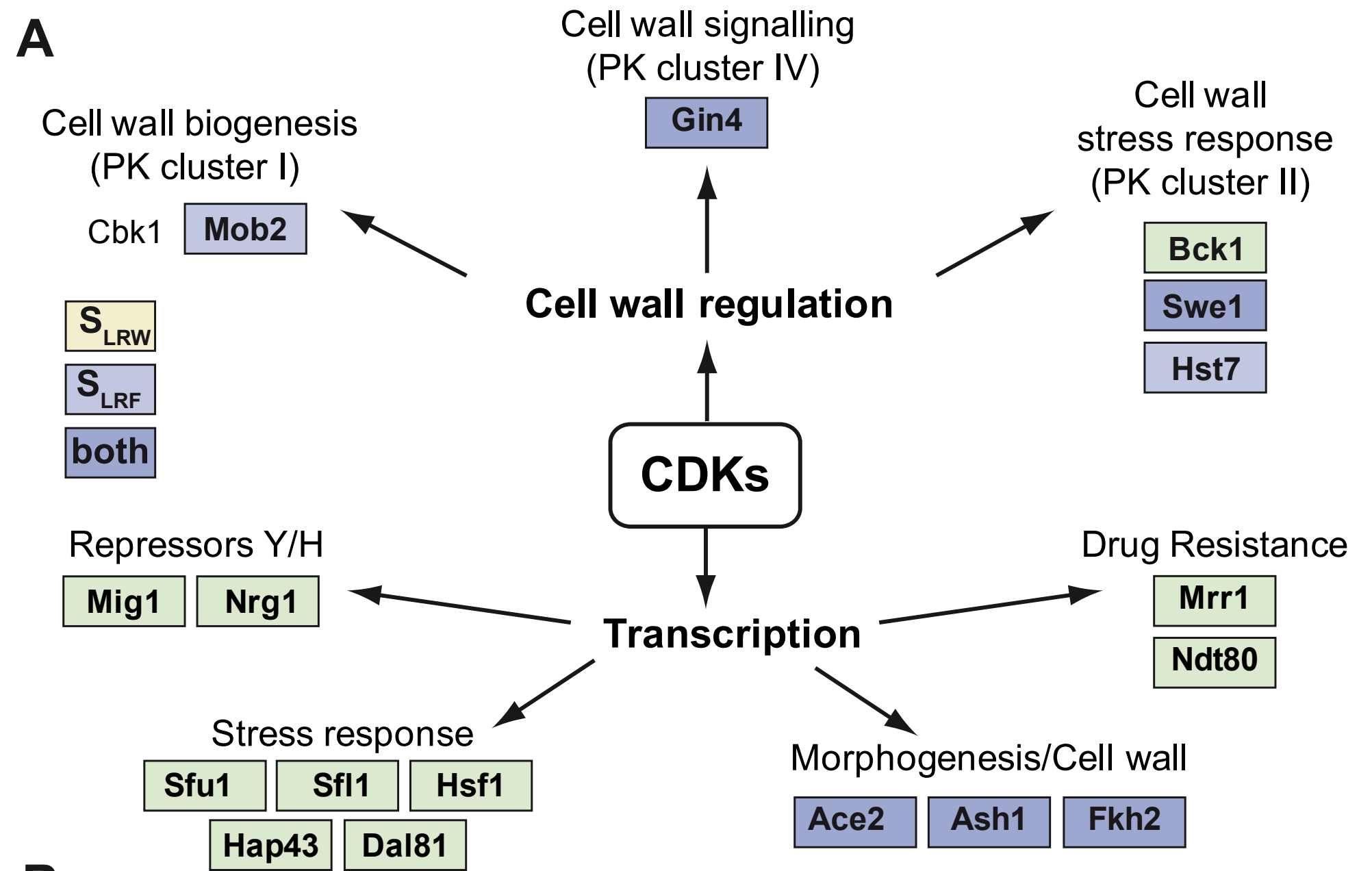
Fig. 1. Selected proteins with Cdk1 regulatory clusters grouped by GO cellular process. The color of the box indicates whether a protein was identified in the strong phosphorylation site search (S_{LRF}), in the weak phosphorylation site analysis (S_{LRW}) or in both. Proteins in red show Cdk-dependent phosphorylation in *S. cerevisiae* and/or *C. albicans* (Ubersax et al., 2003; Beltrao et al., 2009; Holt et al., 2009). The inset shows a graphic representation of the results obtained in the SLRF or SLRW analysis.

Fig. 2. (A) Predicted Cdk1 substrates in cell wall and transcriptional regulation. In the cell wall regulation pathway, many protein kinases involved in CWR or the regulatory subunit of the Cbk1-Mob2 complex contain putative Cdk1 regulatory clusters. Cdks can also control transcription activation or repression through the phosphorylation of transcription factors in different pathways. (B) Alignment of the N-terminal region of *C. albicans* Hap43 and *S. cerevisiae* Hap4. Weak Cdk sites are indicated in blue, while the rectangle shows the conserved CBC domain.

Fig. 3. Evolution of clusters of Cdk regulatory sites in Hemiascomycota: examples of the appearance of clusters of regulatory sites in the *Candida* clade. At the top, a schematic representation of Mob2 and Gin4 with their different domains. Strong (red) and weak (blue) Cdk phosphorylation sites, regulatory regions predicted by SLR (dark grey) and disordered regions (brackets below the sequence) are also indicated. The sequence of the regions containing the predicted regulatory clusters (indicated with dashed lines) is aligned below with their orthologs from the *Candida* and *Saccharomyces* clades. The dashed line indicates gaps introduced to maximize the alignment.

Fig. 4. The Cph1-mediated MAPK pathway can be regulated by Cdk1 at different levels. Putative Cdk1 regulatory clusters are present in different components of the Cst20-Cek1 pathway and in two transcriptional repressors. To the right, a schematic representation of the six putative targets of the pathway, indicating the different domains in each protein, the position of the Cdk phosphorylation sites, the putative regulatory regions, and disordered regions.


Chromatin structure
DNA replication
orf19.1253 (ScPho4)
orf19.4301 (ScSpt21)
orf19.470 (ScTaf12)
orf19.5105 (ScGal11)
orf19.5799 (ScCaf120)
Rpo21
Tel1
Orc1
Orc2
orf19.4412 (ScRev1)
orf19.6155 (ScCdc9)
orf19.6291 (ScFun30)
orf19.652 (ScYen1)
orf19.7023 (Scloc4)
Rad9
Orc6
Pif1
Tfg1
Transcriptional activators
orf19.3407 (Sc Rad18)
Brg1
Hap43
Sfl1
Cta8
Mrr1
Sfu1
Dal81
Ndt80
Sko1
Fhl1
Sef1
Swi1
Gzf3
Ace2
Ash1
Fkh2
Hcm1
Zcf20
Transcriptional represors
Mig1
Nrg1
Rxt3
Ume6
Yox1
Signal Transduction
orf19.730 (ScRgd2)
Hst7
Bck1
Lrg1
Cpp1
Sko1
Cst5
Ssk1
Cst20
Rga2
Cell Cycle
orf19.267 (ScNet1)
Cln3
Smc2
Hgc1
Smc3
Mih1
Sol1
Swe1
Cdh1
Cdc6
Response to chemical stimulus
Bck1
Ndt80
Boi2
Ptk2
Cst20
Rim15
Cst5
Sko1
Gzf3
Ssk1
Mig1
Hst7
Ace2
Transport
Rsv167
Wal1
Sol1
orf19.2867 (ScVps5)
orf19.871 (ScLst4)
Pep7
Cell Polarity
orf19.177 (ScBem1)
Bni4
Boi2
Ase1
Bni1
Cyk3
Hof1
Int1
Mob2
Rgd2
Rgd3
Bud14
Chs4
Gin4
Iqg1
Rga2
Spa2
Filamentous growth
Bni4
Brg1
Cln3
Cpp1
Cst20
Hgc1
Nrg1
Rvs167
Bni1
Hst7
Mob2
Ace2
Ash1
Gin4
Rga2
Spa2
S_LRw
S_LRF
both
S_LRF>3.5
S_LRw>4.8
53
38
137



B

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Hap43 MPAKGPNIIP-KQAPSPIAIAGSPPSSSASTPRSAASPPVSVNTKYSPNTNSNTIMPRQV 59
Hap4  MTAKTFLLQASASRPRSNHFKNEHNNIPLAPVPIAPNTNHHNNSSLEFENDGSKKKKKSS 60
      *.**   : .  . * .  : ..  . . :.   *...    *:. . :.:...   :.

Hap43 MSIQTSKEWVLPPRPKPGRKPSVDTPASKRKAQNRAAQRAFRERRATRVQELEQKLMEVE 119
Hap4  LVVRTSKHWVLPPRPRPGRRSSSHNTLPANNTNN--ILNVGPNSRNSSNNNNNNNIISNR 118
      : ::***.*******:***:.* ... . .:::*    ..  : * :  :: :::::. .
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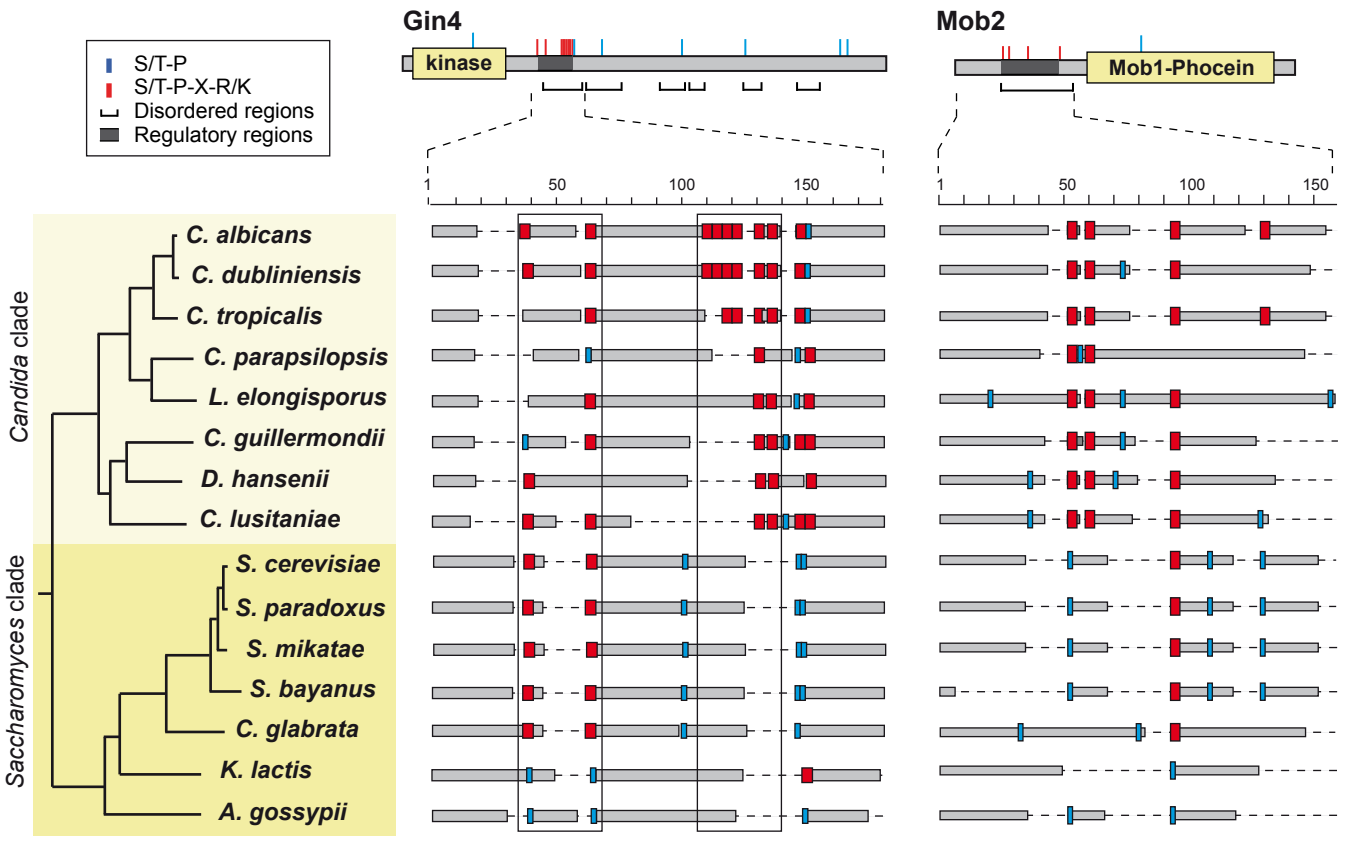
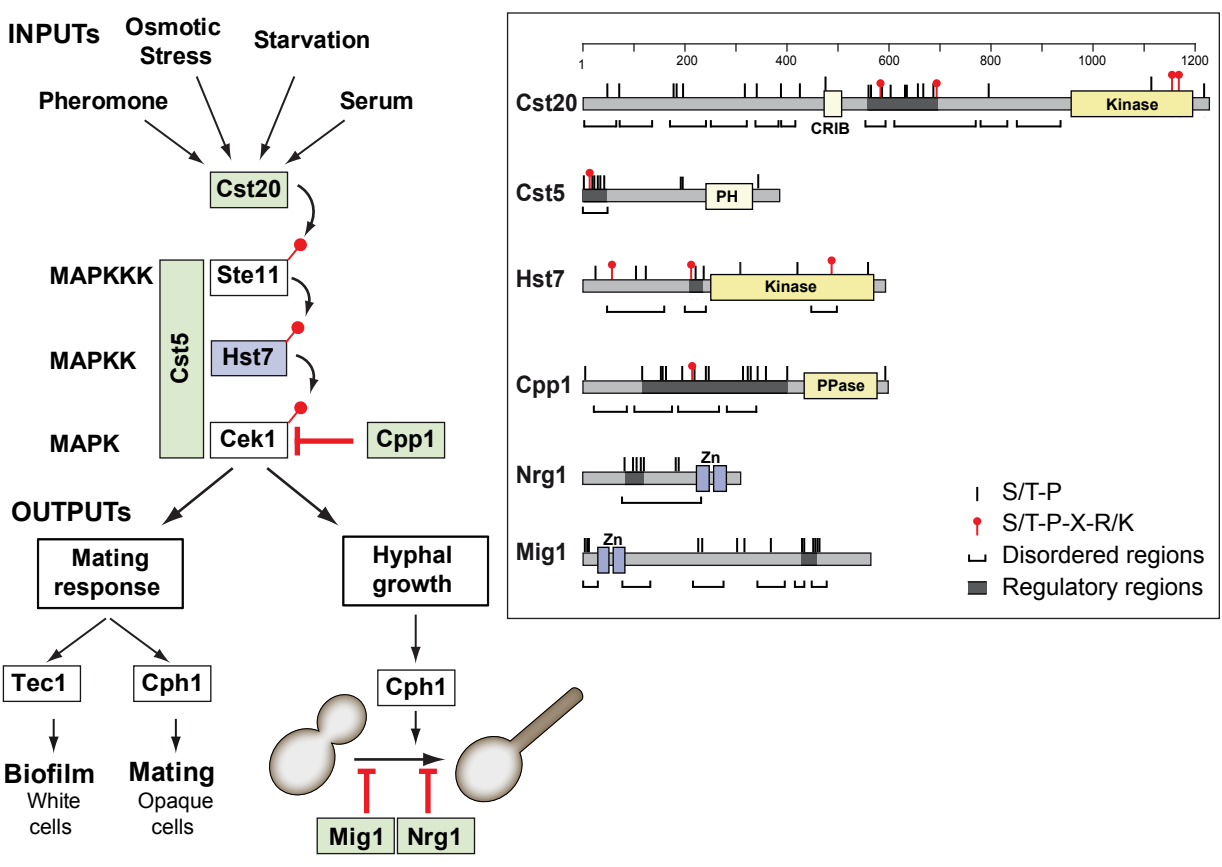

S/T-P
S/T-P-X-R/K
Disordered regions
Regulatory regions
Gin4
kinase
Mob2
Mob1-Phocein
1
50
100
150
Candida clade
C. albicans
C. dubliniensis
C. tropicalis
C. parapsilopsis
L. elongisporus
C. guillermondii
D. hansenii
C. lusitaniae
Saccharomyces clade
S. cerevisiae
S. paradoxus
S. mikatae
S. bayanus
C. glabrata
K. lactis
A. gossypii


INPUTs
Osmotic Stress
Starvation
Pheromone
Serum
Cst20
MAPKKK
Ste11
MAPKK
Hst7
MAPK
Cek1
Cpp1
Cst5
OUTPUTs
Mating response
Hyphal growth
Tec1
Cph1
Cph1
Biofilm
White cells
Mating
Opaque cells
Mig1
Nrg1
1
200
400
600
800
1000
1200
Cst20
CRIB
Kinase
Cst5
PH
Hst7
Kinase
Cpp1
PPase
Nrg1
Zn
Mig1
Zn
S/T-P
S/T-P-X-R/K
Disordered regions
Regulatory regions