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Dissecting Cell Cycle Protein Complexes using the Optimized Yeast Cytosine Deaminase Protein-fragment Complementation Assay

"You too can play with an edge" [1]

par

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Cette thèse intitulée :

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Résumé

Les protéines sont les produits finaux de la machinerie génétique. Elles jouent des rôles essentiels dans la définition de la structure, de l'intégrité et de la dynamique de la cellule afin de promouvoir les diverses transformations chimiques requises dans le métabolisme et dans la transmission des signaux biochimique. Nous savons que la doctrine centrale de la biologie moléculaire: un gène = un ARN messager = une protéine, est une simplification grossière du système biologique. En effet, plusieurs ARN messagers peuvent provenir d'un seul gène grâce à l'épissage alternatif. De plus, une protéine peut adopter plusieurs fonctions au courant de sa vie selon son état de modification post-traductionelle, sa conformation et son interaction avec d'autres protéines. La formation de complexes protéiques peut, en elle-même, être déterminée par l'état de modifications des protéines influencées par le contexte génétique, les compartiments subcellulaires, les conditions environmentales ou être intrinsèque à la croissance et la division cellulaire. Les complexes protéiques impliqués dans la régulation du cycle cellulaire sont particulièrement difficiles à disséquer car ils ne se forment qu'au cours de phases spécifiques du cycle cellulaire, ils sont fortement régulés par les modifications post-traductionnelles et peuvent se produire dans tous les compartiments subcellulaires. À ce jour, aucune méthode générale n'a été développée pour permettre une dissection fine de ces complexes macromoléculaires. L'objectif de cette thèse est d'établir et de démontrer une nouvelle stratégie pour disséquer les complexes protéines formés lors du cycle cellulaire de la levure *Saccharomyces cerevisiae* (*S. cerevisiae*).

Dans cette thèse, je décris le développement et l'optimisation d'une stratégie simple de sélection basée sur un essai de complémentation de fragments protéiques en utilisant la cytosine déaminase de la levure comme sonde (PCA OyCD). En outre, je

décris une série d'études de validation du PCA OyCD afin de l'utiliser pour disséquer les mécanismes d'activation des facteurs de transcription et des interactions protéine-protéines (IPPs) entre les régulateurs du cycle cellulaire. Une caractéristique clé du PCA OyCD est qu'il peut être utilisé pour détecter à la fois la formation et la dissociation des IPPs et émettre un signal détectable (la croissance des cellules) pour les deux types de sélections.

J'ai appliqué le PCA OyCD pour disséquer les interactions entre SBF et MBF, deux facteurs de transcription clés régulant la transition de la phase G1 à la phase S. SBF et MBF sont deux facteurs de transcription hétérodimériques composés de deux sous-unités : une protéine qui peut lier directement l'ADN (Swi4 ou Mbp1, respectivement) et une protéine commune contenant un domain d'activation de la transcription appelée Swi6. J'ai appliqué le PCA OyCD afin de générer un mutant de Swi6 qui restreint ses activités transcriptionnelles à SBF, abolissant l'activité MBF. Nous avons isolé des souches portant des mutations dans le domaine C-terminal de Swi6, préalablement identifié comme responsable dans la formation de l'interaction avec Swi4 et Mbp1, et également important pour les activités de SBF et MBF. Nos résultats appuient un modèle où Swi6 subit un changement conformationnel lors de la liaison à Swi4 ou Mbp1. De plus, ce mutant de Swi6 a été utilisé pour disséquer le mécanisme de régulation de l'entrée de la cellule dans un nouveau cycle de division cellulaire appelé « START ». Nous avons constaté que le répresseur de SBF et MBF nommé Whi5 se lie directement au domaine C-terminal de Swi6.

Finalement, j'ai appliqué le PCA OyCD afin de disséquer les complexes protéiques de la kinase cycline-dépendante de la levure nommé Cdk1. Cdk1 est la kinase essentielle qui régule la progression du cycle cellulaire et peut phosphoryler un grand nombre de substrats différents en s'associant à l'une des neuf protéines cycline régulatrice (Cln1-3, Clb1-6). Je décris une stratégie à haut débit, voir à une échelle génomique, visant à identifier les partenaires d'interaction de Cdk1 et d'y

associer la cycline appropriée(s) requise(s) à l'observation d'une interaction en utilisant le PCA OyCD et des souches délétées pour chacune des cyclines. Mes résultats nous permettent d'identifier la phase(s) du cycle cellulaire où Cdk1 peut phosphoryler un substrat particulier et la fonction potentielle ou connue de Cdk1 pendant cette phase. Par exemple, nous avons identifié que l'interaction entre Cdk1 et la γ-tubuline (Tub4) est dépendante de Clb3. Ce résultat est conforme au rôle de Tub4 dans la nucléation et la croissance des faisceaux mitotiques émanant des centromères. Cette stratégie peut également être appliquée à l'étude d'autres IPPs qui sont contrôlées par des sous-unités régulatrices.

Mots-clés : *Saccharomyces cerevisiae*, sélection positive et négative, essai de complémentation des fragments protéiques, cytosine désaminase de levure, 5-fluorocytosine, cycle cellulaire, facteurs de transcription, Swi6, activités transcriptionnelles, mécanisme de régulation, phase G1/S du cycle cellulaire, kinase dépendante des cyclines (CDK), cycline, complexes entre substrats et protéines, interactions protéine-protéines.

Abstract

Proteins are the end-products of gene interpretative machinery. Proteins serve essential roles in defining the structure, integrity and dynamics of the cell and mediate most chemical transformations needed for everything from metabolic catalysis to signal transduction. We know that the central dogma of molecular biology, one gene = one mRNA = one protein is a gross simplification and that a protein may do different things depending on the form in which its mRNA was spliced, how and where it is post-translationally modified, what conformational state it may be in or finally, which other proteins it may interact with. Formation of protein complexes may, themselves, be governed by the states in which proteins are expressed in specific cells, cellular compartments or under specific conditions or dynamic phases such has growth or division. Protein complexes involved in mitotic cell cycle regulation are particularly challenging to dissect since they could only form during specific phases of the cell cycle, are highly regulated by post-translational modifications and can be found in any subcellular compartments. To date, no general methods have been developed to allow fine dissection of these protein complexes. The goal of this thesis was to establish and demonstrate a novel strategy for dissecting protein complexes regulating the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) mitotic cell cycle.

In this thesis, I describe my development and optimization of a simple survival-selection Protein-fragment Complementation Assay using the prodrug-converting enzyme, yeast cytosine deaminase as reporter (OyCD PCA). I further describe, in a series of proof of principle studies, applications of the OyCD PCA to dissect the mechanism of transcriptional activation by key mitotic transcription factors and to dissect protein-protein interactions (PPIs) among regulators of the mitotic cell cycle.

A key feature of the OyCD PCA is that it can be used to detect both formation and disruption of PPIs by virtue of having positive readouts for both assays.

I applied the OyCD PCA in a strategy to dissect interactions between the key transcription factors of the G1/S phase: SBF and MBF. These two heterodimeric transcription factors are composed of, respectively, two distinct DNA-binding subunits named Swi4 and Mbp1 and a common transcription activation subunit called Swi6. I took advantage of the dual selection by OyCD PCA to engineer a specific mutant of Swi6 in order to demonstrate the rewiring of a transcriptional network. We isolated Swi6 with mutations found in its C-terminal domain previously identified for binding Swi4 and Mbp1 and important for SBF and MBF activities. Our results support a model where Swi6 undergoes a conformational change upon binding to Swi4 or Mbp1. In addition, this Swi6 mutant was used to dissect the regulatory mechanism that governs the entry of *S. cerevisiae* to a new round of cell division also known as START. We found that the SBF and MBF repressor Whi5 directly binds to the C-terminal domain of Swi6.

Finally, I applied the OyCD PCA to dissect the yeast cyclin dependent kinase Cdk1-protein complexes. Cdk1 is the essential kinase that regulates cell cycle progression and can phosphorylate a large number of different substrates by teaming up with one of nine cyclin regulatory proteins (Cln1-3, Clb1-6). I describe a strategy to identify interaction partners of Cdk1 that can easily be scaled up for a genome-wide screen and associate the complexes with the appropriate cyclin(s) required for mediating the interaction using the OyCD PCA and deletion of the cyclin genes. My results allow us to postulate which phase(s) of the mitotic cell cycle Cdk1 may phosphorylate proteins and what function potential or known substrates of Cdk1 may take on during that phase(s). For example, we identified the interaction between Cdk1 and the γ-tubulin (Tub4) to be dependent upon Clb3, consistent with its role in mediating nucleation and growth of mitotic microtubule bundles on the

spindle pole body. This strategy can also be applied to study other PPIs that are contingent upon accessory subunits.

Key words:

Saccharomyces cerevisiae, positive and negative selection, Protein-fragment Complementation Assay, yeast cytosine deaminase, 5-fluorocytosine, cell cycle, transcription factors, Swi6, rewiring transcriptional activities, mechanism of regulation, G1/S phase, cyclin dependent kinase Cdk1, cyclin, substrates and protein complexes, protein-protein interactions.

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List of abbreviations

S. cerevisiae:	*Saccharomyces cerevisiae*
Escherichia coli:	*E.coli*
DNA:	Deoxyribonucleic acid
RNA:	Ribonucleic acid
mRNA:	messenger Ribonucleic acid
ATP:	Adenosine triphosphate
C:	Cytosine
U:	Uracil
5-FC:	5-fluorocytosine
5-FU:	5-fluorouracil
yCD:	Yeast cytosine deaminase
PCA:	Protein-fragment Complementation Assay
TF:	Transcription factors
Cdk:	Cyclin dependent kinase
Cln or Clb:	Cyclin
YPD:	Yeast extract Peptone Dextrose
SC:	Synthetic complete
PCR:	Polymerase chain reaction
OD:	Optical density
PAGE:	Polyacrylamide gel electrophoresis
Kb:	Kilo bases
KDa:	Kilo Dalton
ml:	milliliter
µl:	microliter
mg:	milligram
µg:	microgram
mM:	millimolar
µM:	micromolar

To Casandro and Leopaul, for making me
understand the value of time.

"I don't know anything, but I do know that everything is interesting if you go into it deeply enough." Richard Feynman

* **Death PCA Pumpkin.** Sculpted by Vincent Messier. Winner of the Université de Montréal, Biochemistry Department Pumpkin Competition 2011.

Everyone can play with an edge. Built by members of the Michnick laboratory, 2011.

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Introduction

1.1 Protein complexes governing cellular activity

The simplest entity of life is the cell, whether those that make up complex metazoa such as ourselves or unicellular organisms such as bacteria or fungi. Cells are mainly composed of water and four classes of macromolecules: proteins, nucleic acids, lipids and polysaccharides (Alberts, Bray et al. 1994). Interactions among these macromolecules mediate all of the molecular transformations between matter, energy and information transfers necessary to sustain life (Monod 1968).

Proteins play a very important role among the four classes of macromolecules. Not only do they participate in their own synthesis, they are also involved in the synthesis of the other three classes of macromolecules (nucleic acids, lipids and polysaccharides). How can proteins accomplish all these different functions? Often they interact with other proteins and form functional complexes for relaying information from the extracellular to the intracellular environment or regulate their enzymatic activity. Particularly important classes of complexes are those whose functions are regulated during the cell cycle. Since a cell is constantly growing, dividing or dying at a given time, the functions of these protein complexes are intrinsically coupled to the mitotic cell cycle. However, understanding the functions of protein complexes during the cell cycle remains challenging due to the lack of existing tools for dissecting diverse protein complexes. In this thesis, I will present the development of a novel tool that allowed us to systematically dissect the budding yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*) G1-S cell cycle transcription factors (SBF and MBF) and cyclin-dependent protein kinase complexes in order to understand their different functions.

1.2 Protein-protein interaction networks (PINs) and the understanding of protein functions

Studying protein-protein interactions (PPI) is a general strategy for understanding the function(s) of an unknown protein or discovering novel function(s) of a characterized protein. In recent years, massive genome sequencing projects has given rise to the identification of thousands of genes. Many of these genes still have unknown function(s). This has motivated the design of methods to systematically detect protein-protein interactions on a large scale in order to define potential functions of genes.

The genome of the unicellular organism *S. cerevisiae* was sequenced in 1996 (Goffeau, Barrell et al. 1996). It has 5798 consensus open reading frames (ORFs) that potentially represent the number of genes in its genome (Goffeau, Barrell et al. 1996). What are the functions of these genes? Several protein-protein interaction networks (PINs) have been generated using the yeast two-hybrids screens (Y2H) (Ito, Tashiro et al. 2000; Uetz, Giot et al. 2000; Ito, Chiba et al. 2001), tandem affinity purification followed by mass-spectrometry analysis (TAP-MSs) (Gavin, Bosche et al. 2002; Ho, Gruhler et al. 2002; Gavin, Aloy et al. 2006; Krogan, Cagney et al. 2006) and Protein-fragment Complementation Assays based on the murine dihydrofolate reductase as reporter enzyme (mDHFR PCA) (Tarassov, Messier et al. 2008) in order to better assign functions to all the yeast genes. The results of these studies yield protein interaction network (PIN) data that serve as a valuable treasure chest of information for discovering mechanisms that regulates protein complexes and for inferring the functions of uncharacterized proteins according to the "guilt-by association" concept (Oliver 2000). This means that the function of an unknown protein can be assigned to it by grouping it with its interacting partners. The established PINs allow us to identify many new components of protein complexes that govern basic cellular processes such as transcription, translation, signal

transduction and cell cycle regulation and their mechanism of regulation. Despite all these efforts, 866 ORFs remain uncharacterized.

Ultimately biochemists engaged in understanding some cellular process begin with the supposition that process is somehow mediated by individual or groups of complexes and thus the goal is to identify the component subunits that are necessary to a process and then dissect out the roles of the individual subunits (Alberts 1998).

1.3 Reagents for dissecting protein complexes

The functions of protein complexes can be modulated using small molecules or genetic perturbations. However, such small molecules for inhibiting protein activity or protein-protein interaction are rare. Thus, it is a major challenge to identify novel molecules specific to a particular protein (Arkin and Wells 2004). Genetic perturbations represent an attractive avenue for dissecting protein complexes. With the availability of its genomic sequences, a systematic single gene deletion of almost 5000 *S. cerevisiae* non-essential genes has been accomplished (Giaever, Chu et al. 2002). Many mutant strains carrying two deleted genes have been reported (Tong, Lesage et al. 2004; Costanzo, Baryshnikova et al. 2010) and efforts to obtain the entire array of double deletion strains are on going.

A finer level of protein complex dissection can be achieved by using truncation, deletion or point mutations of individual subunits (also know as a missense mutation). Like gene deletion, mutations in a protein can be used to dissect protein complexes and their functions. A C-terminal truncation variant of a protein can be obtained by simply introducing a nonsense mutation to the DNA sequence of the gene in order to generate a premature stop codon. Mutating the first ATG codon and introducing another ATG codon further on in the gene sequence can obtain an N-terminal truncation variant of a protein. Truncation mutants are useful for

identifying interacting domains (independently folding regions) or binding motifs (small linear sequences) of a protein. Point mutations can provide more fine-detailed information about the chemical basis of an interaction or, in cases of sites that are post-translationally modified, study the effects of these modifications on binding. Yet, there is a drawback to using specific mutants to systematically dissect protein complexes since they cannot be easily predicted or identified from the primary amino acid sequence of the protein of interest.

1.3.1 Fishing for binding mutants by random mutagenesis

Aside from step by step approaches to identify mutants that will disrupt a binding interface, random approaches have been found to be effective. Libraries of gene point, truncation or internal deletion mutants can be generated. There are various ways to introduce mutation(s) into a gene for generating a library (Bonsor and Sundberg 2011). Many laboratories use mutagenesis generated by error-prone polymerase chain reaction (ePCR) to introduce mutations into the gene sequence due to its simplicity and low cost. This PCR method uses a variation in the amount of manganese (Mn) or magnesium (Mg) and an unequal amount of nucleotide concentration to force a low fidelity DNA polymerase from *Thermus aquaticus* (TAQ) to introduce mutation during a PCR reaction (Cadwell and Joyce 1992). Although this technique does not allow for an exhaustive coverage of the amino acid sequence, it has the potential to generate a mutant of desired characteristics when used with the appropriate binding selection strategy. Such methods have been used to disrupt complexes, for example, to increase the fluorescent properties of fluorescent proteins including green and red fluorescent protein (Campbell, Tour et al. 2002) and infrared fluorescent protein (Shu, Royant et al. 2009) .

Error-prone PCR can be scalable for high-throughput screening and has been the method of choice to generate libraries of mutants in genome-wide projects. For

example, error-prone PCR was used to generate libraries of temperature-sensitive mutant for over one thousand essential *S. cerevisiae* genes (Ben-Aroya, Coombes et al. 2008). Similar approaches could be envisioned for screening for disruption of protein-protein interactions using yeast two-hybrid or PCA screening methods.

1.4 A dual selection assay to engineering proteins with specific interaction: A matter of life and death

In order to identify mutation(s) that affect a PIN, an ideal tool to screen for a missense or nonsense mutations should provide a positive output for detecting both the interaction itself and disruption of that interaction. Additional features of the tool would be that full-length proteins can be expressed in their native cellular compartments with appropriate post-translational modifications and since the readout is direct and independent of transcription, the strategy even can be applied to transcription factors. Finally, the tool should allow for easy recovery of the mutant of interest. In addition, this tool should be sensitive and allow detection of conformational changes between the two interacting proteins (Remy and Michnick 1999; Tarassov, Messier et al. 2008).

A simple solution to a dual selection system that can be used to re-engineer interactions between specific proteins or to dissect protein complexes is to use a reporter protein that is bifunctional. Ideally, such a reporter protein would provide simple dual readouts: for example, cell survival or cell death. A selection strategy based on a simple output such as cell growth is favored for library screening since these are inexpensive and require no sophisticated equipment for signal detection. An additional feature of such a tool is that it could also be used to identify contingent interactions. Specifically if an interaction between proteins X and Y is mediated by a third protein Z, then a detector of the interaction of X and Y would give a positive signal in the death assay if Z were deleted.

There are a small number of known reporter proteins that can regulate both cell survival and cell death. They often fall into the category of prodrug-converting enzymes. These enzymes regulate cell survival by participating in pathways that lead to the synthesis of essential metabolites. However, in addition to modifying their natural substrates, they can also convert unnatural and benign compounds (so called prodrugs) to products that are toxic to cells. The most well known prodrug-converting enzymes used in positive-negative clonal selection strategies include the herpes simplex virus thymidine kinases (HSV-TK1 and HSV-TK2), yeast cytosine deaminase (yCD), and orotidine 5-phosphate decarboxylase (Ura3 of *S. cerevisiae*) (Capecchi 1980; Nishiyama, Kawamura et al. 1985; Boeke, Trueheart et al. 1987).

Enzymes that regulate key metabolic pathways and have known inhibitors cannot act as a reporter for a dual PPI selection assay since blocking the activities of the enzymes will automatically inhibit cell survival. An example of a reporter from this category is the imidazoleglycerol-phosphate dehydratase of *S. cerevisiae* (His3). His3 can be used as an auxotrophic marker yet it can also be inhibited by a competitive inhibitor called 3-Amino-1,2,4-triazole (3-AT) (Brennan and Struhl 1980).

1.5 Existing positive and negative selection assays for screening interaction specific mutant(s)

There are two *S. cerevisiae* based PPI assays that can be used for both survival and death selection assay. They are based on the gene product of *URA3* as reporter protein. Ura3 is an enzyme of the pyrimidine *de novo* synthesis pathway that converts orotidine 5-phosphate to uridine monophosphate. In addition, Ura3 can also convert 5-Fluoroorotic acid (5-FOA) to 5-fluorouracil (5-FU) (Boeke, Trueheart et al. 1987), a compound that can further be modified to 5-fluorouridine-triphosphate (5-FUTP) which can induce cell death (Fang, Hoskins et al. 2004).

1.5.1 Reverse yeast two-hybrid assays

The reverse yeast two-hybrid (rY2H) system is a yeast two-hybrid (Y2H) assay using Ura3 as a reporter protein and 5-FOA as prodrug for inducing cell death when two proteins of interest bind to each other (Leanna and Hannink 1996; Vidal, Brachmann et al. 1996). It is based on the reconstitution of the Gal4 transcription factor from its split DNA binding (DB) and transactivating (TA) domains fused to two test proteins (Fields and Song 1989). When the two test proteins interact, the transactivation domain of Gal4 is brought into proximity with its DNA binding domain resulting in the activation of the URA3 reporter gene in the nucleus. In the presence of the prodrug 5-FOA, cells expressing the URA3 gene will be sensitive and cannot grow. Equally, a rY2H with a ribosomal protein of the large (60S) ribosomal subunit (Cyh2) as reporter gene has been developed (Leanna and Hannink 1996). In the presence of cycloxehamide, cells that express the CYH2 gene do not grow. A disadvantage of Cyh2 over Ura3 is that this reporter protein can only be used for death selection.

Since the output of the rY2H system is cell death, a mutation or small molecular that disrupts the interaction between two test proteins will result in cell survival. This particular strategy facilitates the screening process of library of mutant proteins for non-binding mutant(s) or small molecules that can inhibit a specific PPI without the tedious replica-plating step. It has successfully been used to identify mutants for proteins such as the yeast MAPK scaffold protein Ste5 (Inouye, Dhillon et al. 1997).

A central limitation of Y2H assays is that since they work at the level of transcriptional machinery, they cannot be used in a trivial way to study protein complexes involved in transcription itself or perhaps other complexes involved in chromatin dynamics in general (**Figure 1**). First, the Y2H assays cannot be used for

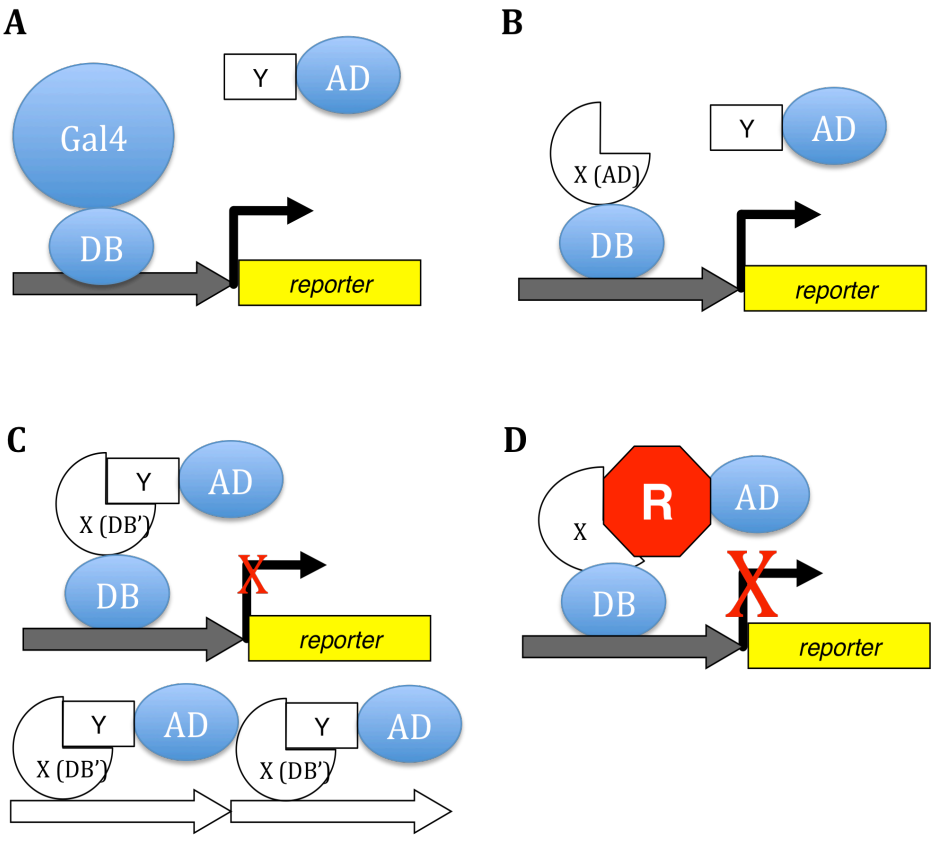

Figure 1. Limitation of the yeast two-hybrid (Y2H) assays. The Y2H system is based on splitting Gal4 into its DNA binding (DB) and activation (AT) domains and fusing these to two proteins of interest (X and Y). The interaction between protein X and protein Y will activate the transcription of the reporter gene. (**A**) Y2H assay cannot be used to study the interaction between Gal4 and its interaction partner(s). (**B**) If protein X has a transcriptional activation (TA) domain fused to DB domain of Gal4, it will activate the transcription of the reporter gene. C) If protein X contains a DB domain, it could bind other genomic DNA sequence and result in a decreased of transcription of the reporter genes. D) If protein X binds to a transcriptional repressor, it can repress the transcriptional activity of Gal4.

studying the interactions of Gal4 and other transcriptional regulators of the GAL genes since Gal4 itself is the split reporter of the Y2H assays. Second, a protein of interest that has a transcriptional activation (TA) domain must not be fused to the DB domain of Gal4 since this will automatically activate the transcription of the reporter gene and give a false positive signal. Hence, the interaction between two proteins each having a TA domain cannot be tested using the Y2H assays. Third, a protein that contains a DB domain fused to the DB domain of Gal4 could preferentially binds to its own DNA motif and not to the Upstream Activation Sequence motif of the GAL promoter resulting in a decreased of transcription of the reporter genes. Finally, proteins that can repress transcription can repress the transcriptional activity of Gal4 and generate a false negative result.

1.5.2 Split-ubiquitin assay using Ura3 as reporter

To overcome some limitations of the Y2H assay that detects PPI in the nucleus, the split-ubiquitin (split-ubi) assay was developed (Johnsson and Varshavsky 1994) and allows detection of PPIs occurring at the cell membrane or in the cytosol. Ubiquitin is a highly conserved small protein of 76 amino acid residues that is covalently attached to a target protein for targeting it for degradation by the 26S proteosome. During this process, the target protein will be degraded but ubiquitin can be recycled since an ubiquitin-specific protease (UBP) will remove ubiquitin from the target protein prior to the degradation event. The split-ubi technique is based on the fragmentation of ubiquitin into an N-terminal peptide (N_{ub}) and a C-terminal peptide (C_{ub}) and fusing them to a pair of proteins of interest. When the proteins of interest interact, the N_{ub} and C_{ub} are brought into proximity and can refold to generate a native-like ubiquitin. A transcription factor fused to the C-terminal end of ubiquitin will rapidly be cleaved off *in vivo* by the UBP and activates the expression of a reporter gene. When URA3 is used as a reporter gene for the split-ubi system, both survival and death selection can be established (Johnsson and

Varshavsky 1994). Since this assay depends on the activity of UBP, it cannot be used to study PPIs that occur in compartmentalized organelles such as the mitochondria and transport vesicles. In addition, interactions that are ubiquitin dependent could give a false negative signal since the UBP can remove the ubiquitin tag and abolish the interaction.

1.6 Towards a Protein-fragment Complementation Assay for life and death selection

Protein-fragment Complementation Assays (PCAs) are direct methods for studying protein-protein interactions in the context of living cells that do not require any transcriptional regulator or ubiquitin-specific protease for generating a signal. PCA consists of rationally dissecting a reporter protein into two fragments and fusing these fragments to two proteins of interest (**Figure 2**). Once the recombinant genes are expressed and the two fusion proteins interact with each other, the reporter fragments are brought into proximity and refold to generate activity of the native reporter protein. PCAs based on reporter proteins that provide a variety of readouts including survival, fluorescence, luminescence and colorimetric (Remy and Michnick 1999; Michnick, Ear et al. 2007). Fluorescent, luminescent and colorimetric PCAs have been developed based on variants of the Green Fluorescent Protein, *Renilla* luciferase (Stefan, Aquin et al. 2007) and *Gaussia* luciferase (Remy and Michnick 2006), and beta-lactamase (Galarneau, Primeau et al. 2002). Survival selection PCAs have been achieved with various reporter proteins including, murine Dihydrofolate Reductase (mDHFR) (Pelletier, Campbell-Valois et al. 1998), aminoglycoside phosphotransferase, hygromycin B phosphotransferase and glycinamide ribonucleotide transformylase (Michnick, Remy et al. 2000). A PCA based on a reporter for both survival and death selection has not been reported prior to the work of this thesis.

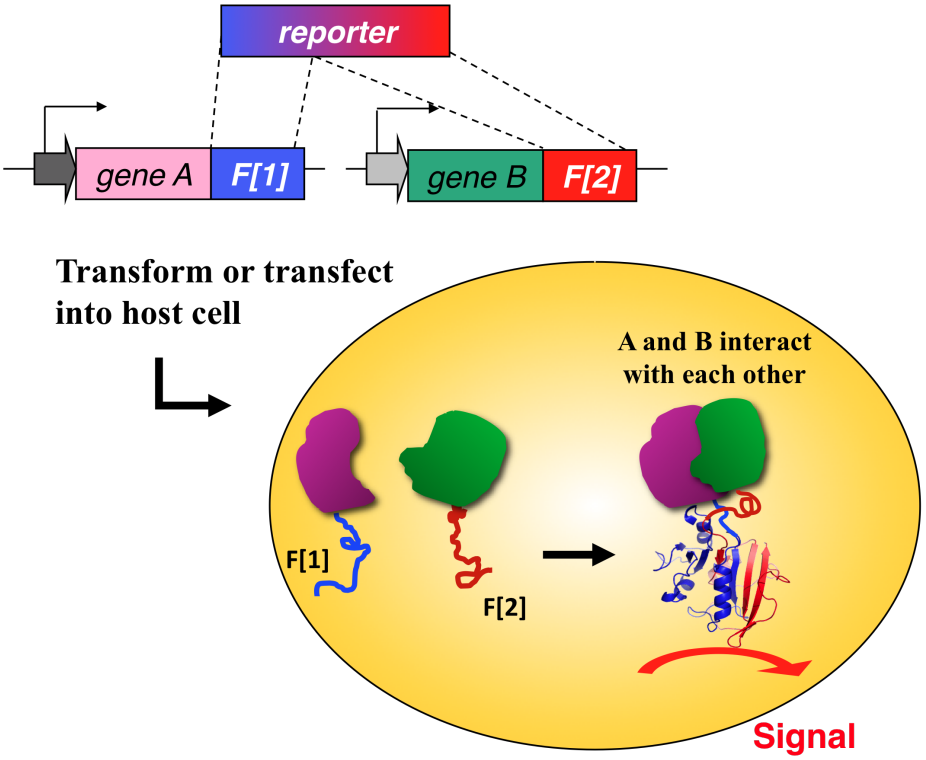

Figure 2. Protein-fragment Complementation Assays (PCA). Fragments of a reporter gene are fused downsteam of the genes of interest and expressed in a host cell. The interaction between the two proteins of interest brings the unfolded reporter-protein fragments into proximity allowing them to fold and restore the activity of the reporter protein. The signal detected depends on the nature of the reporter protein.

The incentive for developing a survival and death selection PCA is to circumvent the limitation of the rY2H and split-ubi assays. Like all PCAs, the following characteristics are some advantages of the survival and death PCA (Michnick 2001): First, it allows genes to be expressed in the relevant cellular context and recombinant proteins can appropriately undergo post-translational modifications. Second, it allows the direct detection of molecular interactions rather than *via* indirect cellular processes, such as transcriptional activation (Fields and Song 1989) or ubiquitin-specific protease (Johnsson and Varshavsky 1994). Third, interactions can be detected in any compartment of the cell (Remy and Michnick 2001). Finally, one of the most interesting features of PCA is that both fragments of the reporter protein do not spontaneously interact to regenerate the full-length reporter protein. The reporter protein fragments only fold to complement the activity of the native reporter when the proteins of interest interact (Pelletier, Campbell-Valois et al. 1998).

1.6.1 Yeast cytosine deaminase as a reporter for life and death PCA

Cytosine deaminase (CD) is an enzyme involved in the pyrimidine salvage pathway (Erbs, Exinger et al. 1997; Kurtz, Exinger et al. 1999) and was initially discovered in yeast and *Escherichia coli* (*E. coli*) in 1925 by Hahn et al. (Hahn and Schafer 1925). CD is not present in higher eukaryotes, including plants and mammals (Nishiyama, Kawamura et al. 1985). The yeast cytosine deaminase (yCD) is an interesting candidate for survival and death selection since the prodrug 5-fluorocytosine is widely available, inexpensive, and a small amount is required to cause cytotoxicity in yeast (33 μg/ml) and mammalian cells (Wera, Degreve et al. 1999). The HSV1-TK requires more than 1000 μg/ml of nucleoside analogs in order to temporarily inhibit cell growth in yeast (Wera, Degreve et al. 1999). The yeast cytosine deaminase (yCD), has been used as a reporter protein for both positive and

negative selection in both yeast (Erbs, Exinger et al. 1997) and mammalian cells (Wei and Huber 1996; Gallego, Sirand-Pugnet et al. 1999; Xiaohui Wang, Viret et al. 2001).

1.6.2 The role of yCD in the pyrimidine salvage pathway and utilization in a survival selection assay

yCD is encoded by the *FCY1* gene of *S. cerevisiae*. This gene product allows yeast to use cytosine found in the surrounding environment as a source of pyrimidine in the same way as bacteria (Grenson 1969). Cytosine enters into the yeast *via* a purine-cytosine transporter encoded by the *FCY2* gene. Cytosine is deaminated to uracil by yCD (Erbs, Exinger et al. 1997) (**Figure 3**). Once cytosine is converted to uracil, it can be phosphoribosylated to uridine 5'-monophosphate (UMP) by uracil phosphoribosyltransferase (encoded by the *FUR1* gene) (Kern, de Montigny et al. 1990). UMP can be further phosphorylated to become uridine 5'-triphosphate (UTP) by uridine kinase (encoded by the *URK1* gene). UTP can then be converted by thymidylate synthase (encoded by the *CDC21* gene) to thymidine monophosphate (TMP). Thus, yeast can recycle cytosine using enzymes of the pyrimidine salvage pathway for the synthesis of ribose and deoxyribose nucleotides.

Under normal condition, S. *cerevisiae* can also synthesize ribose and deoxyribose nucleotides using the *de novo* pyrimidine synthesis pathway. However, when the *de novo* pyrimidine synthesis pathway is inhibited, cells become uracil auxotrophs, requiring the nucleoside uracil for the synthesis of RNA. When uracil is absent and cytosine is present, the pyrimidine salvage pathway becomes the key pathway for generating uracil. yCD thus becomes essential to cell survival. Understanding this pathway allows the establishment of a survival selection assay using yCD as a reporter in yeast (Erbs, Exinger et al. 1997).

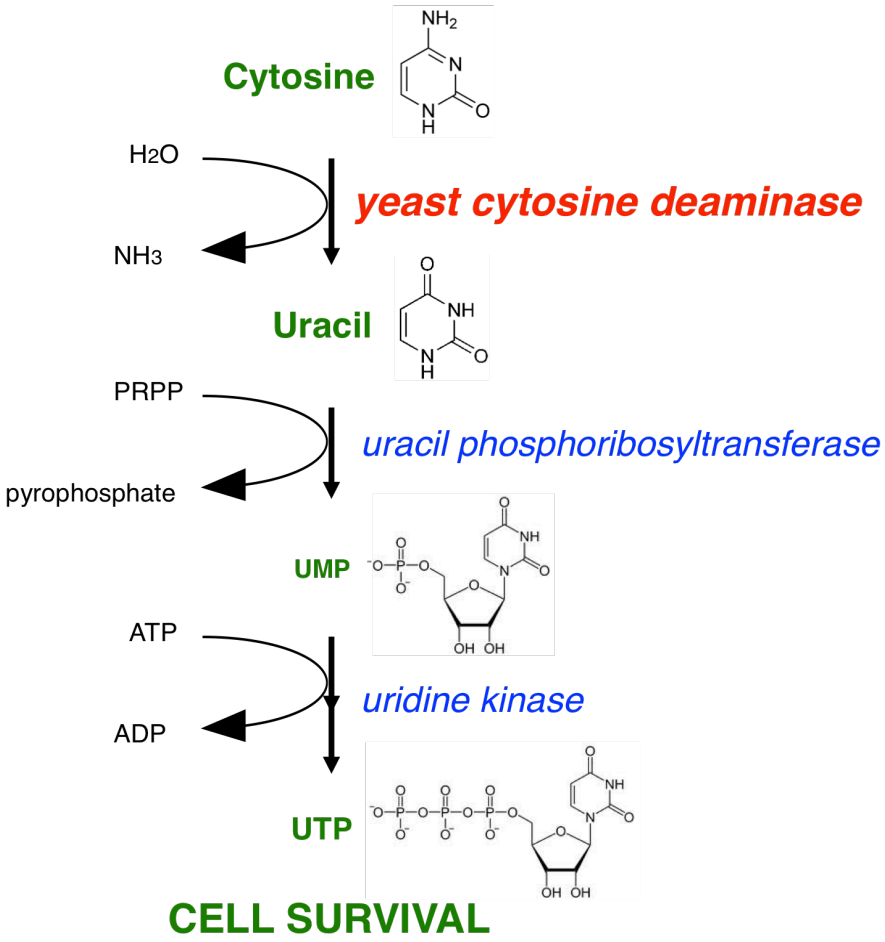

Figure 3. Pyrimidine Salvage Pathway in *S. cerevisiae*. In a yeast strain where a gene of the pyrimidine *de novo* pathway is disrupted, the cell cannot produce uridine 5'-triphosphate (UTP). In order to survive, cells must use the pyrimidine salvage pathway to convert cytosine to UTP. The yeast cytosine deaminase enzyme converts cytosine to uracil. Uracil is converted to uridine 5'-monophosphate (UMP) by the uracil phosphoribosyltransferase and the uridine kinase phosphorylates UMP to UTP.

Wild-type strains of S. *cerevisiae* have genes that encode for yCD (*FCY1*) and the orotidine 5-phosphate decarboxylase (*URA3*). Since Ura3 is the key enzyme for the *de novo* synthesis of pyrimidine, disruption of *URA3* will make the cell dependent on yCD to deaminate cytosine to uracil for cell survival. Many *FCY1* knockout strains of *S. cerevisiae* have been reported in small-scale (Erbs, Exinger et al. 1997) and large-scale studies (Giaever, Chu et al. 2002). The latter large-scale gene disruption project used S. *cerevisiae* BY4741 (*MAT**a** ura3Δ0 leu2Δ0 his3Δ1 met5Δ0*) and BY4742 (*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*) strains. Since the URA3 gene is disrupted in both BY4741 and BY4742 strains, the pyrimidine *de novo* synthesis pathway is blocked.

In mammalian cells, gene knockouts in the pyrimidine *de novo* synthesis pathway also forces cells to utilize the pyrimidine salvage pathway for survival where uracil is phosphoribosylated to UMP. In addition their pyrimidine *de novo* synthesis can also be blocked by an inhibitor known as N-(phosphonacetyl)-L-aspartate (PALA). PALA inhibits aspartate carbamyl transferase (CADases), an enzyme in the carbamoyl-phosphate synthetase complex (Swyryd, Seaver et al. 1974) of the pyrimidine *de novo* synthesis pathway. Cells treated with PALA undergo apoptosis (Wei and Huber 1996) but can proliferate when yCD is introduced and cytosine is added to the culture medium as a supplement.

1.6.3 yCD Death Selection Assay

Cytosine is not the only substrate of yCD. 5-methylcytosine and 5-fluorocytosine (5-FC) can also be deaminated by yCD(Erbs, Exinger et al. 1997). Initially synthesized in 1957 as an antitumor agent, 5-FC was instead observed to have anti-fungal activity (Grunberg, Titsworth et al. 1963) since only fungi and bacteria have cytosine deaminase. 5-FC is a non-toxic prodrug but once converted to 5-Fluorouracil (5-FU), it becomes toxic. 5-FU can be ribosylated to 5-fluorouridine monophosphate (5-FUMP) by uracil phosphoribosyltransferase. 5-FUMP is further

phosphorylated by uridine kinase to become 5-fluorouridine triphosphate (5-FUTP). 5-FUTP inhibits cell growth by incorporating into RNA (Fang, Hoskins et al. 2004; Lum, Armour et al. 2004) and directly inhibiting thymidylate synthase (encoded by the *CDC21* gene in budding yeast) (Parker and Cheng 1990; Longley, Harkin et al. 2003). Thus, combining yCD as a selection enzyme with 5-FC or cytosine is a useful strategy for either negative or positive clonal selection. For instance, the death selection assay has been suggested for a plasmid-shuffling assay (Erbs, Exinger et al. 1997) and is being explored for applications in gene therapy (Hamstra, Rice et al. 1999; Kievit, Bershad et al. 1999). That is, since mammalian cells do not have a gene encoding a cytosine deaminase, cells expressing yCD from a vector will be sensitive to 5-FC (Nishiyama, Kawamura et al. 1985).

1.6.4 Molecular Characteristics of yCD

yCD differs significantly from bacterial cytosine deaminase (bCD) in terms of the quaternary structure, primary amino acid sequence, molecular mass, and relative substrate specificities and affinities (Ireton, Black et al. 2003). Various structures of yCD have been solved (Ireton, Black et al. 2003; Ko, Lin et al. 2003). yCD is a homodimer where the 17.5 KDa monomers are arranged in a 2-fold symmetry (**Figure 4A**). The two monomers associate in a head-to-tail orientation. Tyrosine 121 sidechains form a stacking interaction at the dimer interface (Ireton, Black et al. 2003) (**Figure 4B**). Each monomer is composed of six alpha (α) helices and five beta (β) strands (**Figure 4C**). The five β-strands form a β-sheet that is sandwiched between the α1 and α5 helices on one side and the α2, α3, α4, and α6 helices on the other side. Each subunit has an active site that involves amino acids histidine-62, glutamate-64, cysteine-91, and cysteine-94. The active site can bind a tetrahedral catalytic zinc ion, which helps to coordinate the substrate and participates in an acid/base catalytic mechanism. Amino acids histidine-62, cysteine-91 and cysteine-94 are also involved in the coordination of the zinc molecule.

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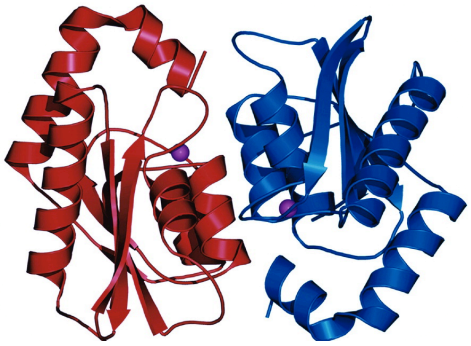

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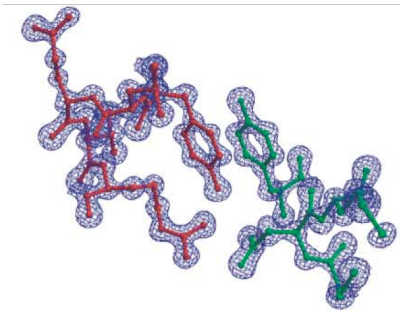

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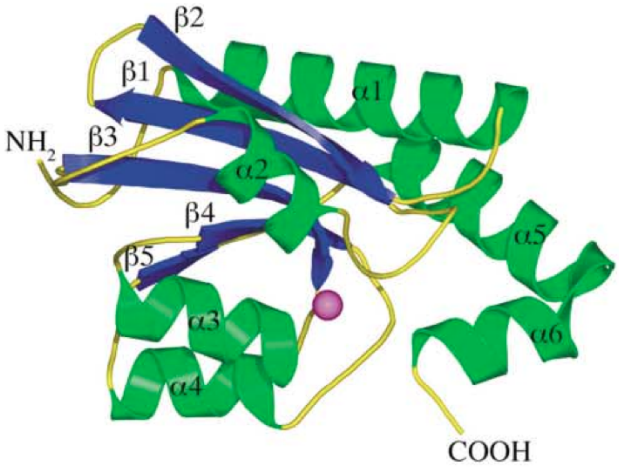

Figure 4. Structural characteristic of the yeast cytosine deaminase (Ireton, Black et al. 2003). A) yCD is a dimeric enzyme. B) Stacking interaction between the tyrosine 121 of one yCD subunit with respect to the tyrosine of the second subunit. C) The yeast cytosine deaminase monomer has a mixed α/β fold. The six α helices are colored in green, five β-strands in blue, and random coil regions in yellow.

1.6.5 Stability of yCD

yCD is stable at 30 °C but not at or above 37 °C. Since yCD is a protein that is of great interest to the field of gene therapy, it has been engineered to have a better stability and consequent increase enzymatic activity when expressed in different type of cells (Korkegian, Black et al. 2005). When residues alanine 23, valine 108 and isoleucine 140 of yCD were mutated to leucine 23, isoleucine108, and leucine 140 (also know as yCD triple mutant), the activity and stability of yCD significantly increased at 37 °C (Korkegian, Black et al. 2005). These residues stabilize yCD by bringing the α1 and α5 helices of yCD together. As described below, we took advantage of these results to engineer a yCD PCA that could work in any cell type, regardless of growth temperature.

1.6.6 Fragmentation of yCD for PCA

As I describe in Chapter two, we dissected yCD based on its structure (Hsu, Hu et al. 2003; Ireton, Black et al. 2003) and simple protein folding and engineering principles. A polypeptide chain contains all information necessary for it to fold into a functional protein (Richards 1958; Anfinsen, Haber et al. 1961; Taniuchi and Anfinsen 1971; Anfinsen 1973). When this polypeptide chain is fragmented into two complementary polypeptide chains, the fragments can fold to restore the activity of the native protein when they are in close proximity (Taniuchi and Anfinsen 1971). In an *in vitro* experiment, this can be achieved by increasing the concentration of the two complementary polypeptide fragments in order to increase the chance for the respective complementary fragments to find each other. Alternatively, the two fragments can be fused to two interacting proteins. PCA is based on this concept. The reporter protein yCD is fragmented and fused to two proteins of interest in such a way that it can refold to its native structure when the test proteins interact. Cutting within secondary structure elements could interfere with the refolding of the

reporter protein and the stability of the fragments. For this reason, cut sites were selected to be in loop regions of the enzyme where there are no secondary structure elements. Since the yCD reporter protein is an enzyme, the goal was also to select cut sites that do not interfere with the active site.

1.6.7 Optimization of yCD PCA activity by fragment shuffling and error-prone PCR mutagenesis

It has previously been shown that two fragments of an enzyme containing overlapping amino acid residues could rearrange to generate a functional enzyme (Taniuchi and Anfinsen 1971; Ostermeier, Nixon et al. 1999). It has also been reported that removing some residues in a peptide fragment could increase or decrease the activity of an engineered protein (Ostermeier, Nixon et al. 1999). By testing yCD fragment 1 versus yCD fragment 2 of the different cut sites, different PCA combinations could be screened in order to increase the activity of the yCD PCA.

1.7 Application of OyCD PCA to study protein complexes that govern the cell cycle

One of my motivations for developing the yCD PCA was a desire to understand complex processes that orchestrate the different phases of the cell cycle. Protein complexes that govern the cell cycle are extremely difficult to study because of the transient nature of their interactions. Many proteins are tightly regulated for degradation by ubiquitylation (Mendenhall and Hodge 1998) and many regulators of the cell cycle are transcription factors (Morgan 2007). Thus, it would be difficult to study these protein complexes using assays based on the Y2H (Fields and Song 1989) or split-ubi system (Johnsson and Varshavsky 1994). The OyCD PCA is ideal for studying *S. cerevisiae* cell cycle protein complexes since it can be used to engineer

specific mutants for dissecting protein functions, identify novel component of a protein complex, and study the contingency among the different subunits.

1.8 The *S. cerevisiae* sexual and mitotic cell cycles

S. cerevisiae can grow and divide in haploid and diploid forms (Morgan 2007) (**Figure 5**). The haploid cells exist in two different mating type: *MAT***a** and *MAT*$\boldsymbol{\alpha}$. Cells from each mating type release a pheromone to attract the opposite mating type. *MAT***a** and *MAT*$\boldsymbol{\alpha}$ cells produce **a**- and $\boldsymbol{\alpha}$-factor respectively (Jackson and Hartwell 1990). When the pheromone binds to the receptor on cells of the opposite mating type, it activates a MAP kinase mating response pathway that causes the cell to arrest at the G1 phase of the cell cycle. Thus, when two cells of the opposite mating type are in close proximity, the MAP kinase cascade initiates the complex morphogenic transformations needed for the cells to fuse and form a diploid (Cross, Hartwell et al. 1988; Jackson and Hartwell 1990). When diploid cells are deprived of nitrogen and carbohydrate, they undergo meiosis, a process known as sporulation in yeast, to produce a tetrad of cells consisting of two *MAT***a** and two *MAT*$\boldsymbol{\alpha}$ spores.

During the mitotic cell cycle, *S. cerevisiae* undergoes an asymmetric cell division, also known as "budding" in order to duplicate itself. This process gives rise to a mother cell and a smaller daughter cell. Like in all eukaryotes, the mitotic cell cycle of budding yeast is divided into four different stages known as G1 (first gap), S (DNA synthesis), G2 (second gap) and M (mitosis) and is governed by the activity of a single cyclin dependent kinase called Cdk1 (also known as Cdc28) (Morgan 2007). Cdk1 regulates all the stages of the cell cycle from START to the completion of mitosis. START is defined as the commitment to a new round of cell division in yeast. One of the most important roles of Cdk1 at START is to regulate the activity of two cell cycle transcription factors (SBF and MBF). SBF and MBF are the key regulators that control the decision of a cell to enter a new round of cell division.

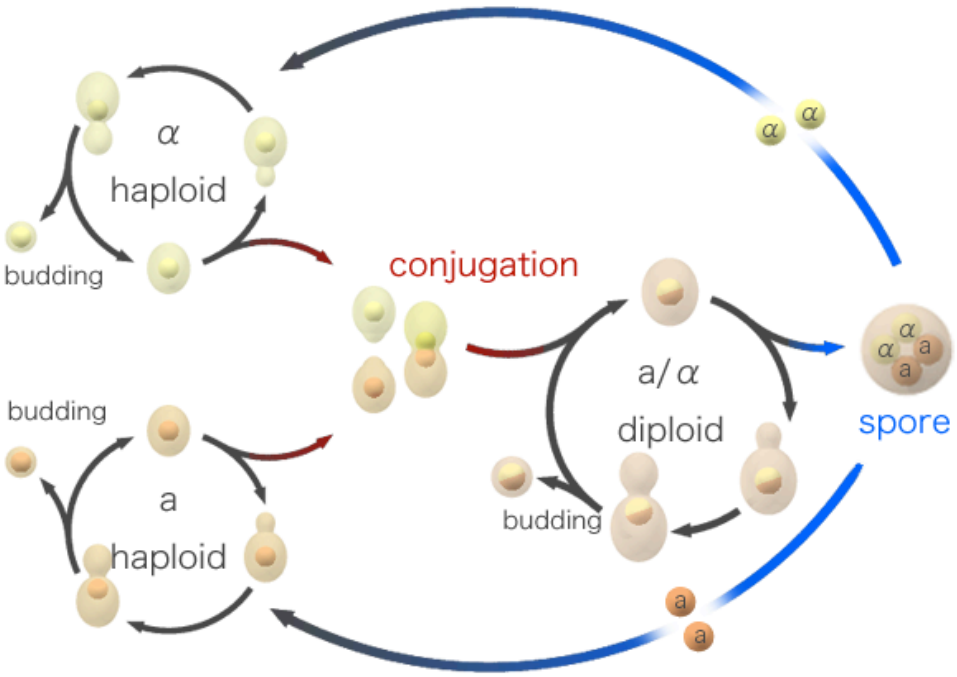

Figure 5. Sexual life cycle of *S. cerevisiae*. Haploid cells of mating type **a** and **α** can undergo mitotic cell division or conjugate to give rise to a diploid cell (a/α). Diploid cells can undergo mitosis or meiosis to form a spore.
Figure taken from
http://www.motherfitness.com/wp-content/uploads/2011/03/Budding_yeast_Lifecycle.png

The transition from one phase of the cell cycle to the next is unidirectional and irreversible. This is orchestrated by the different activities of cyclin dependent kinases (CDKs) in mammalian cells and dfferent Cdk1-cyclin complexes in *S. cerevisiae*. Different regulators of the CDKs are responsible for establishing an intrinsic program where the present CDK-cyclin complex ensures the activation of the next CDK-cyclin complex in line. At the same time, the later CDK-cyclin complex will activate the destruction of the previous CDK-cyclin complex in order to ensure that the transition occurs rapidly (switch-like response) and that the process is irreversible. This is achieved by using positive feedback mechanisms that accelerate changes in activity once a critical threshold is reached (Morgan 1997). An example of this unidirectional and irreversible process is discussed in section 1.9.7.

The extensive field of cell cycle regulation is beyond the scope of this thesis. Since my work specifically addressed the G1/S phase regulation by two key transcription factors (SBF and MBF) and the dissection of the yeast CDK complexes, only these topics are discussed in the next sections.

1.9 Yeast CDKs involved in cell cycle regulation

There are six CDKs in *S. cerevisiae*: Cdk1, Pho85, Kin28, Srb10/Cdk8, Sgv1/Bur1, and Ctk1 (Mendenhall and Hodge 1998). Among these, only Cdk1 and Pho85 play a role in cell cycle regulation and only Cdk1 is essential. Cdk1 forms complexes with one of nine cyclin proteins (Cln1-3, Clb1-6). Binding of Cdk1 to one of the cylins both activates its catalytic activity and directs Cdk1 to specific and distinct substrates (Russo, Jeffrey et al. 1996; Mendenhall and Hodge 1998; Loog and Morgan 2005). Pho85 is the other CDK that contributes to cell cycle regulation when it is bound to Pho85 cyclin (Pcl) of the Pcl1 and Pcl2 subfamily (Pcl1, Pcl2, Pcl5, Pcl9 and Clg1) (Huang, Friesen et al. 2007). The other four CDKs (Kin28, Srb10/Cdk8,

Sgv1/Bur1, and Ctk1) have been reported to be important for regulating transcription (Mendenhall and Hodge 1998).

1.9.1 Cdk1

All protein kinases have similar crystal structures consisting of a small N-terminal lobe formed by beta-strands and a large C-terminal lobe of alpha-helices. The structure of the human Cdk2 shows that ATP is bound between the small and large lobe such that the adenosine base is buried in the structure and the phosphate moiety is positioned at the mouth of the cleft (**Figure 6 A**). There is currently no structure of *S. cerevisiae* Cdk1. However the structure of the yeast Cdk1 should be similar to Cdk2 since these two proteins are homologues and Cdk2 can complement for Cdk1 activity in yeast (Ninomiya-Tsuji, Nomoto et al. 1991).

Cdk1 is a serine/threonine kinase that is the main regulator of the mitotic cell cycle of the budding yeast. Like all CDKs, Cdk1 is inactive by itself. It requires the association of a cyclin subunit in order to become an active kinase. The comparison of the crystal structure of the human Cdk2 (De Bondt, Rosenblatt et al. 1993) and Cdk2-cyclin-A (Jeffrey, Russo et al. 1995) shows that two alpha-helices of Cdk2 undergoes a significant conformational change when bound to cyclin A (**Figure 6 B**). First, the α1-helix also known as the PSTAIRE helix since it contains the highly conserved PSTAIRE amino acid residues moves inward (**Figure 6 B**). Second, the L12 helix changes structure to become a beta-strand (**Figure 6 B**). These structural modifications contribute to the reorganization of the active site of CDK, such that the ATP binds in a correct conformation for the phosphorylation reaction to occur. The minimal Cdk1 consensus motif is S/T-P and the full Cdk1 consensus motif is S/T-P-x-K/R (S/T is the phosphorylatable residue and X is any residue). Substrates of Cdk1 with the minimal Cdk1 consensus motif(s) and the full Cdk1 consensus motif(s) have

been found to be phosphorylated (Ubersax, Woodbury et al. 2003; Holt, Tuch et al. 2009; Keck, Jones et al. 2011).

1.9.2 Regulators of Cdk1

There are four mechanisms by which other proteins regulate CDKs activities and these mechanisms are conserved for Cdk1 in *S. cerevisiae* (Morgan 1995):

1) CDK requires binding to a cyclin for activation. The budding yeast Cdk1 associates with one of the nine cyclin (Cln1, Cln2, Cln3, Clb1, Clb2, Clb3, Clb4, Clb5 or Clb6) subunits in order to be active.

2) In addition to cyclin binding, CDK requires phosphorylation at the position threonine 169 by a CDK activating kinase (Cak1) in order to further increase its kinase activity (Desai, Gu et al. 1992).

3) The activity of the metazoan cyclin-CDK complexes can be inhibited by phosphorylation at residue threonine 15. *S. cerevisiae* Cdk1 is phosphorylated at threonine 18 and tyrosine 19 by Swe1 (Ma, Lu et al. 1996).

4) Cyclin-CDK complexes can be inhibited by CDK inhibitory subunits (CKIs). In budding yeast, Far1 inhibits Cln-Cdk1 complexes (Peter, Gartner et al. 1993; Tyers and Futcher 1993) and Sic1 inhibits Clb-Cdk1 complexes (Mendenhall 1993). Pho81 is an inhibitor of the Pc17-Ph085 and Pho80-Ph085 complexes (Huang, Friesen et al. 2007).

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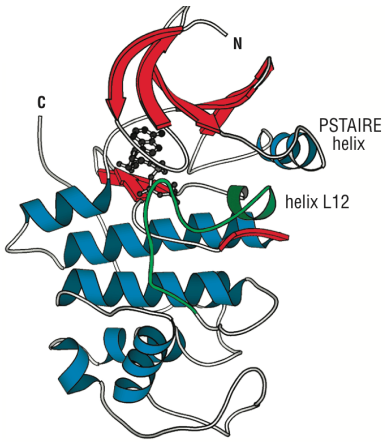

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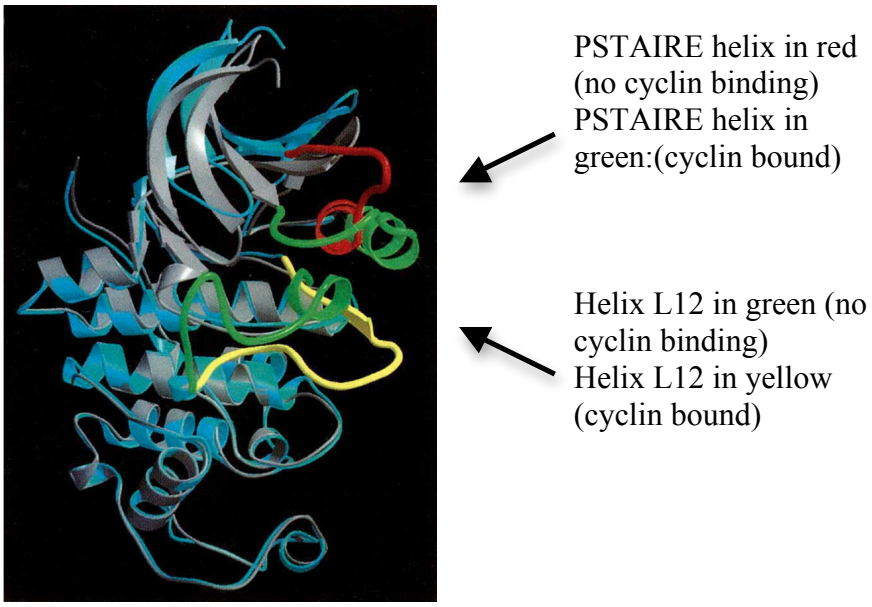

Figure 6. Structural feature of Cdk2. A) Like all protein kinases, Cdk2 has a small N-terminal lobe formed by beta-strands (red) and a large C-terminal lobe composed of alpha helices (blue). ATP, shown in stick representation, binds to Cdk2 between the small and large lobe. The two structural elements that undergo a major conformational change upon cyclin binding are the PSTAIRE and L12 helices in green (Morgan 2007). B) Superposition of the structure of Cdk2 without cyclin binding (grey) and with cyclin binding (blue) (Jeffrey, Russo et al. 1995).

1.9.3 Cyclins and the cell cycle

Cyclins can increase CDK activities by as much as 40,000 fold (Connell-Crowley, Solomon et al. 1993). In addition, cyclins increase the affinity of CDK for binding their substrates. For example, the mammalian Cdk2 binds weakly to INCA1 by itself but when in complex with cyclin A1, increases its binding capacity significantly (Diederichs, Baumer et al. 2004). The interaction between Cdk2 and cyclin A causes the two proteins to undergo a conformational change that creates a new surface for substrate docking. Some cyclins possess a hydrophobic patch that recognizes the RXL motifs on their target proteins. Proteins with the RXL motif are CDK substrates or CDK regulators such as the CKD inhibitor p27 (Russo, Jeffrey et al. 1996).

With the exception of Cln3, all the budding yeast cyclins have a paralog resulted from the duplication of its genome 100 million years ago (Bloom and Cross 2007). Each pair of paralogs shares strong homology and has similar functions during the mitotic cell cycle. This means that in the absence of one cyclin, the paralogs can compensate for its function(s).

The quantity of cyclins in the cell varies in a cyclic pattern during cell division due to regulation in the timing of their expression and destruction. The different cyclin subunits allow Cdk1 to recognize and phosphorylate different substrates at specific times throughout all the phases of the cell cycle. Cyclins are classified into four categories: G1, G1/S, S and M-phase cyclins (**Table 1**) (Morgan 2007).

In *S. cerevisiae*, the only G1 cyclin is Cln3. It is expressed throughout all phases of the cell cycle but its expression level increase by two fold at the G1/S phase (Mendenhall and Hodge 1998). At its peak level of expression, Cln3 activates Cdk1 to phosphorylate the G1/S transcription factors SBF and MBF. In metazoa, there are three G1 cyclins: cyclin D1, D2, D3.

Table 1. Different classes of yeast and human cyclins. Adapted from Figure 3-4 (Morgan 2007).

	Cyclin class (with Cdk partner)			
Species	**G1**	**G1/S**	**S**	**M**
S. cerevisiae	Cln3 (Cdk1)	Cln1,2 (Cdk1)	Clb5,6 (Cdk1)	Clb1 2,3,4 (Cdk1)
H. sapiens	cyclin D1,2,3 (Cdk4,6)	cyclin E (Cdk2)	cyclin A (Cdk2,1)	cyclinB (Cdk1)

The G1 to S-phase of *S. cerevisiae* is a period mainly characterized by bud emergence and spindle pole body duplication and genome replication. For passage through the G1-phase of the cell cycle, the cell requires at least the presence of G1-phase cyclins: Cln1, Cln2 or Cln3. At the end of G1 to S-phase, these cyclins are rapidly degraded since they have a PEST motif in their amino acid sequence (Rogers, Wells et al. 1986). A PEST sequence is characterized by hydrophilic sequences containing at least one proline, one acidic residue, and a serine or a threonine flanked by basic residues. The G1-cyclins lacking their C-terminal fragment containing the PEST sequence was shown to be more stable than the wild-type proteins (Yaglom, Linskens et al. 1995). The PEST sequence targets cyclins and other cell cycle regulatory proteins for phosphorylation. This stimulates the proteins to be ubiquitylated *via* the SCF ubiquitin ligase complexes and consequently to their rapid degradation. As a result, the half-life of the G1/S cyclins is around three to ten minutes (Barral, Jentsch et al. 1995).

The S and M-phase cyclins are also known as B-type cyclins due to their homology with the metazoan cyclin B. During the S-phase, the early-expressed Clb5 and Clb6 are responsible for initiating DNA replication and passage through S-phase. During mitosis, Clb1, Clb2, Clb3 and Clb4 play important roles in spindle morphogenesis and inhibit mitotic exit. At the end of mitosis, the mitotic cyclins are degraded by the ubiquitination pathway via the anaphase promoting complex (APC) (Morgan 2007) and cell division occurs. The half-life of the B-type cyclins is also around 15 minutes (Mendenhall and Hodge 1998).

1.9.4 Cdk1 substrates

Proteomic approaches have been used to identify protein targets of Cdk1. Using *in vitro* cell-extract assays, Ubersax *et al.* identified 181 proteins that are

phosphorylated by Cdk1 in complex with Clb2 (Ubersax, Woodbury et al. 2003). More recently, using mass-spectrometry, over 300 proteins have been identified to be targets of Cdk1 (Holt, Tuch et al. 2009). Interestingly, mass-spectrometry analysis of binding partners of cyclins after an immunoprecipitation yielded a limited list of proteins that interact with cyclins (Archambault, Chang et al. 2004). This is probably due to the short half-life of the cyclins (Mendenhall and Hodge 1998). Thus, to date, it is still difficult to associate a specific cyclin to a specific substrate. Further, no general methods have been developed to identify *in vivo* substrates of Cdk1.

1.9.5 Pho85

Pho85 is approximately 60% identical to Cdk1 and yet is not essential for cell survival (Huang, Friesen et al. 2007). Pho85 can bind one of ten Pho85 cyclin belonging to either the Pcl1 and Pcl2 subfamily (Pcl1, Pcl2, Pcl5, Pcl9 and Cgl1) or Pho80 subfamily (Pho80, Pcl6, Pcl7, Pcl8 and Pcl10) (Measday, Moore et al. 1997). Pho85 bound to a cyclin of the Pho80 family regulates phosphate starvation whereas Pho85 bound to a cyclin of the Pcl1 and Pcl2 family regulates cell cycle progression (Huang, Friesen et al. 2007; Huang, Kaluarachchi et al. 2009). The Pcl1 or Pcl2-Pho85 kinase complexes are required for cell cycle progression in the absence of both Cln1 and Cln2-Cdk1 complexes (Measday, Moore et al. 1997). Although the Pho85 consensus sites (S-P-X-L/I and S/T-P-X-D-L) are suggested to be different from the full consensus site of Cdk1 (S/T-P-X-K/R), these previous results suggest that the Pcl1 or Pcl2-Pho85 complexes could phosphorylate Cdk1 substrates and regulate the G1-S phase of the cell cycle. More recently, the Pho85-Pcl9 complex has been shown to phosphorylate Whi5, a repressor of SBF and MBF thereby regulating the transition through the G1-S phase of the cell cycle (Huang, Kaluarachchi et al. 2009).

1.9.6 START and G1 to S-phase cell cycle transcription factors: SBF and MBF

At the G1-phase of the cell cycle, the cells go through a point where they decide to commit to a new round of cell division or not. This critical point is called START in *S. cerevisiae.* and corresponds to the time when the yeast starts to bud and duplicate their spindle pole body (SPB) prior to DNA replication. The transcription of genes required for progressing from G1 to S phase of *S. cerevisiae* is regulated by two transcription factors, SBF and MBF (**Figure 7**). These key transcription factors regulate the expression of over 200 genes at START and some of these include other transcription factors that regulate the cell cycle such as *HCM1*, *PLM2*, *POG1*, *TOS4*, *TOS8*, *TYE7*, *YAP5*, *YHP1*, and *YOX1* (Horak, Luscombe et al. 2002). SBF activates genes mainly involved in budding and in membrane and cell wall biosynthesis (Igual, Toone et al. 1997), whereas MBF activates genes predominately involved in DNA replication and repair (Iyer, Horak et al. 2001). SBF and MBF seem to have highly redundant function in the mitotic cell cycle and are suspected to have specific function during meiosis (Iyer, Horak et al. 2001).

SBF and MBF are heterodimeric complexes composed of a common transactivation subunit called Swi6 and different DNA binding protein, Swi4 and Mbp1 respectively (**Figure 7**). SBF and MBF were thought to regulate the expression of different cyclin genes. SBF mainly regulates the expression of *CLN1* and *CLN2* (Nasmyth and Dirick 1991) whereas MBF governs the expression of *CLB5* and *CLB6*. However it is particularly challenging to dissect the different functions between SBF and MBF. While some genes are activated by either MBF or SBF, others are activated by both MBF and SBF. Single knockouts of any of these genes are viable whereas double knockouts of any pairs generate a lethal phenotype (Mendenhall and Hodge 1998). This suggests that these two transcription factors could act in parallel pathways and serve redundant functions.

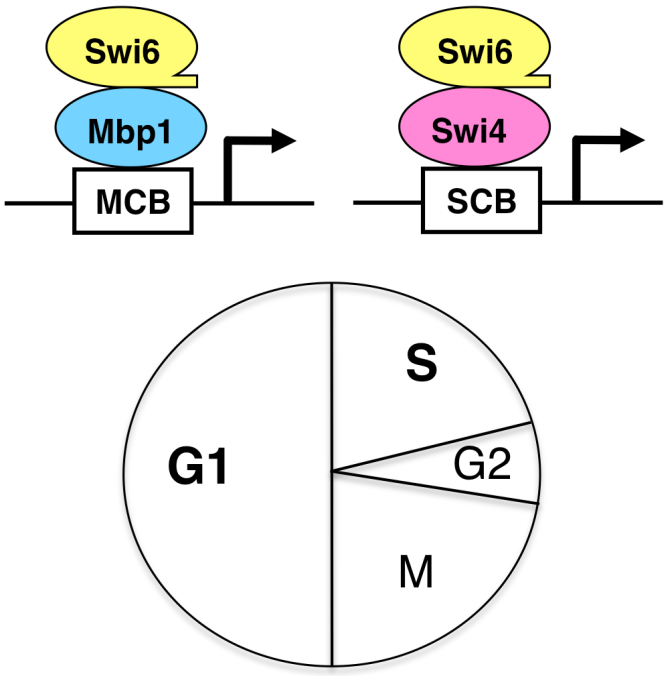

Figure 7. MBF and SBF transcription factors. MBF (Swi6:Mbp1) and SBF (Swi6:Swi4) complexes share the common Swi6 transcriptional activating subunit and regulate the transition of G1-to-S phase of the cell cycle by activating the expression of genes involved in DNA replication, budding and membrane biogenesis. MBF and SBF respectively bind to *MluI* cell-cycle box (MCB) and Swi4/6-dependent cell-cycle box (SCB) in the promoter regions of their target genes.

Swi4 and Mbp1 are structurally similar and belong to the helix-turn-helix DNA binding protein family (Taylor, Treiber et al. 1997; Xu, Koch et al. 1997). The DNA binding domain is found at the N-terminal of the proteins. Swi4 binds to the Swi4-Swi6-dependent cell-cycle box (SCB) DNA sequence (CACGAAAA) and Mbp1 binds to the *MluI* cell-cycle box (MCB) DNA sequence (ACGCGT) (Bahler 2005). Swi6 does not have a DNA binding domain at its N-terminal, but has a repeat of four ankyrin repeats (Foord, Taylor et al. 1999) followed by a heterodimerizing domain at its C-terminal similar to Swi4 and Mbp1 (Primig, Sockanathan et al. 1992; Koch, Moll et al. 1993; Siegmund and Nasmyth 1996). Co-immunoprecipitation experiments have shown that the C-terminal domains of both Swi4 and Mbp1 can interact with the C-terminal domain of Swi6 (Siegmund and Nasmyth 1996).

1.9.7 Activation and inactivation of SBF and MBF

Whi5 is a cell size sensor that acts by directly inhibiting SBF and MBF, ensuring that cells do not pass START before they have grown to a sufficient size (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004). When the cell reaches a critical size at the G1 phase, Cdk1/Cln activates SBF by phosphorylation of Whi5 (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004). Phosphorylation of Whi5 causes it to change its localization from nuclear to cytosolic and consequently it cannot bind and inhibit SBF and MBF activity. MBF is also thought be regulated by another repressor protein called Nrm1 (de Bruin, Kalashnikova et al. 2006). Whi5 acts as the integration centre for processing cellular decision to commit to a new round of cell division. When the cell is found in a nutrient deprive environment, Whi5 does not get phosphorylated and SBF and MBF remain inactive (Huang, Kaluarachchi et al. 2009).

The cyclin genes *CLN1*, *CLN2*, *CLB1*, *CLB2*, *CLB5*, and *CLB6* are among the most important targets of SBF and MBF. Cln1 and Cln2 were demonstrated to associate

with Cdk1 and phosphorylate Whi5 in order to further activate SBF and MBF by forming a positive feedback (Skotheim, Di Talia et al. 2008). Clb5 and Clb6 form complexes with Cdk1 but they are kept inactive by Sic1 (Tyers 1996). During the S-phase of the cell cycle, Sic1 is targeted for protein degradation allowing activation of the Clb5-6/Cdk1 complexes. At the G2/M phase of the cell cycle, Cdk1-Clb1 and Cdk1-Clb2 complexes phosphorylate and inactivate SBF by phosphorylation of Swi4 (Amon, Tyers et al. 1993; Siegmund and Nasmyth 1996). Swi6 is phosphorylated by the Cdk1-Clb6 complex and this phosphorylation promotes its nuclear exit during the M-phase of the cell cycle (Geymonat, Spanos et al. 2004). The inactivation of SBF and MBF serves as a regulatory mechanism to prepare the cell for mitosis and not return to the G1 or S phase. This makes the transition between each phase of the cell cycle rapid and irreversible.

1.9.8 Conservation of cell cycle regulation between yeast and metazoa

The regulation of SBF and MBF by Whi5 is analogous to the mammalian cell E2F and pRB system (Dick 2007). Although there is no sequence homology between the *S. cerevisiae* and metazoan proteins, the architectural design of this regulatory system is strikingly similar. pRB is the repressor that binds and inhibit the activity of the E2F and DP transcription factor in metazoan that regulates the Restriction point of the cell cycle (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004). Whi5 is the functional homologue of pRB. Phosphorylation of Whi5 and pRB abolish their interaction with the transcription factors thereby allowing the transcription factors to activate transcription of genes required for entry into the S-phase of the cell cycle.

1.9.9 Remodeling of the cytoskeleton during the cell cycle and the morphogenesis checkpoint

Cell growth and mitotic cell division are tightly coupled in budding yeast. The size of the bud is a direct indicator of the stage of the cell cycle of the dividing cell. The coordination between the process of bud formation and nuclear events of the budding yeast cell cycle is regulated by a morphogenesis checkpoint. This checkpoint involves the phosphorylation of Swe1 to delay or arrest when condition for normal bud growth is perturbed (Lew 2003). In addition, many proteins are involved in bud formation, cytoskeletal remodeling, and spindle pole bodies are potential substrates of Cdk1. Proteins such as Bud6 and Bem1 have been shown to be substrates of Cdk1 (Ubersax, Woodbury et al. 2003) but many remain uncharacterized due to the dynamic nature of this process. It would therefore be interesting to identify other proteins involved in bud growth using *in vivo* strategies.

1.10 Objectives of this thesis

Dissecting protein complexes has been a challenging task due to the lack of a general tool to engineer and detect mutations in proteins that will disrupt their protein-protein interactions or to probe dependencies for interactions among subunits in a protein complex. Protein complexes that regulate the mitotic cell cycle are particularly difficult to study because their interactions can be transient and regulated by post-translational modifications. These complexes serve different functions in different subcellular compartments at different times, ranging from cell cycle phase-specific transcription factors to metabolic or cytoskeletical structural elements and regulators. The goal of this thesis is to demonstrate that "you too can play with an edge" and dissect the function of cell cycle protein complexes with an all-in one tool.

The first aim of this thesis was to develop a general strategy that can be used to engineer any protein with distinct characteristics. I chose a PCA-based strategy using the yCD reporter enzyme that allows for clonal selection to be established upon the formation or disruption of protein-protein interaction between two proteins of interest.

The second aim was to apply the optimized yCD PCA (OyCD PCA) to dissect the function of Swi6 at the G1-S phase of the cell cycle. We used the OyCD PCA to engineer a specific mutant of Swi6 that has defective MBF activity while its SBF activity remains unchanged.

Finally, the goal was to apply the OyCD PCA to identify potential substrates of the yeast Cdk1 and infer the regulatory cyclin required for associating with Cdk1.

Chapter 2

A generalized life or death selection strategy to detect formation or disruption of protein-protein interactions

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In this chapter, I developed a novel *in vivo* selection strategy based on a Protein-fragment Complementation Assay using the yeast cytosine deaminase as a reporter enzyme (OyCD PCA) to detect protein-protein interactions between any categories of proteins including full-length transcription factors. A particularity of this assay is that it can detect both formation and disruption of protein-protein interactions and the output of the signal is detected as cell growth.

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Author contributions:

PHE and SWM designed the experiments, analyzed the results and wrote the manuscript. PHE performed all the experiments.

Abstract

Selection strategies are central tools in molecular biology. Here, we describe a general *in vivo* strategy to detect protein interactions based on a Protein-fragment Complementation Assay using the reporter enzyme cytosine deaminase from *Sacharomyces cerevisiae* (yCD PCA). We optimized the yCD PCA (OyCD PCA) using rational and random mutagenesis to function at 30 and 37 ^{o}C for applications in various cell types. This assay can detect either formation or disruption of protein-protein interactions. We used the OyCD PCA to detect interactions among the yeast cell cycle transcription factor subunits of SBF and MBF and found a novel interaction between the Swi6 subunit. In addition, we described applications of the OyCD PCA to mutagenic and chemical disruption of protein-protein interactions. This *in vivo* strategy would be ideal for performing fine dissection of transcriptional circuits to understand their basic molecular mechanisms or for applications in synthetic biology.

Introduction

The development of selection assays has facilitated studies in genetics, molecular biology, biochemistry and synthetic biology. Selection assays and strategies have followed a trend of increasing complexity while at the same time simplification of the components used to achieve complex selections. A way to simplify a complex selection system is to use a single reporter protein for both positive and negative selection. Some auxotrophic markers are interesting candidates since in addition to regulating cell survival, they convert non-toxic prodrugs to toxic compounds that kill cells (Hardies, Axelrod et al. 1983; Grimm, Kohli et al. 1988; Erbs, Exinger et al. 1997). These metabolic enzymes are also known as prodrug-converting enzymes (PCEs) and are used in both cell survival and cell death selection assays. The combination of a PCE and prodrug has been widely used in gene replacement (Boeke, Trueheart et al. 1987) strategies and the yeast two hybrid (Y2H) system (Vidal, Brachmann et al. 1996) for studying protein-protein interactions (PPIs).

Y2H using a PCE as a dual reporter protein has proven to be an important technique for identifying mutants that disrupt specific PPIs (Inouye, Dhillon et al. 1997). The mutants can then be used in genetic and biochemical experiments to dissect biochemical pathways. Unfortunately, this strategy is limited to study of soluble proteins or domains that can be transported to the nucleus and therefore excludes applications to membrane or compartmentalized proteins. Since the Y2H assay is based on reconstitution of transcription factors that in turn interact with the transcriptional machinery, it cannot be readily applied to full-length transcription factors or proteins that contribute directly or indirectly to transcription such as the RNA polymerases and mediator complexes, elongation factors or chromatin modifying enzymes. These limitations motivated us to develop an alternative strategy for detection and selection of PPIs based on a Protein-fragment

Complementation Assay (PCA) (Pelletier, Campbell-Valois et al. 1998; Michnick 2001; Remy, Campbell-Volois et al. 2005) using a PCE as reporter enzyme.

As is the case for PCAs based on a number of different reporter proteins, a PCE-based PCA involves the design and selection of two polypeptide fragments of a reporter protein and fusion of these complementary fragments to two interacting proteins. Interaction of the two proteins brings the fragments of the reporter protein into proximity, thus recreating the unimolecular conditions required for the fragments to fold together into an active reporter enzyme (Pelletier, Campbell-Valois et al. 1998; Michnick, Remy et al. 2000). In the case of a PCE-based PCA, the fragments could be reconstituted to restore the activity of the full-length PCE, which would result in positive (cell growth) or negative (cell death) selection for growth, depending on whether two test proteins interact and the conditions under which cells are grown (**Fig. 1A**). A PCE-based PCA would have the following features:

(i) PPIs are detected directly and do not require any other intracellular (e.g. transcriptional) machinery. This means that the PCA could be used in any cell type and could be applied to any aspect of chromosome biology.

(ii) Proteins can be expressed in their relevant cellular context, reflecting their native state and with the correct post-translational modifications. Thus, PPIs could be studied regardless of the compartments in which the interaction occurs.

(iii) Simple life or death selection would allow for the rapid screening for mutants of desired characteristics by using the positive selection to screen for increased affinity and negative selection for increased specificity. In each case, a positive readout is assured. This removes any ambiguity about whether a mutation may result in changes of a protein other than the ability to interact with another partner.

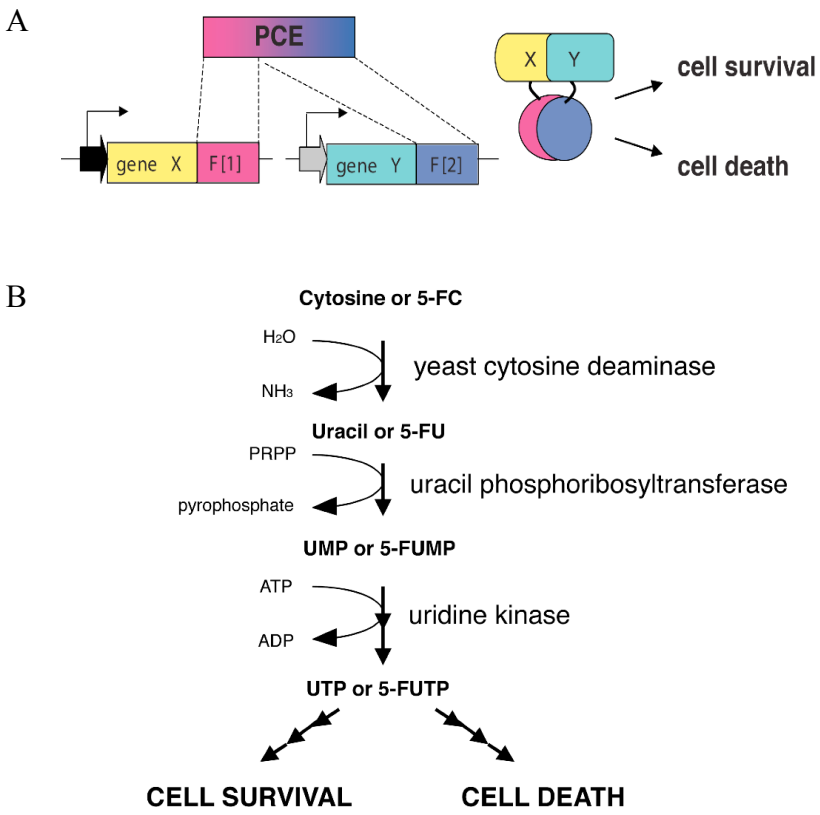


Figure 1. A positive and negative selection PCA based on yCD. **(A)** Dual selection PCA. A reporter gene is dissected into two complementary *N*- and *C*-terminal fragments each fused to one of two interacting proteins, such that the fragments fold into an active enzyme when brought into proximity by the interacting proteins. The PCA can serve as a reporter for formation of a protein-protein interaction provided that the reconstituted reporter enzyme supports growth under one condition (life assay) or no growth under another condition in which case disruption of complexes allows for growth (death assay). **(B)** Enzymes of the pyrimidine salvage pathway in *S. cerevisiae.* Yeast uses this pathway for cell survival when their *de novo* pyrimidine synthesis pathway is inhibited. Yeast cytosine deaminase deaminates (yCD) cytosine to generate uracil. Uracil is phosphoribosylated by uracil phosphoribosyltransferase to uridine monophosphate (UMP). UMP is phosphorylated by uridine kinase to uridine diphosphate and uridine triphosphate (UTP). UMP can converted to thymidine monophosphate and UTP can be incorporated into RNA. 5-fluorocytosine (5-FC) can also be processed by enzymes of the pyrimidine salvage pathway and allow the establishment of a negative selection assay. 5-FUTP is the toxic compound that causes cell death.

Here we describe the development of an optimized PCA based on the *Saccharomyces cerevisiae* prodrug-converting enzyme cytosine deaminase (OyCD PCA) that has all three desired features. Unlike Y2H, it is completely independent of any transcriptional machinery and unlike the split-ubiquitin reporter (Johnsson and Varshavsky 1994) it requires no other cell type- or cell compartment-specific proteins. We applied the OyCD PCA to detect interactions between the subunits of the yeast cell cycle transcription factors SBF and MBF; detect formation and disruption of protein-protein interaction with a positive growth output; and detect disruption of protein-protein interaction with a small molecule.

Results

Selection and design of cytosine deaminase PCA

In a prodrug-converting enzyme-based PCA the reconstituted activity would result in positive (cell survival) or negative (cell death) selection for growth, depending on whether the two test proteins interact and the conditions under which cells are grown (**Fig. 1A**). As for all PCAs, one based on a prodrug-converting enzyme requires the design of two complementary N- and C-terminal polypeptide fragments of a reporter protein fused to two interacting proteins. Interaction of the two proteins brings the fragments of the reporter protein into proximity, thus recreating the unimolecular conditions required for the fragments to fold together into an active reporter enzyme (Pelletier, Campbell-Valois et al. 1998; Michnick 2001).

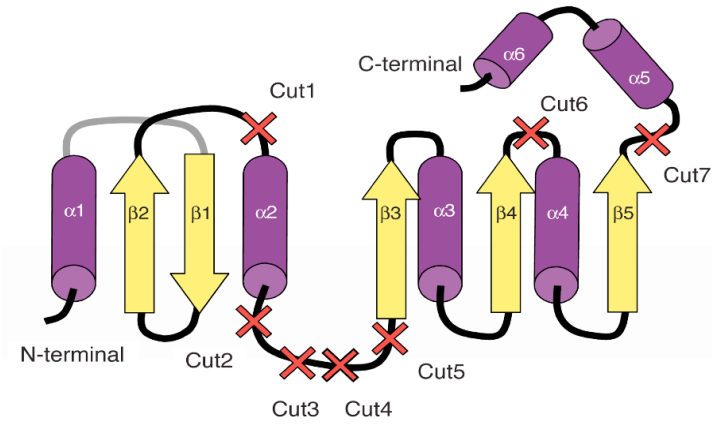
We selected the prodrug-converting enzyme yeast cytosine deaminase (yCD) for our PCA because positive and negative selection assays have been established for it in a broad spectrum of cell types including bacteria (Mahan, Ireton et al. 2004), yeast (Hartzog, Nicholson et al. 2005), and mammalian cells (Wei and Huber 1996). Further, its structural features meet criteria we have previously established for designing PCAs (Pelletier, Campbell-Valois et al. 1998; Michnick 2001). yCD permits cell survival by deaminating cytosine to uracil when the *de novo* pyrimidine pathway is inhibited and uracil is not available in the environment (Kurtz, Exinger et al. 1999). yCD can also deaminate 5-fluorocytosine (5-FC), a non-toxic compound, to 5-fluorouracil (5-FU), which is ultimately converted to 5-fluorouridine triphosphate (5-FUTP). 5-FUTP inhibits DNA and RNA synthesis when incorporated into DNA and RNA leading to cell death (Fang, Hoskins et al. 2004) (**Fig. 1B**).

Analysis of the yCD structure (PDB accession: 1OX7) revealed several possible sites to dissect the enzyme into two fragments based on criteria previously used to identify candidate fragmentation sites(Pelletier, Campbell-Valois et al. 1998). We tested seven different combinations of yCD fragments (referred to as F[1] and F[2], respectively) each fused to the C-terminal of homodimerizing residues (250 to 281) of the GCN4 parallel coiled-coil leucine zipper (ZIP) *via* a 15 amino acid $(GGGGS)_3$ linker sequence (**Fig. 2A**). Plasmids carrying the complementary fusion constructs, under control of the inducible *GAL1* promoter, were respectively transformed in *ura3Δ* and *fcy1Δ* (*FCY1* encodes for yCD) haploid strains since their *de novo* pyrimidine synthesis pathway and pyrimidine salvage pathway were impaired (Giaever, Chu et al. 2002). Cells from each mating type were mated and screened for yCD PCA activity. Only cells that express a functional PCA can deaminate cytosine to uracil and thus allow for cell survival or show sensitivity to 5-FC. We tested overlapping and non-overlapping fragments (**Table 1**) and found that the best yCD PCA activity was observed when both yCD-F[1] and yCD-F[2] contain amino acids 57 to 77 (ZIP-yCD-F$[1]_{1\text{-}77}$ and ZIP-yCD-F$[2]_{57\text{-}158}$) (**Fig. 2B**).

Optimization of yCD PCA

Starting from the optimum fragments we performed both rational and directed evolution to screen for improved yCD PCA activity. We first introduced three mutations (A23L, V108I, I140L) previously shown to increase thermostability of full-length yCD (Korkegian, Black et al. 2005) and found that cells showed increased sensitivity to 5-FC when grown at 37 ˚C (**Fig. 3**). However, for protein interactions other than the leucine zippers, the PCA was not optimal. For example, cells expressing the yCD PCA with the thermostable mutations fused to interacting partners human small GTPase H-Ras (Ras) and the Ras binding domain (RBD) of the serine/threonine kinase c-Raf are only slightly sensitive to 5-FC (**Fig. 4A**). To further improve the activity of yCD PCA, we generated a library of randomly mutated yCD

A



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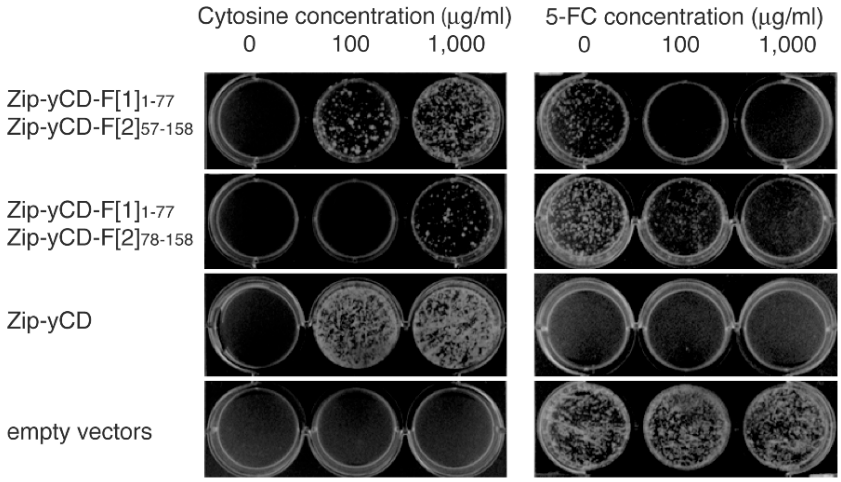


Figure 2. Development of the yCD PCA. **(A)** yCD topology and cut sites. yCD monomer is composed of six alpha (α) helices and five beta (β) strands. All cut sites are in loop regions of the protein. Cut sites are represented as cut1 to cut7 and are between residues K-56 and G-57, R-73 and L-74, G-76 and K-77, K-77 and V-78, K-80 and D-81, N-111 and V-112, and V-132 and D-133 respectively. **(B)** Comparison of yCD PCA activity using the life and death assays on solid medium. For the survival selection life assay, cells were plated on selective medium containing 0, 100 or 1,000 μg/mg of cytosine and incubated for 5 days at 30˚C. For the death assay, cells expressing the fusion proteins were pre-incubated with 100 μg/mg 5-FC in liquid selection medium. After preincubation time, each sample was plated on solid selection medium containing 0, 100 or 1,000 μg/mg of 5-FC and incubated 2 days at 30 ºC.

Table 1. PCA activity of the different yCD fragment combinations. *MAT***a** cells carrying Zip-[F1]yCD cut1 were mated with *MAT*α cells carrying Zip-[F2]yCD of different cut sites (cut1 to cut7). This process was repeated with *MAT***a** cells carrying Zip-[F1]yCD cut2 to Zip-[F1]yCD cut7. 5 X 10^3 cells were assayed for their sensitivity to 5-FC by comparing the OD_{600} of cells grown in the presence and absence of 100 μg/ml of 5-FC after 24 hrs of incubation at 30 °C. Results are qualitatively represented as: **++++**, highest yCD PCA activity; **+++**, high yCD PCA activity; **++**, moderate yCD PCA activity; **+**, low yCD PCA activity.

	Zip-F[1]cut1	Zip-F[1]cut2	Zip-F[1]cut3	Zip-F[1]cut4	Zip-F[1]cut5	Zip-F[1]cut6	Zip-F[1]cut7
Zip-F[2]cut1	+				+	+	
Zip-F[2]cut2	+++				+		
Zip-F[2]cut3	+++		+		+		+
Zip-F[2]cut4	++++			++	+		
Zip-F[2]cut5	++		+	+		+	
Zip-F[2]cut6	++						
Zip-F[2]cut7			+				

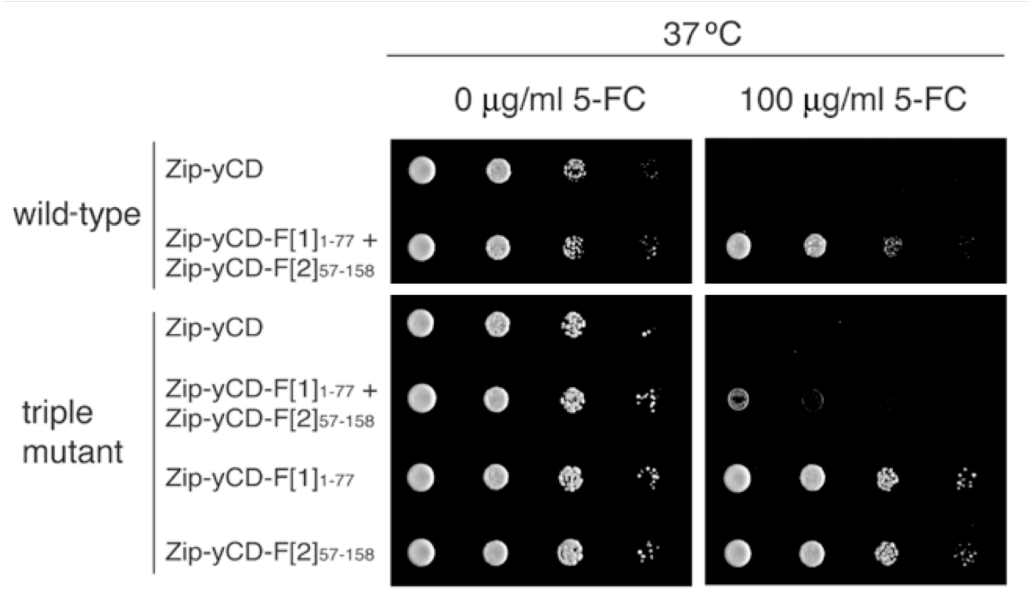
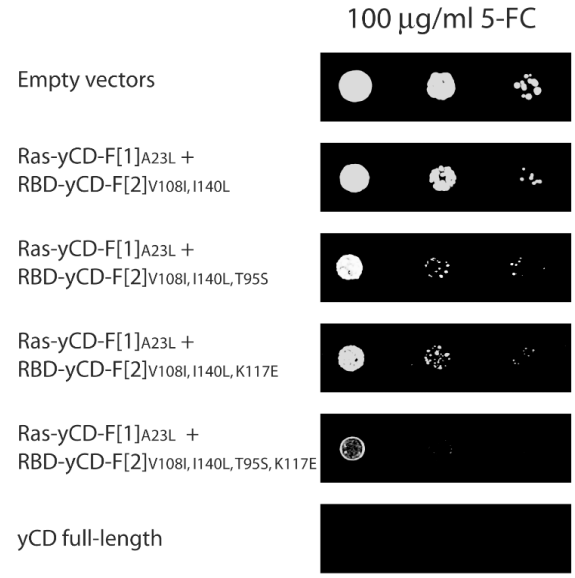


Figure 3. Activity of yCD PCA with the thermostabilizing triple mutations at 37 °C. Cells expressing wild-type Zip-yCD-F[1]$_{1-77}$ and Zip-yCD-F[2]$_{57-158}$ are not sensitive to 5-FC at 37 °C whereas cells expressing the same constructs with the triple mutations are sensitive. Cells carrying Zip-yCD-F[1]$_{1-77}$ or Zip-yCD-F[2]$_{57-158}$ alone are not sensitive to 5-FC at 37 °C.

A



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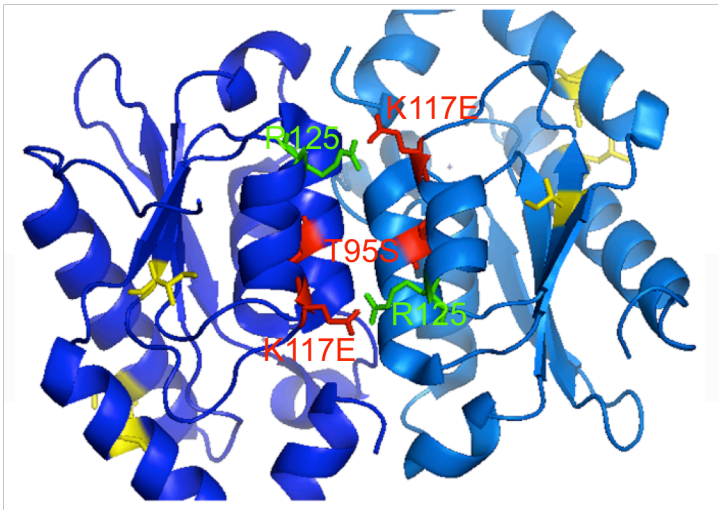


Figure 4. Optimizing yCD PCA activity. **(A)** The effect of different mutations on the activity of yCD PCA as a function of sensitivity to 5-FC at 37 °C. Cells expressing Ras-yCD F[1]$_{A23L}$ and RBD-yCD F[2]$_{V108I, I140L, T95S, K117E}$ are the most sensitive to 5-FC. **(B)** A model of the OyCD structure based on the dimeric yCD triple mutant (PDB accession: 1YSB) with T95S and K117E mutations (modeled in PYMOL (DeLano 2002)). The triple mutations are represented in yellow, T95S and K117E mutations in red and R125 in green.

fragments by error-prone PCR (with 1 to 2 mutations per fragment) fused to the H-Ras and the RBD (Ras-yCD-F[1]ep and RBD-yCD-F[2]ep) and screened for combinations of these with improved yCD cytosine deaminase activity at 37 ˚C using the survival selection assay of yCD PCA (**Fig. 5**). Subsequently, we isolated 276 clones and evaluated them for improved yCD 5-FC deaminase activity at 37 ˚C using the death selection assay of yCD PCA. We found 16 that have increased 5-FC deaminase activity (**Tables 2 and 3**). Clones carrying the T95S and K117E mutations, which in combination with the three thermostable mutations showed the greatest sensitivity to 5-FC. The new optimized fragments are called henceforth, OyCD-F[1] and OyCD-F[2]. It is not obvious how these mutations could improve activity of the PCA. T95S is located in the middle of the α4- facing the α5-helix (**Fig. 4B**) while K117E mutation is at the beginning of the α5 helix with the sidechain oriented towards the dimer interface. The latter mutant may result in a salt bridge that stabilizes the dimer between the mutant E117 (red) carboxylate side chain and the guanidinium sidechain of wild-type R125 (green) of the adjacent subunit. Interestingly, all mutations that showed increased yCD activity for deaminating 5-FC to 5-FU are located at the dimer interface of yCD (**Fig. 6**). This further suggests that the dimerization of yCD is required for its 5-FC deamination activity. To verify this hypothesis, we introduced a mutation at the tyrosine 121 residue, previously suggested to be a key residue for yCD dimerization and determined the activity of the OyCD PCA. By mutating tyrosine 121 to alanine 121, the activity of the OyCD PCA was lost (**Fig. 7**).

The goal for developing the OyCD PCA is to establish a general selection assay for dissecting the functions of any protein complexes including transcription factors. Before we applied the OyCD PCA to studying the interactions between proteins, we needed to determine if the activity of this PCA is induced by protein-protein interactions or results from spontaneous complementation of the fragments. To test

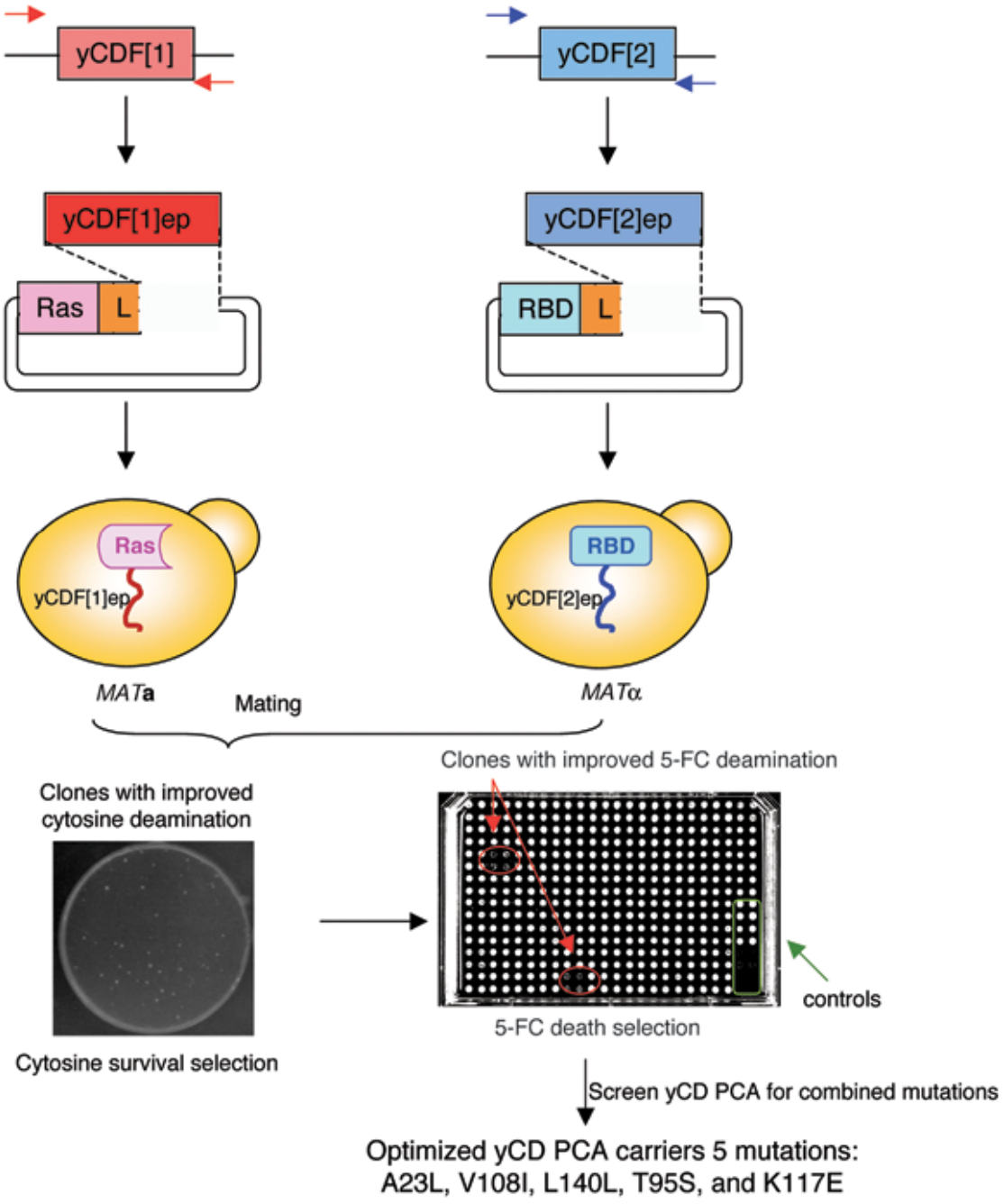

yCDF[1]
yCDF[2]
yCDF[1]ep
yCDF[2]ep
Ras
L
RBD
L
Ras
yCDF[1]ep
RBD
yCDF[2]ep
MATa
Mating
MATα
Clones with improved 5-FC deamination
Clones with improved cytosine deamination
controls
5-FC death selection
Cytosine survival selection
Screen yCD PCA for combined mutations
Optimized yCD PCA carriers 5 mutations:
A23L, V108I, L140L, T95S, and K117E

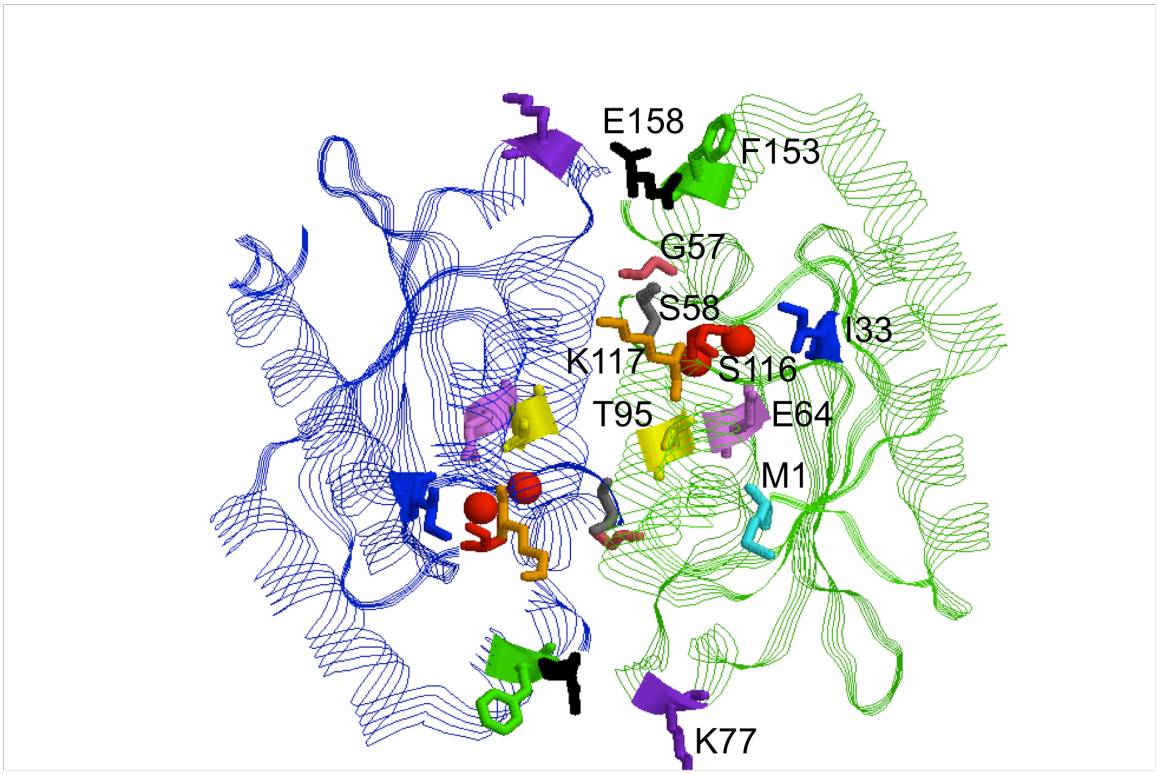
Figure 5. Optimization of yCD PCA. We started with the yCD-F[1]1-77 and yCD-F[2]57-158 (yCD-F[1] and yCD-F[2], respectively) harboring the mutations that resulted in 5-FC sensitivity at 37 °C. We generated error-prone PCR products of yCD-F[1] and yCD-F[2] (yCD-F[1]ep and yCD-F[2]ep) and cloned the fragments downstream of the Ras and RBD gene sequences respectively. The plasmid libraries were transformed in *fcyΔ* mutant strains. Cells from each mating type were mated and screened for improved yCD PCA activity by identifying colonies that grew after 4 days of incubations at 37 °C. These clones were then screened for enhanced yCD deamination of 5-FC to 5-FU as determined by sensitivity to 5-FC resulting in cell death. Different combinations of point mutation(s) located on yCD-F[1] and on yCD-F[2] that improved yCD PCA activity were identified (**Table 2**). We combined the different point mutations identified using site-directed mutagenesis. Clones that were highly sensitive to 5-FC were selected and additional mutations identified are listed in **Table 3**.

Table 2. Mutations identified in yCD fragments from the error-prone PCR library screen.

Clones from epPCR library	Mutation on yCD-[F1] $_{A23L}$	Mutation on yCD-[F2] $_{I140L, V108I}$
1	-	E64A
2	-	T95S
3	-	T95S
4	-	T95S
5	-	T95S
6	-	T95S
7	-	K117E
8	-	F153L
9	M1L	G57R
10	M1L	T95S
11	M1L	T95S
12	M1L	T95S
13	M1L	S116C
14	M1L	S58P + E158E
15	M1L + K77M	T95S
16	I33V	S58P + E158E

Table 3. Mutations identified by combining mutations found in yCD fragments from the error-prone PCR library screen.

Clones from combined mutagenesis library	Mutation on yCD-[F1] A23L, M1L	Mutation on yCD-[F2] I140L, V108I, T95S
1	-	S116C, K117E
2	-	S116C, K117E
3	I33V	K117E
4	I33V, K77M	S116C, K117E
5	I33V, K77M	S116C, K117E
6	I33V, K77M	S116C, K117E



Legend	G57 : pink	S116: red
M1 : cyan	S58: grey	K117: orange
I33: blue	E64: violet	F153: green
K77: purple	T95: yellow	E158: black

Figure 6. Mapping mutations identified in yCD fragments that increase yCD deamination of 5-FC to 5-FU onto the yCD structure. The structure of yCD dimer (PDB accession: 1OX7). The yCD monomers are shown in blue and green. Residues identified to be mutated in clones carrying yCD fragments with increase 5-FC deamination activity are shown in stick representation. The colour of each unique residue corresponds to an amino acid residue on yCD listed below the structure. Among the sixteen clones identified to have increased 5-FC deaminase activity, eleven unique residues were found. All eleven residues are located at the dimer interface of yCD.

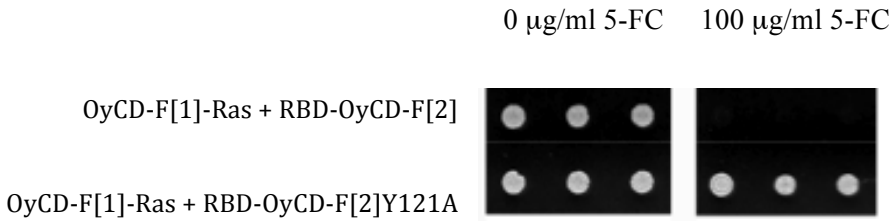


Figure 7. Effect of Y121A mutation on the optimized yCD PCA. Cells expressing interacting proteins fused to OyCD fragments (OyCD-F[1]-Ras and RBD-OyCD-F[2]) are sensitive to 5-FC. Cells expressing the same fusion proteins that contain the Y121A mutation on OyCD-F[2] are resistant to 5-FC. A ten-fold serial dilution was performed and cells were pinned onto selection medium. The first spot corresponds to 10,000 cells.

this, we co-expressed our proteins of interest fused to OyCD-F[1] with OyCD-F[2] alone and observed no change in OyCD PCA activity (**Fig. 8**). This suggests that the OyCD fragments cannot spontaneously complement each other to restore yCD activity. The activity of the OyCD PCA observed are promoted by interacting proteins.

Detection of protein interactions between transcription factor subunits

In the budding yeast *S. cerevisiae*, each round of cell division requires the activity of two important transcription factors, SBF and MBF. SBF and MBF are heterodimeric complexes composed of a common transactivation protein, Swi6 and different DNA binding proteins Swi4 and Mbp1, respectively. We generated fusion proteins of Mbp1, Swi4, and Swi6 with OyCD fragments and tested them for protein-protein interactions (PPIs) using the 5-FC death selection assay of OyCD PCA. Consistent with previous studies (Siegmund and Nasmyth 1996) we observed interactions between Mbp1 and Swi6 and between Swi4 and Swi6 (**Fig. 9A**). We found no interaction between Mbp1 and Mbp1 or Swi4 and Swi4 (**Fig. 9A**). Interestingly, we observed a novel interaction of Swi6 with itself (**Fig. 9B**). The dimerization of Swi6 has been suggested (Foord, Taylor et al. 1999) but has never been shown by any *in vitro* or *in vivo* assay since assays such as the Y2H system is based on the reconstruction of the transcription factor Gal4 from its DNA binding domain (DBD) and transactivation domain (AD) to drive the expression of the reporter protein (Fields and Song 1989). Since Swi6 has a AD, the fusion of Swi6 to the DBD of Gal4 will give a false positive signal using the Y2H assays. Testing for the interaction between Swi6 and Swi6 would not be possible. The homodimerization of Swi6 could suggest a new regulatory mechanism of activation for Swi6. However, the biological significance is yet to be determined. PPIs between transcription factors (TFs) and TF subunits are important for their activation but unfortunately have not been extensively studied *in vivo* since many detection assays are based on TF

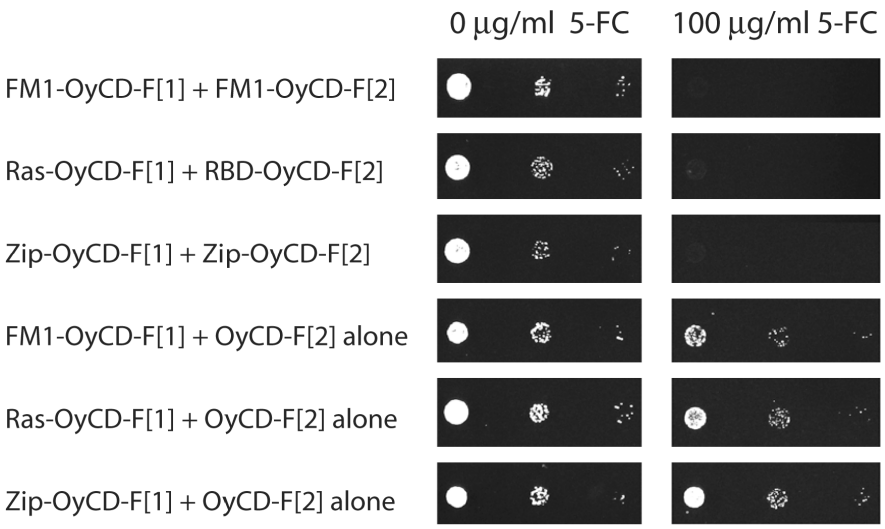


Figure 8. OyCD PCA activity is mediated by protein-protein interaction. Cells expressing interacting fusion proteins fused to OyCD fragments (Ras-OyCD-F[1] and RBD-OyCD-F[2], FM1-OyCD-F[1] and FM1-OyCD-F[2] or Zip-OyCD-F[1] and Zip-OyCD-F[2]) are sensitive to 5-FC. Cells expressing Ras-OyCD-F[1], FM1-OyCD-F[1] or Zip-OyCD-F[1] with OyCD-F[2] alone are resistant to 5-FC. A ten fold serial dilution was performed and cells were pinned onto selection medium. The first spot corresponds to 10,000 cells.

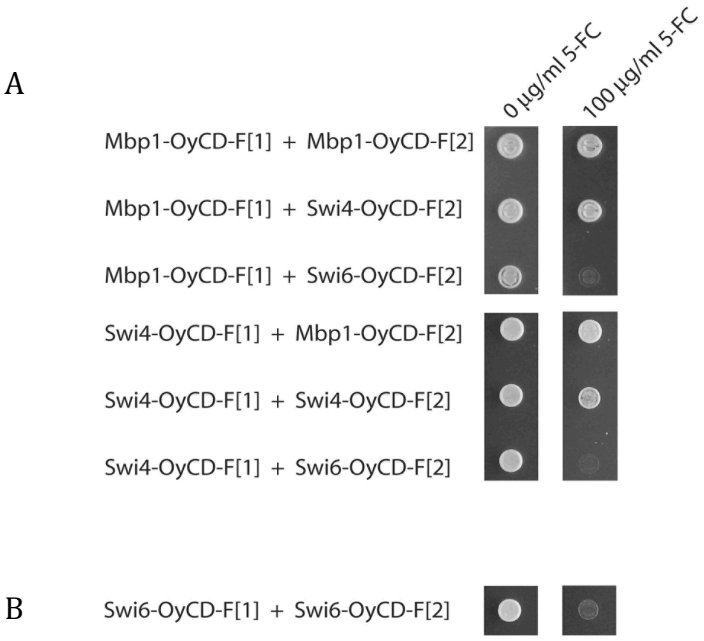


Figure 9. Detecting interactions among transcription factor subunits. **(A)** Interactions among subunits of SBF and MBF transcription factors. Cells expressing interacting proteins fused to OyCD fragments (Mbp1-OyCD-F[1] and Swi6-OyCD-F[2] or Swi4-OyCD-F[1] and Swi6-OyCD-F[2]) are sensitive to 5-FC. Cells expressing non-interacting fusion proteins (Mbp1-OyCD-F[1] and Mbp1-OyCD-F[2], Mbp1-OyCD-F[1] and Swi4-OyCD-F[2]), Swi4-OyCD-F[1] and Mbp1-OyCD-F[2], or Swi4-OyCD-F[1] and Swi4-OyCD-F[2]) are resistant to 5-FC. **(B)** Interaction observed between Swi6 and Swi6.

as readout of the signal. Often only fragments of TFs are investigated and not the full-length TFs. With our OyCD PCA, we showed that potential novel interactions between TFs can easily be identified.

Detecting formation and disruption of protein-protein interactions

For purposes of designing specificity of PPIs, one would want to positively select for mutants that bind with high affinity, while sequentially selecting against non-specific interactions. Usually it is the second step that is complicated by the need to replicate positive clones and then perform necessary negative-selection (Havranek and Harbury 2003). In the case of the OyCD PCA, both selection for and against interaction results in a positive selection for cell growth. To demonstrate this principle, we used OyCD PCA to detect the interaction of Ras and binding affinity mutants of the RBD. We found that Ras interacts with the *wild-type* RBD and mutants of RBD possessing K_D up to 14 μM (**Fig. 10**) but not with the R89L mutant for which a K_D could not be measured in *in vitro* and *in vivo* assays (Block, Janknecht et al. 1996; Campbell-Valois, Tarassov et al. 2005). Thus, these results show that the OyCD PCA provides positive selection for both formation or disruption of a PPI and with an upper limit for detection of positive PPIs with K_D around tens of micromolar for Ras and RBD. The sensitivity of the OyCD PCA will depend on the number of reconstituted enzymes in the cell and not just the affinity. Thus, the sensitivity of the OyCD PCA will also depend on the quantity of each fusion protein expressed.

Disruption of protein-protein interaction with a small molecule

Much effort is now devoted to creating novel chemical or protein probes for manipulation of cellular regulatory networks. In a final demonstration of broad interest, we show that OyCD PCA could be used as a positive genetic screening tool for identifying molecules that disrupt the interactions of a specific PPI (Cochran

2001; Toogood 2002; Yin and Hamilton 2005). The constitutive interaction between the homodimeric protein FM1 was challenged with the macrolide natural product FK506 (Rollins, Rivera et al. 2000). FK506 specifically binds to and disrupts the homodimeric complex of FM1. We first determined an optimal concentration of FK506 by titration (**Fig. 11**) and found it to be similar to that obtained with a three-hybrid transcriptional reporter assay (Licitra and Liu 1996). Cells expressing FM1 fused to OyCD fragments were sensitive to 5-FC and did not form colonies (**Fig. 12**). However, colonies were observed when these cells were grown in the presence of 5-FC and FK506. In contrast, cells expressing interacting proteins, Ras-OyCD-F[1] and RBD-OyCD-F[2] or OyCD-F[1]-Ras and RBD-OyCD-F[2] did not grow in the presence of 5-FC and FK506. Cells expressing non-interacting proteins, FM1-OyCD-F[1] and RBD-OyCD-F[2], and cells carrying empty vectors remained resistant to either 5-FC alone or 5-FC and FK506. These results show that OyCD PCA can be used to detect disruption of a direct interaction between FM1 homodimer using FK506. Therefore, OyCD PCA could potentially be used as a positive screen of libraries of small molecules or genetically encoded nucleotides and linear or cyclic peptides (Scott, Abel-Santos et al. 1999) or of intrabodies based on simple protein scaffolds (Koide, Gilbreth et al. 2007; Gilbreth, Truong et al. 2011) for those that disrupt specific PPIs.

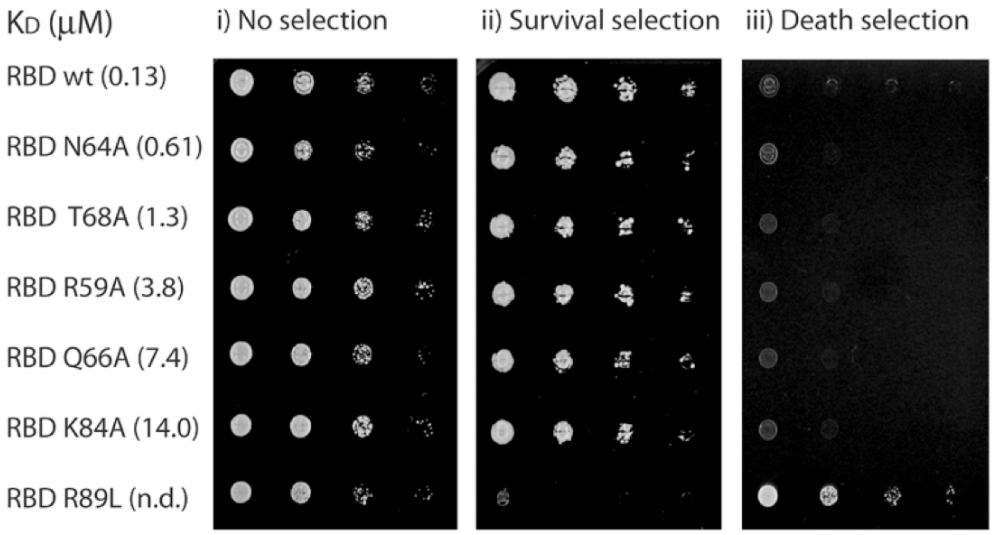


Figure 10. Dual selection properties of OyCD PCA. Cells were transformed with OyCD-F[1]-Ras and wt RBD-OyCD-F[2] or mutated RBD-OyCD-F[2]. Serial dilution was performed and cells were pinned onto medium with i), no selection, ii), 50 μg/mg cytosine for survival selection, and iii), 50 μg/mg 5-FC for death selection.

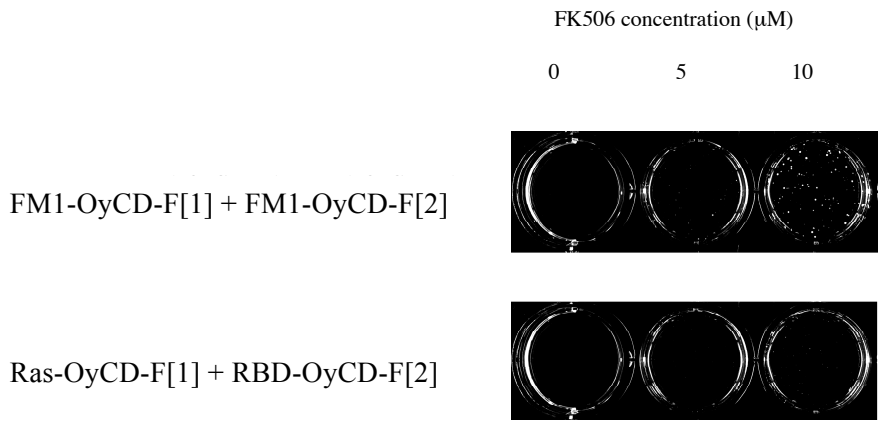


Figure 11. Titration of FK506 concentration required for the disruption of FM1 OyCD PCA. FM1 is homodimeric protein that can be dissociated to form monomeric proteins when bound to FK506. Cells expressing FM1-OyCD-F[1] + FM1-OyCD-F[2] and Ras-OyCD-F[1] + RBD-OyCD F[2] were plated on selection medium containing 100 μg/ml of 5-FC and various concentration of FK506. Only cells expressing FM1-OyCD-F[1] and FM1-OyCD-F[2] grew in the presence of 5 and 10 μM FK506.

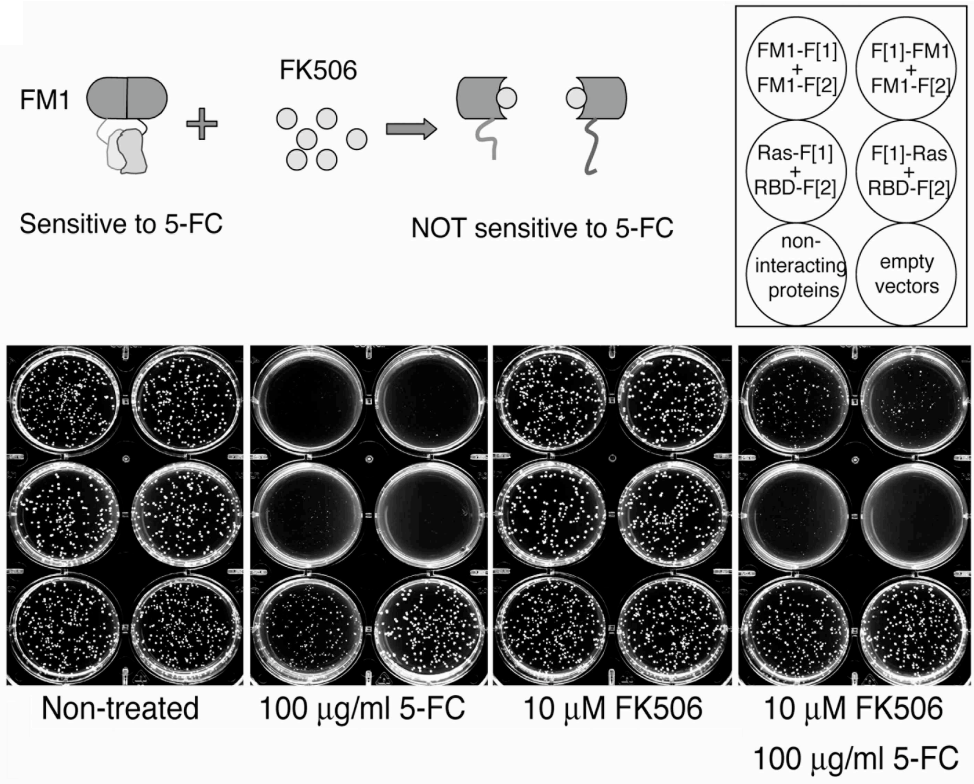


Figure 12. Disruption of interaction between FM1 homodimer using FK506. Dissociation of FM1 OyCD PCA induced by FK506. Cells expressing OyCD PCA fusion proteins and carrying empty vectors were plated on medium with no selection, with 100 μg/ml 5-FC, with 10 μM FK506, and with 100 μg/ml 5-FC and 10 μM FK506 for selection. Cells carrying FM1 OyCD PCA are sensitive to 5-FC but can restore growth in the presence of 5-FC and FK506.

Discussion

Simultaneous positive and negative selection is an invaluable strategy for genetics. Our results demonstrate that the OyCD PCA is a simple life/death, positive and negative molecular genetic selection assay. This multi-purpose selection system can be used to study the interaction or disruption of interaction for all categories of full-length proteins including TFs since it is independent of the transcriptional machinery. In addition, OyCD PCA can be used to generate specific mutants for dissecting the complexity of these pathways. Thus, this novel tool can be used in combination with chromatin immunoprecipitation coupled with microarrays (ChIP-chip) (Pokholok, Zeitlinger et al. 2006), protein microarrays (Ho, Jona et al. 2006) and other methods for understanding the components of gene regulation. Finally, it is a powerful tool for identifying genetically encoded peptides (Norman, Smith et al. 1999), cyclic peptides (Scott, Abel-Santos et al. 1999), or small organic molecules (Cochran 2001; Toogood 2002; Yin and Hamilton 2005) that disrupt the interaction of a specific PPI.

OyCD PCA is particularly attractive for many cell systems and model organisms for two reasons. First, OyCD PCA is functional at various temperatures ranging from 22 ^{o}C (data not shown) to 37 ^{o}C. We took advantage of this property to express mammalian proteins, Ras and different forms of RBDs in yeast at 37 ^{o}C (**Fig. 10**) and showed that the interactions are consistent with previous studies (Block, Janknecht et al. 1996). Other mammalian proteins can therefore be expressed in yeast using this assay and be engineered for the desired characteristics. In addition, the functionality of this PCA at different temperatures allows it to be transferred to other cell systems that are grown under different conditions. Second, the cytosine deaminase enzyme is not present in higher eukaryotes such as mammalian, insect, worm, or plant cells. Hence, OyCD PCA can be directly introduced into these cells and selection for the cytosine deaminase activity can be achieved. Budding yeast and

bacteria possess cytosine deaminase; however, knockout strains of this enzyme are currently available (Mahan, Ireton et al. 2004). Together, these features allow OyCD PCA to be used in any cell or model organism.

Materials and methods

Construction of the yCD PCA. GCN4 leucine zipper (ZIP) and the linker sequence coding for amino acids GGGGS was amplified by PCR with *pfu* polymerase (Fermentas) from pcDNA3.1-ZIP-[F1.2] mDHFR (Remy and Michnick 1999). All oligo sequences are listed in **Table 4.** ZIP was cloned into the multiple cloning sites of p413Gal1 and p415Gal1 vectors (Mumberg, Muller et al. 1995) at the *Xba*I and *Xho*I restriction sites. A unique *BspE*I site was added as part of the linker sequence for cloning downstream of the ZIP sequence. Vectors carrying the ZIP sequence are named p413Gal1-ZIP and p415Gal1-ZIP. yCD gene and yCD fragments were amplified from the genomic DNA of *S. cerevisiae* strain BY4743 (diploid: *ura3Δ0 leu2Δ0 his3Δ1 met5Δ0 lys2Δ0*) using *pfu* polymerase and cloned into p413Gal1-ZIP and p415Gal1-ZIP vectors downstream of the ZIP sequence using *BspE*I and *Xho*I restriction sites. Ras_{1-166} was amplified from pQE30-Ras-DHFR[3](Pelletier, Campbell-Valois et al. 1998) and subcloned upstream of yCD-F[1] to generate p413Gal1-Ras-yCD-F[1]. RBD_{1-131} was amplified from p416Gal1-RBD_{1-133} and subcloned upstream of yCD-F[2] to generate p415Gal1-RBD-yCD-F[2]. The full-length sequence of Ras_{1-189} was amplified from p413ADH1-Ras_{1-189} and subcloned in p413Gal1 using *Xba*I and *Xho*I sites with a primer that introduces a *BspE*I site downstream of *Xba*I. OyCD-F[1] was subcloned upstream of Ras_{1-189} using *Xba*I and *BspE*I sites to generate p413Gal-OyCD-F[1]-Ras_{1-189}. Wild-type RBD_{55-132} and seven mutant RBD_{55-132} (Block, Janknecht et al. 1996; Campbell-Valois, Tarassov et al. 2005) sequences were amplified and subcloned upstream of OyCD-F[2] to give p415Gal1-$RBD_{wt\ or\ mutant}$-OyCD-F[2]. Mbp1, Swi4, and Swi6 were digested from p415ADH-Mbp1-vF[2], p415ADH-Swi4-vF[2], and p415ADH-Swi6-vF[2] (Manderson, Malleshaiah et al. 2008) and sub-cloned in p413Gal1-OyCD-F[1] and p415Gal1-OyCD-F[2]. GST was amplified from pGEX-5X-3 and sub-cloned in p415Gal1 plasmids to generate p415Gal1-GST, p415Gal1-Mbp1-GST and p415Gal1-Swi4-GST. FM1 was

Table 4. List of primers.

Experiments	Primer Information	Primer Sequence 5' to 3'(restriction sites are underlined)
OyCD PCA	3) NterZIP-forward with XbaI site	cgc tctaga ggg ATGAACACTGAAGCCGCCAGGCG
OyCD PCA	4) NterZIP-reverse with BspEI and XhoI sites	ccg ctcgag cta tccgga gccaccgccacc GCGTTCGCCAACTAATTTC
OyCD PCA	5) NterRas-forward with XbaI site	cgc tctaga ggg ATGACAGAaTACAAGCTTG
OyCD PCA	6) NterRas-reverse with BspEI and XhoI sites	ccg ctcgag cta tccgga gccaccgccacc GTGCTGCCGGATCTCACG
OyCD PCA	7) NterRBD-forward with XbaI site	cgc tctaga ggg ATGGAGCACATACAGGGAGC
OyCD PCA	8) NterRBD-reverse with BspEI & XhoI sites	ccg ctcgag cta tccgga gccaccgccaccCAGGAAATCTACTTGAAG
OyCD PCA	9) yCD-forward with BspEI site	ggc tccgga ggt gga ggt tct gga ggt ATGGTGACAGGGGGAATGGC
OyCD PCA	10) yCD-reverse with XhoI site	ccg ccc ccg ctcgag cta CTCACCAATATCTTCAAACC
OyCD PCA	11) Linker-F2cut2-forward with BspEI site	ggc tccgga ggt gga ggt tct gga ggt ggc gga tct TTAGAGGGCAAAGTGTACAAAG
OyCD PCA	12) F1cut2-reverse with XhoI site	ccg ccc ccg ctcgag cta TCTCCCACAGTTTTCCAAAGTGGAG
OyCD PCA	21) Linker-1forward with BspEI site	ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct ATGGTGACAGGGGGAATGGC
OyCD PCA	22) F1cut1-reverse with XhoI site	ccg ccc ccg ctcgag cta CTTTTGAAATCTCATGTT
OyCD PCA	23) F2cut1-forward with BspEI site	ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GGATCCGCCACACTACAT
OyCD PCA	25) F1cut3-reverse with XhoI site	ccg ccc ccg ctcgag cta GCCCTCTAATCTCCCACAG
OyCD PCA	26) F2cut3-forward with BspEI site	ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct AAAGTGTACAAAGATACCAC
OyCD PCA	28) F1cut4-reverse with XhoI site	ccg ccc ccg ctcgag cta TTTGCCCTCTAATCTCCC
OyCD PCA	29) F2cut4-forward with BspEI site	ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GTGTACAAAGATACCACT
OyCD PCA	31) F1cut5-reverse with XhoI site	ccg ccc ccg ctcgag cta TTTGTACACTTTGCCCTC
OyCD PCA	32) F2cut5-forward with BspEI site	ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GATACCACTTTGTATACG
OyCD PCA	34) F1cut6-reverse with XhoI site	ccg ccc ccg ctcgag cta GTTCTCACCGACAACACA
OyCD PCA	35) F2cut6-forward with BspEI site	ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GTTAATTTCAAAAGTAAGGGC
OyCD PCA	38) F1cut7-reverse with XhoI site	ccg ccc ccg ctcgag cta AACAACAACAACCTCGTG
OyCD PCA	39) F2cut7-forward with BspEI site	ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GACGATGAGAGGTGTAAA
OyCD PCA	111) CYC1-reverse with BglII	ctaaac*agatct*AGCTTGCAAATTAAAGCCTTCGAGCG
OyCD PCA	143) yCD-A23L-forward	GACATTGCCTATGAGGAGGCGctgTTAGGTTACAAAGAGGGTGGT
OyCD PCA	144) yCD-A23L-reverse	ACCACCCTCTTTGTAACCTAAcagCGCCTCCTCATAGGCAATGTC
OyCD PCA	145) yCD-I140L-forward	GACGATGAGAGGTGTAAAAAGctgATGAAACAATTTATCGAcGAAAGACCTCAGGATTGG
OyCD PCA	146) yCD-I140L-reverse	CCAATCCTGAGGTCTTTCgTCGATAAATTGTTTCATcagCTTTTTACACCTCTCATCGTC
OyCD PCA	147) yCD-V108I-forward	GCCATCATCATGTATGGTATTCCACGCTGcGTTatcGGTGAGAACGTTAATTTCAAAAG
OyCD PCA	148) yCD-V108I-reverse	CTTTTGAAATTAACGTTCTCACCgatAACgCAGCGTGGAATACCATACATGATGATGGC
OyCD PCA	149) F1epyCD I33V-forward	GGTTACAAAGAGGGTGGTGTTCCTGTTGGCGGATGTCTTATCAATAAC
OyCD PCA	150) F1epyCD I33V-reverse	GTTATTGATAAGACATCCGCCAACAGGAACACCACCCTCTTTGTAACC
OyCD PCA	151) F1epyCD K77M-forward	GAAAACTGTGGGAGATTAGAGGGAATGTAGCTCGAGTCATGTAATTAG
OyCD PCA	152) F1epyCD K77M-reverse	CTAATTACATGACTCGAGCTACATTCCCTCTAATCTCCCACAGTTTTC
OyCD PCA	153) F2epyCD G57R-forward	ggaTCTGGAGGTGGCGGATCTaGaTCCGCCACACTACATGGTGAGATC
OyCD PCA	154) F2epyCD G57R-reverse	GATCTCACCATGTAGTGTGGCGGAtCtAGATCCGCCACCTCCAGAtcc
OyCD PCA	155) F2epyCD S58P-forward	GGAGGTGGCGGATCTGGAcCCGCCACACTACATGGTGAGATCTCCAC
OyCD PCA	156) F2epyCD S58P-reverse	GTGGAGATCTCACCATGTAGTGTGGCGGgTCCAGATCCGCCACCTCC
OyCD PCA	157) F2epyCD E158V-forward	CAGGATTGGTTTGAAGATATTGGTGTGTAGCTCGAGTCAT
OyCD PCA	158) F2epyCD E158V-reverse	CTAATTACATGACTCGAGCTACACACCAATATCTTCAAACCAATCCTG
OyCD PCA	159) F2epyCD E64A-forward	GATCTGGATCCGCCACACTACATGGTGCGATCTCCACTTTGGAAAACTG
OyCD PCA	160) F2epyCD E64A-reverse	CAGTTTTCCAAAGTGGAGATCGCACCATGTAGTGTGGCGGATCCAGATC
OyCD PCA	161) F2epyCD S116C-forward	ATCGGTGAGAACGTTAATTTCAAATGTAAGGGCGAGAAATATTTACAAACT
OyCD PCA	162) F2epyCD S116C-reverse	AGTTTGTAAATATTTCTCGCCCTTACATTTGAAATTAACGTTCTCACCGAT
OyCD PCA	163) F2epyCD K117E-forward	GAGAACGTTAATTTCAAAAGTGAGGGCGAGAAATATTTACAAACTAGA
OyCD PCA	164) F2epyCD K117E-reverse	TCTAGTTTGTAAATATTTCTCGCCCTCACTTTTGAAATTAACGTTCTC
OyCD PCA	165) F2epyCD F153L-forward	GACGAAAGACCTCAGGATTGGTTGGAAGATATTGGTGAGTAGCTCGAG
OyCD PCA	166) F2epyCD F153L-reverse	CTCGAGCTACTCACCAATATCTTCCAACCAATCCTGAGGTCTTTCGTC
OyCD PCA	167) F2epyCD G57R&S58P-forward	CTGGAGGTGGCGGATCTaGgcCCGCCACACTACATGGTGAGATtTCCACTTTGGAAAAC
OyCD PCA	168) F2epyCD G57R&S58P -reverse	GTTTTCCAAAGTGGAaATCTCACCATGTAGTGTGGCGGgcCtAGATCCGCCACCTCCAG
OyCD PCA	169) F2epyCD S116C&K117E-forward	GGTGAGAACGTTAATTTCAAATGTgAGGGCGAGAAATATTTACAAACTAGAGG
OyCD PCA	170) F2epyCD S116C&K117E-reverse	CCTCTAGTTTGTAAATATTTCTCGCCCTcACaTTTGAAATTAACGTTCTCACC
OyCD PCA	171) RBD55-XbaI-forward:	tct cgc tctaga ggg ATG AGC AAC ACT ATC CGT GTT TTC
OyCD PCA	172) r-RBD132-6aa-BspEI:	tct ccg tccgga gcc acc gcc acc ATC CAG GAA ATC TAC TTG AAG TTC
OyCD PCA	56)f-BspEI-10aa-F36M	ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GGAGTGCAGGTGGAAACCATC
OyCD PCA	57)r-XhoI-stop-F36M	ccg ccc ccg ctcgag tta TTCCAGTTTTAGAAGCTCCAC
OyCD PCA	58)f-XbaI-F36M-5aa	cgc ggg tctaga ATG GGAGTGCAGGTGGAAACCATC
OyCD PCA	59)r-BspEI-5aa-F36M	ccc tccgga gcc acc gcc acc TTCCAGTTTTAGAAGCTCCAC
OyCD PCA	Ypd1 5'XbaI	GCGCGTCTAGAATGTCTACTATTCCC
OyCD PCA	Ypd1 3'BspEI Linker	CCCGGG tccggagccaccgccaccTAGGTTTGTGTTGTAATATTT
OyCD PCA	432) f-Xba-Ssk1aa495	CCCGGGtctaga ATG ACCACAAGTGAAAAAGTTTTC
OyCD PCA	Ssk1 3'BspEI linker	CCCGGG tccacctccggagccaccgccaccCAATTCTATTTGAGTGGGCGA
OyCD PCA	433) Xba-Skn7aa361-forward	CCCGGGtctaga ATG AGCCTAACACCAAATGCTCAA
OyCD PCA	231)Skn7-Linker-BspEI-reverse	AAATTTtccggagccaccgccacc TGATAGCTGGTTTTCTTGAAG
OyCD PCA	438) Ypd1-W80A-forward	GCATTAGGCTTACAAAGAATTGCCgcgGTTTGTGAAAGAATTCAAAACTTGGG
OyCD PCA	439) Ypd1-W80A-reverse	CCCAAGTTTTGAATTCTTTCACAAACcgcGGCAATTCTTTGTAAGCCTAATGC
SBF MBP	SpeI Swi4	agagag actagt ATGCCATTTGATGTTTTGATATC
SBF MBP	BspEI Swi4	agagag tccgga gccaccgccacc TGCGTTTGCCCTCAAATCC
SBF MBP	SpeI Swi6	agagag actagt ATGGCGTTGGAAGAAGTGG
SBF MBP	BspEI Swi6	agagag tccgga gccaccgccacc TGAAGCATGCTTTTTTAAAAAATC
SBF MBP	SpeI Mbp1	agagag actagt ATGTCTAACCAAATATACTCAG

amplified from $pC_4EN\text{-}F_M3$ (Ariad) and subcloned upstream of yCD-F[1] $_{A23L}$ (OyCD-F[1]) and yCD-F[2] $_{V108I,\ I140L,\ T95S,\ K117E}$ (OyCD-F[2]) to generate p413Gal1-FM1-OyCD-F[1], p413Gal1-OyCD-F[1]-FM1 and p415Gal1-FM1-OyCD-F[2]. Whi5 was subcloned in p415Gal1 using *BamH*I and *Xho*I sites.

Selection for wild-type yCD PCA activity. *S. cerevisiae* BY4741 (*MAT***a**: *ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*), BY4742 (*MAT*α: *ura3Δ0 leu2Δ0 his3Δ1 met5Δ0*), and BY4743 *fcy1Δ* mutant strains were generated as part of the yeast gene knockout collection (Giaever, Chu et al. 2002), were used to assay for yCD PCA activity since their genomic copies of *fcy1* were disrupted. These deletion strains were propagated in medium containing 200 μg/mg of Geneticin® (G418) (Invitrogen). For assaying yCD PCA activity, BY4741 and BY4742 *fcy1Δ* cells were transformed with p413Gal1 and p415Gal1 vectors carrying respective fusion genes. Cells from each mating type were mated and selected on SC-met-lys-his-leu + 2% glucose. Protein expression was induced by inoculating yeast cells overnight in 1 ml of SC-met-lys-his-leu + 2% raffinose. The next day, 20 μl of the culture was transferred to 1 ml of the same selection medium with 2% galactose for 6 hrs induction at 30˚C. For the survival selection assay, cells were plated on solid selection medium: SC-met-lys-his-leu-ura + 2% agar + 2% raffinose and 2% galactose (with 0, 100 or 1000 μg/mg cytosine). Plates were incubated at 30°C for 6 days. For the 5-FC death selection assay with 5-FC preincubation, approximately 5,000 cells were transferred to 1 ml of SC-met-lys-his-leu + 2% raffinose and 2% galactose + 100 μg/mg 5-FC and grown for 18 hrs at 30 °C with shaking. After the preincubation period, 10 μl of each sample was plated on solid selection medium: SC-met-lys-his-leu + 2% agar + 2% raffinose and 2% galactose (with 0, 100 or 1000 μg/mg 5-FC). Plates were incubated at 30 °C for either 2 or 3 days.

Optimization of yCD PCA activity. To generate the 37 °C stable yCD PCA, site-directed mutagenesis was carried out according to the QuickChange strategy

(Stratagene) in order to introduce the A23L, V108I and I140L triple mutations. For further optimization of yCD PCA activity, error-prone PCR was used to generate a library of yCD-F[1] and yCD-F[2] (yCD-F[1]ep and yCD-F[2]ep) carrying on average one mutation per fragment. PCR was performed with *Taq* polymerase (NEB), unbalanced nucleotides (1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, and 0.2 mM dGTP), 5 mM $MgCl_2$, and 10% DMSO using the following conditions: 95 ^oC (3 min), 30 cycles of [95 ^oC (1 min), 55 ^oC (1 min), 72 ^oC (1 min)], 72 ^oC (5 min). yCD-F[1]ep and yCD-F[2]ep were subcloned downstream of Ras or RBD genes in p413Gal1-Ras and p415Gal1-RBD vectors using *BspE*1 and *Xho*I restriction sites. The ligation products were transformed in DH5α cells by electroporation. The size of each library was calculated to be 1,463 and 1,983 clones, respectively (Reetz 2004). The libraries were transformed into BY4741 and BY4742 *fcy1Δ* strains. Cells from respective haploid types were mated, generating approximately 3 x 10^6 clones, and plated on SDC-met-lys-his-leu-ura (uracil-depleted medium) containing 1000 μg/ml of cytosine. Selection plates were incubated at 37 ^oC. After 4 days, cells with increased cytosine deaminase activity from yCD PCA formed colonies. Two hundred and seventy six colonies were inoculated in SC-met-lys-his-leu + 2% raffinose and 2% galactose and cultured overnight. The next day, cells were pinned onto SC-met-lys-his-leu + 3% agar + 2% raffinose + 2% galactose (with 0 and 100 μg/mg 5-FC). Plates were incubated at 37 ^oC for 2 days. Sixteen clones were identified to have increased sensitivity to 5-FC. PCR was performed on these clones to amplify yCD-F[1] and yCD-F[2]. PCR products were sequenced to identify mutations. Mutations found are listed in **Table 2**.

p413Gal1-Ras-yCD-F[1]$_{A23L,\ M1L}$ and p415Gal1-RBD-yCD-F[2]$_{V108I,\ I140L,\ T95S}$ plasmids were retrieved by isolating DNA from yeast cells using DNeasy Tissue Kit (Qiagen) and transforming the DNA into DH5α cells for amplification of the plasmids. To further increase yCD PCA activity, other mutations were combined with yCD-F[1]$_{A23L,\ M1L}$ or yCD-F[2]$_{V108I,\ I140L,\ T95S}$ by site-directed mutagenesis (Stratagene). Plasmids

were transformed into BY4741 and BY4742 *fcy1Δ* strains, respectively. Cells were mated and selected for cytosine deaminase activity on uracil-depleted medium containing 100 μg/mg of cytosine. Cells were incubated at 37 °C for 2 days. Colonies were assayed for 5-FC sensitivity by pinning onto 5-FC plates as previously described in this section. 5-FC sensitive clones were identified and PCR products containing yCD fragments were sent for sequencing. Mutations found are listed in **Table 3**.

OyCD PCA for cell cycle transcription factors. Plasmids carrying fusion genes were co-transformed into the BY4741 *fcy1Δ* strain. Colonies were grown overnight in 1ml of SC-met-lys-his-leu + 2% raffinose, induced for protein expression with 2% galactose for 6 hrs and assayed for yCD activity by pinning onto SC-met-lys-his-leu + 3% agar + 2% raffinose and 2% galactose with 0 μg/mg 5-FC as control plate and on SC-met-lys-his-leu + 3% agar + 2% raffinose and 2% galactose with 100, 200 or 500 μg/mg of 5-FC for death selection. Plates were incubated at 30 °C for 2 to 3 days.

Dual selection assays for Ras and RBD interaction using OyCD PCA. Plasmids were co-transformed in the BY4743 *fcy1Δ* strain. Colonies were grown overnight in 1 ml of SC-met-lys-his-leu + 2% raffinose and induced for protein expression with 2% galactose for 6 hrs and assayed for yCD activity by pinning onto SC-met-lys-his-leu + 3% agar + 2% raffinose and 2% galactose with 0 μg/mg 5-FC as control plate, on SC-met-lys-his-leu-ura + 3% agar + 2% raffinose and 2% galactose with 50 μg/mg cytosine for survival selection, and on SC-met-lys-his-leu + 3% agar + 2% raffinose and 2% galactose with 50 μg/mg 5-FC for death selection. Plates were incubated at 37˚C for 2 days.

Disruption of interaction between FM1 homodimer using FK506. Empty plasmids and plasmids carrying fusion genes were co-transformed into the BY4743 *fcy1Δ* strain. Colonies were grown overnight in 1ml of SC-met-lys-his-leu + 2% raffinose and induced for protein expression with 2% galactose for 6 hrs. 500 cells

from each sample were plated on different selection media: SC-met-lys-his-leu + 3% agar + 2% raffinose + 2% galactose alone, with 100 μg/mg 5-FC, with 10 μM FK506, and with 100 μg/mg 5-FC and 10 μM FK506. Plates were incubated at 30 ^{o}C for 2 days.

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Chapter 3

Re-engineering the cell cycle transcription factor Swi6 and dissecting its mechanism of activation

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In this chapter, I used the OyCD PCA to re-engineer Swi6 the common subunit of the SBF and MBF transcription factors to rewire its transcriptional activities. Such mutant protein has valuable use in the field of synthetic biology and for dissecting the molecular mechanisms that control cell cycle regulation.

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Author contributions:

PHE and SWM designed the experiments, analyzed the results and wrote the manuscript. PHE performed all the experiments.

Abstract

Dissection of protein interaction networks is key to understanding regulatory transcriptional circuits and to design organisms with desired traits. We used a general *in vivo* strategy based on a Protein-fragment Complementation Assay using the optimized cytosine deaminase as reporter (OyCD PCA) to dissect interactions between pairs of proteins in a complex that allows for positive detection of either formation or disruption of protein-protein interaction. We applied this strategy to engineer a mutant form of Swi6, the common subunit of yeast *S. cerevisiae* G1/S phase transcription factor complexes SBF (Swi6:Swi4) and MBF (Swi6:Mbp1) by specifically perturbing its interaction with Mbp1. Such mutants can be used to alter the transcriptional activities and to dissect the regulatory mechanism of Swi6 at START. We generated a Swi6 mutant carrying the L777V and A780T mutations (2m-Swi6). The 2m-Swi6 shows decreased MBF activity while SBF activity remains unchanged. Furthermore, we used the 2m-Swi6 to dissect the regulatory mechanism of SBF and MBF by Whi5 and identified the C-terminal fragment of Swi6 to be the direct target of Whi5 binding. This demonstrates that the OyCD PCA can be used for rewiring or dissecting transcriptional networks.

Introduction

The creation of synthetic gene transcription circuits holds promise for both uncovering the principles of circuit design and for their redesign for biotechnological applications such as in metabolic engineering (Haseltine and Arnold 2007). However, it is not trivial to redesign transcriptional circuits that both achieve the aim of the design and are not toxic to the organism in which the redesigned circuit is introduced. Methods to dissect and re-engineer transcriptional machinery by directed evolution are limited. For instance, one way to engineer a transcriptional circuit is to change protein-protein interactions made by transcription factors so that one or more interactions are disrupted. To achieve this requires a strategy that allows library screens of mutant transcription factors that can provide a positive readout for either formation or disruption of protein-protein interactions. Such method will facilitate the selection of desired clones that disrupt an interaction while conserving interaction with other binding partners. This dual positive selection has been achieved with development of the optimized yeast cytosine deaminase Protein-fragment Complementation Assay (OyCD PCA) (**Chapter 2**).

In the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), cell cycle regulation requires proper timing of activation and inactivation of transcription factors by the cyclin dependent kinase Cdk1 (also known as Cdc28). Cdk1 is activated by three G1/S-specific cyclins, Cln1-3 and six B-type cyclins, Clb1-6. The transcription of genes required for progressing from G1 to S phase of the cell cycle is regulated by two key transcription factors, SBF and MBF. SBF and MBF are heterodimeric complexes composed of a common transactivation subunit, Swi6 and different DNA binding subunits Swi4 and Mbp1, respectively. SBF and MBF bind to distinct DNA binding motif sequences, respectively SCB and MCB, in gene promoters and activate their transcription (Bahler 2005). As *S. cerevisiae* prepares for a new round of cell division, it relies on the activity of SBF and MBF to activate the

expression of over two hundred genes in order to prepare the cell to enter S-phase (Iyer, Horak et al. 2001).

The Cdk1-Cln3 complex activates SBF and MBF at the G1 phase by phosphorylation of Whi5, an SBF and MBF repressor (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004) while Cdk1-Clb complexes inactivate SBF at the G2/M phase by phosphorylation of Swi4 (Amon, Tyers et al. 1993) and Swi6 (Geymonat, Spanos et al. 2004). Immunoprecipitation assays showed that Whi5 directly binds to the SBF and MBF complexes and not to the individual transcription factor subunits (Swi4, Mbp1 or Swi6) alone (Costanzo, Nishikawa et al. 2004). This suggests that perhaps Whi5 binds to a hybrid surface formed by the SBF or MBF complex or directly binds to a fragment of Swi6 that becomes exposed only upon binding to Swi4 or Mbp1.

Studying protein complexes by dissecting specific protein-protein interaction is a valuable strategy to gain insights into molecular mechanisms that underline a biological process. Here, we chose to re-engineer Swi6 using the OyCD PCA in order to alter its transcriptional activity and better understand its regulation by Whi5. The MBF and SBF gene regulatory network represents an elegant and challenging problem for dissection since first, the extent to which these transcription factors are redundant versus specific to activate target genes is ambiguous and second, there is high sequence homology between Mbp1 and Swi4 making it difficult to deduce what regions of the two proteins confer unique transcriptional activities towards specific genes (Primig, Sockanathan et al. 1992; Koch, Moll et al. 1993). We reasoned that by re-engineering Swi6 such that its binding to Mbp1 is disrupted, but retains binding to Swi4, we could selectively and specifically dissect SBF and MBF transcriptional activities. We applied a positive-negative clonal selection strategy based on the OyCD PCA to generate a mutant form of Swi6 carrying the L777V and A780T mutations (2m-Swi6) that disrupted MBF transcriptional activity while its SBF

activity remains unchanged. In addition, we used the 2m-Swi6 to dissect the mechanism by which Swi6 activity is regulated by Whi5, demonstrating that the C-terminal domain of Swi6 is directly responsible for binding to Whi5.

Results

Selection of SBF and MBF domain structure for mutagenesis

Swi6 is the common subunit of MBF (Mbp1:Swi6) and SBF (Swi4:Swi6) transcription factor complexes (**Fig. 1A**) (Bahler 2005). Swi6 is a modular protein that contains two transcriptional activation regions (N- and C-TAR), an ankyrin repeat domain (AnkRD) and a C-terminal heterodimerizing domain (BD) that can interact with the C-terminal Swi6 binding domains of Mbp1 (mBD) or Swi4 (sBD) (Siegmund and Nasmyth 1996; Sedgwick, Taylor et al. 1998) (**Fig. 1B**). The BD of Swi6 corresponds to residue 663 to 803 of the full-length protein whereas mBD and sBD correspond to residue 650 to 833 and 1017 to 1095 of Mbp1 and Swi4, respectively. We generated fusion proteins of full-length Mbp1, Swi4, and Swi6 with OyCD fragments and tested for interactions among the proteins using the 5-FC death selection assay. Consistent with previous studies (Siegmund and Nasmyth 1996) we observed interactions between Mbp1 and Swi6, and between Swi4 and Swi6 and no interaction between Mbp1 and Mbp1 or Swi4 and Swi4 (**data shown in Figure 9 of Chapter 2**). Since these and other studies showed that the BD of Swi6 interacts with mBD and sBD (Koch, Moll et al. 1993), we randomly mutated the C-terminal fragment of Swi6, which includes the C-TAR and the BD, from amino acid 570 to 803 ($Swi6_{570\text{-}803}$) by error-prone PCR (Swi6*) (**Fig. 1B**). The C-TAR served as a negative internal control for the screen since it was not previously found to interact with sBD or mBD (Siegmund and Nasmyth 1996).

A

B

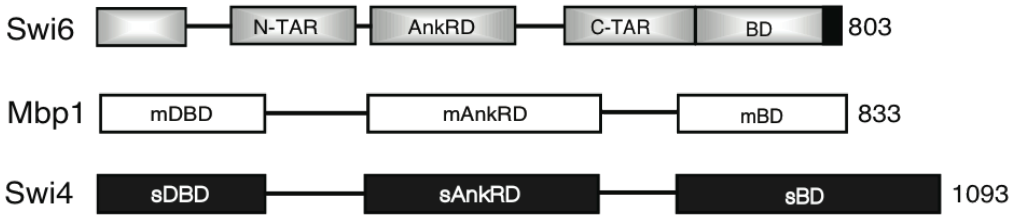


Figure 1. MBF and SBF transcription factors. (**A**) MBF (Swi6:Mbp1 complex) and SBF (Swi6:Swi4 complex) share the common Swi6 transcriptional activating subunit and regulate the transition of G1-to-S phase of the cell cycle by binding *via* unique Mbp1 or Swi4 DNA binding subunits to MCB and SCB DNA sequences, respectively within promoters of target genes. (**B**) Domain structures of Swi6, Mbp1 and Swi4. Swi6: N-, C-TAR: Transactivation domains; AnkRD: ankyrin repeats; BD: Mbp1 and Swi4 binding domain. Mbp1 and Swi4: mDBD and sDBD DNA binding domains; m and sAnkRB: ankyrin repeat domains. m and sBD: Mbp1 or Swi4 Swi6 binding domains.

Dissection of SBF and MBF transcription factor complexes by an OyCD PCA library screening strategy

We next set out to screen for mutants of Swi6 that preferentially interact with Swi4 over Mbp1 (**Fig. 2**). In the first step, we screened a library of ten thousand clones of Swi6* against Mbp1 in the death assay and eight thousand positive clones (non-reconstitution of OyCD activity) were collected. In the second step, these Swi6 mutants were screened against Swi4 in the OyCD life assay (reconstitution of OyCD activity). After the two steps of selection, ninety clones carrying potential Swi6 mutants were re-tested for interactions with Mbp1 or Swi4 using OyCD PCA (**Fig. 3**). Nine clones showed decreased OyCD PCA activity with Mbp1 while the OyCD PCA activity with Swi4 remains unaffected (**Fig. 4A**). Comparison of a set of sequences from the original Swi6* mutant library to those of the clones found after the life and death selection screen showed that mutants in the initial library were randomly distributed throughout the C-TAR and BD whereas the mutants selected after the second Swi4 screen carried mutations located only in the BD (**Fig. 4B**).

Re-engineering the SBF and MBF transcriptional circuit with a Swi6 mutant

We screened the nine Swi6 mutants to identify those that disrupt MBF but not SBF activity (Andrews and Moore 1992). The full-length Swi6 mutants fused to OyCD-F[1] were characterized for MBF or SBF transcriptional activity by using the pBA487 (4X MCB) and pBA251 (4X SCB) reporter plasmids respectively (Andrews and Moore 1992), in a Swi6 complementation assay in the *SWI6* knockout (*swi6*Δ) strain. In this strain, yeast transformed with an empty vector showed no MBF or SBF activity while yeast transformed with Swi6-OyCD-F[1] showed both MBF and SBF activity. Two single Swi6 mutants (Swi6L777V and Swi6A780T) showed decreased MBF activity (**Fig. 5A**) while SBF activity was not affected (**Fig. 5B**). When the two mutations were combined, the double mutant (2m-Swi6) showed a further reduction of MBF activity while the SBF activity remained unchanged (**Fig. 5A and B**). The remaining seven clones showed no change in MBF or SBF activity (**Fig. 6**).

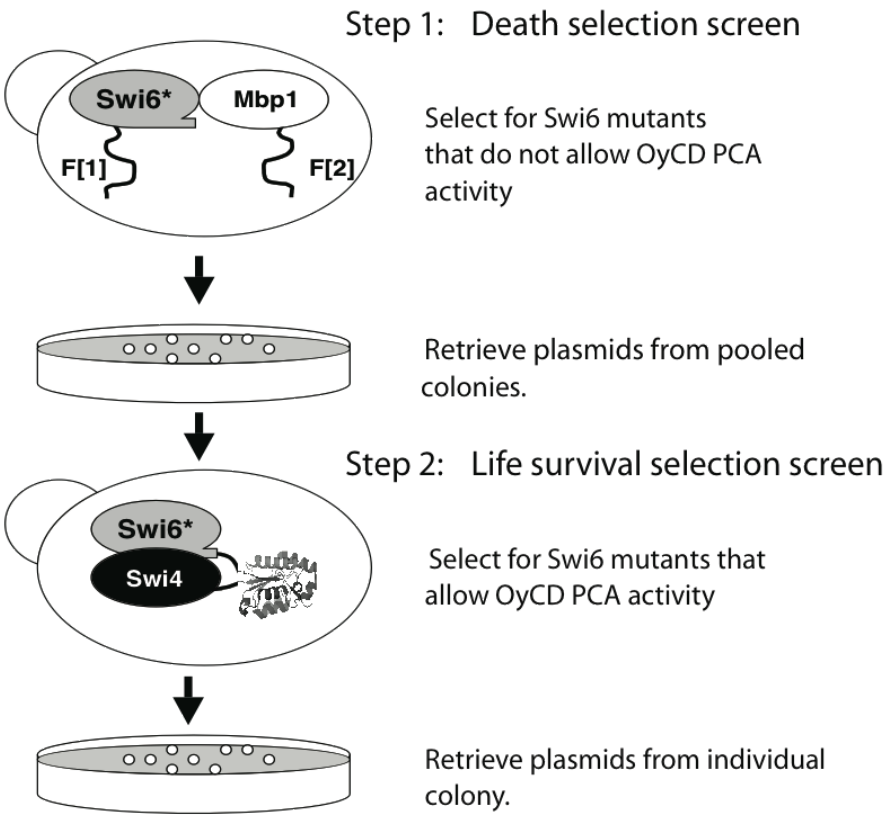


Figure 2. Strategy for engineering a Swi6 mutant. Step 1: Death selection screen of a mutant Swi6 library (Swi6*) co-expressed with Mbp1, both fused to OyCD fragments. Selection is for clones lacking OyCD PCA activity (growth in the presence of 5-FC). Step 2: Life selection of Swi6* clones from Step 1, co-expressed with Swi4, both fused to OyCD fragments. Selection is for clones with OyCD PCA activity (increased growth in the presence of cytosine).

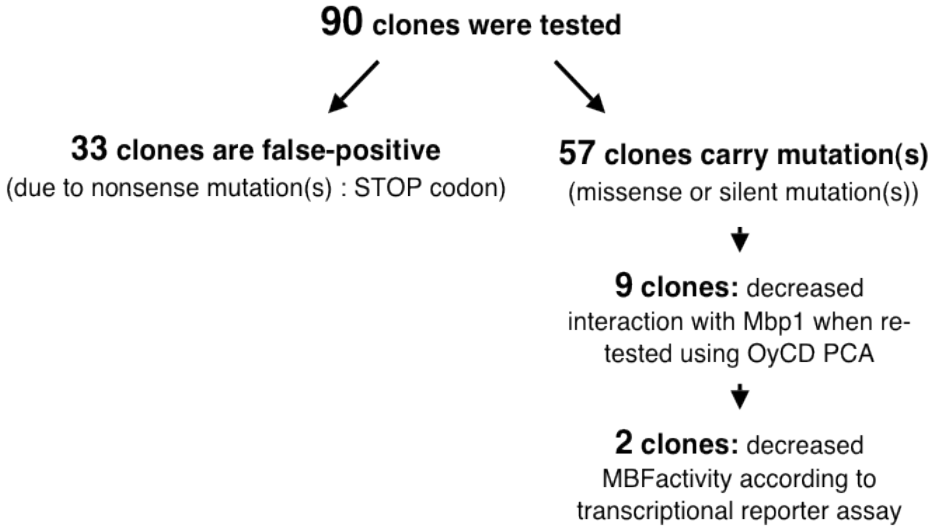


Figure 3. Selection process for re-engineering Swi6. After the two rounds of selection, 90 Swi6* clones were individually characterized for interaction with Mbp1 and Swi4 using OyCD PCA. Thirty-three clones were found to be false positives due to the presence of a stop codon in the Swi6* sequence. These false-positive clones were recovered since cells were plated densely (approximately twenty thousand cells were plated on a ten centimeter Petri dish) during the cytosine survival selection (**Fig. 2**). This occurs because uracil can diffuse out of cells with OyCD PCA activity into the selection medium and allow neighboring colonies to grow despite not having OyCD PCA activity (Griffith and Jarvis 1996; Paluszynski, Klassen et al. 2006). Fifty-seven of the Swi6* clones carry missense or silent mutations. When retested with Mbp1 and Swi4, only nine clones showed a decreased OyCD PCA activity with Mbp1 while the OyCD PCA activity with Swi4 was not affected. The remaining forty-eight Swi6 mutants included three that showed no significant difference from wild-type Swi6 and forty-five that showed loss of OyCD PCA signal with both Mbp1 and Swi4. It is also possible that these false-positive clones were recovered since cells were plated densely during the first or second step of selection. These false-positive clones can be avoided by plating fewer than five thousand clones on a ten-centimeter Petri dish (based on un-published data of other OyCD PCA screens).

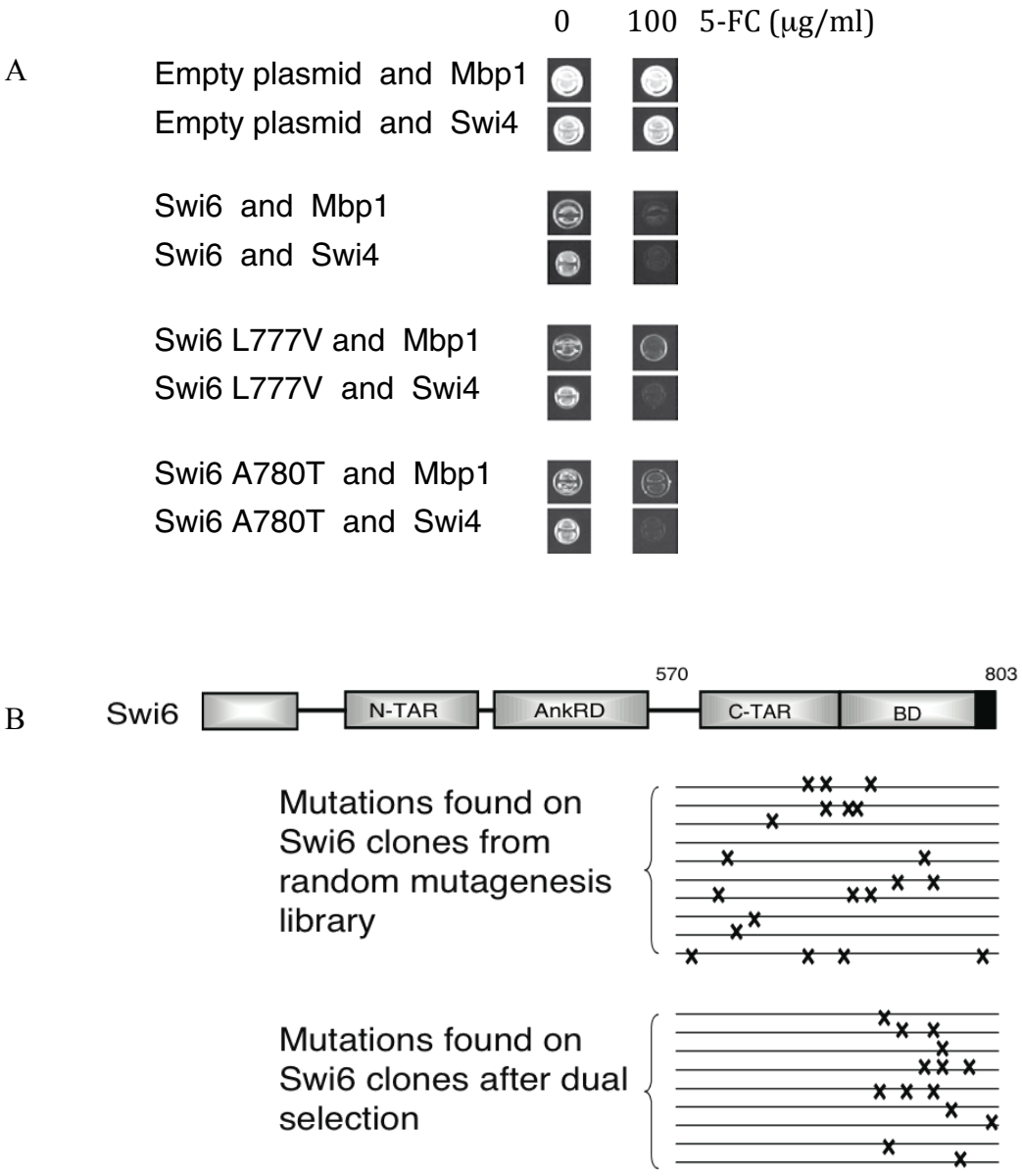


Figure 4. Mutation in Swi6 before and after selection. (**A**) Two examples of the nine Swi6* clones that showed decreased OyCD PCA activity with Mbp1. Cells expressing wild-type Swi6 and Mbp1 or Swi6 and Swi4 fused to OyCD fragments are sensitive to 100 μg/ml of 5-FC. Cells expressing Swi6 L777V or Swi6 A780T and Mbp1 fused to OyCD fragments are resistant to 100 μg/ml of 5-FC. Cells expressing L777V or Swi6 A780T and Swi4 fused to OyCD fragments are sensitive to 5-FC. (**B**) Mutations in $Swi6_{570\text{-}803}$ from the initial library are randomly distributed. After the two-step selection screen, nine of the ten selected Swi6* clones carry mutation(s) located within the C-terminal Mbp1 and Swi4 binding domain of Swi6 and no mutations are found in the activation domain (AD).

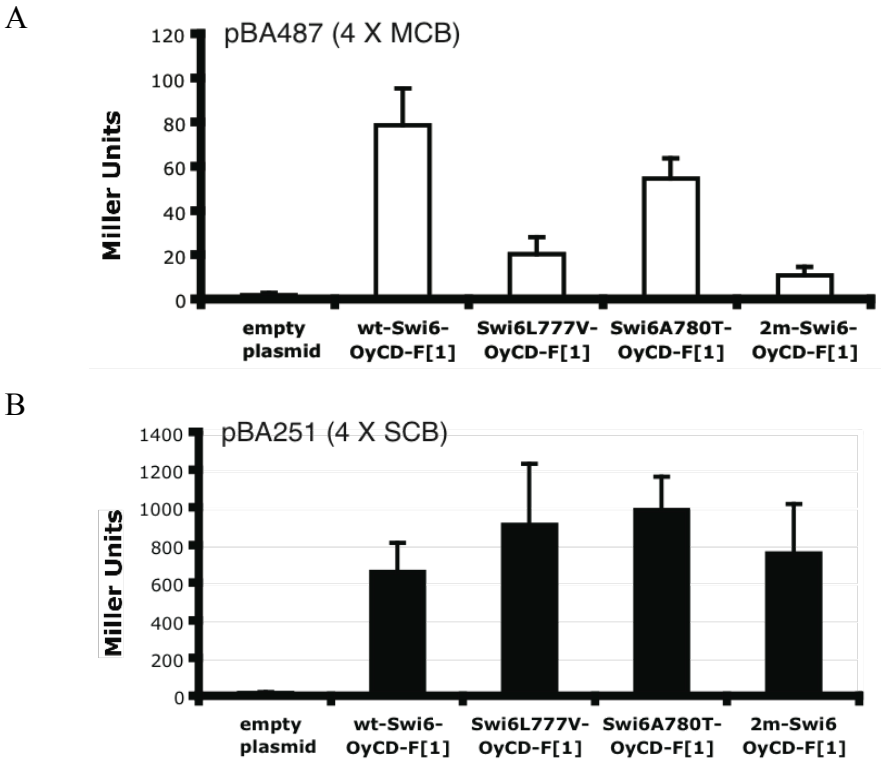
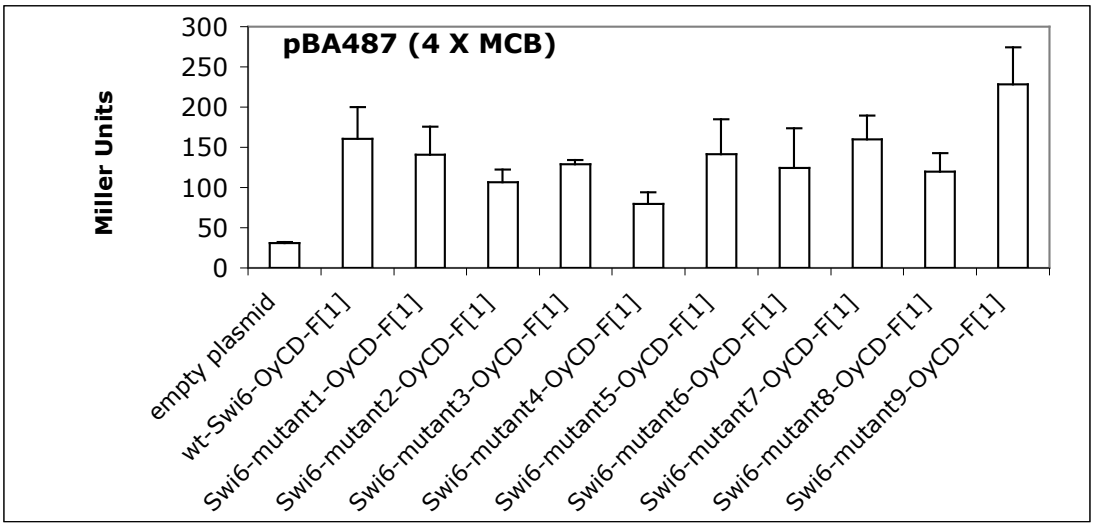


Figure 5. Transcription assays for Swi6 mutants. Full-length Swi6 and Swi6 mutants fused to OyCD-F[1] were tested for MBF (**A**) and SBF (**B**) transcriptional activities in *SWI6* deletion cells using pBA487 (4X MCB) and pBA251 (4X SCB) reporter plasmids respectively. Data are mean ± s.d. (n = 4). Mutants Swi6*L777V, Swi6*A780T, and the combined double mutant Swi6*L777V, A780T (2m-Swi6) show decreased MBF activity in comparison to *wild-type* (wt) Swi6 whereas SBF activity is not affected.



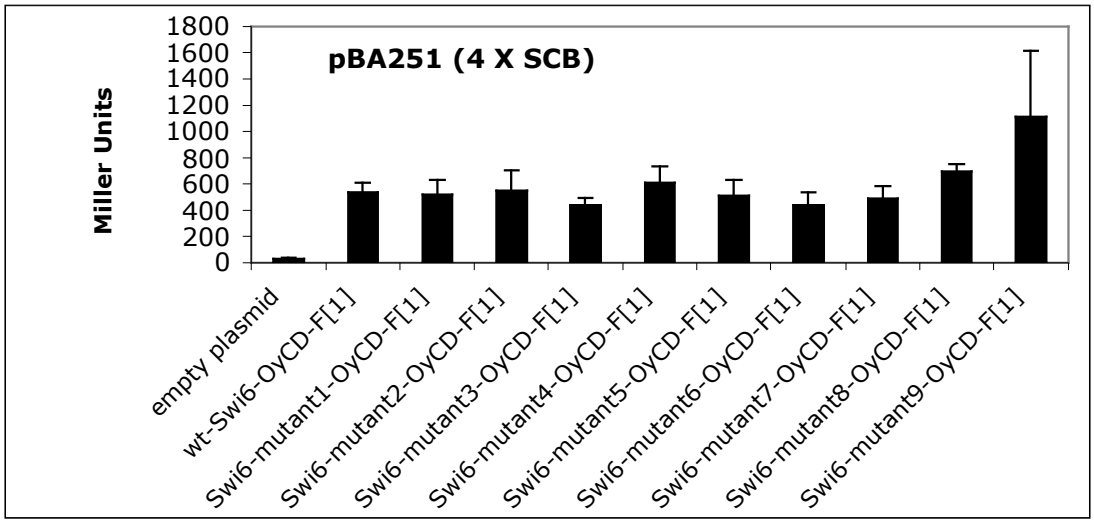


Figure 6. SBF and MBF reporter assays. The nine clones were screened for MBF and SBF transcriptional activities in *swi6* deletion cells using pBA487 (4X MCB) and pBA251 (4X SCB) reporter plasmids of MBF and SBF activity, respectively. Swi6-mutant2 and mutant4 showed significant decreased MBF activity while SBF activity is unchanged in comparison to the wild-type Swi6. Error bars represent the standard deviation calculated for four independent samples. Swi6-mutant2 carries the A780T mutation and Swi6-mutant4 carries the L777V mutation.

Characterization of the 2m-Swi6 mutant

We next performed GST pulldown experiments to test for direct interaction between the 2m-Swi6 mutant and Mbp1 and Swi4. Surprisingly, both full-length 2m-Swi6 and the C-terminal fragments retained the ability to bind to both Mbp1 and Swi4 of *wild-type* Swi6 (**Fig. 7A** and **7B**) although OyCD PCA activity was decreased (**Fig. 4A** and **8**) at similar expression levels of *wild-type* Swi6 and 2m-Swi6 (**Fig. 9**). This contradiction with the OyCD PCA results may be explained by the way the OyCD PCA and PCAs in general work. In a PCA the interaction between the test proteins brings reporter fragment into proximity to allow refolding and reconstitution of the activity of the native enzyme. Thus detection of an interaction by PCA is limited by steric access of the two fragments to each other, a requirement that could be affected by the conformation or topology of a protein complex. This is a feature that has been in fact exploited to distinguish allosteric states and the topologies of protein complexes on a large-scale (Remy, Wilson et al. 1999; Tarassov, Messier et al. 2008). Thus we hypothesize that the 2m-Swi6 mutation does not disrupt its interaction with Mbp1 but somehow renders the conformation of the complex in some unproductive state.

We then asked whether recruitment of 2m-Swi6 to the promoter regions of MBF and SBF target genes is disrupted. We performed a chromatin immunoprecipitation (ChIP) experiment with an affinity purified Swi6 polyclonal antibody (Iyer, Horak et al. 2001). We found that the full-length 2m-Swi6 associated with promoters of MBF and SBF targeted genes such as *CLN2*, *CLN3*, and CDC45 to the same extent as *wild-type* Swi6 **(Fig. 10)**. As a control for the ChIP experiment, both forms of Swi6 are not recruited to the promoter of the *FKS2* gene (Kim, Truman et al. 2008). Furthermore, full-length 2m-Swi6 appears to interact with Swi4 and Mbp1 to the same extent as *wild-type* Swi6 **(Fig. 10)**.

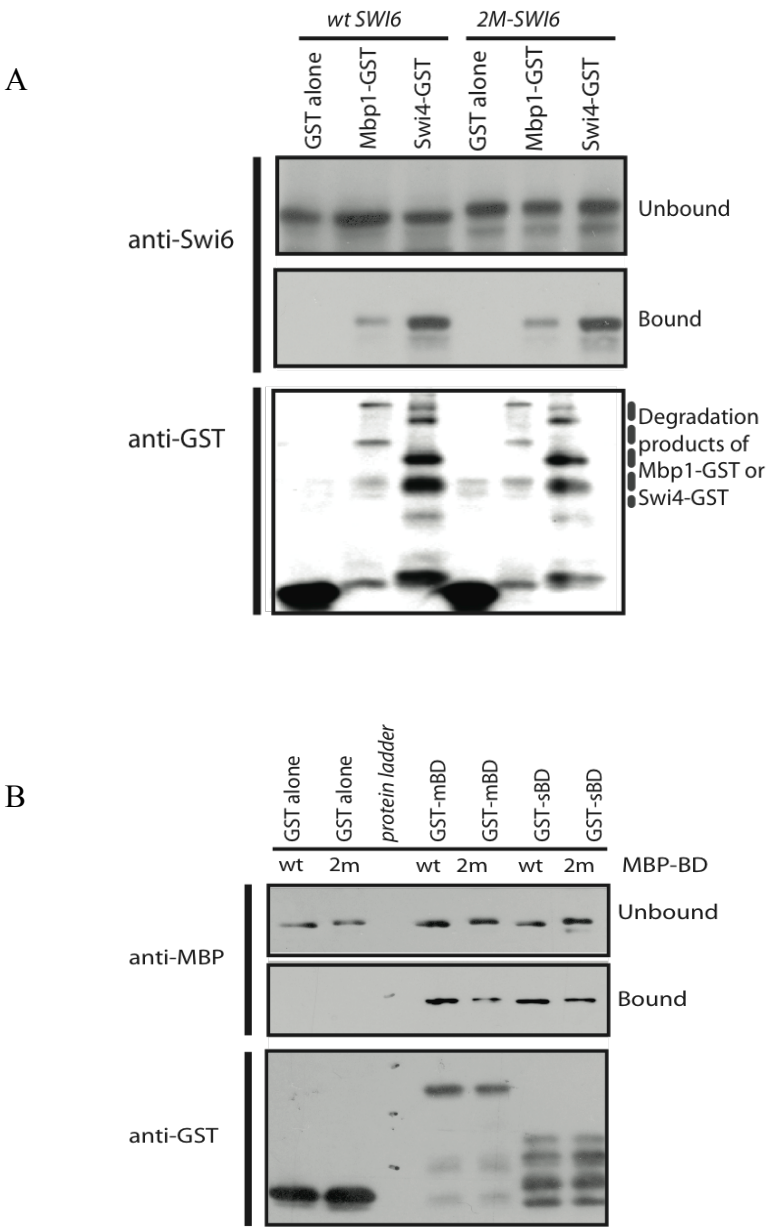


Figure 7. Interaction between the Mbp1, Swi4 and Swi6 by pulldown assays (**A**) GST pulldown assays with full-length Swi6, 2m-Swi6, Mbp1 and Swi4 proteins expressed in yeast in the indicated combinations. The upper panels show unbound and bound fractions of Swi6 to GST fusion proteins detected with a Swi6-specific antibody. The lower panel shows the expression of GST and GST fusion proteins detected with a GST antibody. (**B**) GST pulldown assays with purified C-terminal binding domains of Mbp1, Swi4, and Swi6 individually expressed in bacteria in the indicated combinations. The *wild-type* (wt) or mutant (2m) Swi6 BD was fused downstream of the maltose binding protein (MBP) and Mbp1 and Swi4 BD are fused to glutathione-S-transferase (GST). Fusion proteins were detected by Western blot, using an anti-MBP antibody to detect *wild-type* and 2m-Swi6-BD and an anti-GST antibody to detect GST-mBD and GST-sBD.

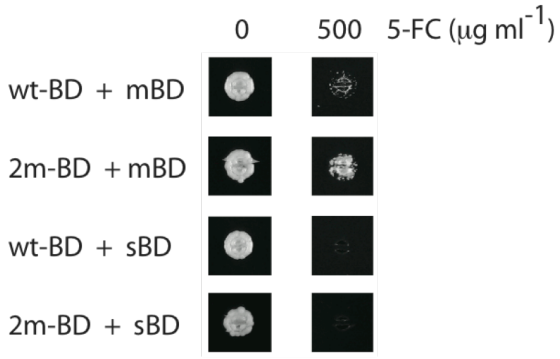


Figure 8. Interaction between the C-terminal domain of SBF and MBF determined by OyCD PCA. Cells expressing interacting fusion proteins pairs (wt-BD with mBD, wt-BD with sBD and 2m-BD with sBD) are sensitive to 5-FC. Cells expressing 2m-BD with mBD are resistant to 5-FC.

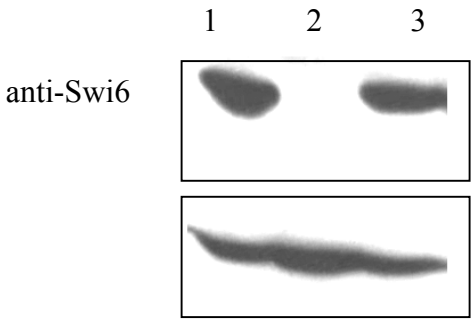


Figure 9. Expression of *wild-type* and 2m-Swi6 fused to OyCD-F[1]. The expression level of Swi6(663-803) fusion protein was detected using the Swi6 antibody. The membrane was re-probed for the expression of Pgk1 as loading control. Cells were transformed with the following plasmid: lane 1, wild-type Swi6-OyCD-F[1]; lane 2, an empty vector; lane 3, 2m-Swi6-OyCD-F[1].

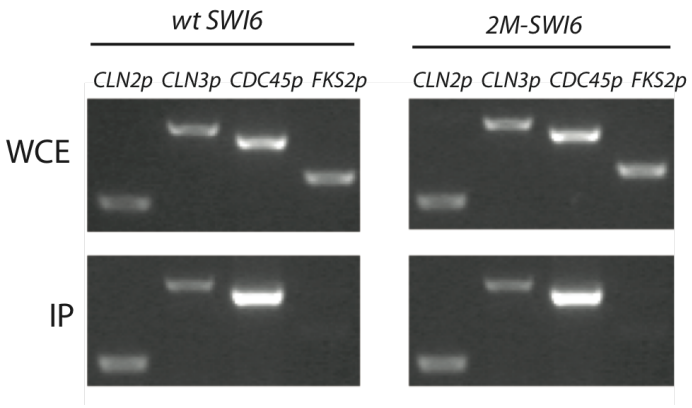


Figure 10. Chromatin immunoprecipitation of MBF and SBF complexes using the affinity purified Swi6 antibody in *wild-type* (*wt*) *SWI6* and *2M-SWI6* strains. The upper panel shows promoter regions of *CLN2*, *CLN3*, *CDC45* and *FKS2* gene (*CLN2*, *CLN3*, *CDC45* and *FKS2*) detected from the whole-cell extract (WCE). The lower panel shows promoter regions immunoprecipitated (IP) with Swi6.

In spite of that fact that 2m-Swi6 does bind to both Swi4 and Mbp1, it does alter its ability to activate gene transcription through MBF but not SBF making it a valuable tool for dissecting MBF and SBF functions. We thus used it in an attempt to determine how the regulator of SBF and MBF, Whi5 may exert its effect. Whi5 is the repressor protein that binds to and inhibits the SBF and MBF complexes at the G1 phase of the cell cycle. Whi5 does not interact with the full-length Mbp1, Swi4, or Swi6 individually (Costanzo, Nishikawa et al. 2004) but it is still not known how it interacts with the SBF and MBF complexes. Since the C-terminal domains of Mbp1, Swi4 and Swi6 correspond to the dimerization domains of these transcription factor subunits, we asked whether Whi5 interacts with the domains using the OyCD PCA (**Fig. 11A**). Interestingly, our results suggest that Whi5 interacts with the C-terminus of Swi6 (BD of Swi6) but not with the C-terminus of Mbp1 or Swi4. However, the C-terminal domain of 2m-Swi6 did not interact with Whi5. We confirmed these interactions by a GST *in vitro* pulldown experiment and observed similar results (**Fig. 11B**) with the OyCD PCA results, supporting our hypothesis that Whi5 directly interacts with the C-terminal domain of Swi6.

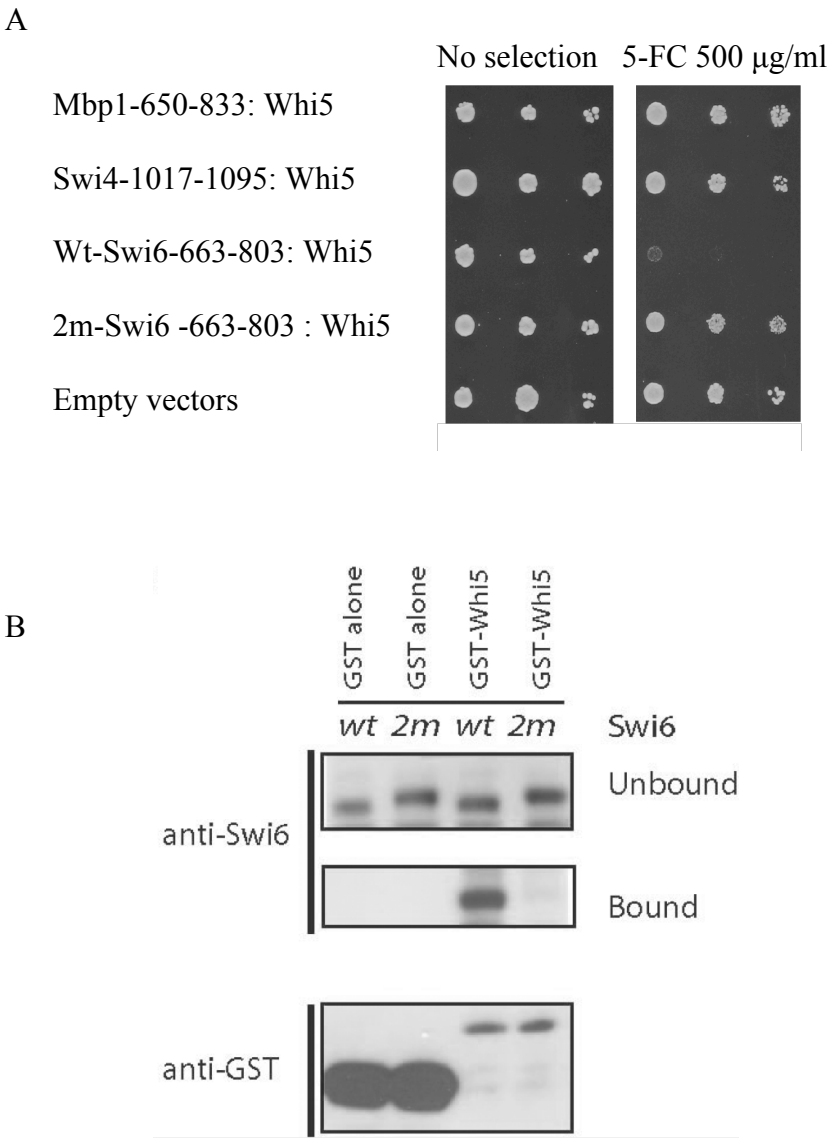


Figure 11. Interactions between Swi6 and Whi5. **(A)** Detecting the interactions between Whi5 and full-length Mbp1, Swi4 and Swi6 using OyCD PCA. **(B)** GST pulldown binding studies of Whi5 and both forms of full-length Swi6. The upper panels show unbound and bound fractions of Swi6 to GST fusion proteins detected by using an antibody against Swi6. The lower panel shows the expression of GST and GST fusion proteins detected by using an antibody against GST.

Discussion

As our understanding of the complexity of protein interaction and genetic networks become more sophisticated, we recognize that proteins neither have single nor even necessarily a small number of interactions or functions. Equally we need better tools to perform fine dissection of these complexes if we hope to understand and engineer new functions in proteins. The OyCD PCA provides a valuable tool to perform such fine dissection. The case of MBF and SBF dissection described here is a clear illustration of the utility of this approach. Through the discovery of just two point mutations in one subunit of a larger protein complex we were able to re-engineer the transcriptional activity of Swi6 to be an SBF specific transcription activator and rewire a cell cycle transcriptional circuit.

In addition, the mutant form of Swi6 that we have created gives us insight into the mechanism of activation by binding Mbp1 and Swi4. As we described above, the OyCD PCA can act as a sensor for detecting conformational changes in a protein complex. Our results suggest that Swi6 undergoes a conformation change upon binding to Mbp1 and Swi4. In its inactive state, the AnkRD of Swi6 could antagonize Swi6 transactivation by direct binding to both N- and C-terminal TARs (Sedgwick, Taylor et al. 1998). Residues 773 to 784 of Swi6 have been shown to play a role in activation of Swi6 (Sedgwick, Taylor et al. 1998). Binding of Swi6 to Mbp1 or Swi4 causes the TARs to dissociate from the AnkRD and Swi6 to open up, allowing the TARs to engage the transcriptional machinery, a transition that requires participation of residues 773 to 784. Since the two mutations in 2m-Swi6 (L777V and A780T) are found in this region, it is possible that the 2m-Swi6 mutations decouple binding of Swi6 to Mbp1 from a change in conformation that is necessary for transactivation. 2m-Swi6 could be locked in the inactive state, whether or not bound to Mbp1 (**Fig. 12**). PCAs are exquisitely sensitive to the topology of protein complexes because the reporter fragments must be free and close enough in space to

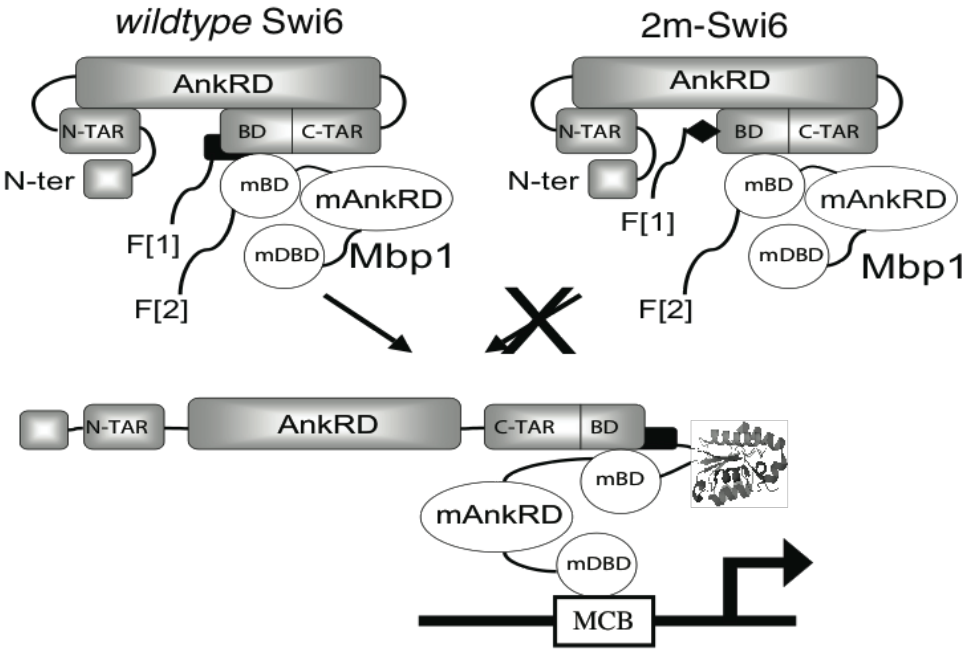


Figure 12. Model for allosteric regulation of Swi6. Swi6 undergoes a conformation change on binding Mbp1 and activates MBF activity. 2m-Swi6 fails to undergo conformation change on binding Mbp1 due to mutation of resides L777V and A780T (black diamond). Abreviations: N- and C-TAR: Swi6 transcriptional activation domains; AnkRD Swi6 ankyrin repeat domain; BD: Swi6 C-terminal heterodimerizing domain (BD) that binds to mBD or sBD, C-terminal Swi6 bidning domains of Mbp1 and Swi4 respectively; mDBD and sDBD: Mbp1 and Swi4 DNA binding domains; mAnkRD and sAnkRD: Mbp1 and Swi4 ankyrin repeat domains.

fold (Remy, Wilson et al. 1999; Tarassov, Messier et al. 2008). We suggest that the OyCD PCA result for the Mbp1: 2m-Swi6 interaction is thus not due to disruption of the interaction, but is caused by sequestering of the PCA fragment that is fused to the C-terminus of Swi6 downstream from residues 773 to 784. Swi4 must engage the conformation change in Swi6 in a different way, thus allowing for formation of an active SBF complex.

We have also shown that we can use this Swi6 mutant to dissect the mechanism of regulation of SBF and MBF by Whi5. It was not previously known how Whi5 interacts with the MBF and SBF complex. Here, we showed that Whi5 directly binds to the C-terminal domain of Swi6 and that this does not occur with 2m-Swi6. Using protein with point mutation(s) is a powerful strategy to dissect the mechanism of regulation in biological systems. The 2m-Swi6 may be used to identify additional proteins that regulate MBF activity.

The unique attribute of the OyCD PCA is that it can be applied to study interactions of any full-length proteins, expressed in appropriate cellular compartments and with posttranslational modifications that reflect their natural state under any specific conditions. While we explored the uses of the OyCD PCA in dissecting transcriptional circuits we can envision many applications to engineering other cellular regulatory circuits that are mediated by protein-protein interactions, including signal transduction or metabolic pathways where disruption of specific protein-protein interactions could prevent substrate-product channeling between enzyme subunits or allosteric regulation of specific reactions.

Materials and methods

OyCD PCA for cell cycle transcription factors. Plasmids carrying fusion genes were co-transformed into the BY4741 *fcy1Δ* strain. Colonies were grown overnight in 1 ml of SC-met-lys-his-leu + 2% raffinose, induced for protein expression with 2% galactose for 6 hrs and assayed for yCD activity by pinning onto SC-met-lys-his-leu + 3% agar + 2% raffinose and 2% galactose with 0 μg/mg 5-FC as control plate and on SC-met-lys-his-leu + 3% agar + 2% raffinose and 2% galactose with 100, 200 or 500 μg/mg of 5-FC for death selection. Plates were incubated at 30˚C for 2 to 4 days.

Re-engineering Swi6 using OyCD PCA. Error-prone PCR was used to generate a library of Swi6*$_{(560-803)}$ carrying on average two to three mutations. PCR was performed with the same conditions as for yCD-F[1]ep and yCD-F[2]ep. Swi6*$_{(560-803)}$ were subcloned in p413Gal1-Swi6-OyCD-F[1] using *EcoR*1 and *BspE*I restriction sites. The ligation products were transformed in DH5α cells by electroporation. The size of each library was 10^4 clones. The library was transformed in BY4741 *fcy1Δ* strain. p415Gal1-Mbp1-OyCD-F[2] and p415Gal1-Swi4-OyCD-F[2] were transformed in BY4742 *fcy1Δ* strain. BY4741 *fcy1Δ* strain carrying p413Gal1-Swi6*-OyCD-F[1] were mated with BY4742 *fcy1Δ* strain carrying p415Gal1-Mbp1-OyCD-F[2], generating approximately 3 x 10^4 clones. Colonies were pooled and plated on SC-met-lys-his-leu 2% agar + 2% raffinose + 2% galactose containing 200 μg/ml of 5-FC. Selection plates were incubated at 30˚C. After 3 days, cells expressing Swi6*-OyCD-F[1] that do not interact with Mbp1-OyCD-F[2] formed colonies. Colonies were pooled for DNA extraction (Qiagen). DNA was electroporated in MC1061 *E. coli* in order to retrieve plasmids. Plasmids were re-transformed in BY4741 *fcy1Δ* strain, colonies were pooled, mated with BY4742 *fcy1Δ* strain carrying p415Gal1-Swi4-OyCD-F[2] and plated on SDC-met-lys-his-leu-ura 2% agar + 2% raffinose + 2% galactose containing 200 μg/ml of cytosine. After 3 days, cells expressing Swi6*-OyCD-F[1] that interacts with Swi4-OyCD-F[2] formed colonies. The survival

selection of OyCD PCA can favor clones with nonsense mutations if cells are plated at high density. This is due to the fact that uracil can diffuse out of cells with OyCD PCA activity into the selection medium and allow neighboring cells to survive despite the absence of OyCD PCA activity (Griffith and Jarvis 1996; Paluszynski, Klassen et al. 2006). Colonies were pooled for DNA extraction and plasmids were retrieved. Individual plasmid was re-transformed in BY4741 *fcy1Δ* strain carrying either p415Gal1-Mbp1-OyCD-F[2] or p415Gal1-Swi4-OyCD-F[2] and assayed for OyCD PCA activity using 5-FC assay. Swi6* clones with a decreased interaction with Mbp1, slightly resistant to 5-FC, while still conserving an interaction with Swi4, sensitive to 5-FC, were identified and sent for sequencing.

Beta-galactosidase transcriptional reporter assay. Plasmids containing Swi6-OyCD-F[1] or mutant forms of Swi6-OyCD-F[1] were transformed in BY4741 *swi6Δ* strain carrying either pBA487 (4X MCB) or pBA251(4X SCB) (gifts from Brenda Andrews). Colonies were assayed for beta-galactosidase activity (Ralser, Goehler et al. 2005).

***Wild-type*, *2M-SWI6* strains and *mbp1*Δ *2M-SWI6* strains.** The SWI6 gene along with its 500 bp upstream sequence (PSWI6) was PCR amplified from genomic DNA and subcloned into pAG25 (Goldstein and McCusker 1999) which carries the nourseothricin N-acetyl-transferase (NAT1) gene that confers resistance to nourseothricin to generate pAG25-PSwi6. The L777V and A780T mutations were introduced intro pAG25-PSWI6 by site-directed mutagenesis according to the QuickChange strategy (Stratagene) to give pAG25-PSWI6-2M. The PSWI6-NAT1 and PSWI6-2M-NAT1 sequences were PCR amplified with forward and reverse oligos that have sequence homology to the SWI6 promoter and terminator sequences. Both PCR products were used to transform the BY4741 *swi6*Δ strain (Gietz and Woods 2002) and cells were selected on YPD + 100 μg/mL nourseothricin (WERNER

BioAgents). Clones were confirmed by diagnostic PCR, sequencing and western blotting using a rabbit polyclonal antibody against Swi6 (gift from Brenda Andrews).

Mbp1 and Swi4 GST pulldown in *wild-type* and *2M-SWI6* strains. *Wild-type* and *2M-SWI6* strains (described below) were transformed with p415Gal1-GST, p415Gal1-Mbp1-GST and p415Gal1-Swi4-GST. Cells were grown in SD-lys-leu + 2% glucose to OD_{600} of 0.5, washed and grown in SD-lys-leu + 2% raffinose + 2% galactose for 1 hr 30 min in order to induce the expression of Mbp1-GST and Swi4-GST. Cells were lysed by bead beating in 50 mM tris pH 7.5, 150 mM NaCl, 15 mM MgCl2, 1% Nonidet P-40, 0.1 mM sodium orthovanade, 1 mM DTT, 1 mM PMSF, 20 mM beta-glycerophosphate and 2 μg/ml of leupeptin. Cell lysates were centrifuged for 10 min and the supernatants were used for GST pulldown with 25 μl of Sephadex-gluthatione. Samples were equilibrated by rotation at 4˚C for 2 hrs and washed 3 times with the same buffer without protease inhibitors. Antibody against Swi6 was used to detect the presence of Swi6 and the anti-GST antibody was used to detect GST, Mbp1-GST and Swi4-GST.

GST-pulldown with interacting domains of Mbp1, Swi4, and Swi6. $Mbp1_{1017-1095}$, $Swi4_{650-833}$, and $Swi6_{633-803}$ were sub-cloned in pGEX-5X-3 and pMAL-2CX (New England Biolabs) using *BamH*I and *Xho*I sites. Plasmids were transformed in BL21 *E. coli* strain for protein expression. Cell lysates were incubated with Sephadex-gluthatione (GE Healthcare) or amylose resin (New England Biolabs) at 4 ^{o}C for 1 hr. The beads were washed 5 times with the lysis buffer and resuspended in pulldown buffer for GST fusion proteins, and in MBP column buffer with maltose for eluting MBP fusion proteins. For pulldown experiments, GST fusion proteins and MBP fusion proteins were mix in 400 μl of PB buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 10% Glycerol, 0.1% Triton X-100, 2 mM DTT) and rotate on a wheel for 2 hrs at 4 ^{o}C. The samples were centrifuge at 4000 rpm for 1 min, the supernatants were collected and labeled as unbound fractions. The pellets were

washed 5 times with 1 ml of PD buffer and the beads were resuspended in 40 μl of sample buffer. 25 μl of protein from the unbound and bound fractions were loaded on a 10% SDS-polyacrylamide gel, transferred on PVDF membrane, and probed with anti-MBP antibodies (New England Biolabs) or GST antibodies (Sigma).

GST-pulldown with Whi5. Whi5 was sub-cloned in pGEX-5X-3 using *BamH*I and *Xho*I sites. Plasmids were transformed in BL21 *E. coli* strain for protein expression. Cell lysate was incubated with Sephadex-gluthatione (GE Healthcare) at 4 ^{o}C for 1 hr. The beads were washed 5 times with the lysis buffer and resuspended in PB buffer. For pulldown experiments, GST and GST-Whi5 was incubated with 500 ml of cell lysate from *wild-type* or *2M-SWI6* strains for 2 hrs at 4 ^{o}C. The samples were centrifuge at 4000 rpm for 1 min, the supernatants were collected and labeled as unbound fractions. The pellets were washed 5 times with 1 ml of PD buffer and the beads were resuspended in 40 μl of sample buffer. 25 μl of protein from the unbound and bound fractions were loaded on a 10% SDS-polyacrylamide gel, transferred on PVDF membrane, and probed with anti-Swi6 antibodies or GST antibodies (Sigma).

Mbp1 and Swi4 GST pulldown in *wild-type* and *2M-SWI6* strains. *Wild-type* and *2M-SWI6* strains were transformed with p415Gal1-GST, p415Gal1-Mbp1-GST and p415Gal1-Swi4-GST. Cells were grown in SD-lys-leu + 2% glucose to OD_{600} of 0.5, washed and grown in SD-lys-leu + 2% raffinose + 2% galactose for 1 hr 30 min in order to induce the expression of Mbp1-GST and Swi4-GST. Cells were lysed by bead beating in 50 mM tris pH 7.5, 150 mM NaCl, 15 mM MgCl2, 1% Nonidet P-40, 0.1 mM sodium orthovanade, 1 mM DTT, 1 mM PMSF, 20 mM beta-glycerophosphate and 2 μg/ml of leupeptin. Cell lysates were centrifuged for 10 min and the supernatants were used for GST pulldown with 25 μl of Sephadex-gluthatione. Samples were equilibrated by rotation at 4˚C for 2 hrs and washed 3 times with the same buffer without protease inhibitors. Antibody against Swi6 was used to detect the presence of Swi6 and the anti-GST antibody was used to detect GST, Mbp1-GST and Swi4-GST.

Chromatin Immunoprecipitation of Swi6. *Wild-type* and *2M-SWI6* strains were grown in YPD and used for chromatin immunoprecipitation using the affinity purified antibody against Swi6 according to Ren B. et al. (Ren, Robert et al. 2000) with the exception that cells were fixed with formaldehyde for only 30 minutes at room temperature. The purified DNA was used for PCR using the TAQ polymerase (Bioshop) with oligos specific for the promoters of the following genes: *CLN2*, *CLN3*, *CDC45*, and *FKS1*. PCR products were loaded on 1% agarose gel.

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Chapter 4

Systematic Screens to Identify and Dissect Cdk1-cyclin Complexes *in Vivo*

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Cdk1 is the essential cyclin dependent kinase in *S. cerevisiae*. Cdk1 phosphorylates many proteins involved in cell cycle regulation by teaming up with one of nine cyclin regulatory proteins. The identification of Cdk1 substrates and the cyclin involved in a Cdk1 protein complex would allow us to place the complex at a specific time point during the cell cycle and consequently reveal the mechanism of regulation. In this chapter, I describe a strategy to identify interaction partners of Cdk1 and associate the complexes to the appropriate cyclins. This method can be used to dissect the specific function of a Cdk1-cyclin protein complex.

Author contributions:

PHE designed the experiments, generated the Gateway construct, performed the OyCD PCA screens with MJB using the manual pintool, analyzed the results and wrote the manuscript.

MJB performed the Gateway reactions and OyCD PCA screens using the manual pintool and wrote the manuscript.

JMK performed the cyclin contingency screen using the robotic pintool with guidance from PHE and SWM.

SWM designed the experiments**,** supervised the project, analyzed the results and wrote the manuscript.

Abstract

Cyclin-dependent protein kinases (Cdk) are regulatory enzymes whose temporal and spatial selectivity for specific substrate proteins are governed by their binding to cell cycle-regulated cyclin subunits. Specific cyclin:Cdk complexes bind to and phosphorylate target proteins involved in cell cycle regulation. The identification of specific cyclin:Cdk substrates is challenging and so far, has largely been achieved using *in vitro* techniques. We used a Protein-fragment Complementation Assay based on the optimized yeast cytosine deaminase (OyCD PCA) to systematically identify potential substrates or regulators of the budding yeast *Saccharomyces cerevisiae* cyclin-dependent kinase (Cdk1) along with the regulatory "cyclin" proteins involved. Cdk1 is the essential kinase in budding yeast responsible for driving cell cycle progression. In a systematic screen, we tested sixty-eight potential substrates and identified interactions between Cdk1 and seventeen known and nine novel potential substrates. We then took advantage of the negative clonal selection form of the OyCD PCA to identify candidate cyclin(s) that could mediate the detected Cdk1:protein interaction. In this assay, clonal survival occurs only when an interaction is disrupted. Based on this screen we were able to infer known and novel cyclin-dependencies for many interactions. As specific examples, we identified long suspected and critical interactions of cyclin Cbl3 with the γ-tubulin (Tub4) and Clb4 and 5 with Kar9, consistent with their known roles in mitotic spindle assembly and dynamics. The strategy we describe is widely applicable to studying any protein-protein interactions that are contingent upon accessory subunits.

Introduction

Biochemical processes are orchestrated by protein complexes. A central problem in biology is to discern the functions of individual subunits and synergistic relationships among them. For example, enzymes that perform post-translational modifications on proteins such as the ubiquitin ligases (Thornton and Toczyski 2006), protein kinases (Morgan 1997) and protein phosphatases (Cohen 1989) require different subunits to perform multiple transfer steps, assure specific subcellular localization or provide additional specificity to substrate recognition (Janssens, Longin et al. 2008). Cyclin-dependent kinases are a case in point and the budding yeast *Saccharomyces cerevisia*e (*S.cerevisia*e) cyclin-dependent kinase (Cdk1) is a very well studied example of an enzyme of this category (Morgan 1995). Cdk1 requires the association of one of nine available cyclin (Cln1, Cln2, Cln3, Clb1, Clb2, Clb3, Clb4, Clb5 or Clb6) partner proteins to recognize and phosphorylate its substrates (Mendenhall 1993). The different Cdk1-cyclin complexes play critical roles in orchestrating all the processes necessary for cellular division.

During the G1 to S-phase transition, Cln3, Cln1 and Cln2 associate with Cdk1 and regulate the phosphorylation of proteins involved in bud emergence and spindle pole body duplication. In S-phase, Clb5 and Clb6 form complexes with Cdk1 to activate DNA duplication. During mitosis, Clb1-4 promote spindle pole body maturation and separation as well as isotropic bud growth. At the metaphase to anaphase transition point, the Cdk1-Clb2 complex phosphorylates the yeast securin Pds1 in order to activate its degradation by the anaphase promoting complex (APC) (Nasmyth 2002).

The crucial role of Cdk1 in cell cycle regulation has prompted three genome-wide studies to identify all Cdk1 substrates (Ubersax, Woodbury et al. 2003; Ptacek, Devgan et al. 2005; Holt, Tuch et al. 2009). Ubersax *et al.* generated an analogue

sensitive Cdk1 (Cdk1-as) that uses a bulky ATP analogue (N6-benzyl-ATP) to phosphorylate its substrates in a crude cell lysate and found 181 substrates of Cdk1 among the 695 proteins tested (Ubersax, Woodbury et al. 2003). Ptacek *et al.* identified 50 purified proteins that were phosphorylated by the Cdk1-Cln2 or Cdk1-Clb5 complexes (Ptacek, Devgan et al. 2005). Holt *et al.* performed a differential phosphoproteomic analysis of a Cdk1-as strain treated or not with the specific Cdk1-as inhibitor 1NM-PP1 and reported 308 proteins phosphorylated by Cdk1 (Holt, Tuch et al. 2009). Interestingly, there are only two substrates phosphorylated by Cdk1 that overlap between the three studies**.** It is not unusual for large-scale experiments based on distinct techniques and conditions to reveal unique and distinct results due to the nature of the experimental strategies. For example, little overlap was observed among protein-protein interactions observed in different large-scale studies (Tarassov, Messier et al. 2008). Limitations of the approaches taken so far is that they were either limited to testing cyclins that can be readily expressed and behave well *in vitro*, or in the case of the phosphoproteomic analysis, no information about the cyclins that might have mediated phosphorylation of potential targets can be inferred.

Identifying the cyclin(s) that mediate specific Cdk1-substrate interactions remains a challenge and is critical for dissecting mechanisms of cell cycle regulation since it places the Cdk complexe(s) at specific phases of the cell cycle; i.e. during phases during which specific cyclins are expressed. This remains a difficult task due to the transient nature of the complexes and the low abundance of certain proteins that form these complexes (Archambault, Chang et al. 2004). Some cyclins are expressed at very low levels and are rapidly degraded (Mendenhall and Hodge 1998), while some Cdk1 substrates are targeted for degradation when they are not needed (eg. Pds1, the yeast securin). Ideally, an *in vivo* strategy would allow us to identify Cdk1 substrates and simultaneously infer which cyclin(s) mediate the Cdk:substrate interaction.

We previously reported a simple survival-selection screening assay for protein-protein interactions *in vivo* based on optimized yeast cytosine deaminase Protein-fragment Complementation Assay (OyCD PCA) (Ear and Michnick 2009). This strategy is ideal to detect specific Cdk:substrate interactions and to infer contingent cyclin:Cdk:substrate interactions. In a systematic screen, we tested sixty-eight known substrates or potential substrates and identified interactions between Cdk1 and seventeen known and nine novel potential substrates. We then took advantage of the negative clonal selection form of the OyCD PCA to identify candidate cyclin(s) that could mediate the detected Cdk1:protein interaction.

Results

Dissecting Cdk1-substrate-cyclin ternary complexes using OyCD PCA

The OyCD PCA is based on the optimized *S. cerevisiae* prodrug-converting enzyme cytosine deaminase (OyCD), where the reporter consists of two complementary N- and C-terminal fragments (OyCD-F[1] or F[2]) of the yCD gene (*FCY1*) fused individually to two proteins of interest and performed in an *FCY1* deletion strain (Ear and Michnick 2009). If the products of the two protein-OyCD fragment fusions interact, OyCD refolds from its complementary fragments, reconstituting its enzymatic activity. The assay provides two potential outputs: positive growth, under cytosine-limited conditions or no growth when cells are treated with a yCD pro-drug called 5-fluorocytosine (5-FC) (Ear and Michnick 2009). Here we used the negative selection form of this assay, where cells are grown on medium containing 5-FC, a non-toxic prodrug that is converted to the toxic compound 5-fluorouracil (5-FU) by yCD (Kurtz, Exinger et al. 1999). The positive selection form of the assay is more sensitive to known factors such as colony density and number of cells plated and composition of media, whereas the negative, death selection results are dependent only on the concentration of 5-FC used.

Our strategy for dissecting Cdk1 complexes *in vivo* was divided into two parts. First, we perform the OyCD screen with Cdk1 as bait and potential substrates as prey in the negative selection assay. Second, we tested the Cdk1-positive prey interactions again in strains in which individual cyclin genes have been knocked out. In the primary screen (*wild-type*; all cyclins expressed) cells grow in the presence of 5-FC if Cdk1 does not interact with the prey protein or do not grow if they do interact (**Fig.1A**). We then repeated the screen with the preys that we observed to interact with Cdk1, but in strains in which one of the nine cyclin gene is deleted (**Fig. 1B**). We can predict three potential outcomes from this screen: i) if none of the cyclins were essential to the interaction we would expect identical results to those in the *wild-type*

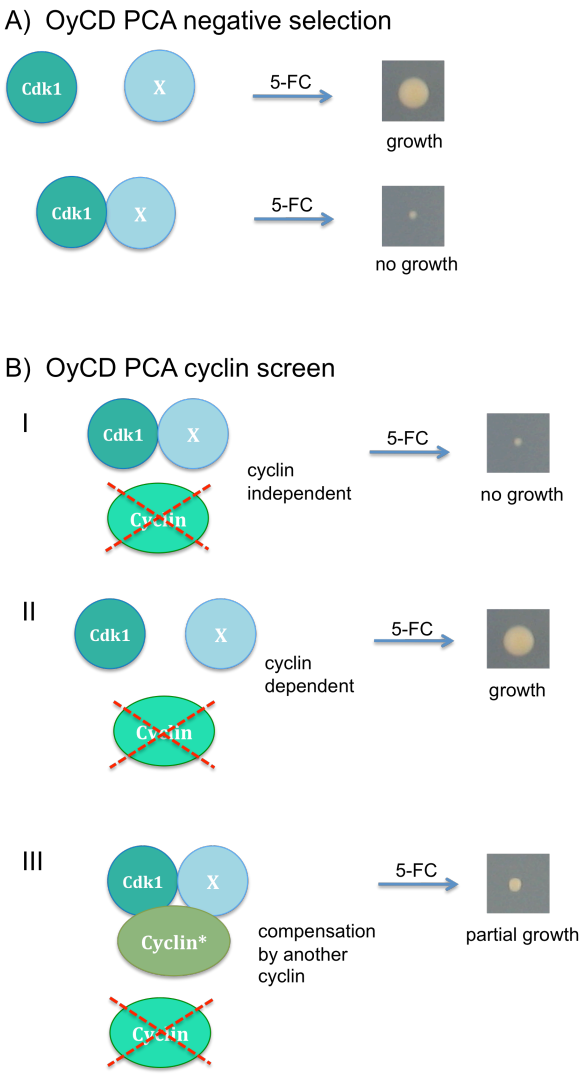
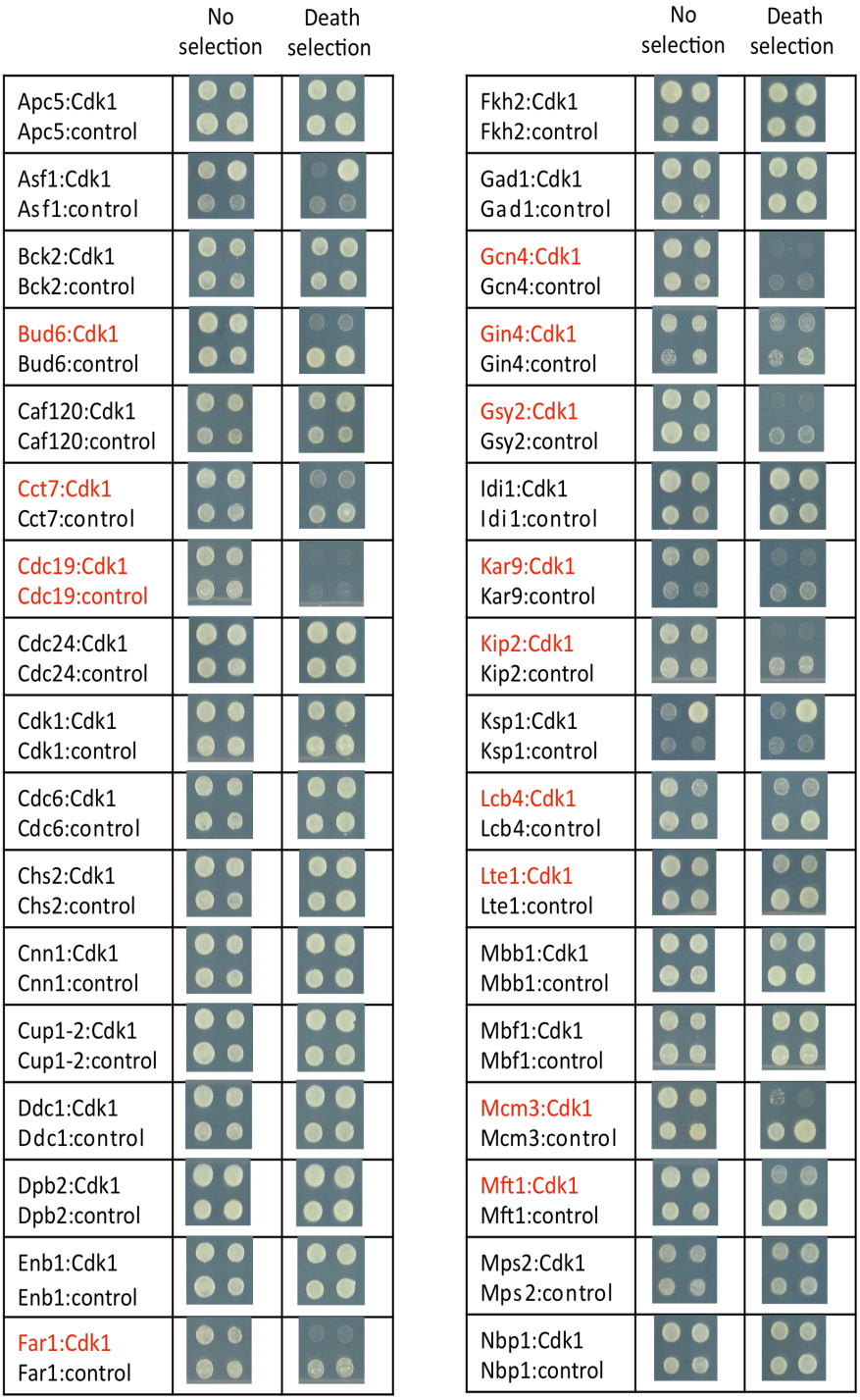


Figure 1. Dissecting Cdk1 complexes using the OyCD PCA. (**A**) Detecting the interaction between Cdk1 and a protein of interest using the OyCD PCA. Cdk1 and proteins of interest (protein X) are fused to OyCD fragments. In the death selection OyCD PCA, the absence of an interaction fails to allow the OyCD reporter enzyme to fold and restore the activity of the native enzyme. Cells expressing these fusion proteins are resistance to 5-FC. If protein X interacts with Cdk1, cells are sensitive to 5-FC. (**B**) Testing for cyclin contingency of a Cdk1 complex. I) The interaction between Protein X and Cdk1 is independent of a cyclin. When the cyclin gene is deleted, protein X can still interact with Cdk1 allowing the reporter fragments to fold and consequently cells are sensitive to 5-FC. II) Interactions are cyclin dependent. In the absence of a specific cyclin, no interaction is observed. III) When another cyclin (Cyclin*) can compensate for the abolished cyclin, a decreased interaction can be observed since the abundance of the cyclin is the limiting factor for mediating the interaction between Cdk1 and Protein X. Cells are partially sensitive to 5-FC.

strain (no growth in cells treated with 5-FC). There may be cases in which Cdk1 interacts with a protein alone, requiring no cyclin partner; ii) in cases where the Cdk1-prey interaction depends on a single cyclin, we would expect to see complete restoration of growth in one cyclin deletion strain grown in 5-FC; iii) a Cdk1-prey interacting may depend on more than one cyclin. In this case we would expect to see restoration of partial growth when several single cyclin knockout strains are grown on 5-FC.

Screening for proteins that interact with Cdk1 using OyCD PCA

As a proof of principle of our strategy, we selected ninety-four known (Ubersax, Woodbury et al. 2003), potential Cdk1 substrates and randomly chosen candidates as preys for determining their interaction with Cdk1. This list contains many proteins involved in transcription, cell cycle regulation and spindle pole assembly (Bloom and Cross 2007). For some of these genes the expression is cell cycle regulated while for others their expression is not (de Lichtenberg, Jensen et al. 2005). Of the ninety-four preys we selected, we successfully generated OyCD PCA fragment F[2] fusion expression vectors for sixty-eight, encoding proteins that are known to regulate the cell cycle (twelve), are metabolic enzymes (seven) or are implicated in phosphate utilization (six), DNA repair (three), protein degradation (two) or budding (fourteen). The remaining candidates were signaling proteins (six), transcriptional and translational regulators (twelve) and six uncharacterized proteins. Expression plasmids were co-transformed into the *FCY1* deletion strain with Cdk1-OyCD-F[2]-encoded or a control plasmid that only expresses the OyCD-F[2]. We performed the OyCD PCA by inoculating two colonies from each transformation and growing them on medium with or without 5-FC (1 mg/ml). Twenty-seven of the sixty-eight preys showed interaction with Cdk1-OyCD-F[2] (**Fig. 2**). As a negative control for spontaneous fragment complementation, we tested all prey for OyCD PCA activity with OyCD-F[2] alone. Only Cdc19 interacts with OyCD-F[2] alone.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Death selection
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Apc5:control
Asf1:Cdk1
Asf1:control
Bck2:Cdk1
Bck2:control
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Bud6:control
Caf120:Cdk1
Caf120:control
Cct7:Cdk1
Cct7:control
Cdc19:Cdk1
Cdc19:control
Cdc24:Cdk1
Cdc24:control
Cdk1:Cdk1
Cdk1:control
Cdc6:Cdk1
Cdc6:control
Chs2:Cdk1
Chs2:control
Cnn1:Cdk1
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Cup1-2:Cdk1
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Ddc1:Cdk1
Ddc1:control
Dpb2:Cdk1
Dpb2:control
Enb1:Cdk1
Enb1:control
Far1:Cdk1
Far1:control
No selection
Death selection
Fkh2:Cdk1
Fkh2:control
Gad1:Cdk1
Gad1:control
Gcn4:Cdk1
Gcn4:control
Gin4:Cdk1
Gin4:control
Gsy2:Cdk1
Gsy2:control
Idi1:Cdk1
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Kar9:Cdk1
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Kip2:Cdk1
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Mps2:Cdk1
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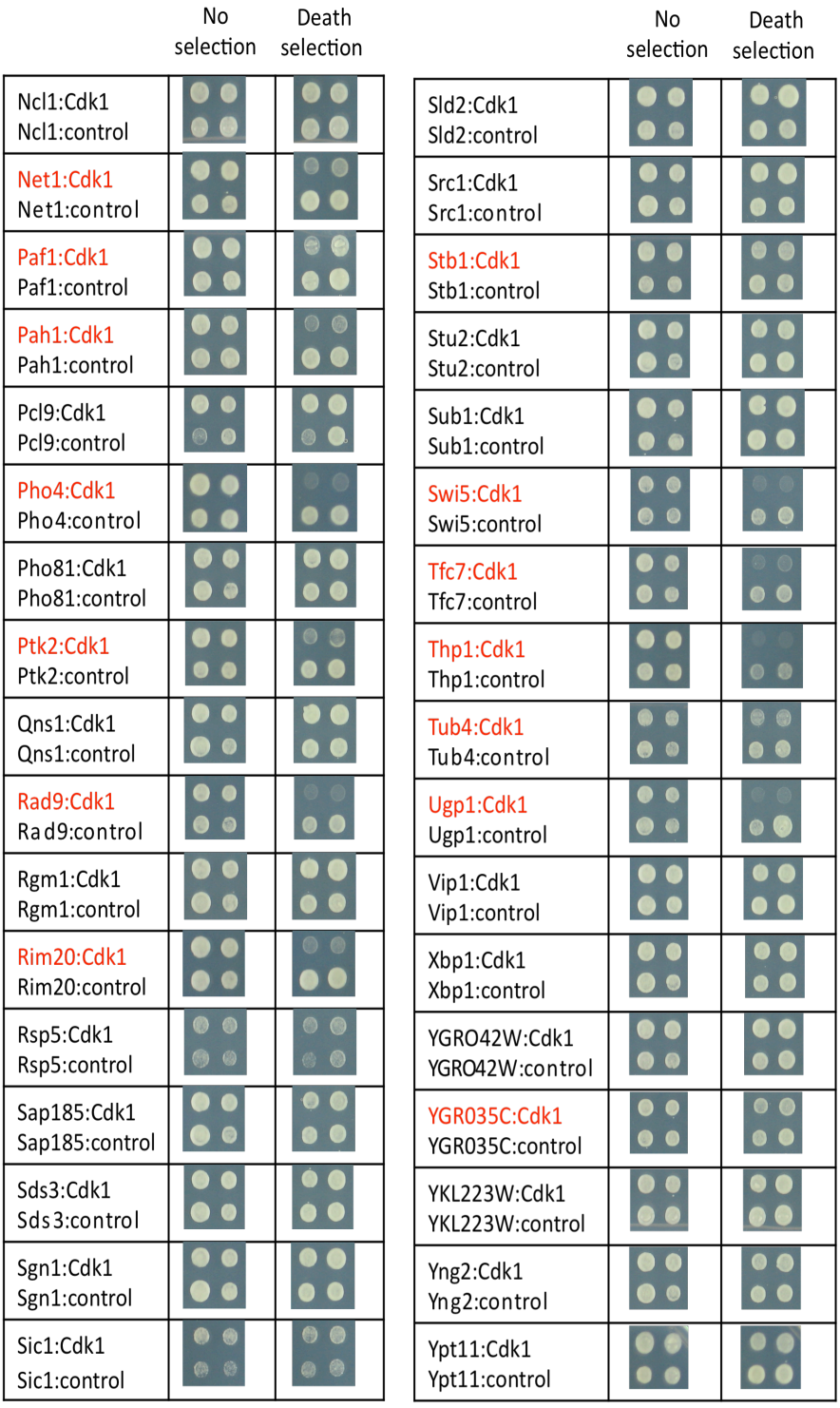
Nbp1:Cdk1
Nbp1:control


No selection
Death selection
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Net1:Cdk1
Net1:control
Paf1:Cdk1
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Pah1:Cdk1
Pah1:control
Pcl9:Cdk1
Pcl9:control
Pho4:Cdk1
Pho4:control
Pho81:Cdk1
Pho81:control
Ptk2:Cdk1
Ptk2:control
Qns1:Cdk1
Qns1:control
Rad9:Cdk1
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Rgm1:Cdk1
Rgm1:control
Rim20:Cdk1
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Rsp5:Cdk1
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Sap185:Cdk1
Sap185:control
Sds3:Cdk1
Sds3:control
Sgn1:Cdk1
Sgn1:control
Sic1:Cdk1
Sic1:control
No selection
Death selection
Sld2:Cdk1
Sld2:control
Src1:Cdk1
Src1:control
Stb1:Cdk1
Stb1:control
Stu2:Cdk1
Stu2:control
Sub1:Cdk1
Sub1:control
Swi5:Cdk1
Swi5:control
Tfc7:Cdk1
Tfc7:control
Thp1:Cdk1
Thp1:control
Tub4:Cdk1
Tub4:control
Ugp1:Cdk1
Ugp1:control
Vip1:Cdk1
Vip1:control
Xbp1:Cdk1
Xbp1:control
YGR042W:Cdk1
YGR042W:control
YGR035C:Cdk1
YGR035C:control
YKL223W:Cdk1
YKL223W:control
Yng2:Cdk1
Yng2:control
Ypt11:Cdk1
Ypt11:control

Figure 2. Protein-protein interaction results between Cdk1 and the sixty-eight test proteins. Plasmids expressing the gene of interest fused to OyCD-F[1] were transformed into *fcy1Δ* yeast strains containing either Cdk1-OyCD-F[2] (labeled as Cdk1) or OyCD-F[2] (labeled as control) and screened for protein-protein interactions using the OyCD PCA. Two colonies from each transformation were grown and printed on medium without 5-FC (no selection) and in the presence of 1mg / ml of 5-FC (death selection). When a protein of interest interacts with Cdk1-OyCD-F[2] or OyCD-F[2], cells are sensitive to 5-FC and show a decreased growth in comparison to cells co-expressing the protein of interest fused to OyCD-F[1] and the control OyCD-F[2] alone. When OyCD PCA activity is observed, the protein names are colored in red.

We examined the sequences of the twenty-six positive preys to determine their potential to be substrates or regulators of Cdk1 (**Table 1**). Seventeen of the proteins were previously identified as Cdk1 substrate (Ubersax, Woodbury et al. 2003; Ptacek, Devgan et al. 2005; Holt, Tuch et al. 2009; Keck, Jones et al. 2011). However, we also identified nine, not previously described to interact with Cdk1. Eight of the nine proteins have full (S/T-P-X-K/R) or minimal (S/T-P) Cdk1 consensus sites. We found one protein (Rim20) that does not have any Cdk1 consensus site. Interestingly, Rim20 has seven cyclin binding motifs (RXL), suggesting that it could be a regulator of the Cdk1-cyclin complexes similar to the mammalian cyclin-Cdk inhibitor p27Kip1 (Russo, Jeffrey et al. 1996) which also has no Cdk consensus site and two cyclin binding motifs.

Cyclin contingency of Cdk1 complexes

The kinase activity of Cdk1 is cyclin dependent (Mendenhall and Hodge 1998)(Ubersax, Woodbury et al. 2003; Ptacek, Devgan et al. 2005)(Koivomagi, Valk et al. 2011). In order to determine if the interactions between Cdk1 and its potential substrates or regulators are contingent upon a particular cyclin *in vivo*, we performed the OyCD PCA in different cyclin deletion strains for twenty-one of the twenty-six proteins that interact with Cdk1 (**Fig. 3**). We were unable to transform five genes (GCN4, LCB4, NET1, PAF1 and TFC1 fused to OyCD-F[1]) into many cyclin deletion strains carrying CDK1-OyCD-F[2] therefore we did not include them in this screen. As a positive control to assure that none of the cyclin knockout strains affect performance of the OyCD PCA, we tested a constitutive homomeric leucine zipper-forming peptide interaction with the OyCD PCA in all the different strains. As a negative control, we tested for the activity of OyCD PCA in the different strains expressing CDK1-OyCD-F[2] alone. All results were evaluated by taking the ratio of integrated colony intensity of each sample grown on selection medium with 1 mg/ml of 5-FC over the intensity of colonies grown on selection medium without 5-FC.

Table 1. List of genes encoding proteins that interact with Cdk1 tested by OyCD PCA. Twenty-six of the sixty-eight candidates that interact with Cdk1 are listed along with the number of full Cdk1 consensus sites (S/T-P-X-K/R), Cdk1 minimal consensus sites (S/T-P) and cyclin binding motifs in their protein sequence. Seventeen genes encoding proteins previously reported to be Cdk1 substrates are in blue. The gene names of the nine novel proteins that interact with Cdk1 are in pink. One gene product (Rim20) interacts with Cdk1 but does not have any Cdk1 consensus sites (shown in red).

list of 26 positive	Full Cdk consensus sites	Minimal Cdk consensus sites	Cyclin binding sites
BUD6	2	10	6
CCT7	0	1	5
FAR1	4	15	5
GCN4	0	5	1
GIN4	2	11	11
GSY2	0	4	6
KAR9	2	15	9
KIP2	2	8	6
LTE1	8	20	6
LCB4	0	6	5
MCM3	5	11	5
MFT1	0	1	4
NET1	3	17	3
PAF1	0	2	4
PAH1	2	15	6
PHO4	0	7	1
PTK2	2	16	5
RAD9	9	20	11
RIM20	0	0	7
STB1	5	18	2
SWI5	8	20	3
TFC7	0	4	1
THP1	0	3	6
TUB4	0	5	1
UGP1	0	2	4
YGR035C	2	2	0

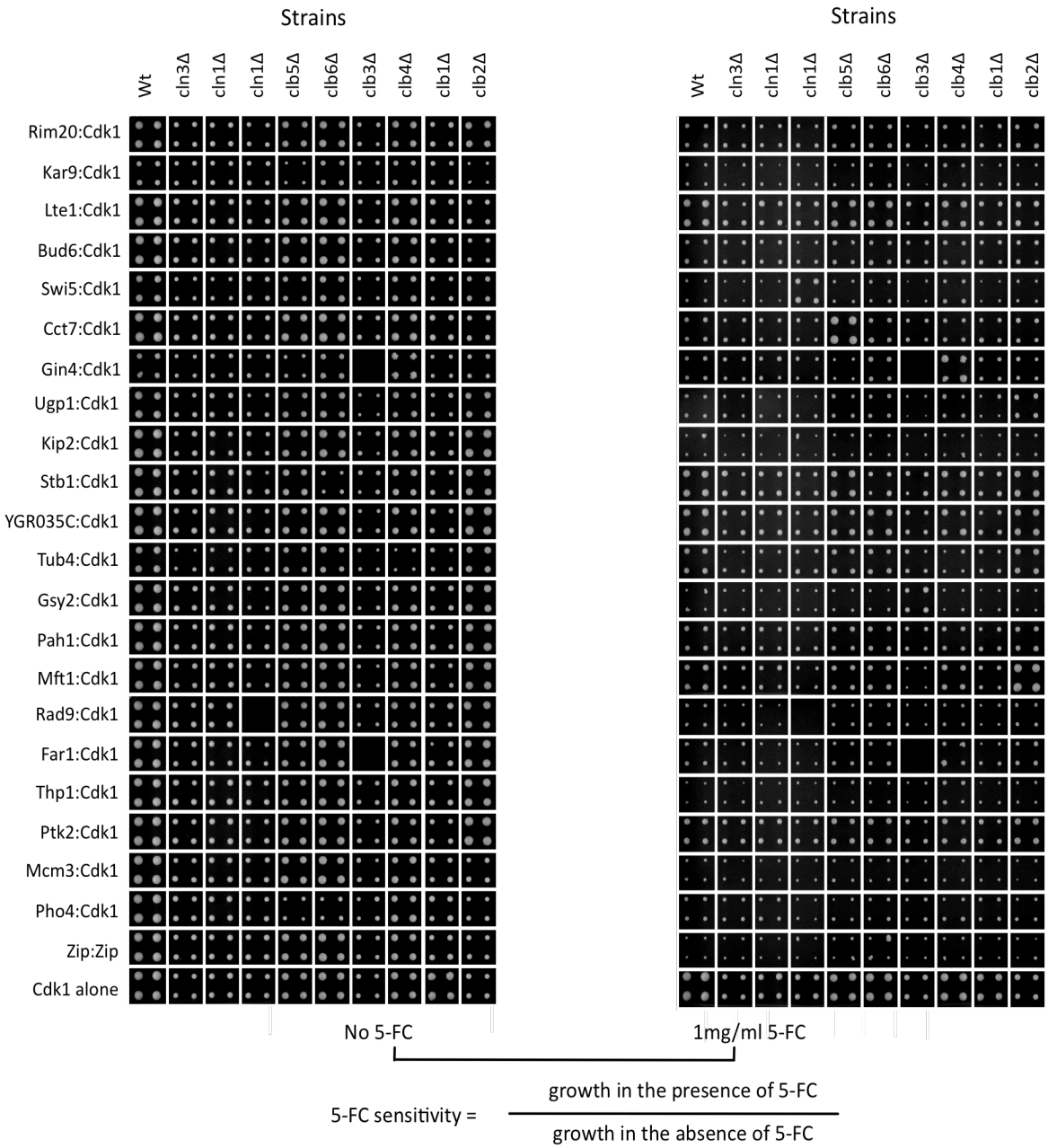

Figure 3. The OyCD PCA results in different cyclin deletion strains. The interaction of Cdk1 and its interacting partners were tested in a yeast strain expressing all the nine cyclins (wt) and in strains with one of the nine cyclins and *FCY1* genes deleted. The cyclin deletion strains are labeled by the gene name followed by a delta symbol (Δ). For example, the *CLN1* deletion strain is named *cln1Δ*. Each row represents the different yeast strains expressing Cdk1 and a protein of interest fused to the OyCD fragments. The two last rows correspond to cells expressing the controls for this experiment. The positive control corresponds to yeast expressing the homodimeric GCN4 coil-coil leucine zipper (Zip:Zip) fused to the OyCD fragments. This interaction is constitutive and is independent of any cyclin. The negative control corresponds to yeast expressing only Cdk1 fused to OyCD fragment 1.

In total, we tested the activity of the OyCD PCA of the twenty-one interaction along with the Zip:Zip and Cdk1 alone controls in the different yeast strains in three different experiments. We observed that the over-expression of Cdk1 in combination with some of the proteins (Thp1, Bud6 and Gin4) did affect the growth of the yeast strains in comparisons to the over-expression of Cdk1 alone or the Zip:Zip (**Fig. 4A**). We also found that the some of the cyclin deletion strains grew poorly in comparison to the *wild-type* strain (**Fig. 4B**). However, the effect of gene over-expression affected the *wild-type* and cyclin deletion strains uniformly and did not affect the OyCD PCA activity, since similar OyCD PCA activity was observed for all the different yeast strains (**Fig. 4C and 4D**). Overall, the effect of the OyCD PCA activity was dominant over the effect of the over-expression of the two genes of interest (**Fig. 4A**) or the different strains (**Fig. 4B**).

In order to compare the activity of the OyCD PCA of each interaction in the ten different yeast strains, a Student t-test was performed using the OyCD PCA activity obtained. For the Zip:Zip control, there was some minor differences in growth in the different strains in comparison to the *wild-type* strain but in no cases did the strain background significantly affect results compared to the assay (Student t-test, $P < 0.04$) (**Fig. 4C**). We did not observe significant differences among strain results for a negative control Cdk1-OyCD-F[2] expressed alone (Student t-test, $P < 0.02$). We considered results with a $P < 0.02$ to be significant since this is the lowest p-value for the activity of cells expressing the Cdk1 alone control in the *CLB1* deletion strain (**Fig. 4C**). Among the twenty-one candidates that interact with Cdk1, we found that the interaction between Kip2 and Cdk1 remains unchanged in all the cyclin deletion strains with respect to the *wild-type* strain, similar to the interaction between Zip:Zip (**Fig. 5**). The interaction between Cdk1 and the remaining twenty proteins decreased in one specific or in several cyclin deletion strains. For example, yeast expressing Tub4 and Cdk1 grew better when the *CLB3* gene was deleted. This suggests that Clb3 mediates the interaction between Tub4 and Cdk1. In contrast, many interactions

between Cdk1 and its partners were dependent on various cyclins. For example, the interaction between Kar9 and Cdk1 was dependent upon Clb2, Clb4 and Clb5. Likewise, the interaction between Rim20 and Cdk1 decreased in the *CLN3*, *CLN1* or *CLB5* deletion strains. The OyCD PCA signal also decreased for Mcm3-Cdk1 interaction in the *CLN2*, *CLB5* and *CLB3* deletion strains.

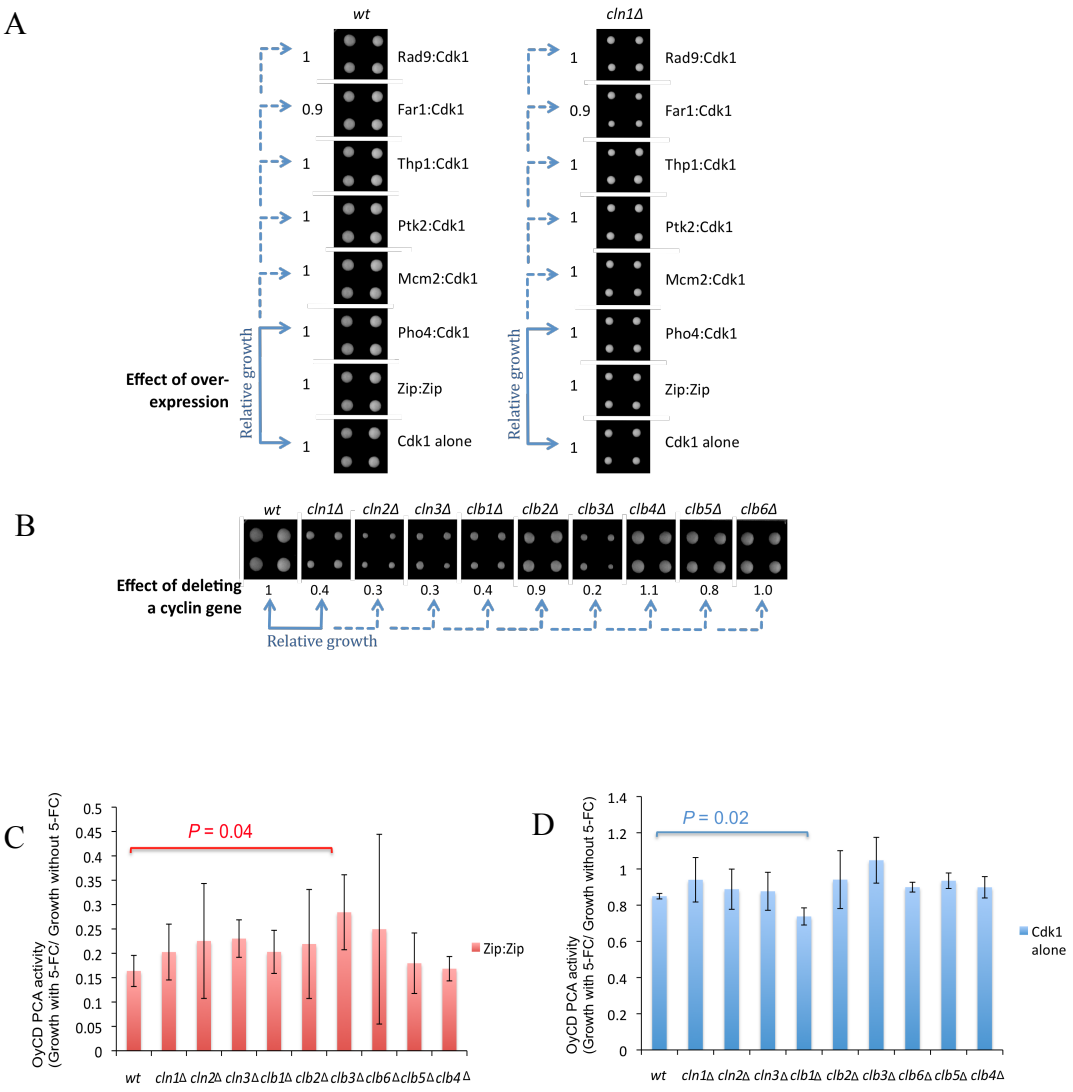

Figure 4. Factors influencing the activity of OyCD PCA. A) The effect of over-expressing Cdk1 and its interacting partners and cyclin gene deletion on growth of the yeast strain. The over-expression of some fusion genes slightly affects the fitness of the yeast strain in comparison to the yeast expressing only Cdk1 or Zip:Zip fused to OyCD fragments. B) The deletion of some cyclin genes affect the ability of the strain to grow in comparison to the *wild-type* (wt) strain. C and D) The effect of the OyCD PCA activity on growth is relatively constant in the different cyclin deletion strains. Statistical significance was assessed using Student *t*- test. P = 0.04 was obtained for wt and *cln3*Δ strains expressing Zip:Zip interaction. P = 0.02 was obtained for wt and *clb1*Δ deletion strains expressing Cdk1 alone.

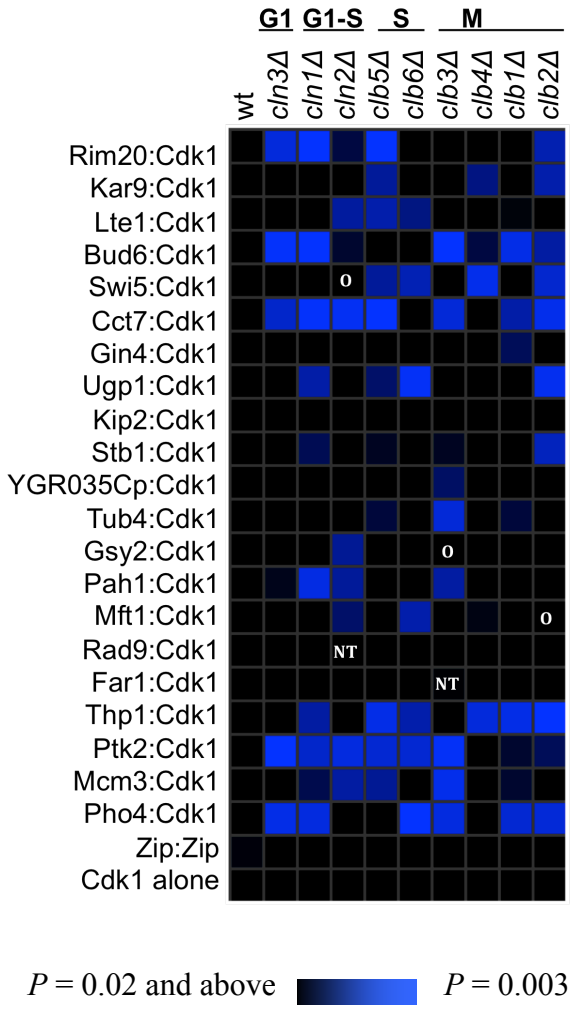

Figure 5. Contingency of the Cdk1 complexes. The gene of interest and Cdk1 fused to OyCD fragments were transformed into the *FCY1* deletion strain which is referred to as the *wild-type* strain (wt) and nine different cyclins and *FCY1* double deletion strains which were represented by their gene name in *italic* followed by a Δ sign (eg. *cln1* Δ). Colonies of each transformation were assayed for OyCD PCA activity in the presence of 1 mg/ml of 5-FC in three different experiments. The growth of each sample was quantified using ImageJ. All strains expressing only Cdk1-OyCD-F[2] (Cdk1 alone) were resistant to the 5-FC death selection assay with $P > 0.02$. All strains expressing the GCN4 leucine zipper domains (Zip:Zip) fused to the OyCD fragments were sensitive to 5-FC in the death selection assay with a $P > 0.04$. The loss of interaction detected in the different cyclin deletion strains is depicted using their p-value. The $P = 0.02$ was used as a cutoff for this experiment. Only $P < 0.02$ are represented on the matrix (Tarassov and Michnick 2005). Two samples have not been tested and are represented by NT. The values of three samples were omitted (O) since they were not consistent with two previous experiments.

DISCUSSION

The *S. cerevisiae* cyclin-dependent kinase (Cdk1) is the main regulator of the cell cycle and was first identified in a genetic screen (Hereford and Hartwell 1974). Yet, its large spectrum of functions is only fully appreciated when the elaboration of its molecular mechanisms was revealed, key mutations were identified and the crystal structures of the enzyme were solved (De Bondt, Rosenblatt et al. 1993; Russo, Jeffrey et al. 1996). Its kinase activity is highly dependent on its interaction with one of nine cyclins or other regulatory protein such as Cak1, Far1, Sic1 and Swe1 (Mendenhall and Hodge 1998). Identifying the ternary complexes between Cdk, cyclin and a substrate or regulator *in vivo* is a difficult task, since these interactions occur during specific phases the cell cycle and in specific compartments of the cell. In addition, with the exception of Cln3, each cyclin has a paralog cyclin that shares high sequence identity and has overlapping functions. We have established an *in vivo* screening system based on the OyCD PCA in order to identify proteins that interact with Cdk1 and associated the regulatory cyclin subunit(s) to various Cdk1 complexes.

Among the twenty-six proteins that we identified to interact with Cdk1 using the OyCD PCA, we found nine proteins previously not reportedly linked to Cdk1. Eight of the nine candidates have the minimal Cdk1 consensus site (S/T-P) (**Table 1**). Since proteins with the minimal Cdk1 consensus site can be phosphorylated by Cdk1-cyclin complexes (Ubersax, Woodbury et al. 2003; Holt, Tuch et al. 2009), these eight proteins could be potential Cdk1 substrates. Rim20 is the only protein we found that interacts with Cdk1, but has no minimal or full consensus Cdk1 site. Rim20 may not be a substrate at all, but potentially a regulatory protein that binds to Cdk1-cyclin complexes. Rim20 was first characterized as a regulator of Ime2, the protein kinase involved in activating meiosis (Su and Mitchell 1993). Our cyclin contingency experiment suggests that Rim20 interacts with Cdk1 in complexes with

G1, G1-S and S phase cyclins (Cln3, Cln1 and Clb3). It would be worth investigating whether Rim20 could inhibit Cdk1 activity in order to stop the mitotic cell cycle when diploid cells are nitrogen starved, driving them into meiosis, similar to how Far1 inhibits Cdk1 activity in the presence of α-factor for haploid *MAT***a** yeast strain (Tyers and Futcher 1993).

Cyclins have previously been reported to have both specific and overlapping functions. For example, Clb5 and Clb6 have different biological functions in terms of promoting S-phase progression, despite the fact that they are closely related (Jackson, Reed et al. 2006). Clb6 is rapidly degraded at the G1/S-phase border, since it has a destruction box motif at its N-terminal recognized by the SCF^{Cdc4} ubiquitin ligase complex (Jackson, Reed et al. 2006). In contrast, Clb5 is stably expressed throughout the entire S-phase into mitosis. In another biological process, such as the regulation of DNA replication, Clb5 and Clb1 to Clb4 were shown to have a redundant role in controlling the pre-replication complex (Ikui, Archambault et al. 2007). It is extremely difficult to distinguish specific *versus* redundant role(s) of a cyclin in Cdk-protein(s) complexes using functional assays. Here, we provide a simple strategy to associate a cyclin to Cdk1-protein complexes based on measuring their protein-protein interaction changes in cyclin deletion strains using the OyCD PCA.

Our cyclin contingency screen results reveal that some Cdk1-protein complexes are independent of a specific cyclin. For example, the Cdk1- Kip2 interaction was not affected when any of the individual nine cyclins was deleted, suggesting that the interaction is independent of any cyclin in particular. In the case of Tub4, one particular cyclin seems to be responsible for mediating its interaction with Cdk1. Our results suggest that Clb3 mediates the interaction between Tub4 and Cdk1. For the majority of the proteins tested (Rim20, Cct7, Ptk2, and Thp1) several cyclins were found to mediate their interaction with Cdk1. *In vitro* binding assays and functional experiments will be required to confirm these results. Nevertheless, our strategy can

provide insights into the mechanism of regulation of Cdk1 and its novel interaction partners. For instance, we found that the novel interaction between Mft1 and Cdk1 is dependent on Clb6. Mft1 is a protein involved in mitotic recombination (Chavez, Beilharz et al. 2000). Cdk1 could interact with Clb6 and phosphorylate Mft1 in order to regulate its activity during the S phase of the cell cycle.

Numerous studies in fission and budding yeast have revealed Cdk1's central function in orchestrating the timing of events during the cell cycle (Coudreuse and Nurse 2010). Many Cdk1 targets are important effectors in the regulation of both the actin and microtubule based cytoskeleton (Holt, Tuch et al. 2009). Prior to mitosis, Cdk1 regulates the dynamic localization and organization of actin during polarized and isotropic growth of the bud (McCusker, Denison et al. 2007). Cdk1 also promotes early spindle positioning to the bud neck by driving the asymmetric distribution of Kar9 to the old spindle pole and associated microtubules (Liakopoulos, Kusch et al. 2003). Cdk1's activity towards Kar9 has been attributed to several cyclins; Clb3, Clb4, and Clb5 (Liakopoulos, Kusch et al. 2003; Moore and Miller 2007). Through a yet to be discovered mechanism, Cdk1 regulates cytoplasmic dynein, ensuring that dynein activity is coupled to the metaphase anaphase transition (Grava, Schaerer et al. 2006). Most recently, the evolutionarily conserved microtubule nucleator γ-tubulin, Tub4 in budding yeast, was shown to be a mitotic target of Cdk1 (Keck, Jones et al. 2011). Mitotic cells were induced through depletion of the APC activator Cdc20 (Tavormina and Burke 1998) which arrests cells at the metaphase-anaphase transition with high levels of B-type cyclin (Clb1-3) Cdk1 activity (Rahal and Amon 2008). Phospho peptide analysis was performed on highly purified preparations of spindle pole bodies (SPBs) enriched in Tub4 and its associated proteins (Keck, Jones et al. 2011). A phospho-mimetic mutation in S360, which lies in an extended and conserved Cdk1 recognition motif, resulted in defects in spindle elongation during anaphase (Keck, Jones et al. 2011). This observation is consistent with Tub4 phosphorylation acting to organize the spindle microtubules in early mitosis, during

spindle assembly. This presents a challenge for deeper investigation into the regulation of Tub4 by Cdk1 as 6 different Cdk1 cyclin complexes (Cdk1:Clb1-6) are candidates. Moreover, detection of phosphorylation at S360 cannot be detected using conventional isolation methods (IP or affinity purification) with protein over production (Lin, Gombos et al. 2011). It is clear that Cdk1 targets the pool of Tub4 that is located on the spindle pole bodies. Thus the OyCD PCA method which sensitively detects protein-protein interactions, was essential to detecting the specific Cdk1:cyclin complex(s) that regulates Tub4.

This assay detected the well-established interaction between Cdk1 and Kar9, as well as Kar9 interaction with three cyclins; the previously reported interaction with Clb4 and Clb5 [4,5], and a new interaction with the late mitotic cyclin Clb2. The assay verified the interaction between Tub4 and Cdk1 (Keck, Jones et al. 2011), and detected a novel interaction between Tub4 and Clb3, an early mitotic cyclin. This suggests that Cdk1-Clb3 targets Tub4 during spindle assembly.

We have established a systematic method to dissect the ternary interaction between a protein of interest and the Cdk1-cyclin complex using the OyCD PCA and Gateway cloning strategy. This strategy has the potential to dissect other natural multi-subunit protein complexes *in vivo*.

Materials and methods

Yeast strains. The *MAT**a*** BY74741 yeast strain along with the *FCY1* (encoding for yeast cytosine deaminase) and all nine single cyclin deletion strains (*fcy1Δ,* cln1*Δ, cln2Δ, cln3Δ, clb1Δ, clb2Δ, clb3Δ, clb4Δ, clb5Δ,* and *clb6Δ*) (Giaever, Chu et al. 2002) were supplied by Dr Jackie Vogel (McGill University). The *FCY1* gene was replaced by the nourceothricin resistance gene in all nine cyclin deletion strains using homologous recombination. The Yeast ORF Collection of over 4900 plasmid based yeast genes in Gateway expression clones was purchased from Open Biosystems (Gelperin, White et al. 2005).

Plasmid Construction. The pAG415GAL1-ccdB-EGFP (LEU2 marker) Gateway destination vector (Alberti, Gitler et al. 2007) was purchased from Adgene. The pAG413GAL1-ccdB-OyCD-F[1] Gateway destination vector was created by cloning an OyCD-F[1] sequence into the position of the EGFP gene in the pAG413GAL1-ccdB-EGFP destination vector using EcoR*V* and Xho*I* restriction sites. The p415Gal1-Linker-OyCD-F[2] was constructed by introducing Linker-OyCD-F[2] sequence in p415Gal1 (ATCC number: 87330) using *BamH*I and *Xho*I sites. The p415GAL1-Cdk1-OyCD-F[2] plasmid was obtained by cloning the CDK1 gene upstream of OyCD-F[2] using *Spe*I and *BamH*I. The negative control p415GAL1-Start-Linker-OyCD-F[2] was created by cloning a Linker-OyCD-F[2], which has an ATG codon before the linker sequence in the position of Cdk1-OyCD-F[2] in p415GAL1-Cdk1-OyCD-F[2] using Spe*I* and Xho*I* restriction sites.

Gateway Cloning. The selected genes from the Yeast ORF Collection were transferred into a Gateway donor vector to obtain ENTRY clones using the Gateway BP reactions according to the manufacturer's protocol (Invitrogen) with the exception that the reaction were scaled down four times and the incubation time was prolonged to sixteen hours at 22 °C. We generated a Destination vector that carries

the first fragment of OyCD (OyCD-F[1]) that we named pAG413GAL1-ccdB-OyCD-F[1]. This vector was used in an LR reaction with an ENTRY clone that carries a gene encoding a protein of interest. The LR reactions were performed according to the manufacturer's protocol (Invitrogen) with the exception that the reactions were scaled down four times and the incubation time was prolonged to sixteen hours at 22 °C. The product of the LR reaction is an Expression clone that contains the gene of interest fused to OyCD-F[1] (pAG413GAL1-GeneX- OyCD-F[1]) and a by-product plasmid that is not recovered. This strategy enabled us to easily create a large number of Gateway expression clones using the selected genes from the Yeast ORF Collection with each fused to the OyCD-F[1] sequence.

Using OyCD PCA to detect protein-protein interactions with Cdk1. The selected genes fused to the OyCD-F[1] sequence in Gateway expression clones were each separately transformed into BY4741 *fcy1Δ* yeast containing either p415GAL1-Cdk1-OyCD-F[2] or p415GAL1-Linker-OyCD-F[2]. After three days of growth two colonies were picked from each transformation and grown in a 96-well plate in 400 μl of synthetic complete medium without histitidine and leucine and with 2 % rafinose for sixteen hours. Galactose was added to each culture at a final concentration of 2% to induce the expression of the OyCD fusion proteins for an hour at 30 °C before pinning the samples on selection raffinose and galactose plates with and without 1 mg/ml of 5-FC (Sigma) using the manual pintool (1.58 mm, 1 ml slot pins, 45 mm, VP 408Sa, V&P Scientific Inc.). Raffinose, galactose and glucose were purchase from Bioshop. Pictures were taken after 3 days of growth.

Detecting protein-protein interactions in the different cyclin deletion strains. Proteins that interacted with Cdk1 were screened in yeast strains expressing all nine cyclin genes or lacking one of the nine cyclin genes. The potential substrate genes fused to the OyCD-F[1] sequence in Gateway expression vectors were co-transformed with p415GAL1-Cdk1-OyCD-F[2] into the *FCY1* deletion (*fcy1Δ*) strain

and the nine single cyclin and *FCY1* double deletion strains. We consider the *fcy1Δ* yeast strain as the control strain in this screen and referred to it as the *wild-type* (wt) strain since it expresses all nine cyclins. The controls for protein-protein interaction detected in the ten yeast strains were the interactions between the homodimeric GCN4 leucine zippers (ZIP) fused to complementary OyCD fragments serving as positive controls and Cdk1 fused to OyCD-F[2] alone serving as negative controls. Four clones were picked and grown to saturation in synthetic complete medium lacking histidine and leucine with 2% glucose and 200 μg/ml of G418 (Sigma). A glycerol stock was prepared with these cultures. All samples from the glycerol stock were printed on plates containing the same medium with 3% agar and allowed to grow for four days. For evaluating the OyCD PCA activity, colonies were pinned on synthetic complete medium lacking histidine and leucine with 2% raffinose and 2% galactose, and 200 μg/ml of G418 (Sigma) and 3% agar plates with and without 1 mg/ml of 5-FC using a robotically manipulated 384 pintool (0.356 mm flat round-shaped pins, custom AFIX384FP8 BMP Multimek FP8N, V&P Scientific Inc.). Pictures were taken after one, two, three and four-day of growth. This experiment has been repeated three times. Results of only one set of experiments taken on day four are shown in **Fig. 4**.

Analysis of the cyclin deletion strains. Cell growth was quantified using ImageJ (Abramoff 2004) by calculating the integrated intensity of each colony. Only the results of one set of experiments taken on day four are represented in **Fig. 5**. The activity of the OyCD PCA was measured by taking the ratio of integrated intensity of each colony grown on 1 mg/ml of 5-FC over the integrated intensity of colonies grown in the absence of 5-FC. A Student t-test was performed using the results of OyCD PCA activity obtained for the wild-type strain and each of the nine single cyclin deletion strains. A minimum $P = 0.04$ was obtained for cells expressing the GCN4 leucine zippers (Zip:Zip) in the Clb3 deletion strain. The minimum p-value *(*$P = 0.02$)

was obtained for cells expressing Cdk1 fused to OyCD-F[1] (Cdk1 alone) in the Clb3 deletion strain. Only P s lower than 0.02 were considered as significant for the interaction between Cdk1 and test proteins in the different yeast strains. Results were represented in a matrix using iVici (Ref). The color scale ranging from black to blue represents $P < 0.02$ to 0.003. Two interactions were not tested and are represented by NT. The values for three interactions were omitted (O) since they were not consistent with two previous experiments.

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General discussion

5.1 Insights into yCD structure and function

In the process of developing the OyCD PCA we also realized new insights into the nature of the yCD structure. Yeast cytosine deaminase is a relatively unstable enzyme that was optimized to become more stable by introducing three residues that help the enzyme pack more tightly (Ireton, Black et al. 2003). Our results suggest that the dimerization of yCD is important for its activity. It is possible that the dimerization of yCD could either help stabilize each monomer or contribute to the correct positioning of the active site of the enzyme. First, all point mutations that we found to have a favorable effect on the activity of the yCD PCA were at the dimer interface of yCD (**Chapter 2, Figure 6**). In addition, the repeated fragment of yCD (the α2 helix) that was found to enhance yCD PCA activity is also mapped to the dimer interface of yCD. Interestingly, other mutations on yCD that increased its activity reported after the development of our PCA are also localized to the dimer interface (Stolworthy, Korkegian et al. 2008). Second, when yCD was mutated at tyrosine 121 to an alanine (Y121A), the activity of yCD was completely abolished. Analysis of the yCD crystal structure shows that Y121 stacks with Y121 of the second yCD subunit. We hypothesized that the stacking of the two tyrosines is the key mechanism for yCD dimerization and activity. This supports the notion that the tyrosine-tyrosine stacking is important for the activity of yCD but it would require additional experimental evidence to determine if the Y121A mutation causes yCD to be a monomer.

Many enzymes have been reported to be only functional as a homodimer. These include the glycogen phosphorylase (Browner and Fletterick 1992), protease of human Kaposi's sarcoma–associated herpes virus (KSHV) (Shahian, Lee et al. 2009) and protein arginine methyl transferase (Zhang and Cheng 2003). It is notable

that in a recent proteomic analysis of protein complexes on the order of fifty percent of proteins were found to exist as homo- or higher order oligomers (Kuhner, van Noort et al. 2009) and we have made similar observations in our lab for *in vivo* protein interactions in *S. cerevisiae* (unpublished results). Further, homo- and higher order homo-oligomers assemble via assembly steps reflected in both their assembly in the cell and their evolution (Levy, Boeri Erba et al. 2008). Thus, it will not be surprising if we find in the future that homomeric enzyme complexes are a common evolutionary development.

Ideally, a reporter for a PCA should be monomeric. A monomeric PCA reporter is a better probe for detecting the protein-protein interactions without forcing proteins to adopt a particular protein complex conformation. Since the OyCD PCA is a dimer, effort should be made in the future to monomerize yCD. It is therefore important to determine if the Y121A mutant of yCD causes the enzyme to be monomeric. If so, we could start re-engineering the monomeric yCD based on this mutation. The challenge will be to identify other mutations that can stabilize or promote the activity of the enzyme following disruption of the interface.

5.2 Development of other biomolecular death assays

Massoud and Gamhbir have developed a PCA based on the Herpes simplex virus thymidine kinase1 (HSV1-TK) as reporter enzyme (Massoud, Paulmurugan et al. 2010). They used the HSV1-TK PCA to detect protein interaction in mice by using a radiolabelled substrates of the enzyme for imaging PPI with a positron emission tomography. Since the HSV1-TK also belong to the family of prodrug converting enzyme and that the prodrug Ganciclovir is available, it could potentially be used to induce cell death when the interaction between two test proteins allow the HSV1-TK fragments to fold and restore the activity of the native enzyme. Additional optimization might be required in order to detect HSV1-TK PCA activity since high

concentration of Ganciclovir is needed to induce cell death in *S. cerevisiae* expressing the full length HSV1-TK (Wera, Degreve et al. 1999).

Chelur and Chalfie have also developed a PCA for inducing cell death in *Caenorhabditis elegans* (Chelur and Chalfie 2007). Their assay is based on reconstituting the activity of caspase-3 (recCaspase) using the anti-parallel leucine-zipper domains to bring the two caspase 3 subunits into proximity and cause cell death in *Caenorhabditis elegans* and in HeLa cells. The application of this strategy to study protein-protein interaction is still limited since this assay has not been tested using full-length proteins as interaction partners. In addition, this assay cannot be used as a dual selection assay since the complementation of the caspase 3 subunit only activates cell death.

5.3 Dissecting cell cycle protein complexes using OyCD PCA

The ideal context to study cell cycle protein complexes is inside the living cell since these complexes are dynamic and subject to various post-translational modifications, including phosphorylation, ubiquitylation glycosylation, acetylation and methylation. In addition, cell cycle regulation involves proteins from all categories ranging from membrane bound proteins to transcription factors. Taking into consideration all these factors, only a limited set of strategies is appropriate for studying these protein complexes and PCA is one of them. Other *in vivo* assays such as Förster resonance energy transfer (FRET) (Browner and Fletterick 1992; Clegg 1995) and Bioluminescence resonance energy transfer (BRET) (Xu, Piston et al. 1999) can be used but scaling up these assays to study many samples would be costly and time consuming. For these reasons, we have developed and optimized the OyCD PCA to study and used it to dissect cell cycle protein complexes *in vivo*. By applying OyCD PCA to study the SBF, MBF and Cdk1-cyclin complexes, we have

highlighted novel features of these complexes potentially important for their functions.

5.3.1 Swi6 dimerization

Using the OyCD PCA, we found that Swi6 can dimerize *via* its C-terminal domain. The dimerization of transcription factor subunits could affect their activity. Many bacterial transcription factors such as the LacR, GalR, AraR repress transcription by binding at various promoter sequences and since they can homodimerize, they form DNA loops (Choy and Adhya 1992; Wong, Guthold et al. 2008). It is possible that Swi6 could bring two distal promoter or enhancer sequences together by dimerizing *via* its C-terminal domain. This could either repress or activate transcription. In one scenario, the DNA loops formed by the Swi6 dimer could sterically hinder the RNA Pol II transcriptional machinery to be correctly recruited to the promoter sequence and activate transcription. Alternatively, the dimerization of Swi6 and DNA looping could promote an efficient and coherent transcription of two different genes. This could be a mechanism of positive feedback at the G1/S transition phase of the cell cycle in order to ensure a rapid and simultaneous expression of genes involved in budding or DNA replication. These hypotheses suggest exciting biological significance of the Swi6 dimerization that would require further investigation.

5.3.2 Swi6 and Whi5 interaction

By generating a mutant form of Swi6 (2m-Swi6) that preferentially binds to Swi4 over Mbp1, we have uncovered the subtle allosteric regulation of the SBF and MBF complexes that play a potentially important role in the transcriptional regulation of their target genes. Interestingly, we found that Swi6 interacts with Whi5 *via* its C-terminal domain. The two point mutations on 2m-Swi6 are located at

the C-terminal domain of Swi6 directly affects the interaction between Swi6 and Whi5. This Swi6 mutant could be used to further understand the role of Whi5 repression and the timing of SBF and MBF activation.

5.3.3 Dissecting cyclin-Cdk1 complexes

Identification of Cdk1 substrates and the Cdk1 enzyme complexe(s) responsible for catalyzing the reaction(s) *in vivo* remains challenging due to the low abundance of these proteins. First, we adapted the OyCD PCA to systematically identify proteins that interact with Cdk1 and found known and potential Cdk1 substrates among the positive candidates. Second, we established a system to identify the cyclin in the Cdk1-protein complexes by performing the OyCD PCA in different single cyclin deletion strains. The combination of these methods represents a powerful *in vivo* strategy to dissect the function of Cdk1 at specific time point during the cell cycle. Since the expression of the regulatory subunit of Cdk1 are cell cycle regulated, knowing the cyclin involved in specific phosphorylation reaction is important for dissecting the mechanism of cell cycle regulation. Coupling protein-protein interaction information with genetic perturbations can give us insights into how cyclins play redundant or specific function(s) in a cellular process.

To date, the question of unique *versus* redundant function of cyclins belonging to the same category remains controversial. While some studies reported that most cyclins have redundant functions (Ikui, Archambault et al. 2007), others showed unique functions (Jackson, Reed et al. 2006) . The emergence of the four pairs of cyclin paralogs 100 million years after the whole genome duplication, suggests that they evolved to perform unique functions (Bloom and Cross 2007). In the wild, *S. cerevisiae* grow and divide despite environmental fluctuations. In the laboratory, we often use the same growth condition for all our experiments. This parameter could contribute to why we detect redundant functions of the cyclins. Perhaps performing

the same experiments under distinct environmental or metabolic conditions would reveal their specific functions. Our strategy is not a functional assay but can provide valuable clues about the roles of specific cyclins and where their functions may overlap with others. In addition, the experiments could be performed under different conditions, perhaps allowing us to more finely dissect the unique functions of these proteins.

5.4 Perspective

The OyCD PCA has been carefully engineering to be functional in multiple biological systems from *S. cerevisiae* to mammalian cells. Here, we have described how it can be used to dissect cell cycle protein complexes in *S. cerevisiae* grown at 30 ^{o}C but it can also be use in bacterial and mammalian cells, which grow at 37 ^{o}C since we optimized it to be functional at 37 ^{o}C. The fact that it can be used to engineer mutant proteins for specific protein-protein interaction means that it can be used to engineer protein binders against specific target proteins. In addition, the OyCD PCA is not just limited to be used for survival or death selections it could have a potential future in the field of imaging.

5.4.1 False positive rate of OyCD PCA in the Swi6 screen

Using the OyCD PCA to engineer a mutant form of Swi6 that disrupts its binding to Mbp1 but still interact with Swi4 was challenging since the C-termini of Mbp1 and Swi4 share high level of sequence identity and similarity (Primig, Sockanathan et al. 1992; Koch, Moll et al. 1993). Taken into consideration, there could also be two additional technical factors that contribute to the high level of false positive (33 of the identified 90 Swi6 clones contain a stop codon that prevent the expression of the OyCD fragment 1) obtained after the two-step selection process. First, the cytosine survival selection assay of OyCD PCA can give rise to false positives since the uracil

that is produced in cells expressing a pair of interacting proteins fused to the OyCD fragments can leak out in to the selection medium allowing neighboring cells to grow even though they do not carry interacting proteins fused to the OyCD fragments (Griffith and Jarvis 1996). In order to overcome this technical problem, less cells should be plated on the selection medium and plates should not be allow to be incubated for more than three to four days. This way, we insure that the colonies that we identified contain only cells expressing interacting proteins fused to the OyCD fragments. Second, the Swi6 mutant and Mbp1 were co-expressed from two plasmids that carry the same antibiotic resistance gene (beta-lactamase gene). Since we extracted the total DNA and transformed it in bacteria in order to retrieve the plasmids carrying the Swi6 mutant gene, there is always the possibility of recovering plasmids carrying Mbp1 fusion gene despite our effort to remove the later plasmids by digestion with specific restriction enzymes. As a solution to this problem, a plasmid carrying the kanamycin resistance gene has been generated and used in other engineering projects and we observed significant improvement.

5.4.2 Engineering specific binders against proteins and post-translationally modified sequences.

OyCD PCA could be applied to screen for artificial binders to target any protein or protein state of interest. In this scenario libraries of for example SH2 domains, fibronectin domains or other binding domains could be screened against specific baits *in viv*o (Koide, Gilbreth et al. 2007; Kaneko, Sidhu et al. 2011). A unique feature of the OyCD PCA is that since *S. cerevisiae* possesses all the machinery for all post-translational modification found in higher eukaryote, it can be used to screen for specific binders against post-translationally modified protein such as phosphorylated, methylated, or acetylated residues. For example, tudor domains only interact with peptides containing methylated arginine or lysine residues (Chen, Nott et al. 2011). These binders could be expressed from plasmids and used to

modulate and study signal transduction pathways by disrupting key post-translational events. The above example is only meant to illustrate the concept of developing a specific probe for methylated arginine residues. Protein methylation and protein acetylation are poorly characterized and there is only a limited set of reagents available for detecting methylated and acetylated proteins. It would therefore be interesting to apply OyCD PCA to identify binders that are specific to methylated and acetylated proteins.

5.4.3 Beyond the survival and death selection assays of OyCD PCA

The activity of yeast cytosine deaminase was previously detected in mice using a non-invasive method based on magnetic resonance spectroscopy where fluorine 19 (^{19}F)-labelled 5-FC was used as substrate (Stegman, Rehemtulla et al. 1999). This study demonstrates the feasibility of using the yCD reporter protein in live animal. It is therefore possible to use the OyCD PCA to detect protein-protein interaction an animal model and study how certain drugs affect signaling pathways.

The OyCD PCA is the fruit of this thesis. Although we have chosen to use this tool for dissecting cell cycle protein complexes, there is no doubt a list of many other applications for this assay. Perhaps further optimization of this strategy will open even more opportunities for it in the near future.

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