DBF4 COORDINATES DNA REPLICATION, CHROMOSOME SEGREGATION, AND CHECKPOINT SIGNAL TRANSDUCTION

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ABSTRACT

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Faithful transmission of genetic information not only requires accuracy in DNA synthesis and chromosome segregation, but also surveillance mechanisms that respond to various stresses in order to coordinate repair and cell-cycle progression. Recent evidence suggests that the Dbf4-dependent Cdc7 kinase (DDK), a two-subunit kinase essential for eukaryotic DNA replication, plays such a role in genomic maintenance. In this study, we demonstrated that Dbf4 inhibits Cdc5 (yeast Polo-like kinase) to prevent premature exit from mitosis. It also regulates late origin firing during replication stress by a direct interaction with the Rad53 checkpoint kinase (the ortholog of mammalian Chk2). Dbf4 is able to simultaneously associate with Cdc7, Cdc5, and Rad53, suggesting that Dbf4 serves as a molecular scaffold to mediate DNA replication, chromosome distribution, and checkpoint responses. We further performed a genome-wide synthetic lethal screen using a *dbf4* mutant, which is defective in binding both Cdc5 and Rad53, to explore the biological relevance of these physical interactions. We globally mapped the genetic interactions of *DBF4* and defined the functional categories for these interactions. These data provide insights into the role of Dbf4 in the convergence of checkpoint signaling and mitotic regulation and prompt us to re-evaluate the role of Dbf4 in cell-cycle regulation.

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KEY TO ABBREVIATIONS

3AT	3 aminotriazole
9-1-1	Rad9-Rad1-Hus1
APC	Anaphase Promoting Complex
ATM	Ataxia Telangiectasia Mutated
ATR	ATM and Rad3 related
BRCT	BRCA1 C-Terminal domain
CDC	Cell Division Cycle
CDK	Cyclin Dependent Kinase
CTF	Chromosome Transmission Fidelity
C-terminal	Carboxy-terminal
Δ	Deletion
DDK	Dbf4-Dependent Kinase
DSB	Double Strand Break
dsDNA	Double Stranded DNA
FEAR	Fourteen Early Anaphase Release
FHA	Fork-Head Associated
GSA	Genetic Synthetic Array
GST	Glutathione-S-transferase
HA	HemAgglutinin
HU	HydroxyUrea

IP	ImmunoPrecipitation
MCM	MiniChromosome Maintenance
MEN	Mitotic Exit Network
MMS	Methyl Methane Sulfonate
N-terminal	Amino-terminal
ORC	Origin Recognition Complex
PAGE	Poly Acrylamide Gel Electrophoresis
PBD	Polo-Box Domain
PCNA	Proliferating Cell Nuclear Antigen
Plks	Polo-like kinases
pre-RC	pre-Replicative Complex
RFC	Replication Factor C
RPA	Replication Protein A
Scm	Synthetic complete medium
Ser	Serine
SPoC	Spindle Position Checkpoint
ssDNA	single stranded DNA
Thr	Threonine
ts	Temperature Sensitive
WT	Wild Type
YPD	Yeast extract Peptone Dextrose

CHAPTER 1

BACKGROUND, RATIONALE, AND PURPOSE

BACKGROUND, RATIONALE, AND PURPOSE

ABSTRACT

The Dbf4-dependent Cdc7 kinase (also known as DDK) is a two-subunit serine/threonine kinase that is essential for the initiation of DNA replication in eukaryotes. The Cdc7 kinase is activated by interacting with the regulatory subunit Dbf4. The expression of Dbf4 oscillates during the cell cycle, analogous to cyclins that activate CDKs (Cyclin-dependent kinases) in a cell-cycle dependent manner. The most characterized function of DDK is to trigger the DNA helicase activity of the Mcm2-7 (Minichromosome maintenance 2-7) complex during S phase. In recent years, accumulating evidence suggests that the Dbf4-dependent Cdc7 kinase not only functions as a DNA replication initiator, but also participates in the regulation of chromosome segregation and the maintenance of genomic integrity. The objective of this study is to determine a novel function of Dbf4 in cell-cycle regulation.

INTRODUCTION

Cdc7 and Dbf4 are essential for the initiation of DNA replication

Chromosome replication in eukaryotic cells is under sophisticated regulation at multiple origins that are distributed throughout the genome (Mechali 2010). In the budding yeast *Saccharomyces cerevisiae*, the heterohexameric Mcm2-7 DNA helicase, part of the pre-replicative complex (pre-RC), is loaded at origins in an inactive form during G1 phase (Bochman and Schwacha 2009). Activation of Mcm2-7 at the onset of S phase depends on both the CDK and Cdc7 kinases. Following the recruitment of other replisome

components, active Mcm2-7 complexes unwind the DNA duplex at replication forks to allow for the bidirectional initiation of DNA synthesis.

The *CDC7* gene was originally identified in Hartwell's "cell division cycle" mutagenesis screens in the budding yeast *Saccharomyces cerevisiae* (Culotti and Hartwell 1971), and the *dbf4* mutant was characterized as another mutant defective in DNA synthesis which formed a large budded cell called "dumbbell former" morphology at the restrictive temperature (Johnston and Thomas 1982). Later genetic studies showed that the temperature sensitive phenotype of *cdc7* mutants was suppressed by overexpression of *DBF4* in high copy-number plasmids (Kitada et al. 1992). *CDC7* was subsequently found to encode a serine/threonine protein kinase (Hollingsworth and Sclafani 1990), and its kinase activity relied on the presence of Dbf4 during the cell cycle (Johnston and Thomas 1982; Patterson et al. 1986; Yoon and Campbell 1991; Yoon et al. 1993; Oshiro et al. 1999). Studies with budding yeast also showed that both Cdc7 and Dbf4 are needed for the firing of origins throughout S phase (Bousset and Diffley 1998; Donaldson et al. 1998; Tanaka and Nasmyth 1998). Together, these observations suggest that Cdc7 and Dbf4 act in concert to trigger DNA synthesis. In the past decade, it has become clear that Dbf4 directly interacts with and activates Cdc7 to regulate the activation of replication origins; thus Cdc7 kinase is also referred to as Dbf4-dependent kinase (DDK) (Johnston et al. 1999; Sclafani 2000).

Mcm2-7 helicase is the physiological substrate of DDK

Both *DBF4* and *CDC7* are essential for viability, but the *mcm5-bob1* mutant, which likely mimics the conformational change and activation of Mcm2-7 helicase, is able to suppress the lethality of *cdc7*Δ or *dbf4*Δ (Hardy et al. 1997; Sclafani 2000; Hoang et al. 2007). This suggests that the Mcm2-7 helicase is the only essential target of DDK. Consistently, *DBF4* has been identified as a suppressor of the *mcm2-1* mutant in an allele-specific manner (Lei et al. 1997) and physically interacts with Mcm2 (Varrin et al. 2005), one of the subunits in the Mcm2-7 hexamer. Furthermore, five of six conserved Mcm subunits (Mcm2, Mcm3, Mcm4, Mcm6, and Mcm7) were found to be the targets of DDK kinase *in vitro* and *in vivo* (Lei et al. 1997; Weinreich and Stillman 1999; Masai et al. 2006; Chuang et al. 2009). However, the biological relevance of DDK-catalyzed Mcm phosphorylation is not completely understood. Based on the studies on the phosphorylation of Mcm4 by DDK (Sheu and Stillman 2006; Sheu and Stillman 2010), it is thought that DDK directly phosphorylates multiple sites on the Mcm2-7 complex and then creates binding sites for other replisome factors, including Cdc45 and the GINS (Sld5-Psf1-Psf2-Psf3, GINS refers to “5-1-2-3” from Japanese “Go-Ichi-Ni-San”) complex. The association of Cdc45 and GINS may drive a structural change in the Mcm2-7 complex and activate its helicase activity (Owens et al. 1997; Weinreich and Stillman 1999; Zou and Stillman 2000; Gambus et al. 2006; Masai et al. 2006; Moyer et al. 2006; Yabuuchi et al. 2006; Francis et al. 2009).

DDK also functions beyond S phase

The kinase activity of Cdc7 is tightly regulated by the cyclical expression of Dbf4 (Jackson et al. 1993; Johnston et al. 1999). In contrast, Cdc7 protein levels remain constant during the cell cycle. Dbf4 expression is low in G1 phase and reaches a peak at the G1-S transition and throughout the S phase. Notably, Dbf4 is continuously present until late mitosis (Cheng et al. 1999; Weinreich and Stillman 1999; Ferreira et al. 2000; Wu and Lee 2002). Since Cdc7 function is correlated with the presence of Dbf4, this suggests that DDK has additional roles in post-replicative cell cycle regulation.

Previous studies have shown that Dbf4 stability during the cell cycle is regulated by APC/C (Anaphase-promoting complex or cyclosome) (Cheng et al. 1999; Weinreich and Stillman 1999; Ferreira et al. 2000), which is a large multisubunit E3 ubiquitin ligase that triggers 26S proteasome-mediated degradation of mitotic regulators (Sullivan and Morgan 2007). In particular, APC/C binds to its substrates by recognizing D-box (Destruction-box) and KEN-box sequences through its adapter protein, Cdc20 or Cdh1 (Cdc20-homologue 1) (Pfleger and Kirschner 2000; Pesin and Orr-Weaver 2008). Dbf4 contains two D-boxes in its N-terminus for both Cdc20 and Cdh1 binding (Weinreich and Stillman 1999; Ferreira et al. 2000; Sullivan et al. 2008; Miller et al. 2009; Chen and Weinreich 2010), and it also contains two putative KEN-boxes in the C-terminus that are specifically recognized by Cdh1. It is known that APC/C-Cdc20 is activated at the metaphase/anaphase transition and subsequently targets Dbf4 for degradation. However, Dbf4 was also found in the later mitotic stages, such as during mitotic exit (Sullivan et al. 2008; Miller et al. 2009; Chen and Weinreich 2010), where APC/C-Cdh1

plays a dominant role in the regulation of proteolysis. These observations suggest that Dbf4 protein persists late into mitosis and is regulated by both Cdc20 and Cdh1. The regulation of Dbf4 stability could provide insight into the role of Dbf4 in late mitosis.

The conserved Dbf4 motif-N is not required for DNA replication

A human homolog of Dbf4, called *ASK* (activator of S-phase kinase), was identified by its association with human Cdc7 in yeast two-hybrid screens (Jiang et al. 1999; Kumagai et al. 1999). A second *DBF4*-like gene was found in the mammalian genome called *DRF1* (Dbf4-related factor 1) /*DBF4B*/*ASKL1* (ASK-like protein 1) (Yoshizawa-Sugata et al. 2005), but the *DRF1* homolog was not found in *S. cerevisiae* or *S. pombe.* The Cdc7 kinase and the regulatory subunit of Dbf4 are both evolutionarily conserved, but they show different degrees of sequence conservation from yeast to humans (Johnston et al. 2000). All Cdc7 homologs share well-conserved residues for serine/threonine kinase activity, but much less conservation is observed in its binding partner Dbf4, which contains no catalytic domain. Sequence alignment among *S. cerevisiae* Dbf4, *S. pombe* Dbf4/dfp1+, and human ASK shows three short conserved motifs, termed motif-N (N-terminal), -M (Middle), and -C (C-terminal) (Masai and Arai 2000). The most conserved motif-M and zinc-finger motif-C of Dbf4/Dfp1 are sufficient to bind and activate the Cdc7/Hsk (in *S. pombe*) kinases in yeasts and thus are essential for viability (Ogino et al. 2001; Harkins et al. 2009; Jones et al. 2010). In contrast, motif-N is dispensable for viability, but could be involved in targeting DDK to stalled replication forks during replication stress (Ogino et al. 2001; Duncker et al. 2002; Gabrielse et al. 2006). Deletion of the Dbf4 N-terminus causes increased sensitivity to

DNA-damaging agents such as hydroxyurea (HU), ultraviolet (UV) light, and methyl methanesulfonate (MMS). These observations suggest that the Dbf4 N-terminus plays a role in cellular response to environmental stresses.

The Dbf4 N-terminus has been reported to contain a BRCT (*BRCA1* C-terminal)-like domain, which generally forms tandem repeats and functions as a phospho-peptide binding module in signal transduction (Gabrielse et al. 2006; Mohammad and Yaffe 2009; Matthews et al. 2012). The BRCT domain is often found in proteins involved in checkpoint response and DNA repair. It was been shown that the BRCT domain of Dbf4 interacts with the Rad53 checkpoint kinase, the ortholog of the human tumor suppressor Chk2 (Checkpoint kinase 2) (Duncker et al. 2002; Chen et al. 2012; Matthews et al. 2012). During replication stress, Rad53 activation coincides with the hyperphosphorylation of Dbf4 and subsequently attenuates DDK kinase activity at late replication origins (Gabrielse et al. 2006; Yabuuchi et al. 2006; Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010; Chen et al. 2012). An attractive model is that the Dbf4 N-terminus is a direct target of the Rad53 kinase during S-phase checkpoint signaling and that the phosphorylated Dbf4 prevents the replicative function of Cdc7, allowing DNA repair to take place before further S phase progression.

Dbf4 N-terminus interacts with the mitotic Polo-like kinase Cdc5

Earlier reports have shown that Dbf4 N-terminus physically associates with the Cdc5 kinase (Hardy and Pautz 1996; Miller et al. 2009; Chen and Weinreich 2010), a homolog of mammalian Polo-like kinases (Plks). The *Polo* gene was named for a

Drosophila melanogaster mutant that exhibited aberrant mitotic spindle and spindle pole bodies (equivalent to the centrosomes in higher eukaryotes), implying that Polo had a crucial role in chromosome segregation (Sunkel and Glover 1988; Barr et al. 2004; Archambault and Glover 2009). Polo kinases are now known to form a large protein family, and they regulate centrosome maturation and duplication, mitotic entry, chromosome segregation, spindle dynamics, and mitotic exit (Lee et al. 2005). Budding yeast (*CDC5*), fission yeast (*plo1+*), and *Drosophila* (*Polo*) each have a single *Polo* ortholog, but mammalian cells have four Polo-like kinases (*Plk1, Plk2, Plk3,* and *Plk4/SAK*) (Dai 2005). The function of Cdc5 closely resembles the human Plk1 (Lee and Erikson 1997; Ouyang et al. 1997), which is frequently associated with malignant types of cancers (Eckerdt et al. 2005; Takai et al. 2005). Different molecular compounds are being developed to inhibit Plk1 kinase activity and its non-catalytic substrate binding domain (Strebhardt and Ullrich 2006; de Carcer et al. 2007; Reindl et al. 2008; Watanabe et al. 2009).

Like the other Polo family members, Cdc5 plays multiple essential roles in mitosis and meiosis. Cdc5 is required for the phosphorylation of Swe1 (*Saccharomyces* Wee1), which is a negative regulator of S-phase CDKs at the G2/M transition (Bartholomew et al. 2001; van Vugt and Medema 2004; Asano et al. 2005). A deficiency in Cdc5-mediated Swe1 phosphorylation delays mitotic entry. Similarly, Cdc5-mediated phosphorylation of cohesins (Mcd1/Scc1; Mitotic chromosome determinant) is crucial in triggering the onset of the metaphase to anaphase transition (Alexandru et al. 2001; Hornig and Uhlmann 2004). In later stages of mitosis, Cdc5 regulates the FEAR (Cdc

fourteen early anaphase release) network and MEN (Mitotic exit network) to facilitate the activation of Cdc14 (Lee et al. 2001; Shou et al. 2002; Stegmeier et al. 2002; Visintin et al. 2003; Rahal and Amon 2008), which is an essential phosphatase that antagonizes mitotic CDK functions during mitotic exit (Stegmeier and Amon 2004). Cdc5 is also implicated in signal transduction during cytokinesis (Valentin et al. 2006; Katis et al. 2010). Intriguingly, recent studies demonstrated that DDK controls meiosis-specific transcription (Lo et al. 2008; Lo et al. 2012), the separase-mediated cleavage of Rec8 (a meiotic cohesin component) (Valentin et al. 2006; Katis et al. 2010), and the disjunction of homologous chromosomes at meiosis I in budding yeast (Valentin et al. 2006; Matos et al. 2008; Wan et al. 2008), suggesting that DDK has additional roles in orchestrating meiotic events. Therefore, the physical interaction between Dbf4 and Cdc5 raises interesting questions regarding whether Dbf4 and Cdc7 participate in mitotic or meiotic events through the interaction with Cdc5.

THESIS OVERVIEW

Saccharomyces cerevisiae DBF4 encodes an essential regulator for the S-phase kinase Cdc7. While the function of Dbf4 in DNA replication has been studied over the past decade, the role of Dbf4 in post-replicative cell-cycle regulation is less understood, and will be the focus of this study. Genetic and biochemical observations suggest that the non-essential N-terminus of Dbf4 interacts with the mitotic Polo-like kinase Cdc5 and checkpoint kinase Rad53. We hypothesized that Dbf4 participates in the post-replicative cell-cycle regulation through direct interactions with Cdc5 and Rad53, and aimed to

identify the molecular mechanism of Dbf4 function in mitotic regulation and checkpoint signal transduction.

In Chapter 2 and 3, we characterized two distinct motifs within the Dbf4 N-terminus that physically interact with Cdc5 and Rad53, separately. By examining the *dbf4* mutants that are unable to interact with Cdc5 or Rad53, we elucidated the novel roles of Dbf4 in the mitotic exit network and the regulation of origin firing during replication stress. Furthermore, we found that Dbf4 simultaneously forms a stable complex with Cdc7, Cdc5 and Rad53 kinases, suggesting that Dbf4 functions as a scaffold that coordinates DNA replication, chromosome segregation and checkpoint signaling during cell cycle. In Chapter 4, we performed a genome-wide synthetic lethal screen by using a *dbf4* mutant, which is defective in binding both Rad53 and Cdc5, to further explore the biological relevance of physical interactions. We globally mapped the genetic interactions of *DBF4* and defined the functional categories for these interactions, including the maintenance of genomic stability, DNA damage or checkpoint signaling, and chromosome segregation. These data suggest that Dbf4, Cdc5, and Rad53 operate in parallel pathways to repair damaged or stalled forks and also to block inappropriate mitotic progression in response to damaged or partially replicated chromosomes.

In Chapter 5, we review recent studies on the molecular basis of Dbf4-Cdc5 and Dbf4-Rad53 interactions and also discuss the potential role of ternary complex (Dbf4-Cdc7-Cdc5-Rad53) outside the S phase. Taken together with the data of synthetic lethal screen, our results contribute to a comprehensive understanding of Dbf4 function in cell-

cycle regulation and we propose a model in which Dbf4 acts as a multifaceted cell-cycle regulator.

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CHAPTER 2

PART A:

CDC7-DBF4 REGULATES MITOTIC EXIT BY INHIBITING POLO KINASE

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Part A was published by *PLoS Genetics, 5(5):e1000498 (2009).*

Ying-Chou Chen identified that the Dbf4 residues 82-88 are necessary for the interaction with Cdc5 and also demonstrated that the Dbf4-mediated Cdc5 inhibition depends on the association of Cdc7. These discovery contributed to Figure 1A, 1B, 3A, 4A, 4B, and 4C in this paper.

PART B:

DBF4 REGULATES THE CDC5 POLO-LIKE KINASE THROUGH A DISTINCT NON-CANONICAL BINDING INTERACTION

Ying-Chou Chen and Michael Weinreich

Part B was published by *Journal of Biological Chemistry,*

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PART A: CDC7-DBF4 REGULATES MITOTIC EXIT BY INHIBITING POLO KINASE

ABSTRACT

Cdc7-Dbf4 is a conserved protein kinase required for the initiation of DNA replication. The Dbf4 regulatory subunit binds Cdc7 and is essential for Cdc7 kinase activation, however the N-terminal third of Dbf4 is dispensable for its essential replication activities. Here we define a short N-terminal Dbf4 region that targets Cdc7-Dbf4 kinase to Cdc5, the single Polo kinase in budding yeast that regulates mitotic progression and cytokinesis. Dbf4 mediates an interaction with the Polo substrate-binding domain to inhibit its essential role during mitosis. Although Dbf4 does not inhibit Polo kinase activity it nonetheless inhibits Polo-mediated activation of the mitotic exit network (MEN), presumably by altering Polo substrate targeting. In addition, although *dbf4* mutants defective for interaction with Polo transit S-phase normally, they aberrantly segregate chromosomes following nuclear misorientation. Therefore, Cdc7-Dbf4 prevents inappropriate exit from mitosis by inhibiting Polo kinase and functions in the spindle position checkpoint.

PART B: DBF4 REGULATES THE CDC5 POLO-LIKE KINASE THROUGH A DISTINCT NON- CANONICAL BINDING INTERACTION

ABSTRACT

Cdc7-Dbf4 is a conserved, two-subunit kinase required for initiating eukaryotic DNA replication. Recent studies have shown that Cdc7-Dbf4 also regulates the mitotic exit network (MEN) and monopolar homolog orientation in meiosis I. Both activities likely involve a Cdc7-Dbf4 interaction with Cdc5, the single Polo-like kinase in budding yeast. We previously showed that Dbf4 binds the Cdc5 polo-box domain (PBD) via a ~40 residue N-terminal sequence, which lacks a PBD consensus binding site (S(pS/pT)P/X), and that Dbf4 inhibits Cdc5 function during mitosis. Here we identify a non-consensus PBD binding site within Dbf4 and demonstrate that the PBD-Dbf4 interaction occurs via a distinct PBD surface from that used to bind phospho-proteins. Genetic and biochemical analysis of multiple *dbf4* mutants indicate that Dbf4 inhibits Cdc5 function through direct binding. Surprisingly, mutation of invariant Cdc5 residues required for binding phosphorylated substrates has little effect on yeast viability or growth rate. Instead, *cdc5* mutants defective for binding phospho-proteins exhibit enhanced resistance to microtubule disruption and an increased rate of spindle elongation. This study therefore details the molecular nature of a new type of PBD binding and reveals that Cdc5 targeting to phosphorylated substrates likely regulates spindle dynamics.

INTRODUCTION

Cell cycle progression requires the highly accurate replication and segregation of chromosomes. Although these two events occur at different times, several cell cycle kinases regulate both DNA synthesis and chromosome segregation (Blow and Tanaka 2005). In budding yeast, the Cdc7-Dbf4 kinase (also called Dbf4-dependent kinase or DDK) plays such a dual role in the cell cycle. The Dbf4 regulatory subunit binds to and activates Cdc7 kinase to initiate DNA replication (Johnston et al. 1999; Jares et al. 2000). DDK also promotes other aspects of chromosome biology including cohesin loading during early S-phase in *X. laevis* (Takahashi et al. 2008), centromeric cohesion in *S. pombe* (Takahashi et al. 2008), and meiotic recombination (Sasanuma et al. 2008; Wan et al. 2008) and the Ndt80 (early meiotic) transcriptional program in *S. cerevisiae* (Lo et al. 2008). Budding yeast DDK also promotes monopolar orientation of homologs in meiosis I and inhibits chromosome segregation in the mitotic cycle (Matos et al. 2008; Sullivan et al. 2008; Marston 2009; Miller et al. 2009). Both activities are likely mediated through an interaction with Cdc5, the single Polo-like kinase in *Saccharomyces cerevisiae*. Polo-like kinases (Plks) regulate mitotic events and are also involved in the response to DNA damage and checkpoint adaptation (Lee et al. 2005; Petronczki et al. 2008; Trenz et al. 2008). Genetic and physical interactions between Dbf4 and Cdc5 were described many years ago (Kitada et al. 1993; Hardy and Pautz 1996) raising the possibility that DDK acted beyond S phase. The DDK-Cdc5 interaction raises interesting questions regarding how these distinct kinases interact and coordinate accurate cell cycle progression.

The *Polo* gene was named for a *Drosophila melanogaster* mutant that exhibited abnormal spindle pole behavior (Sunkel and Glover 1988), implying that Polo had a critical role in mitotic organization. Polo kinases are now known to comprise a large protein family that regulate centrosome maturation and duplication, mitotic entry, chromosome segregation, spindle dynamics, and mitotic exit (Archambault and Glover 2009). Budding yeast, fission yeast and *Drosophila* each have a single Polo ortholog but there are four Polo-like kinases (Plk1-4) in mammalian cells (Archambault and Glover 2009). Consistent with Polo's diverse functions, individual Plks show different and sometimes dynamic subcellular localization (Barr et al. 2004). Polo kinases share a two-domain structure consisting of an N-terminal kinase domain and a C-terminal substrate-binding domain. A unique C-terminal Polo-box domain (PBD) comprised of one or two polo-box (PB) motifs was found in all Polo family members by multiple sequence alignment (Lowery et al. 2005), and is required for Plk subcellular localization and substrate targeting (Lee et al. 1998; Seong et al. 2002; Lee et al. 2005). The PBD is one of many domains that bind phosphorylated substrates (Yaffe and Smerdon 2004). The interaction between an optimal phospho-threonine peptide and the PBD of Plk1 has been defined by structural and mutational studies (Cheng et al. 2003; Elia et al. 2003b). The polo-box domains of Plk1-3 orthologs are constituted from two highly conserved polo-box sequences, called PB1 and PB2, together with a polo cap (Pc) region that stabilizes the folded domain. Over 600 Plk substrates were suggested in proteomic study using the phosphorylation-recognition feature of the PBD (Lowery et al. 2007) suggesting that Plks regulate many substrates. Since Plk1 overexpression occurs in human tumors, Polo kinases are attractive targets for cancer therapy (Strebhardt and

Ullrich 2006). In fact, different molecular approaches are being developed to inhibit both Plk1 kinase activity and its noncatalytic substrate-binding domain (Strebhardt and Ullrich 2006; de Carcer et al. 2007; Reindl et al. 2008; Watanabe et al. 2009).

The *CDC5* gene was first described in a cell division cycle mutant screen by Hartwell and colleagues through the isolation of a single *cdc5-1* temperature sensitive allele (Hartwell et al. 1970). Like the other Polo family members, Cdc5 has multiple roles in mitosis and cytokinesis (Lee et al. 2005). Human Plk1 can complement the growth defect of the yeast *cdc5-1* mutant, which provided further evidence that Polo functional interactions were conserved during evolution (Lee and Erikson 1997; Ouyang et al. 1997). Despite a broad spectrum of potential Cdc5 substrates, only a few PBD-binding interactions have been characterized in detail (Geymonat et al. 2003; Hornig and Uhlmann 2004; Lowery et al. 2004; Asano et al. 2005; Snead et al. 2007; Crasta et al. 2008). We recently performed a two-hybrid screen using the Dbf4 N-terminus and defined a Dbf4 interaction with the Cdc5 PBD (Miller et al. 2009). We further found that Dbf4 residues 66-109 were necessary and sufficient for this interaction. However, this Dbf4 region did not contain a recognizable PBD consensus binding sequence, i.e. Ser-pSer/pThr-Pro/X ("p" denotes phosphorylation and "X" indicates any amino acid), and mediated an interaction with the PBD without a requirement for phosphorylation. Similarly, Glover and colleagues reported that the PBD of *Drosophila* Polo mediates an interaction with Map205 (a microtubule-associated protein) that occurs in the absence of Map205 phosphorylation (Archambault et al. 2008).

Here we systematically map Dbf4 residues required for binding the PBD using genetic and direct peptide binding assays. Although targeted deletion of Dbf4 residues 83-88 or 89-93 completely abrogates Dbf4-Cdc5 binding *in vivo*, only residues 83-88 are critical for a direct PBD interaction and comprise the core of a new type of PBD binding sequence. Furthermore, the PBD interacts with Dbf4 independently of residues that mediate its interaction with phosphorylated proteins using a distinct molecular surface. Surprisingly, highly conserved Cdc5 residues (W517, H641, K643) in the PBD, required for binding proteins with an S(pS/pT)P/X consensus sequence, are not required for yeast viability or wild-type growth rates. This strongly suggests that Cdc5 binding to phosphorylated (primed) substrates is not essential in yeast. Instead, the *cdc5-HK* and *cdc5-WHK* mutants exhibit enhanced resistance to spindle poisons and display altered spindle dynamics. These data define an alternative mode for PBD-protein interactions, and raise the possibility that Cdc5 may bind essential mitotic substrates through a Dbf4-like consensus sequence.

RESULTS

Dbf4 residues 82-96 are required to interact with the Cdc5 PBD

We previously recovered multiple clones of the Cdc5 PBD in a two-hybrid screen using the Dbf4 N-terminus as bait and found that residues 66-109 are necessary and sufficient for a direct interaction with Cdc5 PBD (Miller et al. 2009). Since Dbf4 N-terminal residues 1-109 are dispensable for DNA replication (Gabrielse et al. 2006), the Dbf4 N-terminus interacts with Cdc5 to perform non-essential functions in budding yeast. To define the exact molecular basis of the Dbf4-Cdc5 interaction, we constructed a series of N-terminal Dbf4 deletion mutants and tested their ability to interact with the Cdc5 PBD using a two-hybrid assay. Deletions to residue 82 did not significantly affect the PBD two-hybrid interaction, however, N-terminal deletions extending beyond residue 82 lost the ability to interact with the PBD (Figure 1A, B). We then truncated the C-terminus and found that Dbf4 residues 66-96 were sufficient for PBD binding. Deletion of residues 82-88 (as shown previously (Miller et al. 2009)), 89-93, or 82-96 eliminated PBD binding (Figure 1A, B). This data indicated that sequences between residues 82 and 96 were essential for the Dbf4-Cdc5 interaction but did not define which residues directly contact the PBD.

Figure 1. Mapping the interaction between Dbf4 and the Cdc5 PBD

(A) N-terminal Dbf4 deletion mutants were tested for a two-hybrid interaction with the PBD. 10-fold serial dilutions of saturated cultures were spotted onto SCM-Trp-Leu plates to visualize total cells and SCM-Trp-Leu-His + 2 mM 3AT plates, to score the two-hybrid interaction. (B) Schematic of the features in Dbf4 N-terminus are shown, including two potential destruction-boxes (D-boxes), a conserved BRCT-like domain and motifs N, M and C, along with a summary of the Dbf4-PBD two-hybrid data. (C) Two-hybrid results for various point mutants spanning Dbf4 residues 82-96 are summarized. R83, I85, G87, and A88 are critical for PBD binding. (D) HA-Cdc7-Dbf4 complexes were immunoprecipitated from baculovirus-infected Sf9 cells and examined for co-immunoprecipitation of 3Myc-Cdc5. Cdc5 was co- immunoprecipitated by wild-type Dbf4 but not by Dbf4-Δ82-88 and Dbf4-NΔ109 mutant proteins.

Figure 1. (cont'd)

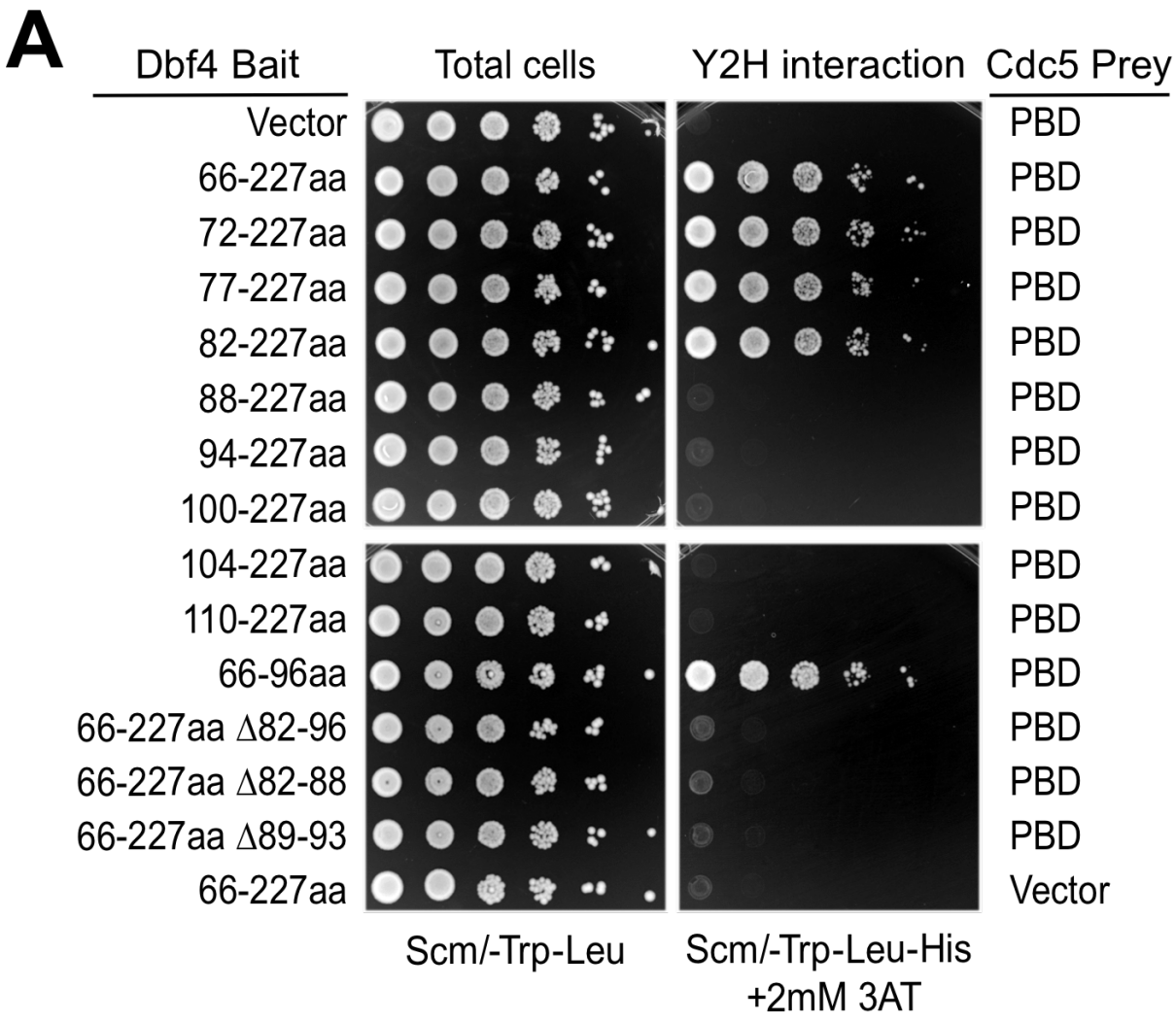


Figure 1. (cont'd)

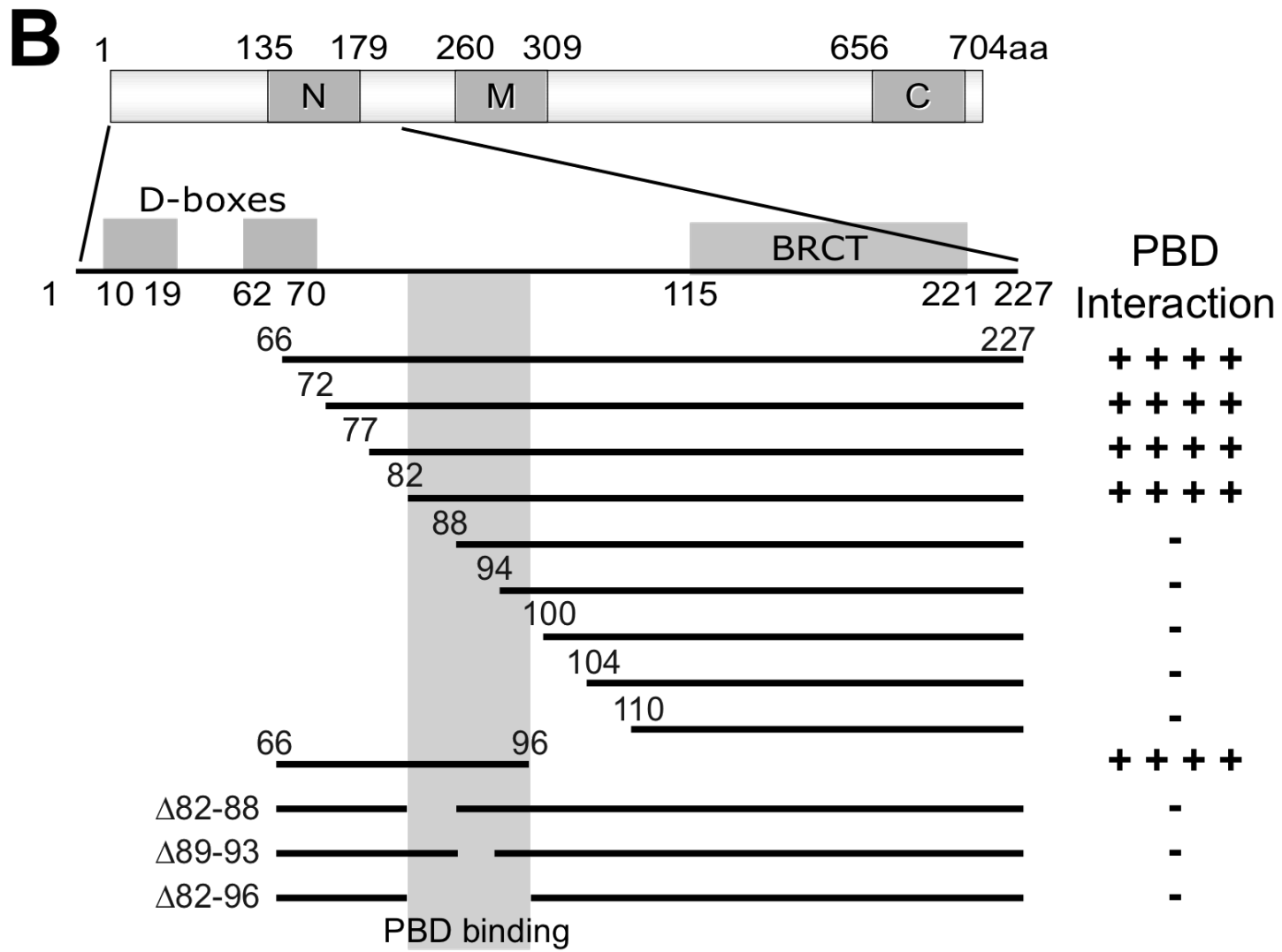


Figure 1. (cont'd)

C

	*		*		*	*									
82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	PBD interaction
A	**R**	**S**	**I**	**E**	**G**	**A**	**V**	**Q**	**V**	**S**	**K**	**G**	**T**	**G**	**+ + + +**
V															**+ + + +**
	A														**+ +**
	E														**-**
		A													**+ + + +**
		E													**+ + + +**
			A												**-**
				A											**+ + + +**
				K											**+ + + +**
					A										**-**
						V									**-**
							A	**A**	**A**						**+ +**
										A	**A**				**+ + + +**
												A	**A**	**A**	**+ + + +**
A	**R**	**S**	**I**	**E**	**G**	**A**	**V**	**Q**	**V**	**S**	**K**	**G**	**T**	**G**	**+ + + +**
82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	PBD interaction

Figure 1. (cont'd)

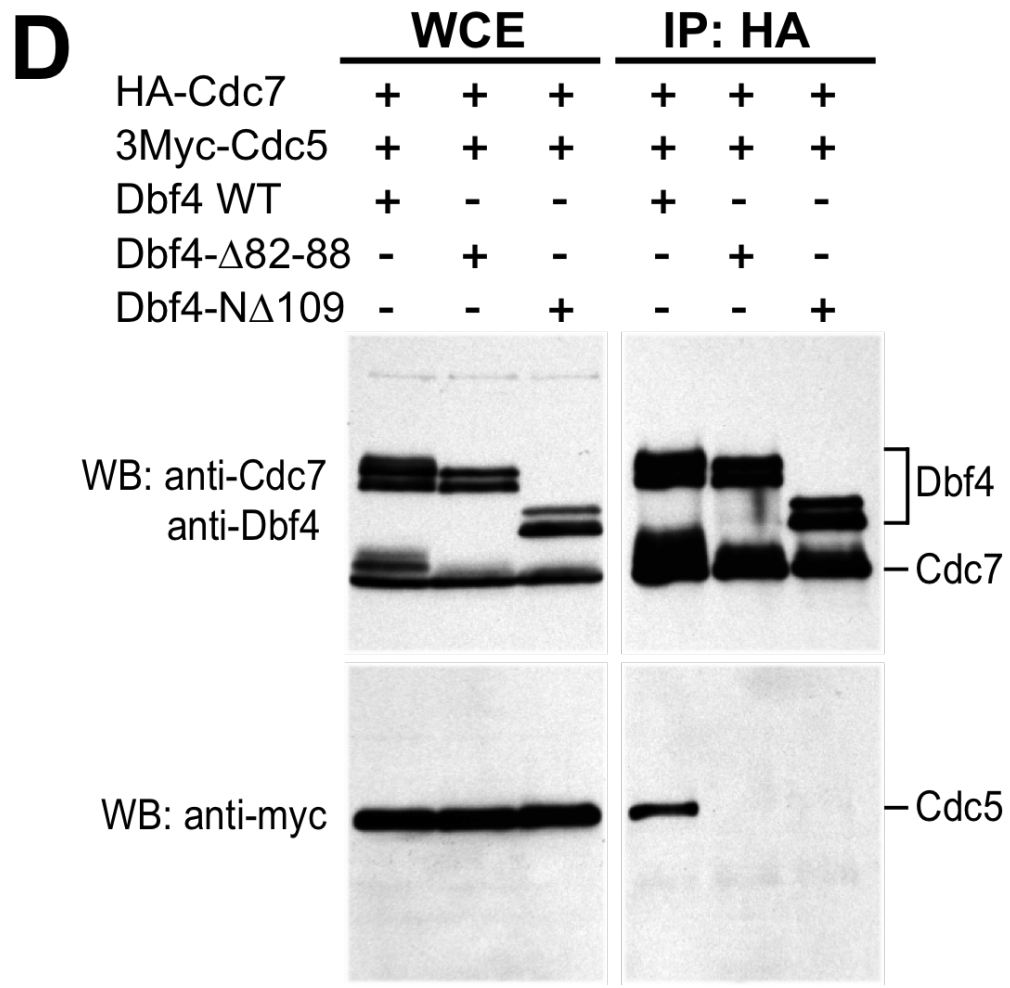
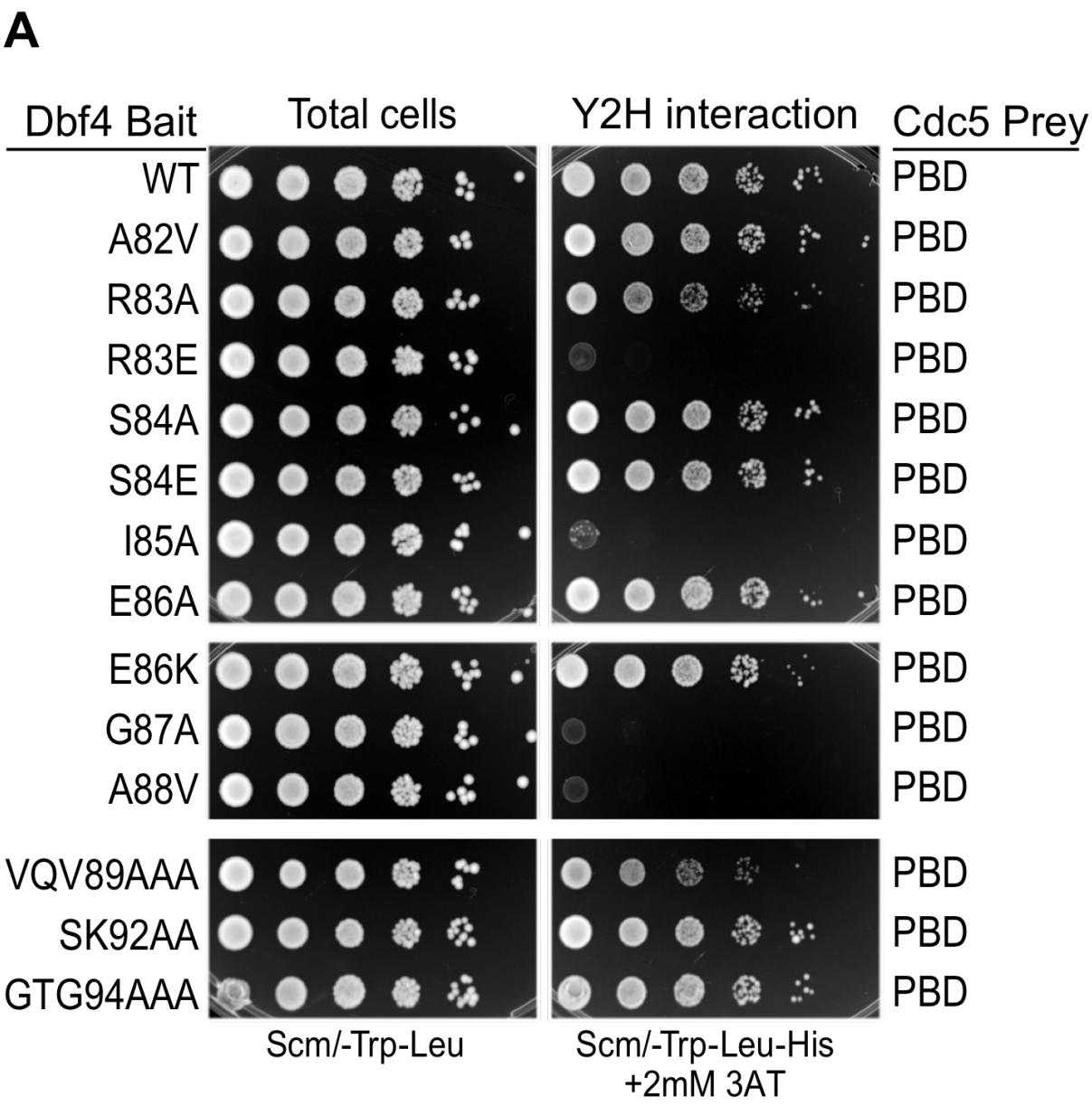


Figure 2. Analysis of Dbf4 residues required for interaction with the PBD

(A) The indicated Dbf4 (66-227) bait constructs were assayed for a two hybrid interaction with the Cdc5 PBD by spotting serial dilutions of cultures onto the indicated media to visualize the total number of cells (left) and the two hybrid interaction (right). (B) Although deletion of Dbf4 residues 89-93 abolishes the PBD interaction, deletion of residues 89-91 (ΔVQV) has only a modest effect on the PBD interaction and deletion of residues 92-93 (ΔSK) has no effect. This strongly suggests that deletion of residues 89-93 indirectly affects the Dbf4-PBD interaction. (C) The VQV89AAA triple point mutant has a similar effect on the PBD interaction as deletion of these same residues, as shown in panel B. However, the V89A, Q90A, and V91A single mutants have no effect on the PBD interaction.

Figure 2. (cont'd)



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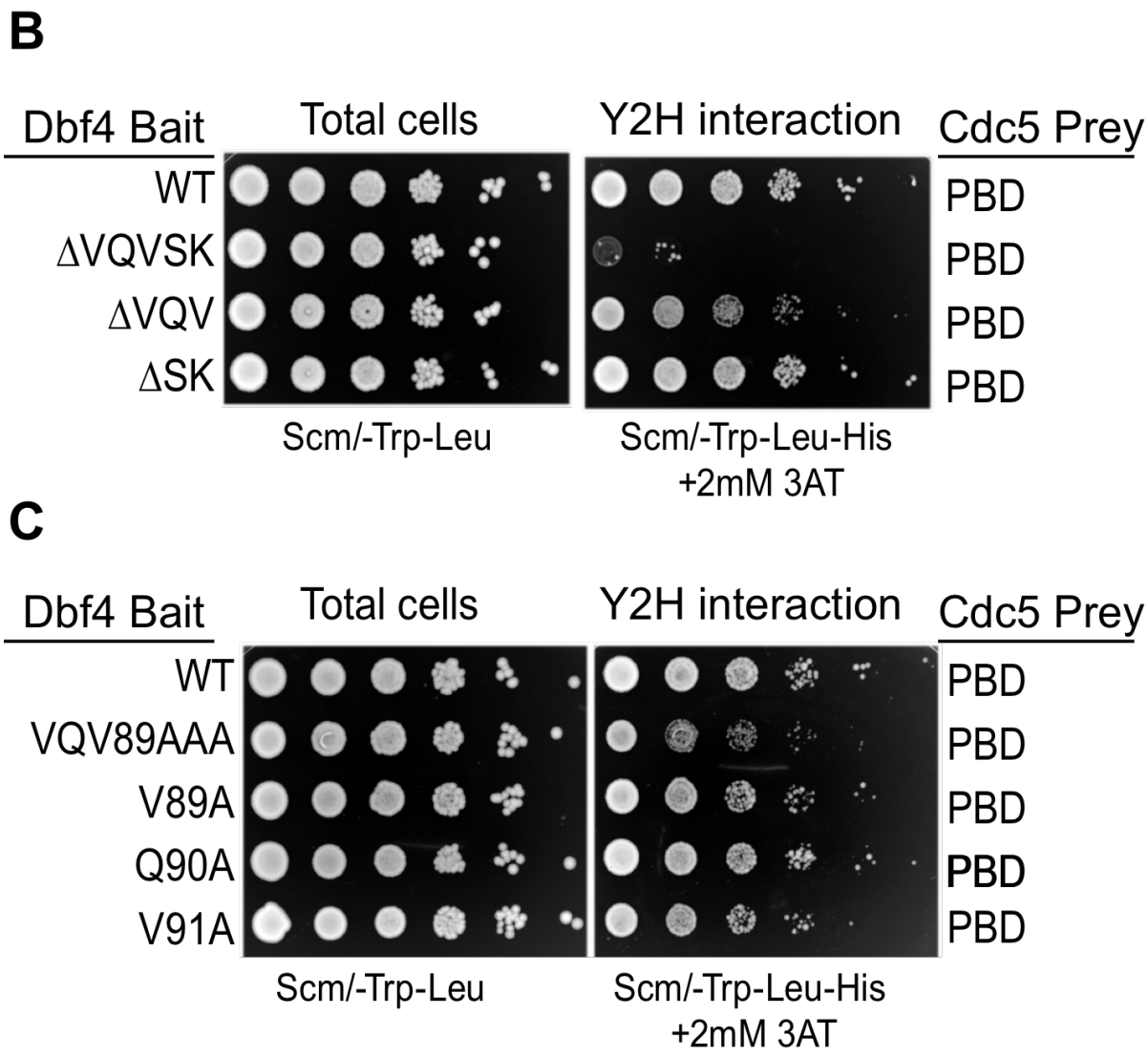
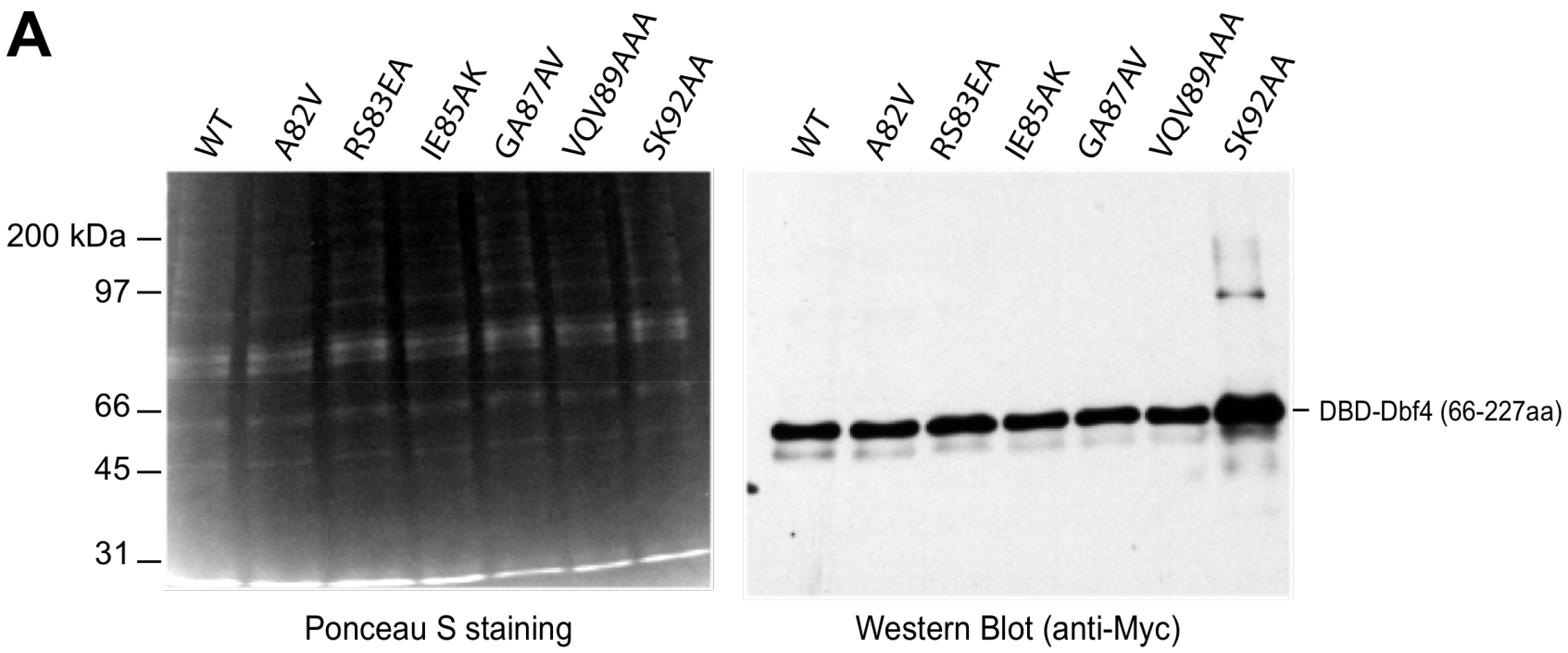


Figure 3. Protein expression of Dbf4 constructs used in two-hybrid and *cdc5-1* suppression assays

(A) The protein expression level of selected Gal4 DNA binding domain (DB) fusions to Dbf4(66-227) and representative point mutants spanning residues 82-93 were visualized by immunoblotting. Ponceau S staining (left) of whole cell extracts verified equal loading in each lane. Dbf4 bait constructs (DBD-Dbf4) contained a Myc tag and were detected using anti-Myc antibody (9E10). (B) Protein expression level of full length Dbf4 wild type and critical point mutants expressed in M2600 (*dbf4Δ::kanMX6 cdc5-1*).

Figure 3. (cont'd)



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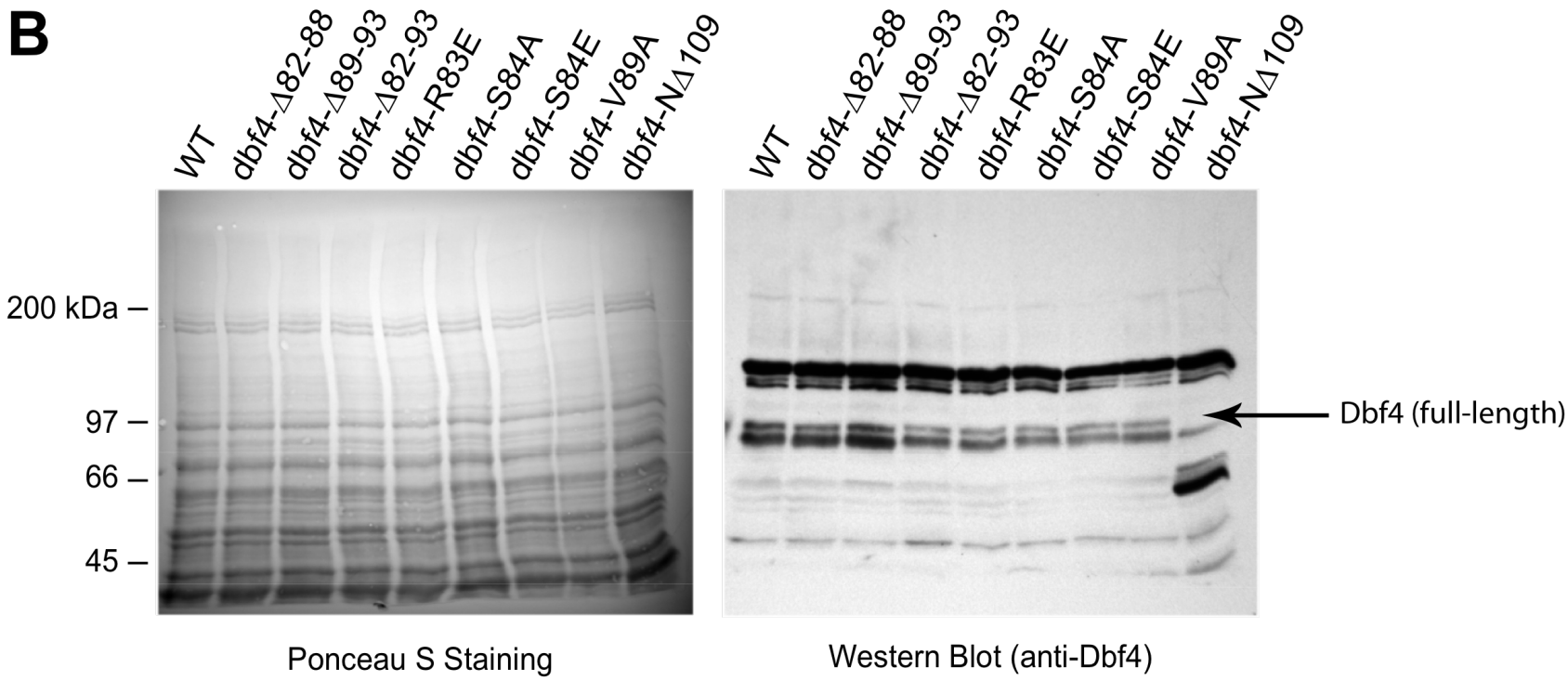


A novel binding motif for the Cdc5 PBD

Cdc5 is most closely related to the Plk1 family and its C-terminal PBD shares about 36% identity with the PBD of human Plk1 (Lee et al. 2005). Using an oriented peptide-library screen, the PBD of Cdc5 and Plk1 were found to preferentially bind Ser-pSer/pThr-Pro/X peptides (Elia et al. 2003b). For both Plk1 and Cdc5, the serine preceding the phosphorylated residue is absolutely required for PBD binding *in vitro* (Elia et al. 2003a; Elia et al. 2003b). The current model for Polo targeting suggests that a priming kinase, such as a cyclin-dependent kinase (Cdk) or MAP kinase, phosphorylates selected Ser/Thr residues on Polo substrates to create a high-affinity PBD recognition motif (Elia et al. 2003b; Barr et al. 2004). Plk1 also uses self-priming to create its own high-affinity binding site on PBIPB1 (Kang et al. 2006). However, several Cdc5 or Plk substrates apparently do not require the priming kinases for PBD binding (Garcia-Alvarez et al. 2007; Archambault et al. 2008; Rahal and Amon 2008) and Dbf4 residues 82-96 do not contain a match to the PBD consensus-binding site.

In order to determine individual Dbf4 residues required for PBD binding, we constructed a series of point mutants spanning residues 82-96 and quantitated the Dbf4 two-hybrid interaction with the PBD. Mutations of residues R83, I85, G87 and A88 completely abrogated the interaction with the PBD similar to deletion of residues 82-88 (Figure 1C; see Figure 2 for two-hybrid spotting data), although the mutant proteins were expressed similarly to the wild type (Figure 3, and data not shown). In contrast, the A82V, S84A, S84E, E86A, and E86K mutations had little effect on the Dbf4-PBD interaction (Figure 1C). Although the VQV89-91AAA mutation had a modest effect on PBD binding, the

SK92,93AA and GTG94-96AAA mutations had no effect (Figure 1C). The V89A, Q90A, and V91A single point mutations also had no effect on the Dbf4-PBD interaction (Figure 2). Together, these observations suggest that Dbf4 residues 83-88 directly bind the Cdc5 PBD in a phosphorylation-independent manner. Since deletion of residues 89-93 eliminated the PBD interaction but mutation of individual amino acids within this sequence had no effect on binding, it is likely that residues 89-93 do not directly contact the PBD or they make non-essential contacts. Based on this point mutant analysis we suggest that Dbf4 residues 83-RSIEGA-88 comprise the core of a novel PBD binding motif.

Lastly, we tested whether residues 82 and following were required for PBD binding in the context of full length Dbf4. Although full length Dbf4 interacted with the PBD, *dbf4* mutants deleting past residue 82 failed to interact (Figure 4) indicating that these residues were critical for the interaction in full length Dbf4. To examine Dbf4-Cdc5 in the context of functional Cdc7-Dbf4 kinase (DDK), we tested the ability of wild type Dbf4 and Dbf4-Δ82-88 proteins to co-immunoprecipitate Cdc5 using a baculovirus expression system. HA-Cdc7, Dbf4 and Myc-Cdc5 proteins were co-expressed in Sf9 cells and the HA-Cdc7-Dbf4 complex was immunoprecipitated using an anti-HA monoclonal antibody. Cdc5 was co-immunoprecipitated by DDK complexes containing wild type Dbf4, but not by DDK complexes containing the Dbf4-Δ82-88 and Dbf4-NΔ109 mutant proteins (Figure 1D). These data indicate the DDK-Cdc5 interaction requires Dbf4 residues 82-88.

A 14-mer Dbf4 peptide containing residues 83-88 is sufficient for the PBD interaction

We next defined the minimal Dbf4-interacting peptide using the two-hybrid assay. A short Dbf4 peptide containing only residues 78-96 was sufficient for PBD-binding (Figure 5A). Although residues 82-96 did not bind the PBD, this was likely due to assay constraints and not due to loss of critical residues from 78-81, i.e. a quadruple alanine mutant of residues 78-81 ("RIER" to "AAAA") bound the PBD as well as wild-type Dbf4 66-96 (Figure 5A). This demonstrates that a 19 amino acid peptide (78-96) containing the Dbf4 sequence 83-RSIEGA-88 is sufficient for PBD binding.

To verify that Dbf4 residues 82-88 comprise a unique PBD binding motif, we first tested the ability of Dbf4 peptides to directly interact with the purified Cdc5 PBD using the AlphaScreen assay (Ullman et al. 1994). In this assay, a biotinylated Dbf4 peptide and purified His_6-PBD are bound to streptavidin (donor) and Ni++ (acceptor) beads, respectively. Excitation with 680 nm light causes donor beads to emit singlet oxygen, which activates fluorophores in proximally bound acceptor beads to emit light at 520-620 nm. A biotinylated Dbf4 peptide (73-96), but not an unrelated peptide, interacted with purified Cdc5 polo-box domain (residues 357-705) in a dose dependent manner (Figure 5B).

Figure 4. Residues required for full length Dbf4 binding to the PBD

(A) Two hybrid assays indicate that deletion of residues 82-88 within full-length Dbf4 completely disrupts the Dbf4-PBD interaction. Although deletion of N-terminal 65 residues did not affect the PBD two-hybrid interaction, the interaction was lost by the addition of the 82-88 deletion. (B) Diagram of full-length (FL) Dbf4 constructs used in two-hybrid assays. The *dbf4-NΔ65* mutant disrupts two destruction boxes at residues 10-19 and 62-70.

Figure 4. (cont'd)

A

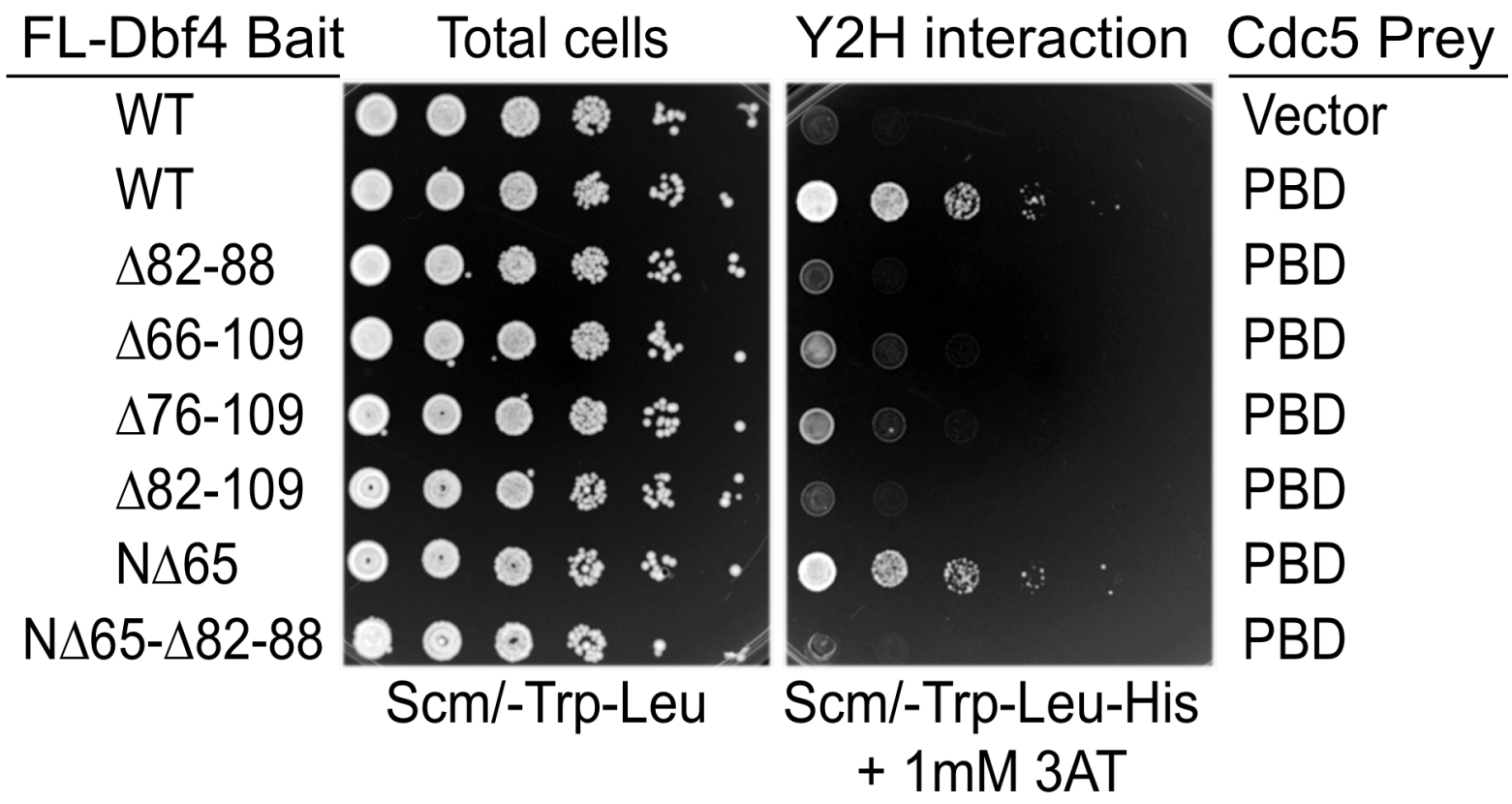


Figure 4. (cont'd)

B

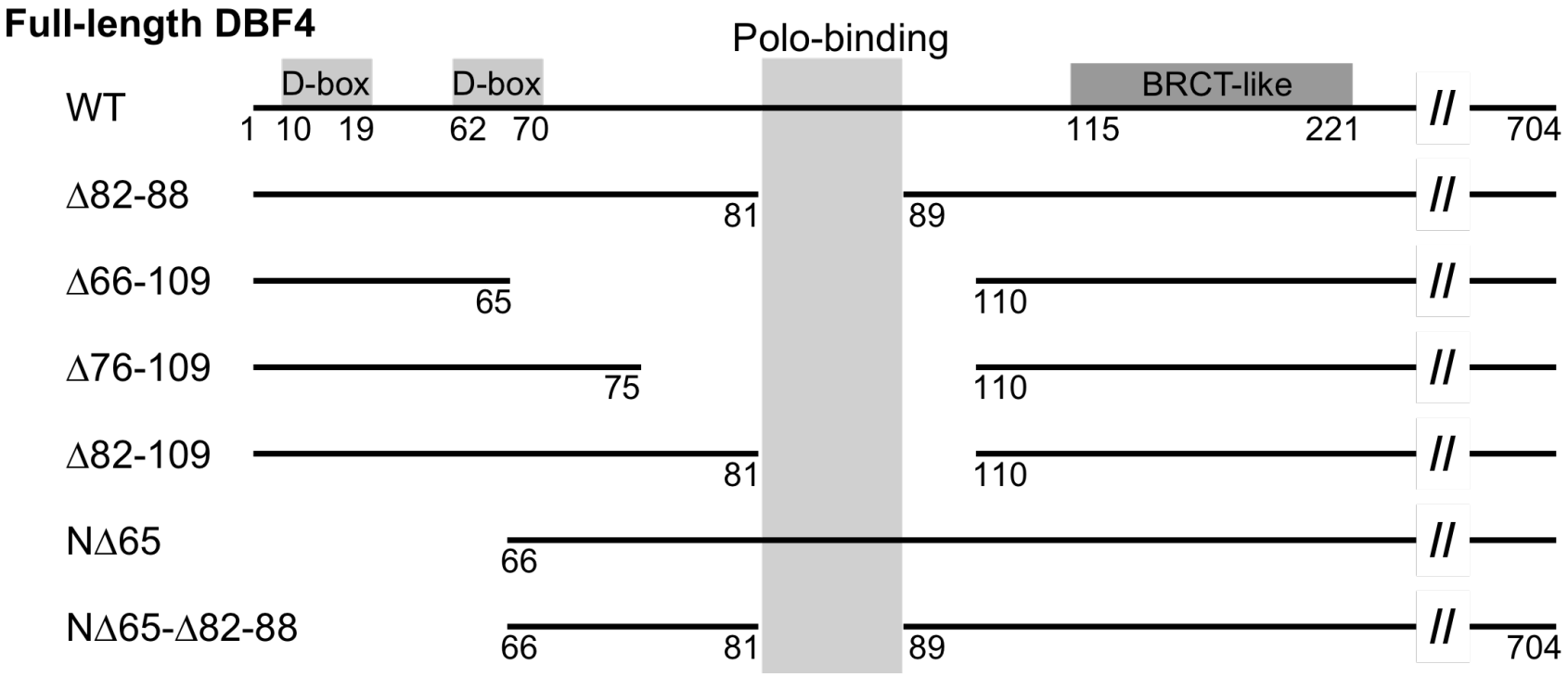


Figure 5. Analysis of Dbf4 residues required for interaction with the PBD

(A) The indicated Dbf4(66-227) bait constructs were assayed for a two hybrid interaction with the Cdc5 PBD by spotting serial dilutions of cultures onto the indicated media to visualize the total number of cells (left) and the two hybrid interaction (right). (B) Although deletion of Dbf4 residues 89-93 abolishes the PBD interaction, deletion of residues 89-91 (ΔVQV) has only a modest effect on the PBD interaction and deletion of residues 92-93 (ΔSK) has no effect. This strongly suggests that deletion of residues 89-93 indirectly affects the Dbf4-PBD interaction. (C) The VQV89AAA triple point mutant has a similar effect on the PBD interaction as deletion of these same residues, as shown in panel B. However, the V89A, Q90A, and V91A single mutants have no effect on the PBD interaction.

Figure 5. (cont'd)

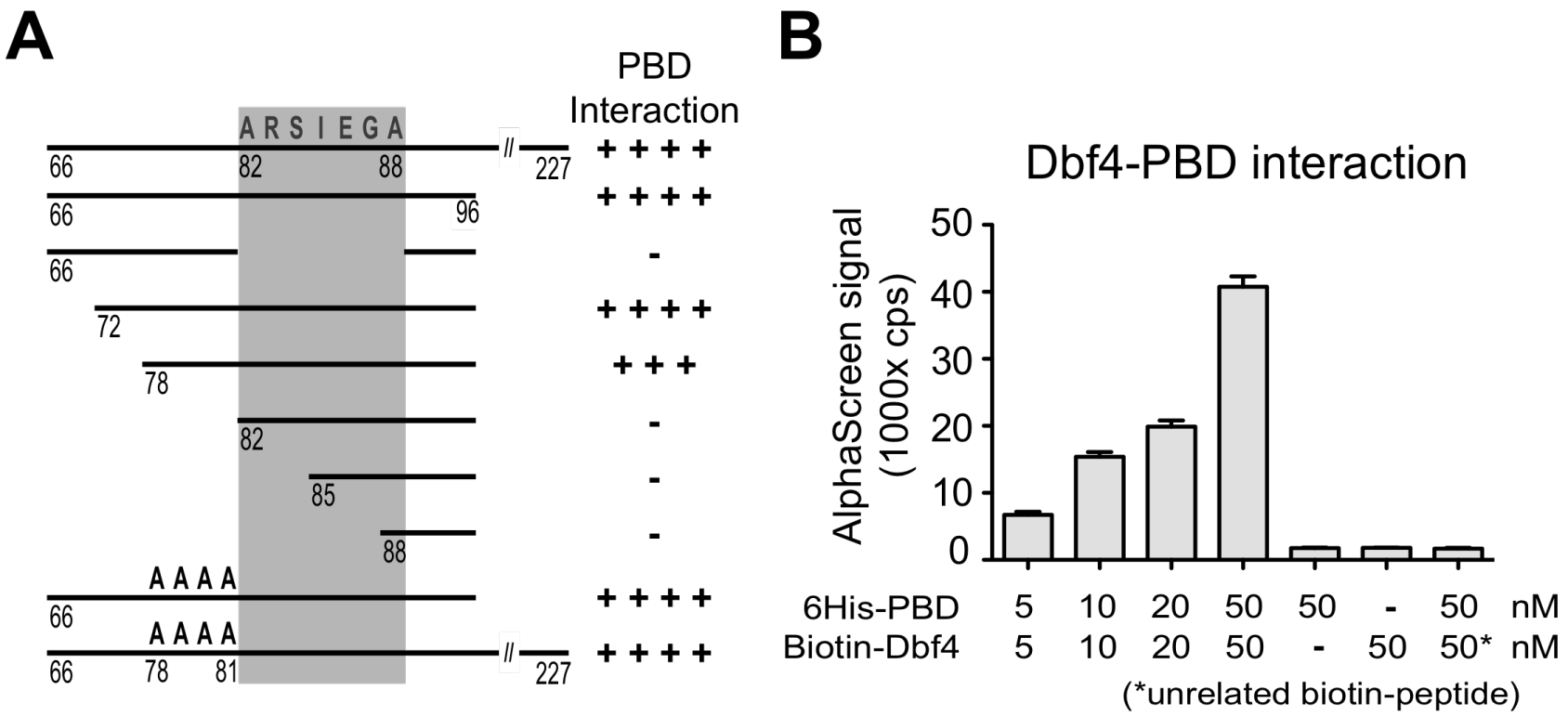
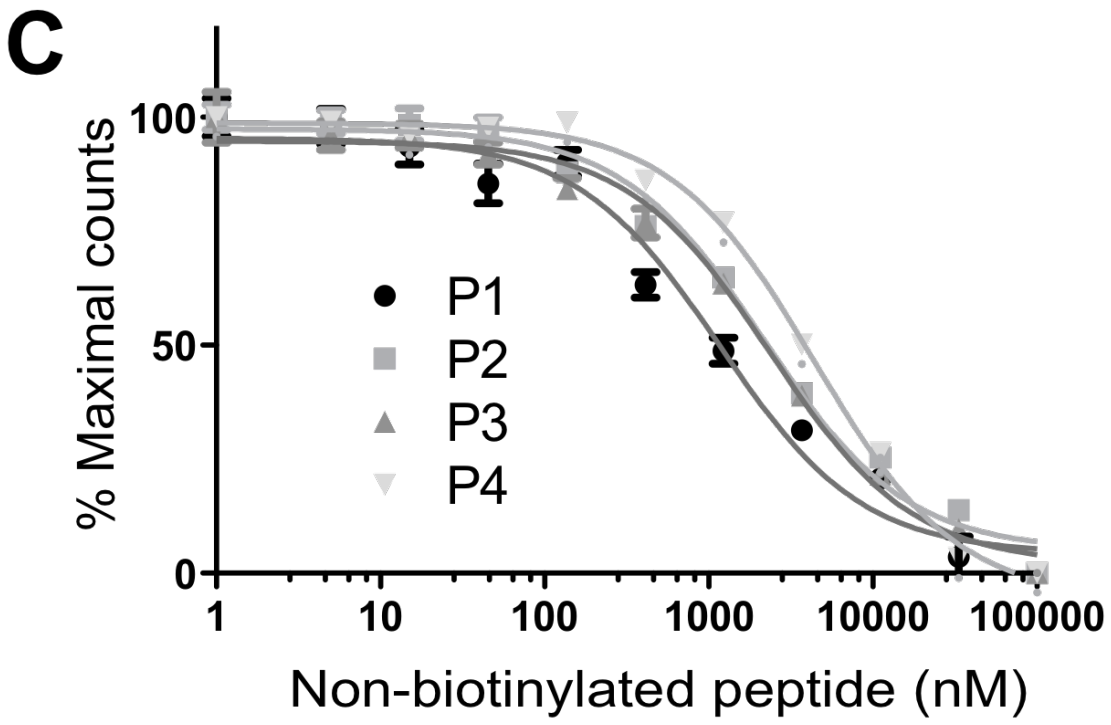
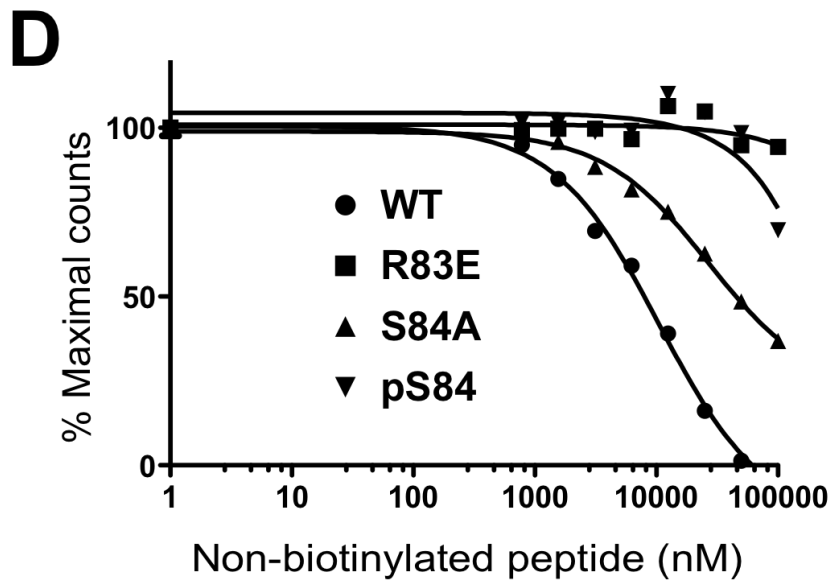


Figure 5. Analysis of Dbf4 residues required for interaction with the PBD

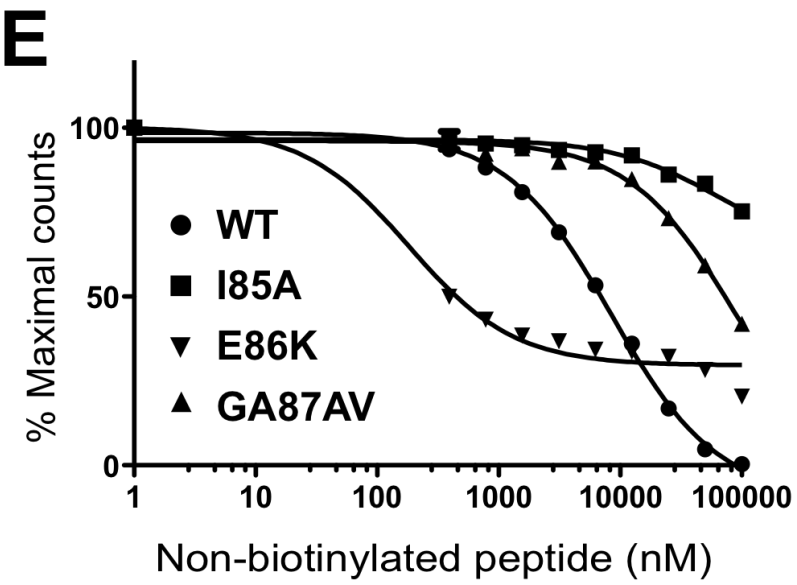


Peptide name		Peptide sequence	IC50 (μM)
Biotin-Dbf4 73-96	Biotin-	EKK RAR IER A**RS IEG A**VQ VSK GTG	
Dbf4 73-96	P1	EKK RAR IER A**RS IEG A**VQ VSK GTG	1.169
Dbf4 78-96	P2	RIE RA**R SIE GA**V QVS KGT G	2.171
Dbf4 78-93	P3	RIE RA**R SIE GA**V QVS K	2.371
Dbf4 80-93	P4	ERA **RSI EGA** VQV SK	4.408

Figure 5. (cont'd)



Peptide name	Peptide sequence
Dbf4 80-93 WT	ERA RSI EGA VQV SK
Dbf4 80-93 R83E	ERA **E**SI EGA VQV SK
Dbf4 80-93 S84A	ERA R**A**I EGA VQV SK
Dbf4 80-93 pS84	ERA R(**pS**)I EGA VQV SK



Peptide name	Peptide sequence
Dbf4 80-93 WT	ERA RSI EGA VQV SK
Dbf4 80-93 I85A	ERA RS**A** EGA VQV SK
Dbf4 80-93 E86K	ERA RSI **K**GA VQV SK
Dbf4 80-93 GA87AV	ERA RSI E**AV** VQV SK

To confirm the specificity of the binding assays, non-biotinylated Dbf4 peptides of differing lengths but containing the Dbf4-RSIEGA sequence were tested for their ability to compete the biotinylated-peptide PBD interaction. Four different peptides ranging from 24 to 14 residues showed a similar ability to compete the Dbf4-PBD interaction (Figure 5C), strongly suggesting that the RSIEGA residues directly bind the PBD. The affinity of Dbf4 peptide binding to the PBD in this assay was 1-5 μM as determined by competition with an unlabeled Dbf4 peptide (Figure 5C).

Dbf4 uses four key residues to bind the PBD and binding is inhibited by phosphorylation

We used peptide competition assays to determine how mutations in the Dbf4-RSIEGA sequence affected PBD binding. Dbf4-R83E, -I85A and -GA87AV peptides (containing mutations that disrupted the Dbf4-PBD two-hybrid interaction) lost the ability to compete with the Dbf4-PBD interaction even when the peptide concentration was increased to 10 μM (Figure 5D, E). However, the Dbf4-S84A and -E86K peptides, which still interacted with Polo in the two-hybrid assay, competed the Dbf4-PBD interaction *in vitro* (Figure 5D, E). These data are in complete agreement with the interaction map produced by two-hybrid data.

Interestingly, the Dbf4-E86K peptide bound to the PBD with higher affinity than the wild-type peptide (10-100 nM, Figure 5E) and importantly, the Ser84 phosphorylated peptide lost the binding ability to PBD (Figure 5D). Therefore, although the S84A and S84E (phospho-mimetic) mutants did not noticeably affect the Dbf4-PBD interaction in the

two-hybrid assay (Figure 1C), a pS84 residue blocked the interaction *in vitro*. These data indicate that S84 phosphorylation is not simply dispensable for the Dbf4-PBD interaction, it blocks the interaction *in vitro*, suggesting that an entirely different type of PBD-protein interaction is occurring.

Mutants altering critical residues in the Dbf4 PBD-binding motif suppress the cdc5-1 temperature sensitivity

Deletion of the Dbf4 N-terminal 109 amino acids suppresses the temperature-sensitive (ts) lethal phenotype of the *cdc5-1* mutant ((Miller et al. 2009), Figure 6A). Cdc5-1 protein contains a P511L missense mutation immediately preceding the PB1 motif and retains significant kinase activity at the non-permissive temperature (Pintard and Peter 2001) but is unable to promote mitotic exit (Park et al. 2003). The suppression data suggests that Dbf4 (DDK) might inhibit Cdc5-1 binding to critical targets required for mitotic exit. Using integrated alleles, we found *dbf4-Δ82-88* and *dbf4-R83E* that are defective for the Polo interaction suppressed the *cdc5-1* ts. The *cdc5-1* mutant grew poorly at 30ºC, but the double mutants grew well at 30°C and also suppressed the *cdc5-1* ts at 32ºC (Figure 6A). Although the *dbf4-Δ82-88* and *dbf4-R83E* mutants suppressed the *cdc5-1* ts at 32°C, there was little suppression at 34°C compared to the *dbf4-NΔ109* mutant. This suggests that additional residues in the Dbf4 N-terminal 109 contribute to robust suppression of the *cdc5-1* ts at 34°C.

Figure 6. Dbf4-RSIEGA mutants suppress the *cdc5-1* temperature sensitivity

(A) The indicated strains W303-1A, *dbf4-Δ82-88* (M2805), *cdc5-1* (M1614), c*dc5-1 dbf4-Δ82-88* (M3112, and M3114), *cdc5-1 dbf4-R83E* (M3116, M3117), and *cdc5-1 dbf4-NΔ109* (M2655, M2656) were spotted onto YPD plates and scored for growth at the indicated temperatures. (B) Various *dbf4* deletions on an *ARS CEN* plasmid (pMW489) were introduced into M2600 (*cdc5-1 dbf4Δ::kanMX6*) and scored for growth by spotting serial dilutions on YPD media at the indicated temperatures. (C) Summary of *dbf4* mutations, their effect on the Dbf4-PBD interaction, and suppression of the *cdc5-1* ts. *dbf4* mutants were scored for growth in the M2600 (*cdc5-1 dbf4Δ::kanMX6*) background by spotting serial dilutions on YPD media at increasing temperatures (Figure S4). (D) High copy plasmids expressing wild type *DBF4* and the indicated mutants were transformed into M1614 (*cdc5-1*). Cultures were spotted onto SCM-Leu plates at 25^{o}C indicating that high copy *dbf4-NΔ65* lethality is alleviated by deleting residues 82-88. (E) Expression of the Dbf4 N-terminus from the *GAL1, 10* promoter is lethal to *cdc5-1* cells only if Dbf4 retains the ability to interact with Cdc5 as occurs in the WT, S84A, S84E, E86A and E86K mutants.

Figure 6. (cont'd)

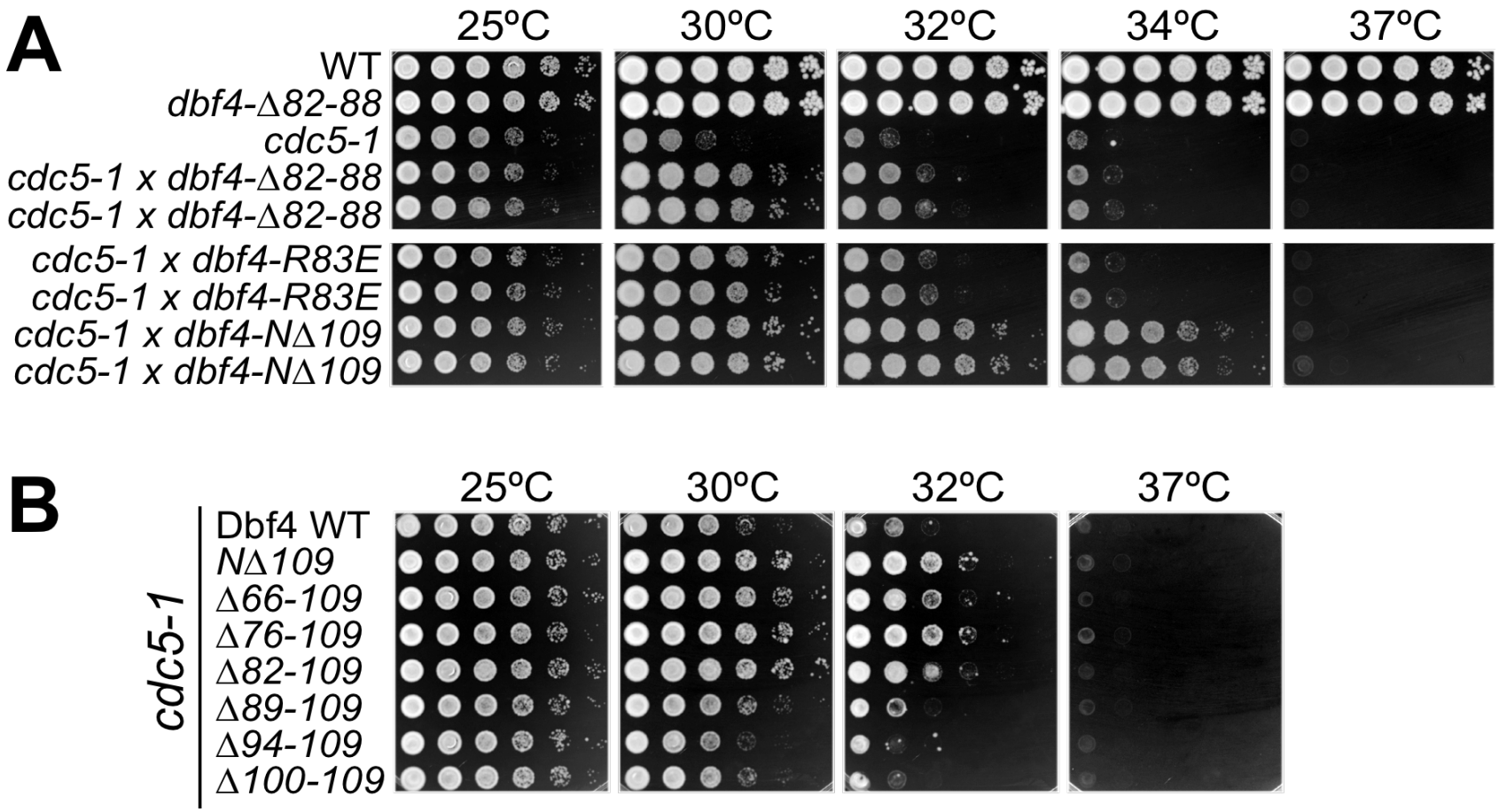


Figure 6. (cont'd)

C

82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	PBD	*cdc5-1*
A	R	S	I	E	G	A	V	Q	V	S	K	G	T	G	interaction	ts suppression
V															+ + + +	-
	E	A													-	+ +
			A	K											-	+ +
					A	V									-	+ +
							A	A	A						+ +	+ +
										A	A				+ + + +	-
												A	A	A	+ + + +	-
	A														+ +	+ +
	E														-	+ +
		A													+ + + +	-
		E													+ + + +	-
			A												-	+ +
				A											+ + + +	-
				K											+ + + +	-
					A										-	+ +
						V									-	+ +
Δ	Δ	Δ	Δ	Δ	Δ	Δ									-	+ +
							Δ	Δ	Δ	Δ	Δ				-	+ +
A	R	S	I	E	G	A	V	Q	V	S	K	G	T	G	PBD	*cdc5-1*
82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	interaction	ts suppression

Figure 6. (cont'd)

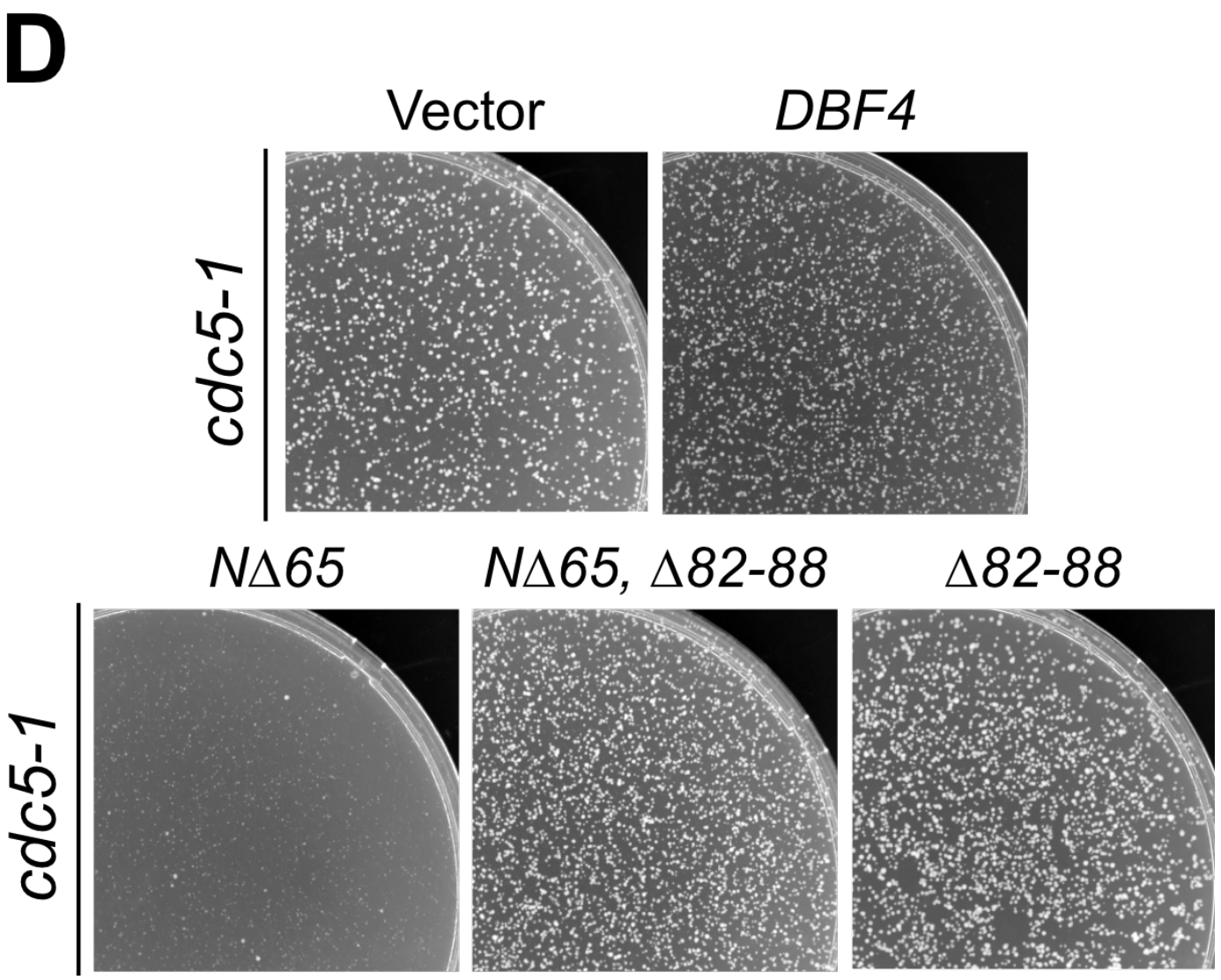


Figure 6. (cont'd)

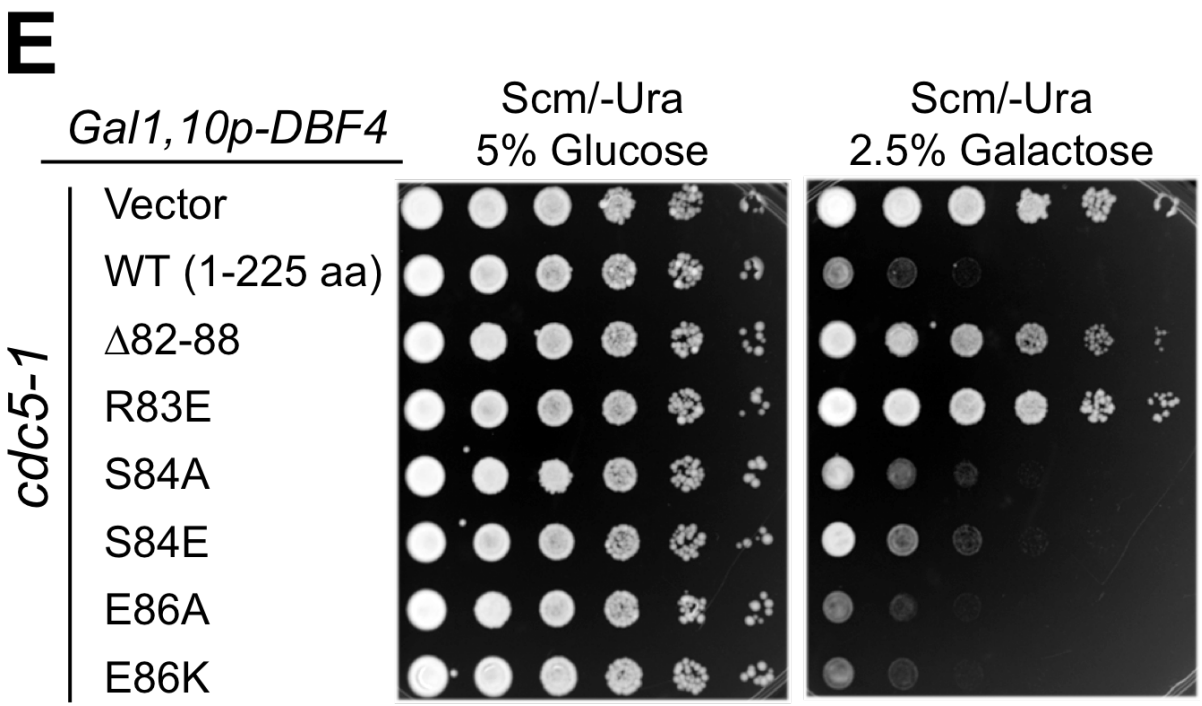
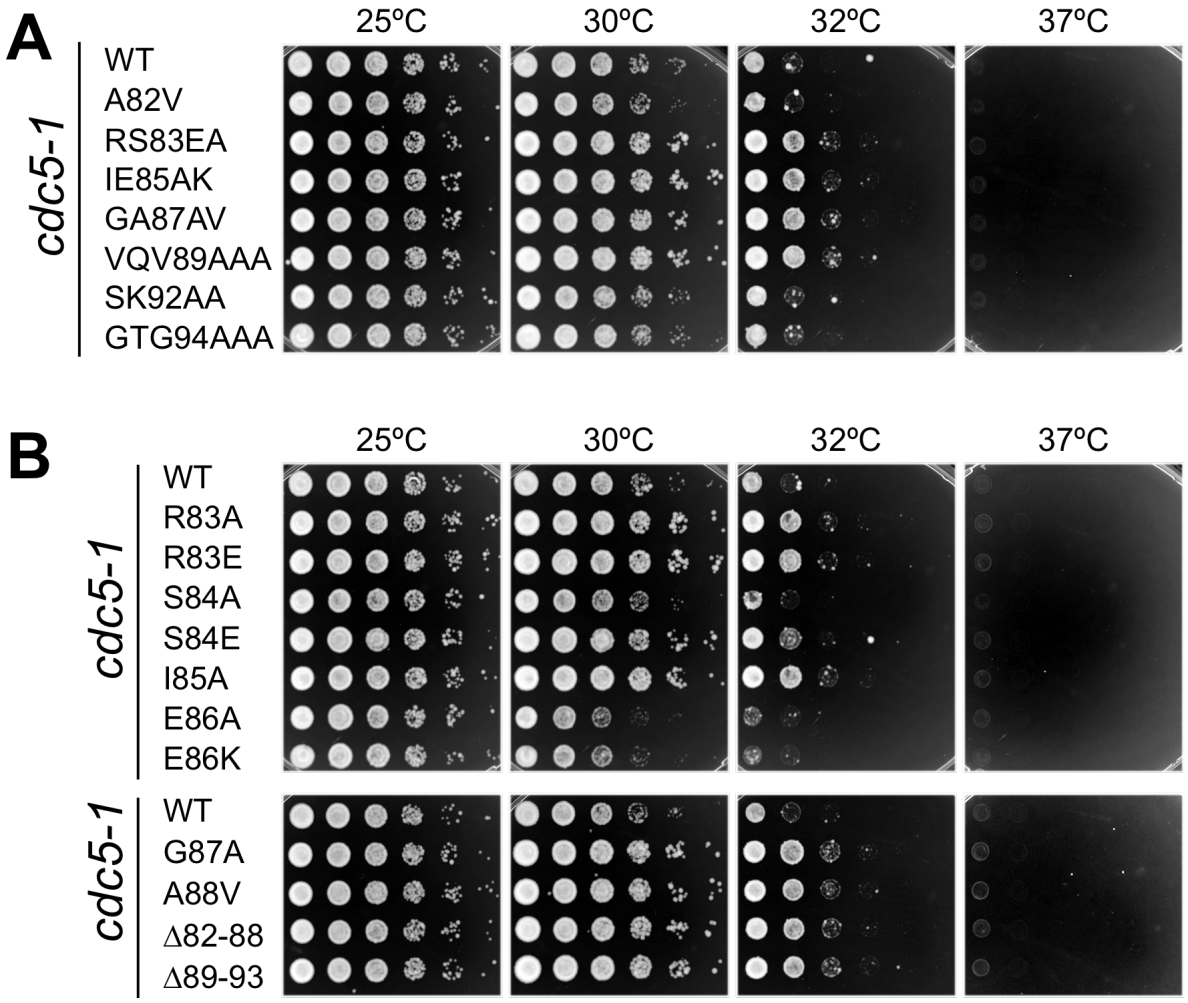


Figure 7. Mutations of Dbf4 residues required for the PBD interaction also suppress the *cdc5-1* ts

(A, B) A series of *dbf4* mutants were shuffled into M2600 (*dbf4Δ::kanMX6 cdc5-1*) on plasmids and then cured of the wild type *DBF4* plasmid. Serial dilutions of the resulting cultures were spotted at increasing temperatures to score the growth phenotype. Only those mutations that disrupt the PBD interaction suppress the *cdc5-1* temperature sensitivity. Mutations such as SK92AA or S84A that have a wild type Dbf4-PBD interaction retain the *cdc5-1* ts. The *dbf4-E86K* mutant actually causes enhanced *cdc5-1* temperature sensitivity at 30oC, consistent with the increased interaction between the Dbf4 E86K peptide and the PBD (Figure 2E).

Figure 7. (cont'd)



To more closely examine the correlation between loss of the Polo interaction and the ability to suppress the *cdc5-1* ts, we created a *DBF4* plasmid shuffle system in the *cdc5-1* background, and tested the ability of various *dbf4* mutants (expressed from the endogenous *DBF4* promoter) to suppress the *cdc5-1* ts. We found that the *dbf4-Δ66-109*, *dbf4-Δ76-109*, and *dbf4-Δ82-109* alleles suppressed the *cdc5-1* ts similar to the *dbf4-NΔ109* allele. However, *dbf4-Δ94-109* or *dbf4-Δ100-109* that retain residues 83-88 did not (Figure 6B). These latter two mutants retain the ability to bind Cdc5 in the two-hybrid assay (data not shown). In the *cdc5-1* plasmid shuffle strain, the *dbf4-NΔ109*, *dbf4-Δ66-109*, *dbf4-Δ76-109*, and *dbf4-Δ82-109* mutants exhibit very similar growth properties at 32^{o}C (Figure 6B) but did not grow at 34^{o}C (not shown). So in this system, the larger N-terminal deletion is phenocopied by smaller deletions removing the Cdc5 binding site. We then examined *dbf4-R83A*, *-R83E*, *-I85A*, *-G87A*, and *-A88V* alleles, which alter residues critical for PBD binding in the two-hybrid and AlphaScreen assays. As expected, these mutants suppressed the *cdc5-1* ts at 30^{o}C and 32ºC like the *dbf4-Δ82-88 cdc5-1* mutant (summarized in Figure 6C). In contrast, we observed no ts suppression by the *dbf4-S84A* and *dbf4-E86K* alleles, which still interacted with the PBD. We observed a strict correlation among mutants in residues 82-88 between loss of Polo binding and suppression of the *cdc5-1* ts (Figure 6C). This indicates that loss of the Dbf4 physical interaction with Cdc5 suppresses the *cdc5-1* growth defect at restrictive temperatures.

The *dbf4-S84A* and *dbf4-S84E* mutants that still interact with Cdc5 did not suppress the *cdc5-1* ts. Accordingly, the *dbf4-E86K* mutant exacerbated the *cdc5-1* growth defect

(Figure 7B), consistent with the fact that the Dbf4-E86K peptide bound with higher affinity to the PBD than the wild type. These observations underscore that although alterations of Dbf4 residues S84 and E86 within the PBD-binding motif can influence the PBD interaction, the S84 and E86 residues do not make essential contacts required for the PBD interaction.

Dbf4 inhibits Cdc5 by directly binding the PBD

We previously found that a chromosomal *dbf4-NΔ65* mutant that interacts with Cdc5 but is stabilized by deleting several D-boxes was synthetic sick or lethal in combination with *cdc5-1* (Miller et al. 2009). This supports the model that Dbf4 inhibits the essential function of Cdc5. This hypothesis is supported by a recent report that a *dbf4-NΔ65* mutant can inhibit ribosomal DNA segregation under certain circumstances (Sullivan et al. 2008), since rDNA segregation during anaphase is regulated by Cdc5 activation of the FEAR (Cdc14 Early Anaphase Release) pathway (Stegmeier and Amon 2004). We investigated whether Dbf4 residues 82-88 are required to inhibit Cdc5 activity by over-expressing Dbf4 from high-copy plasmids. Increased expression of wild-type *DBF4* was deleterious to *cdc5-1*, but over-expression of *dbf4-NΔ65* was lethal to *cdc5-1* (Figure 6D). Deletion of residues 82-88 rescued the synthetic lethality between *dbf4-NΔ65* and *cdc5-1*, strongly suggesting that Dbf4 inhibits Cdc5 function through a direct interaction. Similarly, overproduction of the isolated Dbf4 N-terminus (residues 1-225) from the *GAL1, 10* promoter was lethal to *cdc5-1* cells (Figure 6E) (Miller et al. 2009). In contrast, overproduction of the Dbf4-Δ82-88 and Dbf4-R83E peptides that fail to interact with Cdc5 was not lethal. Taken together with the *cdc5-1* ts suppression results, these data

indicate that the Dbf4-RSIEGA sequence is required to inhibit Cdc5 activity by direct binding to the PBD.

The PBD interacts with Dbf4 using a surface distinct from its phospho-peptide binding surface

Among the Cdc5 PBD substrates that have been described in detail, the spindle pole body protein Spc72, was found to bind the PBD through its S-pS-P motif (Snead et al, 2007). We confirmed that purified His_6-PBD bound the Spc72 phosphopeptide *in vitro* (Figure 8A) with an IC_{50} of ~2mM (data not shown) that is very similar to the Dbf4-PBD interaction. To test whether Dbf4 and Spc72 peptides bound to distinct surfaces of the PBD, we performed competition assays using a non-biotinylated Spc72 phosphopeptide to compete the Dbf4-PBD interaction. Although a wild type Dbf4 peptide spanning residues 80-93 effectively competed the Dbf4-PBD interaction, the phosphorylated Spc72 peptide (containing “S-pS-P”) did not (Figure 8B). This non-competitive result strongly suggests that two specific binding sites exist on the PBD, one for Dbf4 and another for phosphorylated substrates.

Figure 8. Dbf4 binds a surface on the PBD distinct from its phospho-protein binding site

(A) A biotinylated Spc72 phospho-peptide (residues 223-242) bound the PBD in the AlphaScreen assay. (B) The same (non-biotinylated) Spc72 phospho-peptide did not compete the Dbf4-PBD interaction. (C) Purified wild type PBD and PBD-HK proteins interact with Dbf4 in the AlphaScreen assay, but the PBD-HK mutant protein fails to interact with Spc72 phospho-peptide. (D) Two-hybrid $Spc72_{(1-400)}$ and $Dbf4_{(66-227)}$ interactions with the PBD were tested on the indicated plates. As in (C), mutation of the PBD "pincer" residues (H641A, K643M) or (W517F, H641A, K643M) had no affect on the Dbf4-PBD interaction, but eliminated the Spc72-PBD interaction.

Figure 8. (cont'd)

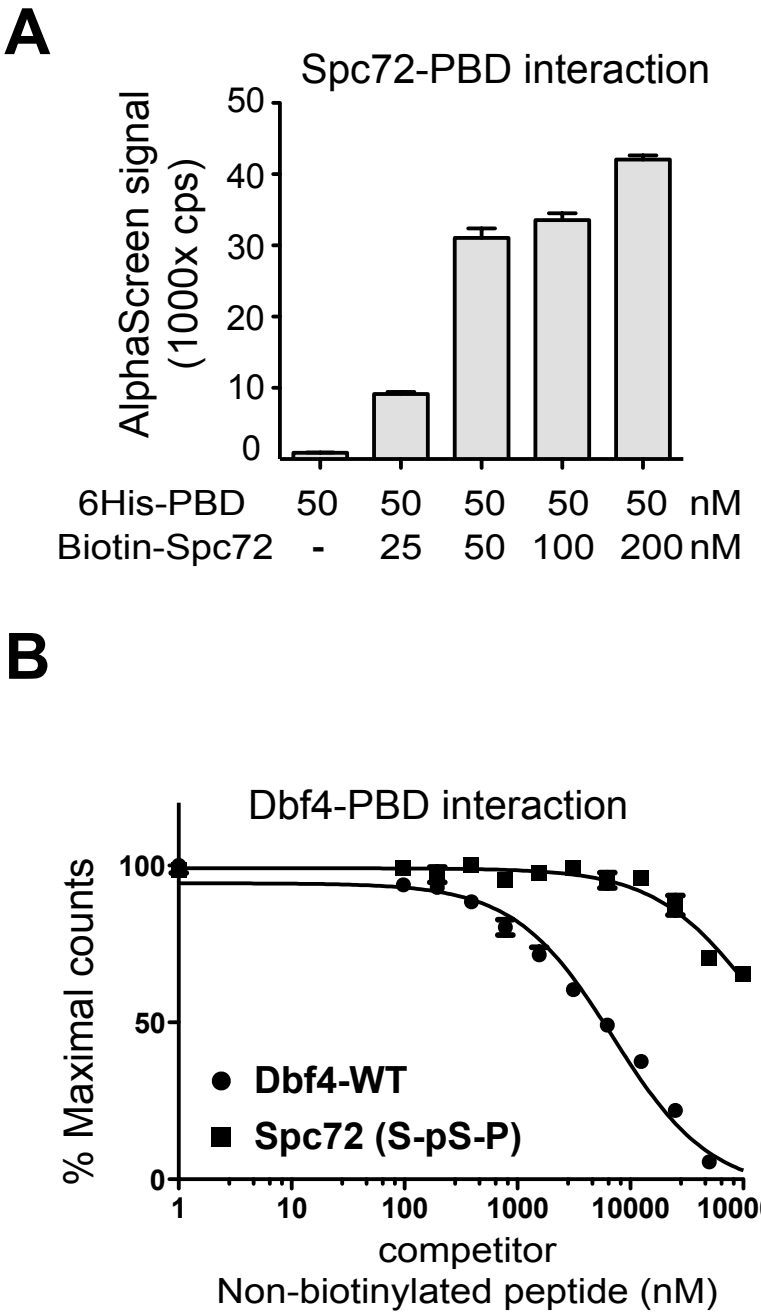


Figure 8. (cont'd)

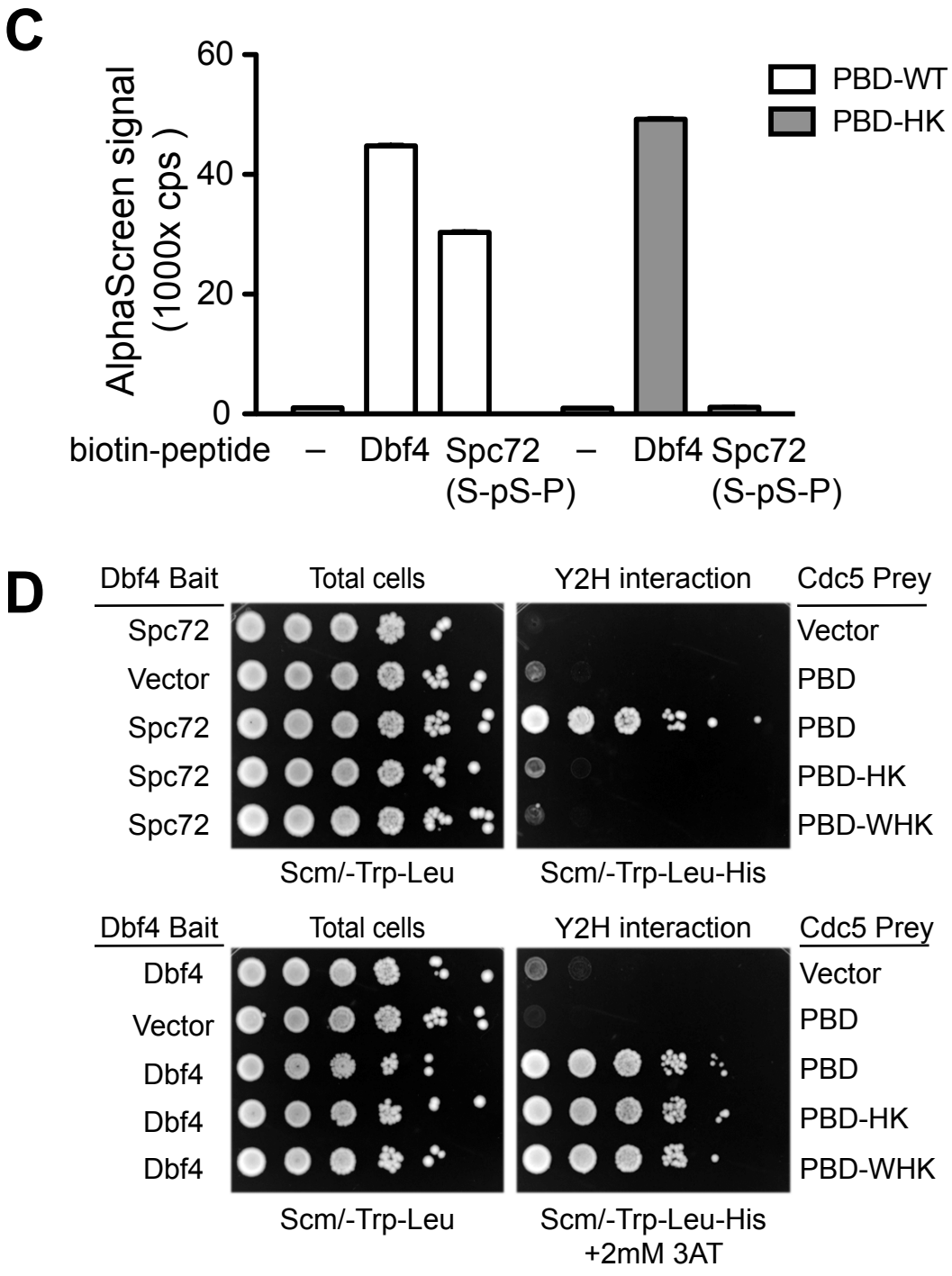
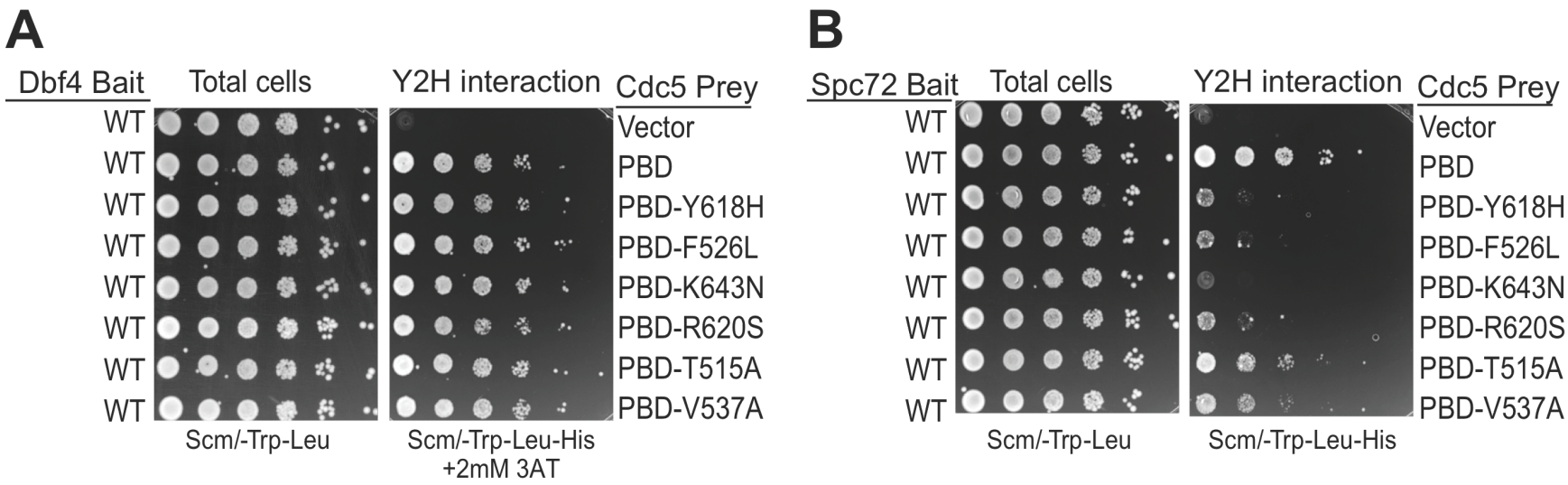


Figure 9. Identification of additional Cdc5 PBD mutations that disrupt the PBD-Spc72 interaction

The Cdc5 PBD in pGAD-Cdc5.3 was randomly mutagenized using Taq polymerase. (A, B) PBDs were screened for their two-hybrid interaction with Dbf4 and Spc72 and this identified six discrete mutations that disrupt (Y618H, F526L, K643N, R620S) or impair (T515A, V537A) the interaction with Spc72 but have no effect on the PBD-Dbf4 interaction. (C) Description of mutations in Cdc5 and the corresponding amino acids in human Plk1. In the Plk1-phosphopeptide structures, L540 and R516 directly contact the phospho-threonine residue and peptide side chains, respectively (ref 24, 25) and S412 makes a water-mediated hydrogen bond with the phospho-threonine (ref 25). L423, V434, and W514 do not directly contact the peptide but are closely positioned to each other in space to make a hydrophobic region. Mutation of these residues may indirectly affect the phospho-peptide binding pocket.

Figure 9. (cont'd)



C

Cdc5	(hsPlk1)		Y2H interaction with Dbf4	Y2H interaction with Spc72	
			+ + + +	+ + + +	
Tyr618	(Trp514)	Y618H	+ + + +	-	this study
Phe526	(Leu423)	F526L	+ + + +	-	this study
Lys643	(Lys540)	K643N	+ + + +	-	Cheng et al., Elia et al.
Arg620	(Arg516)	R620S	+ + + +	-	Cheng et al., Elia et al.
Thr515	(Ser412)	T515A	+ + + +	+ +	Elia et al.
Val537	(Val434)	V537A	+ + + +	+ +	this study

The co-crystal structure of the Plk1 PBD with a phosphothreonine peptide revealed that the “pincer” residues H538 and K540 (which are invariant among human and mouse Plk1, Polo, Plo1 and Cdc5) directly interact with the phosphate group on threonine (Cheng et al. 2003; Elia et al. 2003b). However, the purified PBD-H641A, K643M protein, containing the analogous mutations to Plk1-H538A, K540M, interacted with Dbf4 like the wild type *in vitro* (Figure 8C). In contrast, although the wild-type PBD interacted with Spc72 the PBD-HK protein did not. Similarly, the PBD-HK mutant interacted with Dbf4 but not with Spc72 in yeast cells (Figure 8D). The additional mutation of a conserved hydrophobic residue W517F, analogous to W414 in Plk1 that interacts with the preceding serine (S*-pT-P) of the phosphopeptide, also did not disrupt the two-hybrid interaction with Dbf4 (Figure 8D). Using random mutagenesis we isolated additional PBD mutations that abrogate the PBD-Spc72 interaction but retain the PBD-Dbf4 interaction (Figure 9). The effects of these new PBD mutants on phospho-substrate binding are consistent with structural studies of Plk1-phosphopeptide molecular interactions (Cheng et al. 2003; Elia et al. 2003b). Together, these data indicate that the Cdc5 PBD contains a second binding surface that recognizes non-phosphorylated sequences, like the Dbf4 residues 83-RSIEGA-88.

cdc5-HK “pincer” mutant has normal growth rate but shows increased resistance to microtubule disruption

Very surprisingly, mutation of the pincer residues (H641A and K643M), which eliminated interaction with the Spc72 phospho-peptide *in vitro* (Figure 8C), was tolerated in yeast. In fact the *cdc5-HK* allele completely complemented yeast viability and growth in a

*cdc5*Δ plasmid shuffle strain (Figure 10A, B), and when integrated at the endogenous *CDC5* locus (Figure 10C). Similarly, the *cdc5-W517F, H641A, K643M* (*cdc5-WHK*) allele also fully complemented yeast viability and growth rate (Figure 10A, B) and exhibited no temperature sensitivity up to 37^{o}C (Figure 10A). These data indicate that the invariant pincer residues are not required for Cdc5 to bind essential substrates in yeast.

Although there were little growth phenotypes, the *cdc5-HK* and *cdc5-WHK* mutants exhibited increased resistance over the wild type to microtubule disruption by benomyl (Figure 10C). All three strains grew well on plates containing 15 mg/ml benomyl but the mutant strains grew about 100-fold better in the presence of 30 and 37.5 mg/ml benomyl. This phenotype was also observed with the integrated *cdc5-HK* allele but not with the temperature sensitive (hypomorphic) *cdc5-5* or *cdc15-4* mutants, which were more sensitive or as sensitive to benomyl compared to the wild type strain (Figure 10C). These data raise the possibility that the PBD pincer residues target Cdc5 to a substrate (perhaps a microtubule associated protein) that regulates spindle dynamics.

In M-phase, Cdc5 promotes loss of sister chromatid cohesion, regulates spindle dynamics, and is essential to promote mitotic exit (Archambault and Glover 2009). Therefore, we tested for *cdc5-HK* synthetic growth interactions with spindle checkpoint mutants and the *cdc15*, *dbf2*, and *cdc14* ts MEN mutants. The *cdc5-HK* allele exhibited no synthetic growth interaction with the *mad1D*, *mad2D*, *bub1D*, or *bub2D* spindle checkpoint mutants (data not shown). Although we saw no synthetic growth interaction

with *cdc14-1*, *cdc5-HK* was synthetically lethal with *cdc15-4*, (*cdc15-2*, not shown) and *dbf2-1* alleles (Figure 10D). These synthetic lethal interactions were alleviated by *TAB6-1*, a dominant *cdc14* mutant that suppresses some MEN defects (Shou and Deshaies 2002). Importantly, *TAB6-1* did not significantly affect the growth of the *cdc15-2* or *cdc15-4* mutants at 25^{o}C (data not shown) arguing that *TAB6-1* bypasses the synthetic lethality we observe by suppressing a *cdc5-HK* defect. This data therefore suggests that *cdc5-HK* is defective in promoting mitotic exit.

Since *cdc5-HK* exhibited increased resistance to benomyl, we also directly examined spindle length in asynchronous wild type and *cdc5-HK* cells. The cell cycle distribution of *cdc5-HK* cells revealed a larger percentage of cells in G2/M phase relative to the wild type suggesting a mitotic delay in the mutant (Figure 11A). We quantitated spindle length in large-budded, mitotic cells and observed that the fraction of cells with short spindles (<2mm) was the same in both strains, indicating there was no defect in mitotic entry (Figure 11B). In contrast, the average spindle length in the mutant was about 38% greater than the wild type (7mm versus 5.1mm) (Figure 11B). This could indicate a defect in exiting mitosis (spindle disassembly) or a defect in properly restraining spindle elongation. To address whether spindle elongation occurred with faster kinetics in the mutant, we measured the rate of spindle growth following release from a G2/M block using nocodazole. Wild type and *cdc5-HK* cells were arrested for three hours using 15 mg/ml nocodazole and then released into the cell cycle in the absence of nocodazole at 30^{o}C. Flow cytometry profiles are shown in Figure 11C. The spindles in both strains were depolymerized at 0 minutes, however spindle length increased more rapidly for the

cdc5-HK mutant varying from 300% over wild type lengths at early time points to 40% greater length at 60 minutes (Figure 11D, E). These data indicate that mutation of the pincer residues also causes aberrant spindle elongation.

Figure 10. The Cdc5 pincer residues are not required for yeast viability

(A) The *cdc5-HK* and *cdc5-WHK* mutants complemented a *cdc5*Δ by plasmid shuffle into M1672 (*cdc5*Δ*::kanMX6*/pMW536[*CDC5 URA3*]) evidenced by growth on FOA plates, and at various temperatures on YPD plates following loss of pMW536. (B) Growth curves of M1672 strains containing only the indicated *CDC5* alleles in YPD at 30^{o}C (C) The M1672-transformed strains in panel (A) were spotted onto YPD plates +/- benomyl (top). WT (M138), *cdc5-HK* (M3502), *cdc5-5* (M1680), and *cdc15-4* (M1999) strains containing integrated alleles were similarly spotted onto YPD +/- benomyl (bottom). (D) Representative tetrads from diploid strains of genotype *cdc5-HK/CDC5 dbf2-1/DBF2*, *cdc5-HK/CDC5 cdc15-4/CDC15 TAB6-1/CDC14*, or *cdc5-HK/CDC5 cdc14-1/CDC14* that were sporulated and dissected onto YPD plates at 25^{o}C. Recombinant genotypes are indicated.

Figure 10. (cont'd)

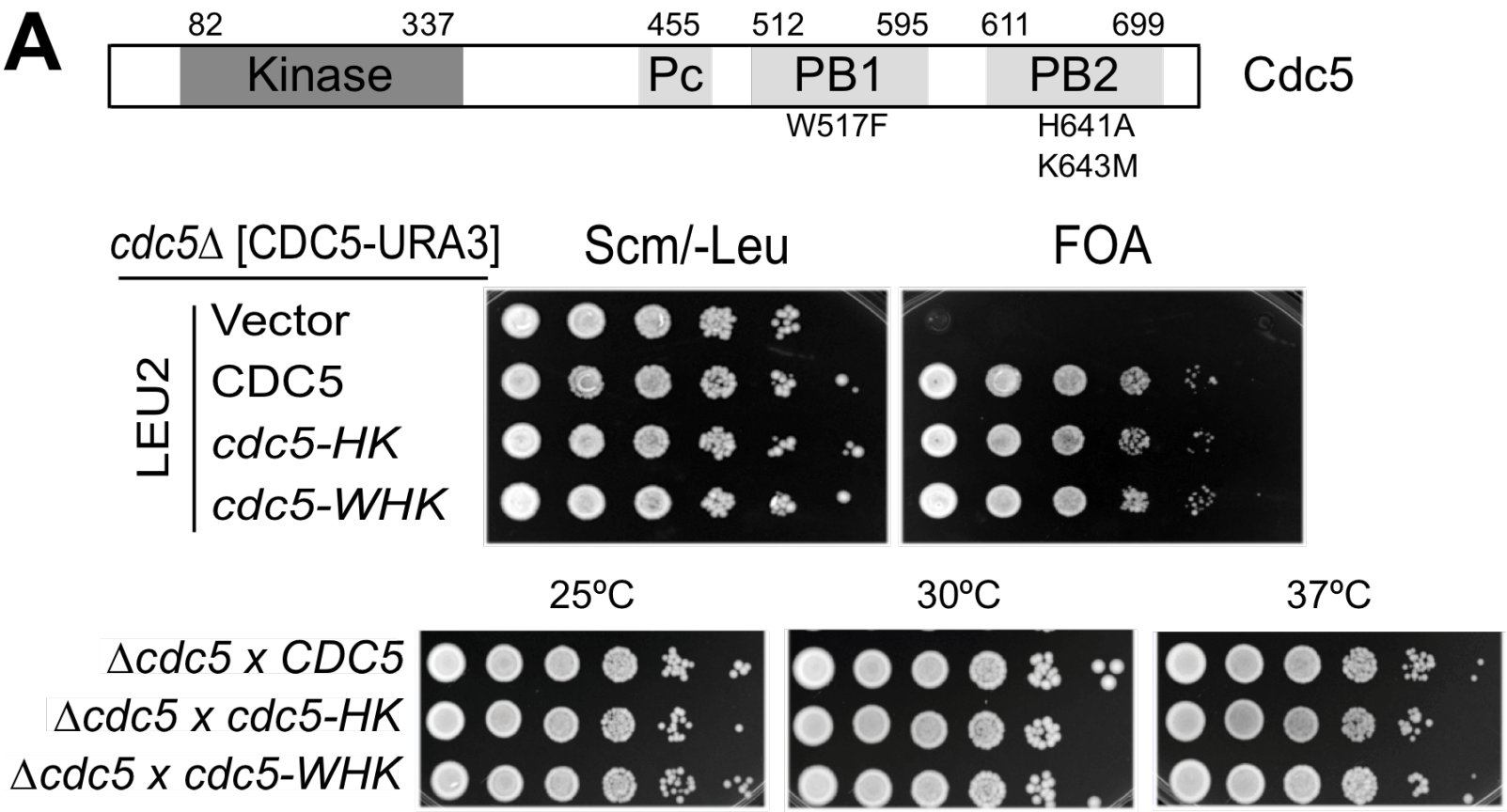
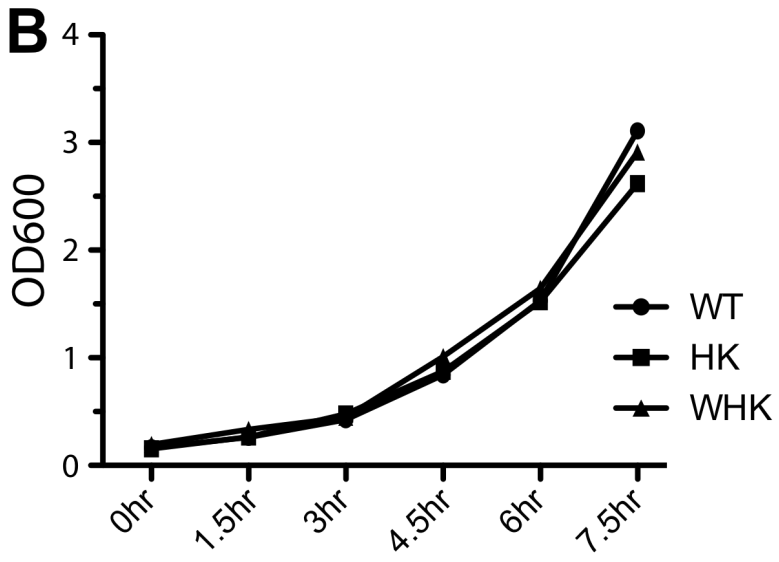


Figure 10. (cont'd)



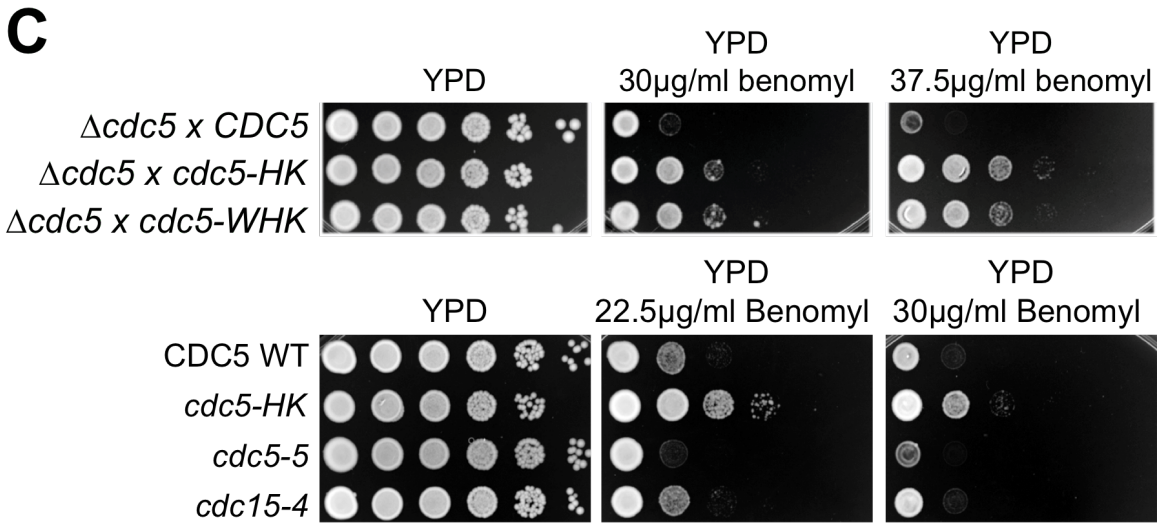


Figure 10. (cont'd)

D

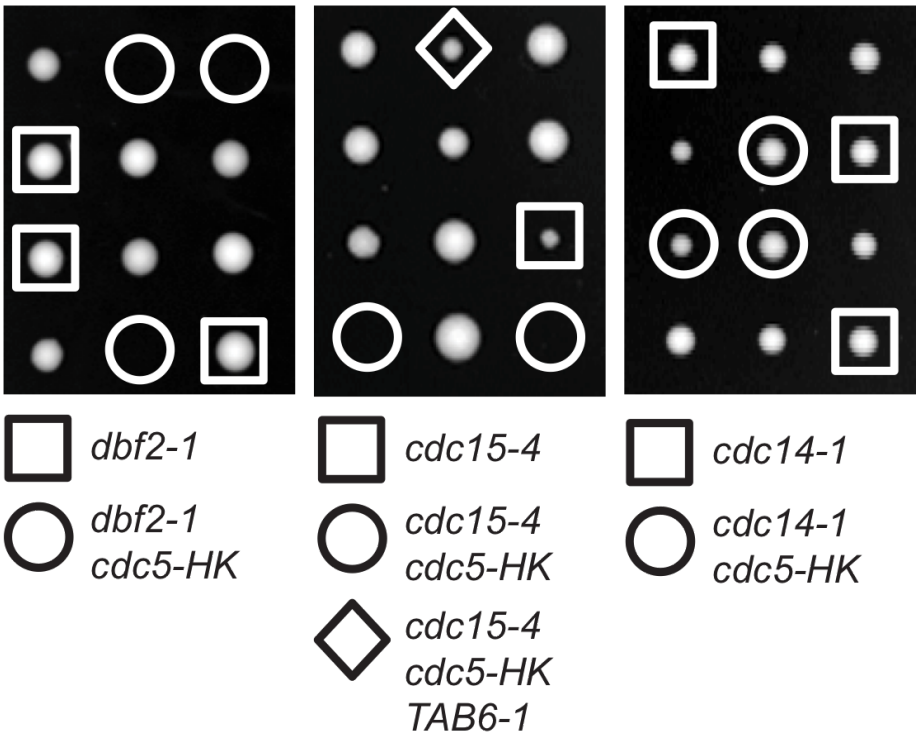


Figure 11. Mutation of the Cdc5 pincer residues causes a G2/M delay and alters spindle dynamics

(A) Flow cytometry profiles of asynchronous W303-1A (WT) and M3502 (*cdc5-HK*) strains. (B) Average spindle length was quantitated in large-budded cells of the same strains, shown with the range that includes 25-75% of spindle lengths. Inset shows fraction of cells with short spindles, <2 mm. (C) Flow cytometry profiles of W303-1A and M3502 arrested in G2 with nocodazole for 3 hours (t=0) and following release at 30^{o}C. (D) Quantitation of spindle length at the indicated times following nocodazole release. Standard errors were all less than 1%. (E) Tubulin staining of representative photomicrographs of cells at 40 minutes following nocodazole release.

Figure 11. (cont'd)

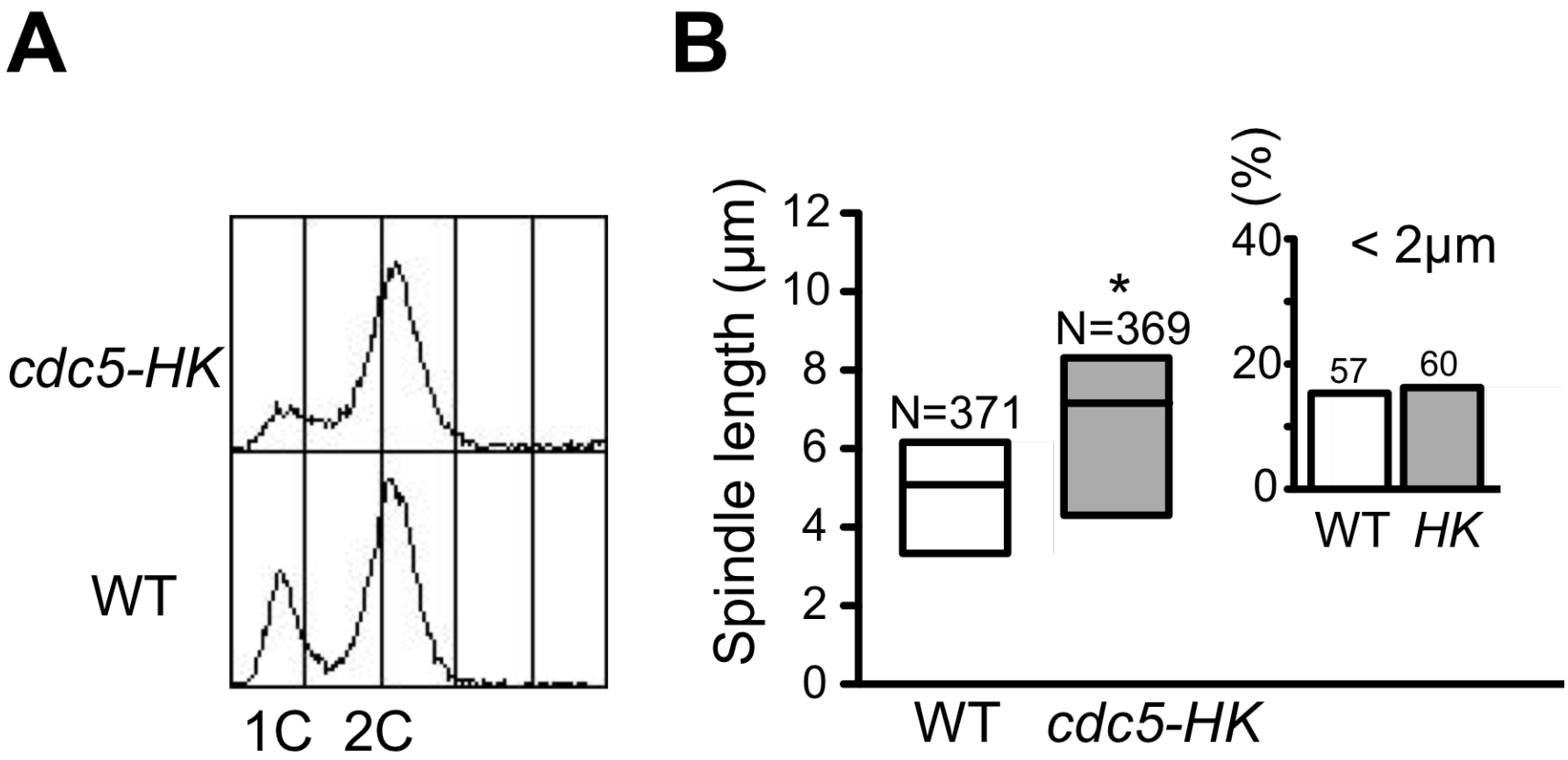


Figure 11. (cont'd)

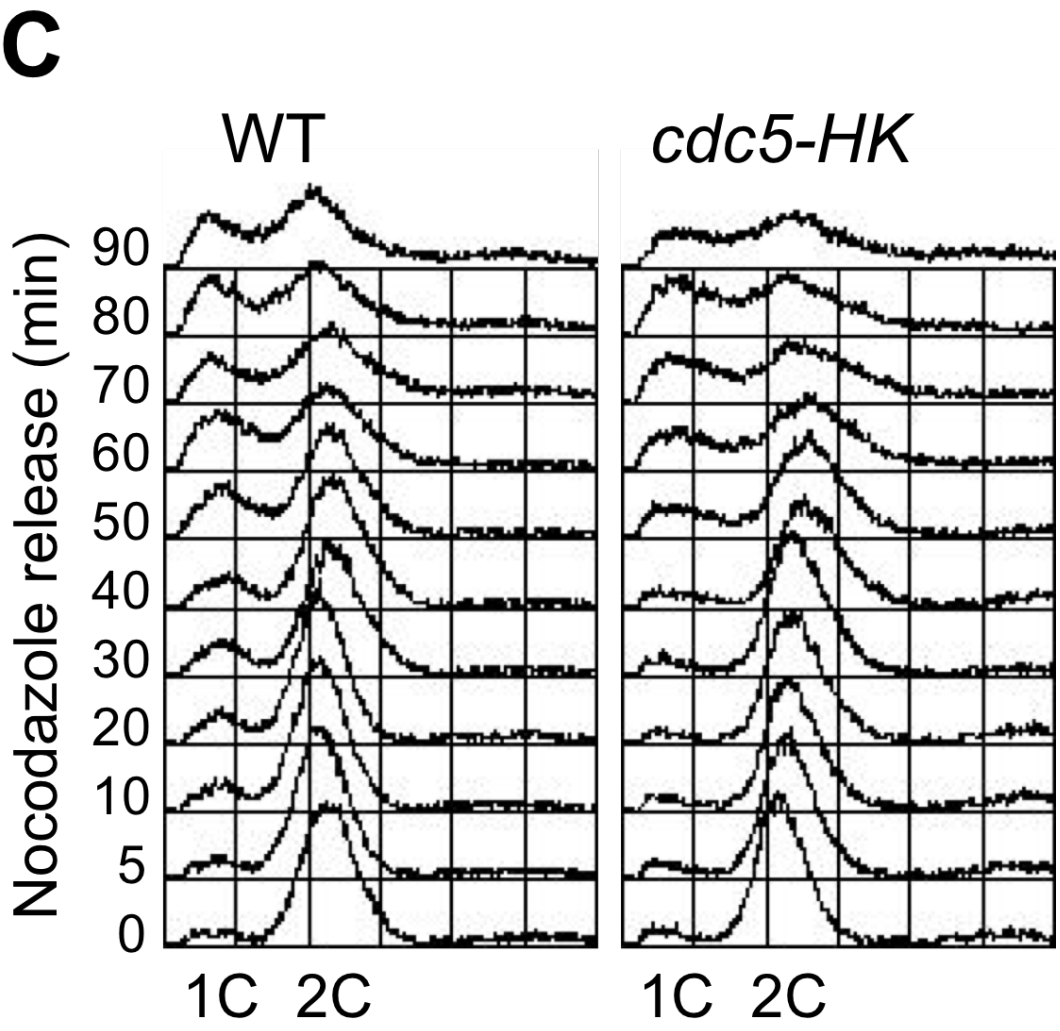
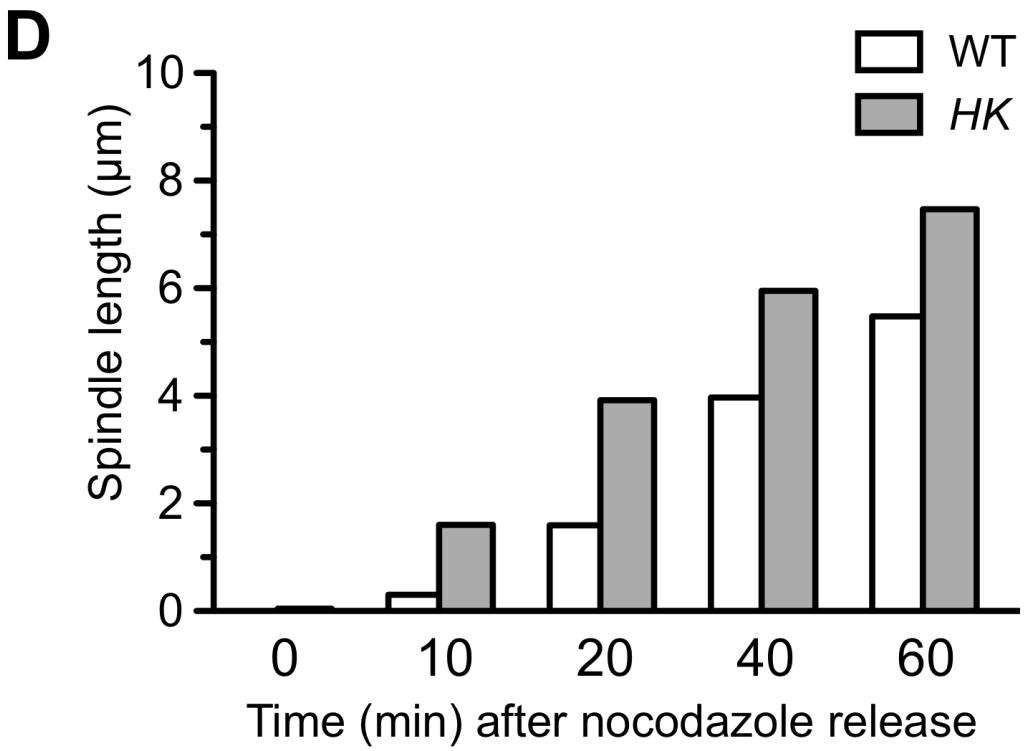


Figure 11. (cont'd)



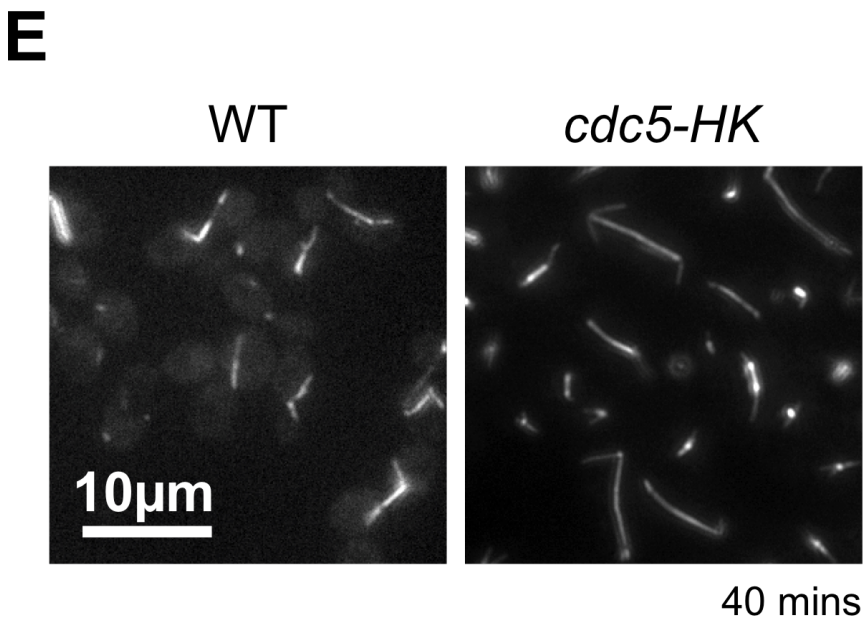
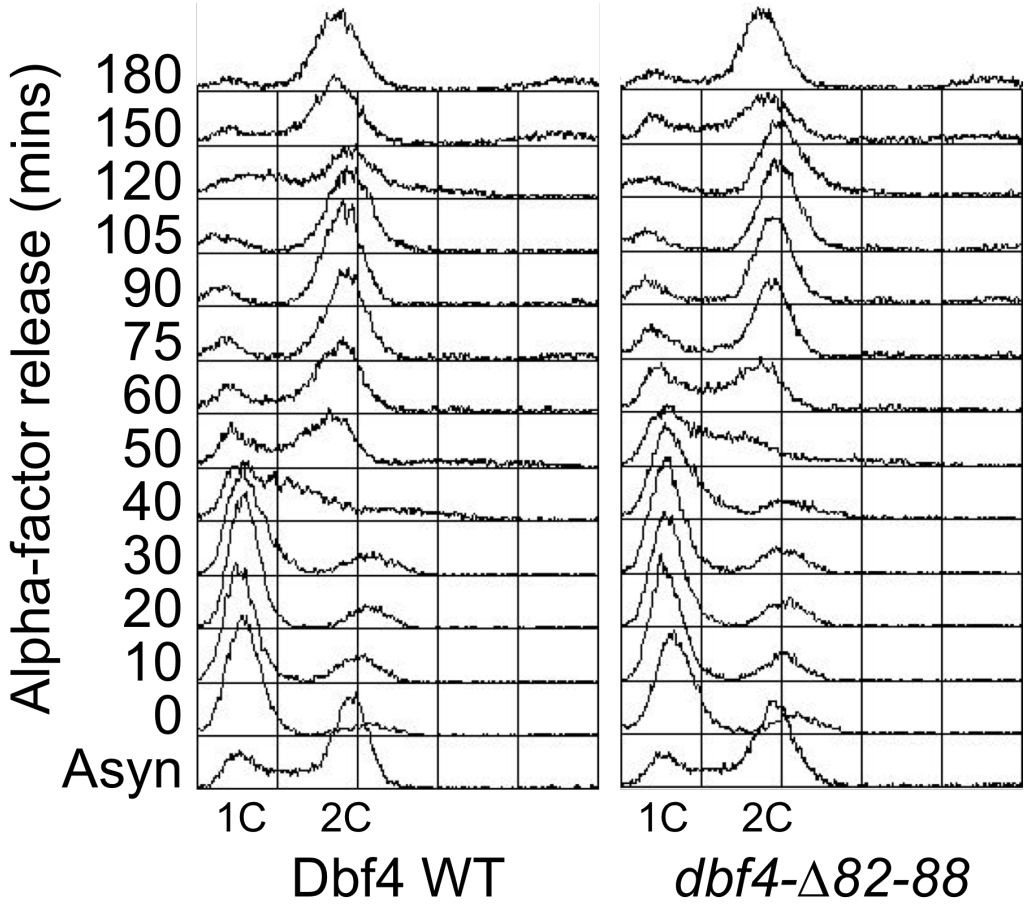


Figure 12. The *dbf4-*Δ*82-88* mutant exhibits normal cell cycle progression.

Wild-type W303- 1A (M138) and *dbf4-*Δ*82-88* (M2804) strains were arrested in G1 phase with alpha factor, and then released into cell cycle at 25oC. Samples were collected at indicated time points and analyzed by flow cytometry.



DISCUSSION

In this study, we determined the Dbf4 residues required for a physical interaction with Cdc5. This analysis revealed a novel Dbf4 sequence (83-RSIEGA-88) directly binds the PBD. Unlike most identified PBD-binding sites, the Dbf4-PBD interaction did not require Ser/Thr phosphorylation and notably, bound to a distinct binding surface within the PBD. Our results establish that Dbf4 residues 83-88 are critical for binding the polo-box domain and mediate an inhibition of Cdc5 function. Surprisingly, the ability of Cdc5 (PBD) to interact with phosphorylated substrates is apparently not required for normal yeast growth but is required for full MEN activation and for regulating spindle dynamics during mitosis.

An alternative mode of PBD binding

The polo-box domains of human Plk1 and yeast Cdc5 bind proteins containing phospho-serine or phospho-threonine consensus sites. In the co-crystal structure of the Plk1-PBD with a phosphorylated peptide, the peptide binds to a shallow pocket at the interface between two polo-box motifs, called PB1 and PB2 (Cheng et al. 2003; Elia et al. 2003b). Two highly conserved residues, H538 and K540 in PB2, directly contact the phosphate group. Further mutational and biological studies have confirmed that the PBD binds phosphorylated substrates *in vivo* and that the PBD is required for Plk1 function in human cells and in yeast, where it complements *CDC5* activity (Lee and Erikson 1997; Ouyang et al. 1997).

The optimal phosphopeptide binding motif containing Ser-[pSer/pThr]-[Pro/X] was described for Cdc5 substrates but only a few of these binding sequences have been characterized or mapped in detail (Yoshida et al. 2006; Lowery et al. 2007; Crasta et al. 2008). Although a subset of Cdc5 substrates examined in one study were found to be phosphorylated by Cdks (Lee et al. 2005), additional Cdc5 substrates might be primed by other kinases or by Cdc5 itself, as has been recently shown for Plk1 (Lee et al. 2008). In contrast, the PBD of *Drosophila* Polo was recently shown to mediate an interaction with the microtubule-associated protein Map205 without a requirement for Map205 phosphorylation (Archambault et al. 2008). In addition, the Cdc5 PBD can bind to Cdc14 independently of a consensus PBD recognition site (Rahal and Amon 2008).

We defined a unique Dbf4 sequence (83-RSIEGA-88) that directly interacts with the PBD. This Dbf4 sequence differs from the PBD consensus-binding sequence in two critical regards. Firstly, this sequence does not contain the absolutely conserved serine preceding a potential phospho-serine or -threonine residue. More importantly, serine phosphorylation is not required for the PBD interaction since mutation of S84 to alanine had little effect on PBD binding *in vitro* or *in vivo*. In fact, a peptide containing phosphorylated S84 lost the ability to compete the Dbf4-PBD interaction *in vitro* (Figure 5D). Therefore, S84 phosphorylation actually inhibited interaction with the PBD. If S84 phosphorylation occurs *in vivo*, this might negatively regulate the DDK-Cdc5 interaction. Whether a similar “RSIEGA” sequence exists in Cdc5 substrates remains to be determined, but our data strongly suggests that the PBD utilizes a second mode of interaction to bind non-phosphorylated proteins. The PBD-HK mutant protein bound

Dbf4 as the wild type, but was defective for interaction with the consensus (S-pS-P) Spc72 peptide. Mutation of six additional PBD residues (three of which mediate Plk1::phospho-peptide contacts in the co-crystal structure) disrupted the PBD-Spc72 canonical interaction but had no effect on the PBD-Dbf4 interaction (Figure 9). Furthermore, the Dbf4-PBD interaction was not competed by a phosphorylated consensus peptide (Figure 8B). These data indicate that the Cdc5 PBD can interact with proteins using two different binding surfaces, one that recognizes phosphorylated substrates and one that recognizes Dbf4.

Although the Plk1-HK mutant is defective for phospho-peptide binding *in vitro* and Plk1 activity *in vivo*, we found that the analogous *cdc5-H641A, K643M* mutant in budding yeast had a wild type growth rate and exhibited no temperature sensitivity (Figure 10A, B). This mutant instead exhibited a G2/M delay by flow cytometry, increased resistance to benomyl, and had an elongated spindle phenotype. These data suggest that Cdc5-HK protein is defective for interactions that restrain spindle elongation or that promote spindle disassembly. Since spindle disassembly follows Cdc5 activation of the MEN (Stegmeier and Amon 2004), a defect in MEN activation could account for the longer spindle length in the mutant. The synthetic lethality of *cdc5-HK* with *cdc15* or *dbf2* ts mutants supported the idea that *cdc5-HK* has a defect in MEN activation. However, arguing against the MEN defect *per se* causing increased resistance to benomyl, we found that the *cdc5-5* and *cdc15-4* MEN mutants had similar or greater sensitivity to benomyl than wild type. The *cdc5-1* and *cdc5-2* mutants also exhibited a greater sensitivity to benomyl than wild type (data not shown). The wild type growth rate and

unique resistance to benomyl argue that the HK mutation caused another defect in Cdc5 activity and that *cdc5-HK* was not simply another hypomorphic MEN mutant. The increased rate of spindle elongation in the mutant compared to the wild type following release of arrested G2/M cells strongly suggests that the *cdc5-HK* mutant has altered spindle dynamics. These data indicate that the pincer residues are not required for the essential function of Cdc5 and since the PBD-HK mutant does not bind phospho-peptides *in vitro*, strongly suggest that PBD interactions with phospho-proteins are not essential in yeast.

Dbf4 is a scaffold for Cdc5 inhibition

When a Cdc5-Dbf4 two-hybrid interaction was first described it was proposed that Cdc5 might have a novel role in DNA replication, and that Dbf4 possibly functioned as a scaffold between these two essential kinases (Hardy and Pautz 1996). Although Cdc5 is absent in G1 and early S-phase phase (Charles et al. 1998; Cheng et al. 1998; Shirayama et al. 1998), it could potentially influence DNA replication during the preceding mitosis. However when cells are released from a G1 block in the absence of Cdc5 expression, DNA synthesis occurs on schedule and cells arrest in telophase with segregated chromatids (Hu et al. 2001; Yoshida et al. 2006; Liang and Wang 2007). The *Xenopus* Plk1 ortholog, Plx1, was recently shown to influence DNA replication in response to DNA damage raising the possibility that additional Polo orthologs might have a similar role (Trenz et al. 2008). We recently proposed that DDK inhibits Cdc5 function during mitotic exit through a direct Dbf4-Cdc5 interaction (Miller et al. 2009). Here we show that multiple *dbf4* mutants defective the Dbf4-Cdc5 interaction suppress

the *cdc5-1* temperature sensitivity. Furthermore, increased Dbf4 expression is lethal to *cdc5-1* cells at the permissive temperature but only if Dbf4 can bind to Cdc5 (Figure 11D). These data indicate that Dbf4 inhibits Cdc5 function by direct association with the PBD. Since DDK phosphorylates Cdc5 *in vitro* (Miller et al. 2009), these findings suggest that Dbf4 may serve as scaffold in late S-phase so that Cdc7 kinase can inhibit Cdc5 by phosphorylating Cdc5 or an essential Cdc5 substrate. It is also possible that Dbf4 inhibits Cdc5 simply by binding to the PBD to prevent access to essential mitotic substrates, since overproduction of a Dbf4 N-terminal peptide is lethal to *cdc5-1* (Figure 11E, and ref (Miller et al. 2009)).

The DDK regulation of Cdc5 is not required for cell division control under normal conditions, since cell cycle progression occurs normally in *dbf4* mutants defective for the Cdc5 interaction in otherwise wild-type cells (Figure 12 and ref (Gabrielse et al. 2006)). This suggests redundant mechanisms to delay Cdc5 activation until anaphase onset. For instance, Kin4 kinase antagonizes Cdc5 function when mitotic spindle positioning errors occur, although this may occur through inhibitory phosphorylation of Cdc5 substrates and not direct Cdc5 phosphorylation (D'Aquino et al. 2005; Pereira and Schiebel 2005). DNA damage and the Rad53 checkpoint kinase also block mitotic exit by directly or indirectly affecting Cdc5 activity (Sanchez et al. 1999; Liang and Wang 2007). Interestingly, the *dbf4-NΔ109* deletion mutant that lacks the Polo binding site is synthetically lethal with *rad53-1* (Gabrielse et al. 2006) raising the possibility that Dbf4 and Rad53 have redundant, but together essential, roles to inhibit Cdc5 during an unperturbed cell cycle.

In summary, we have uncovered a novel PBD binding motif in Dbf4 that may be conserved in other PBD binding proteins. We suggest that DDK uses this unique motif to bind Cdc5 and inhibit its essential function in the mitotic cell cycle. Presumably the Dbf4-PBD interaction does not preclude binding to substrates containing a phosphorylated consensus site based on our peptide competition studies. Therefore, the DDK-Cdc5 ternary complex could in principal interact with Cdc5 substrates containing a phospho-PBD consensus sequence. This model agrees with the finding that DDK and Cdc5 interact during meiosis and that both proteins phosphorylate the Cdc5 substrate Mam1 to promote monopolar spindle orientation during meiosis I (Matos et al. 2008). Defining the Dbf4-PBD physical interaction allows a rigorous investigation of how DDK regulates mitotic and meiotic events.

MATERIALS AND METHODS

Construction of Yeast Strains, Plasmids and Baculoviruses

Strains and plasmids used in this study are listed in Tables 1 and 2. PJ69-4a cells (*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met::GAL7-lacZ*) were used for two-hybrid experiments. All other strains were derivatives of W303 (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3*). The *CDC5* shuffle stain M1672 (*cdc5Δ::kanMX6*/pMW536 [*CDC5 URA3 ARS-CEN*]) was constructed using the same procedure for the *DBF4* shuffle strain, M895, as previously described (Gabrielse et al. 2006). To integrate *dbf4* mutants, *HindIII-XbaI* fragments containing full length *dbf4-Δ82-88* or *dbf4-R83E* were co-transformed into M895 (*dbf4Δ::kanMX6*/pMW490 [*DBF4 URA3 ARS-CEN*]) together with pRS415. Leu-positive transformants were replica plated on FOA. Multiple FOA resistant colonies were recovered to YPD plates and then tested on YPD plates containing 0.2 mg/ml Geneticin to score loss of the *kanMX6* marker. The resulting Geneticin-sensitive candidates were confirmed as correct recombinants following PCR amplification of the *DBF4* locus and then backcrossed to W303. The epitope-tagged Cdc5 strains were made by the method of Longtine (Longtine et al. 1998).

Deletions and point mutations within *DBF4* and *CDC5* were generated by site-directed mutagenesis using the QuikChange system (Stratagene). PCR amplified *NcoI-PstI* fragments containing the full-length *DBF4* coding sequence or various *dbf4* mutants were cloned into the same sites of pGBKT7 (Clonetech) to give the Gal_{DBD}-Dbf4 fusions. *CDC5* (-332 to +2360) was PCR amplified from genomic DNA and cloned into the

HindIII-XbaI sites of pRS415 and pRS416 to give pMW535 and pMW536, respectively. Spc72 residues 1-400 were PCR amplified from genomic DNA and cloned into the *Nde*I-*Bam*HI sites of pGBKT7 to give pYJ356. For high-copy number plasmids, *HindIII-NotI* fragments containing entire WT *DBF4* or various *dbf4* mutants were cloned into the same sites of pRS425. Cdc5 residues 357-705 were cloned on a *Bam*HI-*Xho*I into pET24a-GST (gift of Eric Xu, Van Andel Research Institute) for expression of His_6-GST-PBD.

Construction of baculovirus plasmids encoding WT Dbf4, Dbf4-NΔ109, HA-Cdc7, and 3Myc-Cdc5 was previously described (Gabrielse et al. 2006). An *NcoI-NotI* fragment containing *dbf4-Δ82-88* was cloned in the baculovirus transfer vector, pAcSG2. High-titer baculoviruses were generated by transfection of Sf9 cells using the BaculoGold kit (BD Biosciences) followed by plaque purification and virus amplification.

Growth Conditions, Cell Cycle Synchronization, and Immunofluorescence Cells were cultured in YPD (1% yeast extract, 2% bacto peptone and 2% glucose). Synthetic Complete Medium (SCM) (Sherman et al. 1986) was supplemented with 5% glucose or 2.5% galactose. Benomyl (Sigma) was added directly to plates immediately before pouring (final 0.2% DMSO (v/v)). Synchronous G1 or G2/M cultures were obtained after addition of 5μg/ml of alpha-factor or 15mg/ml nocodazole, respectively, for 3 hours. DNA content was analyzed by flow cytometry as previously described (Weinreich and Stillman 1999). Tubulin and DAPI staining was previously described (Soues and Adams

1998). Spindle length was measured by 60x objective using a Nikon Eclipse TE300 fluorescence microscope and OpenLab version 3.1.7 image analysis software.

Two-hybrid Analysis

Various *DBF4* bait constructs containing Gal4 DNA binding domain (DB) were transformed with pGAD-Cdc5.3 (Gal activation domain (AD) fusion to $Cdc5_{357-705}$) in PJ69-4a and selected on SCM plates lacking tryptophan and leucine. These were spotted at ten-fold serial dilutions on the same plates and also on plates also lacking histidine but containing 2 mM 3-aminotriazole (3AT) at 30°C and cultured for 2-3 days.

Yeast Whole-cell Extracts, IP from Sf9 cells, and Western Blotting

Yeast protein extracts were prepared for Western blotting by trichloroacetic acid extraction (Foiani et al. 1994). Blots were probed in phosphate-buffered saline containing 0.1% Tween containing 1% dried milk. Dbf4 bait constructs contained a Myc tag were detected using anti-Myc monoclonal antibody (9E10, 1:2000) followed by anti-mouse-HRP secondary antibody. Sf9 cells were co-infected with HA-Cdc7, 3Myc-Cdc5 and Dbf4 mutants as previously described (Gabrielse et al. 2006). Whole cell extracts and IPs were probed with polyclonal antibodies against Cdc7 (1:4000) and Dbf4 (1:1000). 3Myc-Cdc5 was detected with 9E10.

Protein Purification and Peptide Binding Assays

His_6-GST-Cdc5 (PBD) was induced in BL21 cells for 3 hours at 30°C using 0.5 mM IPTG. Cells were sonicated in PBS containing 1% Triton X-100 and GST proteins were

purified from soluble extracts by binding to glutathione-agarose (Amersham) and eluted in the buffer (20 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA and 10% glycerol) containing 5mM glutathione and dialyzed against 50 mM MOPS (pH 7.4), 100 mM NaCl, and 10% glycerol.

Dbf4 peptide-PBD binding was quantitated using the AlphaScreen luminescence proximity assay (PerkinElmer Life Sciences) using a histidine detection kit as described (Li et al. 2008). Binding mixtures containing 50 nM N-terminally biotinylated Dbf4 peptide (Biotin-EKKRARIERARSIEGAVQVSKGTG), 50 nM 6_{His}-GST-PBD, 15 µg/ml of streptavidin-coated donor beads, and Ni-chelate-coated acceptor beads, were incubated in buffer containing 50 mM MOPS (pH 7.4), 100 mM NaCl, 0.1 mg/ml BSA for 1 h. Luminescence was recorded in a 384-well plate using an Envision 2104 plate reader (PerkinElmer Life Sciences). For competition assays, titrated unlabeled peptides were added and incubated at room temperature for 1 hour before measurement. Nonlinear regression as implemented in Prism 5.0 (GraphPad Software, San Diego) was used to fit the data to a variable slope dose-response inhibition equation to determine IC_{50} values. All peptides used in this study are listed in Table 3.

Table 1. Plasmids

Plasmid	Description	Source
pAcSG2		BD Biosciences
pCG10	pRS415-*DBF4*$_{110-704}$	Gabrielse et al., 2006
pCG40	pAcSG2-*DBF4*$_{110-704}$	Miller et al., 2009
pCG53	pGBKT7-*Dbf4*$_{66-227}$	Miller et al., 2009
pCG60	pCG53 *ADH1 promoter-Δ(-732)-(-802)*	Miller et al., 2009
pCG74	pGBKT7-*Dbf4*$_{110-704}$	Miller et al., 2009
pCG162	pRS416-*pGAL1,10*	Miller et al., 2009
pCG166	pCG162-*DBF4*$_{1-225}$	Miller et al., 2009
pCG213	pCG162-*DBF4*$_{1-225\ \Delta 82-88}$	Miller et al., 2009
pCM1	pGAD-C1-*CDC5*$_{421-705}$ H641A K643M	This study
pCM16	pAcSG2-3myc-*CDC5*$_{65-705}$	Miller et al., 2009
pET24a-GST		Eric Xu, Van Andel Institiute, MI
pGAD-C1		James et al. 1996
pGAD-Cdc5.3	pGAD-C1-*CDC5*$_{421-705}$	Miller et al., 2009
pGBKT7		Clontech
pJK17	pGAD-Cdc5.3 Y618H	This study
pMW1	pAcPK30-*DBF4*	Gabrielse et al., 2006
pMW47	pAcSG2-HAHIS6-*CDC7*	Gabrielse et al., 2006
pMW489	pRS415-*DBF4*$_{1-704}$	Gabrielse et al., 2006
pMW490	pRS416-*DBF4*$_{1-704}$	Gabrielse et al., 2006
pMW526	pRS415-*DBF4*$_{66-704}$	Gabrielse et al., 2006
pMW535	pRS415-*CDC5*$_{1-705}$	This study
pMW536	pRS416-*CDC5*$_{1-705}$	This study
pMW541	pMW535 H641A K643M	This study
pRS415	*LEU2 ARS-CEN*	Sikorski and Hieter, 1989
pRS416	*URA3 ARS-CEN*	Sikorski and Hieter, 1989
pRS425	*LEU2 2μm*	Sikorski and Hieter, 1989
pYJ1	pCG60-*DBF4*$_{72-227}$	This study
pYJ2	pCG60-*DBF4*$_{77-227}$	This study
pYJ3	pCG60-*DBF4*$_{82-227}$	This study
pYJ4	pCG60-*DBF4*$_{88-227}$	This study
pYJ5	pCG60-*DBF4*$_{94-227}$	This study
pYJ6	pCG60-*DBF4*$_{100-227}$	This study
pYJ7	pCG60-*DBF4*$_{104-227}$	This study
pYJ8	pCG60-*DBF4*$_{108-227}$	This study
pYJ9	pCG60-*DBF4*$_{110-227}$	This study
pYJ10	pCG60 R83E S84A	This study

Table 1. (cont'd)

pYJ11	pCG60 I85A E86K	This study
pYJ13	pCG60 S92A K93E	This study
pYJ14	pCG60 V89A Q90A V91A	This study
pYJ15	pCG60 S84A	This study
pYJ16	pCG60 R83A S84A	This study
pYJ17	pCG60 I85A E86A	This study
pYJ18	pCG60 G87A A88V	This study
pYJ19	pMW489 V89A Q90A V91A	This study
pYJ20	pMW489 S92A K93E	This study
pYJ21	pMW489 I85A E86K	This study
pYJ22	pCG60-*DBF4*$_{66\text{-}96}$	This study
pYJ26	pCG60 A82V	This study
pYJ28	pCG60 R83A	This study
pYJ30	pCG60 R83E	This study
pYJ32	pCG60 I85A	This study
pYJ33	pCG60 E86K	This study
pYJ34	pCG60 G87A	This study
pYJ36	pCG60 A88V	This study
pYJ38	pCG60-*DBF4*$_{66\text{-}227\ \Delta 82\text{-}88}$	Miller et al., 2009
pYJ40	pCG60 E86A	This study
pYJ46	pMW489 R83E S84A	This study
pYJ47	pYJ22-*DBF4*$_{72\text{-}96}$	This study
pYJ49	pYJ22-*DBF4*$_{88\text{-}96}$	This study
pYJ53	pMW489 S84A	This study
pYJ56	pMW489 G87A A88V	This study
pYJ59	pYJ22-*DBF4*$_{77\text{-}96}$	This study
pYJ61	pYJ22-*DBF4*$_{66\text{-}96\ \Delta 82\text{-}88}$	This study
pYJ65	pMW489 A82V	This study
pYJ67	pMW489 I85A	This study
pYJ68	pMW489 G87A	This study
pYJ74	pMW489-*DBF4*$_{\Delta 82\text{-}88}$	This study
pYJ79	pMW489 A88V	This study
pYJ83	pET24a-GST-*CDC5*$_{357\text{-}705}$	This study
pYJ84	pMW489 R83E	This study
pYJ88	pMW489 E86K	This study
pYJ100	pCG60-*DBF4*$_{66\text{-}227\ \Delta 82\text{-}96}$	This study
pYJ111	pMW489 R83A	This study
pYJ114	pMW489 E86A	This study
pYJ123	pCG60 S84E	This study
pYJ124	pMW489 S84E	This study
pYJ126	pYJ22-*DBF4*$_{66\text{-}96\ 78\text{-}81x4A}$	This study

Table 1. (cont'd)

pYJ128	pCG60-*DBF4*$_{66\text{-}227\ 78\text{-}81x4A}$	This study
pYJ136	pCG60 V89A	This study
pYJ137	pCG60 Q90A	This study
pYJ139	pCG60 G94A T95A G96A	This study
pYJ141	pMW489 V89A	This study
pYJ143	pMW489 G94A T95A G96A	This study
pYJ145	pMW489 Q90A	This study
pYJ148	pMW489 S92A	This study
pYJ150	pRS425-*DBF4*$_{110\text{-}704}$	Miller et al., 2009
pYJ152	pRS425-*DBF4*$_{1\text{-}704\ \Delta 82\text{-}88}$	Miller et al., 2009
pYJ153	pRS425-*DBF4*$_{1\text{-}704\ G87A\ A88V}$	This study
pYJ154	pRS425-*DBF4*$_{1\text{-}704}$	Miller et al., 2009
pYJ157	pRS425-*DBF4*$_{66\text{-}704}$	This study
pYJ160	pRS425-*DBF4*$_{1\text{-}704\ R83E}$	This study
pYJ165	pCG60 S92A K93A	This study
pYJ167	pCG60 S92A	This study
pYJ169	pCG60 K93E	This study
pYJ171	pMW489 K93E	This study
pYJ174	pRS425-*DBF4*$_{1\text{-}704\ E86K}$	This study
pYJ182	pAcSG2-*DBF4*$_{1\text{-}704\ \Delta 82\text{-}88}$	This study
pYJ189	pRS425-*DBF4*$_{66\text{-}704\ \Delta 82\text{-}88}$	This study
pYJ193	pMW489-*DBF4*$_{\Delta 76\text{-}109}$	This study
pYJ195	pMW489-*DBF4*$_{\Delta 82\text{-}109}$	This study
pYJ198	pMW489-*DBF4*$_{\Delta 66\text{-}109}$	This study
pYJ201	pMW489-*DBF4*$_{66\text{-}704\ \Delta 82\text{-}88}$	This study
pYJ204	pGBKT7-*Dbf4*$_{1\text{-}704}$	Miller et al., 2009
pYJ206	pGBKT7-*Dbf4*$_{1\text{-}704\ \Delta 82\text{-}88}$	Miller et al., 2009
pYJ210	pGBKT7-*Dbf4*$_{1\text{-}704\ \Delta 66\text{-}109}$	This study
pYJ211	pGBKT7-*Dbf4*$_{1\text{-}704\ \Delta 76\text{-}109}$	This study
pYJ212	pGBKT7-*Dbf4*$_{1\text{-}704\ \Delta 82\text{-}109}$	This study
pYJ215	pGBKT7-*Dbf4*$_{66\text{-}704\ \Delta 82\text{-}88}$	Miller et al., 2009
pYJ218	pMW489-*DBF4*$_{\Delta 89\text{-}109}$	This study
pYJ221	pMW489-*DBF4*$_{\Delta 100\text{-}109}$	This study
pYJ222	pMW489-*DBF4*$_{\Delta 94\text{-}109}$	This study
pYJ231	pMW489 S92A K93A	This study
pYJ236	pMW489-*DBF4*$_{\Delta 89\text{-}93}$	This study
pYJ237	pMW489-*DBF4*$_{\Delta 89\text{-}91}$	This study
pYJ238	pMW489-*DBF4*$_{\Delta 91\text{-}93}$	This study
pYJ260	pRS425-*DBF4*$_{1\text{-}704\ S84A}$	This study
pYJ263	pRS425-*DBF4*$_{1\text{-}704\ S84E}$	This study
pYJ272	pRS425-*DBF4*$_{1\text{-}704\ V89A}$	This study

Table 1. (cont'd)

pYJ274	pRS425-*DBF4*$_{1\text{-}704\ Q90A}$	This study
pYJ276	pRS425-*DBF4*$_{1\text{-}704\ S92A}$	This study
pYJ278	pRS425-*DBF4*$_{1\text{-}704\ K93E}$	This study
pYJ292	pCG166 R83E	This study
pYJ294	pCG166 S84A	This study
pYJ296	pCG166 S84E	This study
pYJ297	pCG166 E86A	This study
pYJ298	pCG166 E86K	This study
pYJ302	pCG60-*DBF4*$_{66\text{-}227\ \Delta 82\text{-}93}$	This study
pYJ303	pMW489-*DBF4*$_{\Delta 82\text{-}93}$	This study
pYJ314	pCM1 W517F	This study
pYJ316	pMW541 W517F	This study
pYJ326	pCG60-*DBF4*$_{66\text{-}227\ \Delta 89\text{-}93}$	This study
pYJ327	pCG60-*DBF4*$_{66\text{-}227\ \Delta 89\text{-}91}$	This study
pYJ328	pCG60-*DBF4*$_{66\text{-}227\ \Delta 92\text{-}93}$	This study
pYJ356	pGBKT7-Spc72$_{1\text{-}400}$	This study
pYJ365	pYJ83 H641A K643M	This study
pYJ368	pYJ83 W517F H641A K643M	This study
pYJ415	pCG60 V91A	This study
pYJ439	pGAD-Cdc5.3 F526L	This study
pYJ441	pGAD-Cdc5.3 K643N	This study
pYJ443	pGAD-Cdc5.3 R620S	This study
pYJ445	pGAD-Cdc5.3 T515A	This study
pYJ447	pGAD-Cdc5.3 V537A	This study

Table 2. Yeast strains

Stain	Genotype	Source
W303-1A	*MAT**a** ade2-1, ura3-1 his3-11, -15 trp1-1 leu2-3, -112 can1-100*	Thomas and Rothstein, 1989
PJ69-4A	*MAT**a** trp1-901 leu2-3, -112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al., 1996
M319	W303 *MAT**a** dbf2-1*	Miller et al., 2009
M331	W303 *MAT**a** cdc15-2*	Miller et al., 2009
M895	W303 *MAT**a** dbf4Δ::kanMX6* [pMW490; pRS416-*DBF4 URA3*]	Cabrielse at al., 2006
M1614	W303 *MAT**a** cdc5-1*	Miller et al., 2009
M1649	W303 *MAT**a** cdc14-1*	Miller et al., 2009
M1656	W303 *MAT**a** dbf4-NΔ109-kanMX6*	Miller et al., 2009
M1678	W303 *MAT**a** cdc5-2(msd2-1)-URA3*	This study
M1680	W303 *MAT**a** cdc5-5(msd2-4)-URA3*	This study
M1672	W303 *MAT**a** cdc5Δ::kanMX6* [pMW536; pRS416-*CDC5 URA3*]	This study
M1800	W303 *MAT**α** dbf4-NΔ109-kanMX6*	Miller et al., 2009
M1999	W303 *MAT**a** cdc15-4*	This study
M2600	W303 *MAT**a** cdc5-1 dbf4Δ::kanMX6* [pMW490; pRS416-*DBF4 URA3*]	This study
M2655	W303 *MAT**a** cdc5-1 dbf4-NΔ109-kanMX6*	Miller et al., 2009
M2657	W303 *MAT**α** cdc5-1 dbf4-NΔ109-kanMX6*	Miller et al., 2009
M2804	W303 *MAT**a** dbf4-Δ82-88-kanMX6*	Miller et al., 2009
M2805	W303 *MAT**α** dbf4-Δ82-88-kanMX6*	This study
M2806	W303 *MAT**a** dbf4-R83E-kanMX6*	This study
M2807	W303 *MAT**α** dbf4-R83E-kanMX6*	This study

Table 2. (cont'd)

M3112	W303 *MAT**a** cdc5-1 dbf4-∆82-88-kanMX6*	Miller et al., 2009
M3114	W303 *MAT**α** cdc5-1 dbf4-∆82-88-kanMX6*	This study
M3116	W303 *MAT**a** cdc5-1 dbf4-R83E-kanMX6*	This study
M3117	W303 *MAT**α** cdc5-1 dbf4-R83E-kanMX6*	This study
M3376	W303 *MAT**a** cdc5∆::kanMX6* [pMW535; pRS415-*CDC5 LEU2*]	This study
M3377	W303 *MAT**a** cdc5∆::kanMX6* [pMW541; pRS415-*cdc5-H641A-K643M LEU2*]	This study
M3378	W303 *MAT**a** cdc5∆::kanMX6* [pYJ314; pRS415-*cdc5-W517F-H641A-K643M LEU2*]	This study
M3486	W303 *MAT**a** cdc5-H641A-K643M*	This study
M3490	W303 *MAT**a** TAB6-1*	D'Aquino et al., 2005
M3502	W303 *MAT**a** cdc5-H641A-K643M-kanMX6*	This study
M3526	W303 *MAT**a** TAB6-1-TRP1*	This study

Table 3. Peptides

Peptide name	Abbr.	Peptide sequence	Length	MW
Biotin-Dbf4 73-96	Biotin-p	Biotin-EKK RAR IER ARS IEG AVQ VSK GTG	Biotin + 24	2854
Dbf4 73-96	p1	EKK RAR IER ARS IEG AVQ VSK GTG	24	2627.9
Dbf4 78-96	p2	RIE RAR SIE GAV QVS KGT G	19	2015.3
Dbf4 78-93	p3	RIE RAR SIE GAV QVS K	16	1799.2
Dbf4 80-93	p4	ERA RSI EGA VQV SK	14	1530
Dbf4-R83E	R83E	ERA ESI EGA VQV SK	14	1503.1
Dbf4-S84A	S84A	ERA RAI EGA VQV SK	14	1514.1
Dbf4-pS84	pS84	ERA R(pS)I EGA VQV SK	14	1610.1
Dbf4-I85A	I85A	ERA RSA EGA VQV SK	14	1487.7
Dbf4-E86K	E86K	ERA RSI KGA VQV SK	14	1529.2
Dbf4-GA87AV	GA87AV	ERA RSI EAV VQV SK	14	1572.2
Biotin-Spc72	Biotin-Spc72	Biotin-EEF LSL AQS (pS)PA GSQ LES RD	Biotin + 20	2457.6
Spc72	Spc72	EEF LSL AQS (pS)PA GSQ LES RD	20	2231.3

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CHAPTER 3

RAD53 BINDS DBF4 THROUGH AN N-TERMINAL T-X-X-E MOTIF AND THIS INTERACTION IS REQUIRED TO SUPPRESS LATE ORIGIN FIRING

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RAD53 BINDS DBF4 THROUGH AN N-TERMINAL T-X-X-E MOTIF AND THIS INTERACTION IS REQUIRED TO SUPPRESS LATE ORIGIN FIRING

ABSTRACT

Cdc7-Dbf4 (DDK) and cyclin dependent kinase (CDK) are essential to initiate DNA replication at individual origins. During replication stress, the S-phase checkpoint inhibits the DDK- and CDK-dependent activation of late replication origins. The Rad53 kinase is a central effector of the replication checkpoint, and both binds to and phosphorylates Dbf4 to prevent late origin firing. The molecular basis for the Rad53-Dbf4 physical interaction is not clear but occurs through the Dbf4 N-terminus. Here, we have characterized the molecular interaction between Dbf4 and Rad53. Surprisingly, both Rad53 FHA domains bind Dbf4 through the same N-terminal T^{105}-x-x-E motif, which closely resembles an optimal pT-x-x-D FHA1 binding site. This sequence precedes a conserved BRCT domain in Dbf4, which is also required for the interaction with Rad53. The Rad53 FHA1 domain correspondingly binds pT-x-x-E (but not T-x-x-E) Dbf4 peptides *in vitro*. Abrogation of the Rad53-Dbf4 physical interaction allows late origin firing during replication checkpoint activation. One model to explain these data is that activated Rad53 uses both its FHA domains to bind two separate pT^{105}-x-x-E sequences within a Dbf4 multimer. Rad53-Dbf4 docking then allows Rad53 to phosphorylate Dbf4 at critical C-terminal sites, which in turn block late origin firing during periods of genome stress.

INTRODUCTION

The fidelity of chromosome replication depends on checkpoint mechanisms to stabilize stalled forks, regulate origin activation, and repair DNA damage (Hartwell and Weinert 1989; Bartek et al. 2004; Segurado and Tercero 2009). In response to replication stress, the replication checkpoint maintains replisome stability and prevents late origins from firing, which allows time for DNA repair and the completion of DNA replication prior to chromosome segregation. Incomplete DNA replication or uncoordinated origin firing following DNA damage can result in genomic instability, cancer predisposition, and premature aging (Branzei and Foiani 2010).

In the budding yeast *Saccharomyces cerevisiae*, activation of the checkpoint sensor kinase, Mec1 (vertebrate ATR), is triggered at stalled forks or sites of DNA damage (Majka et al. 2006; Labib and De Piccoli 2011). Subsequent signal amplification through the Mrc1 or Rad9 adaptors leads to activation of the checkpoint kinase Rad53 (the ortholog of the human tumor suppressor Chk2) (Branzei and Foiani 2009). Rad53 is an integral transducer of various cellular responses to replication stress or DNA damage. Rad53 induces a series of transcriptional responses through MBF-regulated genes (Bastos de Oliveira et al. 2012; Travesa et al. 2012) and also activates the Dun1 kinase, which promotes the expression of ribonucleotide reductase (RNR) subunits and additional DNA repair genes (Huang et al. 1998). In parallel, Rad53 down-regulates the RNR inhibitor Sml1 to increase deoxyribonucleotide levels and facilitate DNA synthesis (Zhao et al. 2001). In response to replication fork stalling, Rad53 prevents the activation of late replication origins by phosphorylating two proteins required for the initiation of

DNA replication: Dbf4 and Sld3 (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010; Duch et al. 2011). Dbf4 is the regulatory subunit of Cdc7 kinase, which is required for initiating DNA replication at individual origins by phosphorylating the replicative MCM helicase (Tsuji et al. 2006; Francis et al. 2009; Randell et al. 2010; Sheu and Stillman 2010). Sld3 is also required for activating the MCM helicase by promoting Cdc45-MCM association (Fu and Walter 2010; Boos et al. 2011).

Cdc7 requires the Dbf4 regulatory subunit for kinase activity. Dbf4 is expressed in late G1-phase, peaks during S-phase, and is present until early to mid-mitosis, when it is destroyed by ubiquitin-mediated proteolysis (Cheng et al. 1999; Weinreich and Stillman 1999; Ferreira et al. 2000; Miller et al. 2009), The timing of Dbf4 destruction suggests that Dbf4 has post-replicative functions. Indeed, recent work has shown that Dbf4 prevents premature exit from mitosis and also controls the segregation of homologous chromosomes in meiosis I by a direct interaction with Cdc5, the only Polo-like kinase in budding yeast (Matos et al. 2008; Miller et al. 2009; Chen and Weinreich 2010). Rad53-mediated phosphorylation of Dbf4 postpones late origin firing during replication stress (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010; Duch et al. 2011) but Cdc7-Dbf4 kinase activity is only reduced 2-fold by Rad53-dependent Dbf4 phosphorylation (Weinreich and Stillman 1999). It is clear that Dbf4 is an *in vivo* target of Rad53 and interacts with Rad53 (Kihara et al. 2000; Duncker et al. 2002; Matthews et al. 2012), but the molecular details of the Rad53-Dbf4 interaction and how Rad53 phosphorylation of Dbf4 prevents late origin activation are unclear.

Rad53 is unique in budding yeast in that it contains two FHA (fork-head associated) domains, termed FHA1 and FHA2, which flank a central kinase domain. FHA domains comprise a ubiquitous class of protein-protein interaction modules found in more than 200 different proteins from yeast to mammals (Mahajan et al. 2008). Structural studies show that FHA domains fold into a ß-sandwich composed of 6-stranded and 5-stranded ß sheets (Durocher et al. 2000). Four of the five most conserved residues in the domain are situated in substrate binding loops that selectively recognize a phosphorylated threonine (Liang and Van Doren 2008). Orientated peptide library screening identified consensus phospho-threonine peptides for the FHA1 and FHA2 domains and the structural basis of their interaction with the Rad53 FHA domains were also determined (Liao et al. 1999; Durocher et al. 2000; Byeon et al. 2001). The FHA1 domain preferentially binds peptides containing the consensus sequence pTxxD but the FHA2 domain prefers isoleucine at the +3 position, pTxxI.

Here we have mapped the Dbf4 residues required for a physical interaction with Rad53. We found that a short sequence from residues 100-109 that contained a potential FHA1 binding site consensus (T-x-x-E) and the adjacent BRCT (BRCA1 carboxyl-terminal) domain were both required for Rad53 binding. Interestingly, both Rad53 FHA domains were required to bind Dbf4 and depended on a critical threonine 105 residue, which differs from a previous report (Matthews et al. 2012). This suggested that the FHA1 and FHA2 domains bind to the same Dbf4 sequence containing a pT105 residue. Biochemical assays confirmed that the FHA1 domain bound to a Dbf4 pT^{105}-X-X-E peptide in a phosphorylation dependent manner. However the FHA2 domain did not

bind the same isolated peptide suggesting that additional contacts with Dbf4 are required for stable binding of the FHA2 domain. Lastly, abrogation of the Rad53-Dbf4 physical interaction blocked Dbf4 phosphorylation by Rad53 and allowed late origin firing in the presence of HU. We suggest that Dbf4 is phosphorylated on T105 and in response to replication fork arrest, the pT^{105}-x-x-E FHA1 binding site together with the BRCT domain cooperate to form a docking site for Rad53. The Rad53 physical interaction then promotes Dbf4 phosphorylation at critical downstream sites to inhibit late origin firing.

RESULTS

Rad53 interacts with a sequence preceding the Dbf4 BRCT domain

Dbf4 is a downstream substrate of the Rad53 kinase in the DNA replication checkpoint (Masai et al. 1999; Weinreich and Stillman 1999; Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010; Duch et al. 2011). In the presence of HU, Rad53 phosphorylates multiple sites within Dbf4 to inhibit the late origin firing. Our previous study showed that deletion of Dbf4 residues from 66-109 prevented Rad53-mediated Dbf4 phosphorylation in HU (Gabrielse et al. 2006), suggesting that these residues, which are N-terminal to a conserved BRCT domain, played a critical role in the Rad53-Dbf4 interaction.

We used a two-hybrid assay to map the Rad53 binding site within Dbf4. Using a series of Dbf4 N-terminal truncations we found that a deletion through residue 65 retained the Rad53-Dbf4 interaction (Figure 13A). However, further deletions to residue 109 (just prior to the BRCT domain) or to residue 221 resulted in a complete loss of Rad53 binding. The *dbf4-NΔ221* mutant was still capable of associating with Cdc7 through its C-terminal motifs M and C (Figure 13A and (Ogino et al. 2001; Harkins et al. 2009). In addition, Dbf4 residues 66-227 were sufficient to interact with Rad53 (Figure 13B). Therefore, Dbf4 residues 66-227 contain a separate domain (or domains) that interacts with the Rad53 kinase. These data also indicate that a sequence within Dbf4 residues 65-109, which is poorly conserved among Dbf4 orthologs (Masai and Arai 2000; Gabrielse et al. 2006), is required for the Rad53 interaction.

Figure 13. Mapping the interaction between Dbf4 and Rad53

(A) Deletion mutants in otherwise full-length Dbf4 were tested for a two-hybrid interaction with full-length Rad53. 10-fold serial dilutions of saturated cultures were spotted onto SCM-Trp-Leu plates to visualize total cells and SCM-Trp-Leu-His + 2mM 3AT plates to score the two-hybrid interaction. (B) The Dbf4 N-terminal fragment (residues 66-227) was sufficient for the Rad53 interaction and this interaction requires both the FHA domains. (C) Dbf4 residues 100-227 comprised the minimal region for Rad53 FHA1 domain binding. (D) Schematic of the features in Dbf4 are shown, including the Polo-like kinase (Cdc5) binding site, a conserved BRCT domain, motifs M and C, along with a summary of the Dbf4-FHA1 domain interaction.

Figure 13. (cont'd)

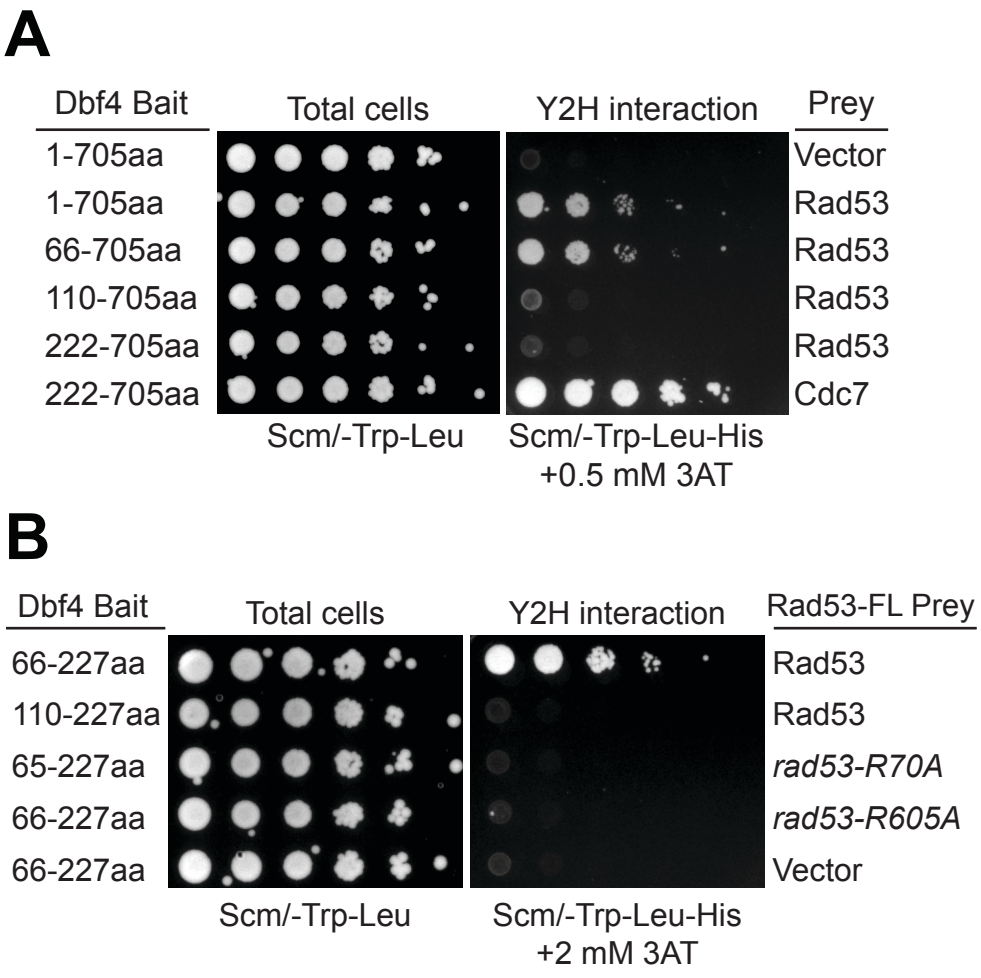


Figure 13. (cont'd)

C

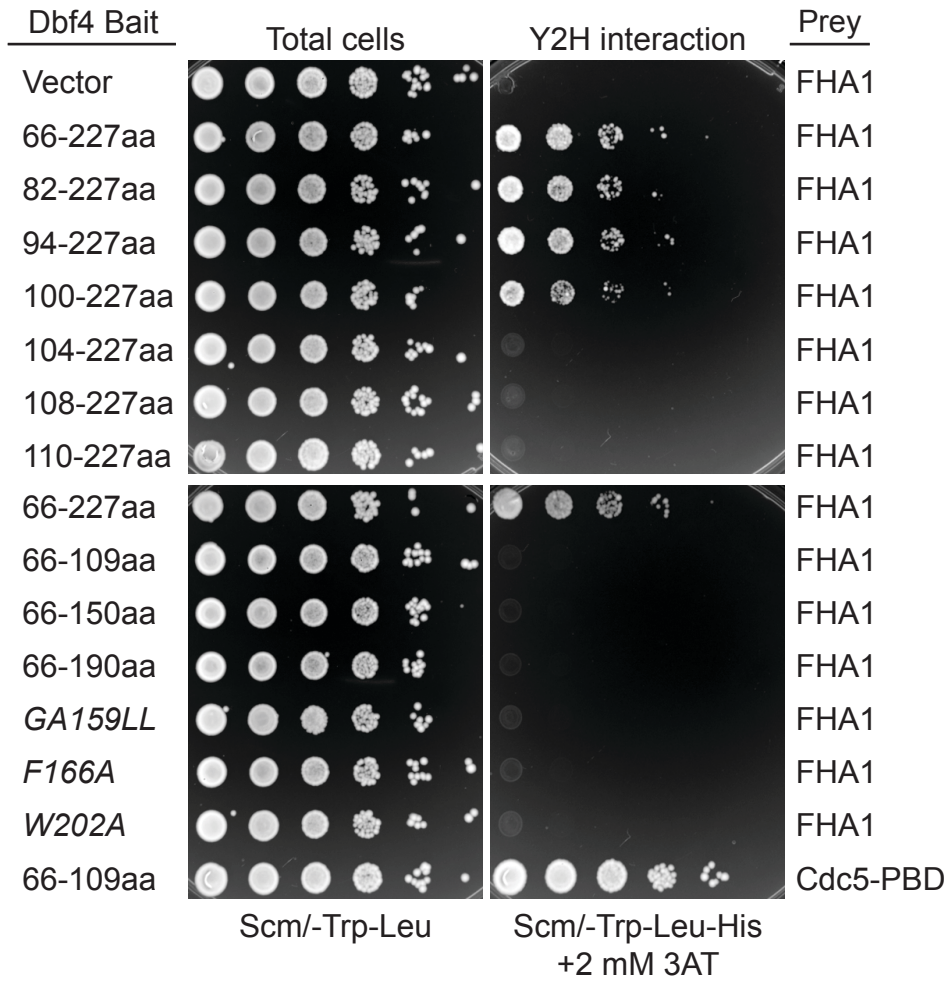


Figure 13. (cont'd)

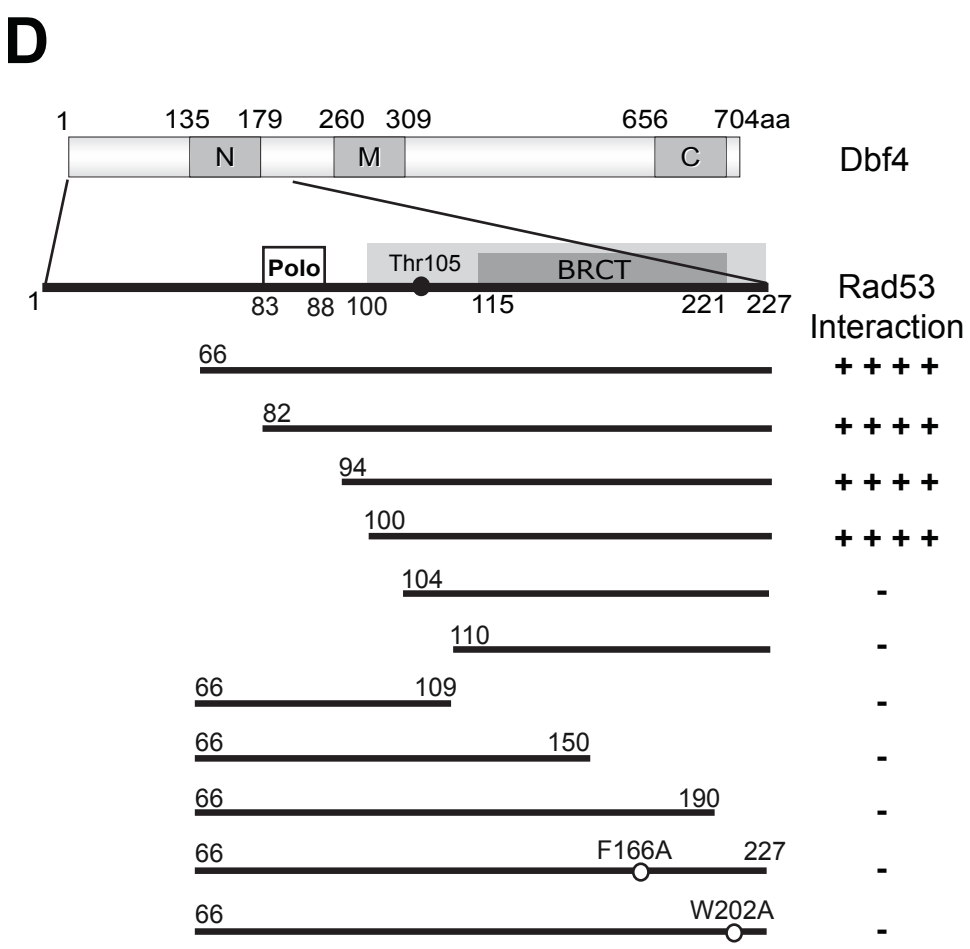


Figure 14. Analysis of FHA domain-Dbf4 interactions including a screen of all T/Y residues in Dbf4 residues 100-227.

(A) The indicated substitutions of Dbf4 threonines were assayed for a two-hybrid interaction using Dbf4 N-terminus (66-227) as bait with the Rad53 FHA2 domain by spotting serial dilutions of cultures onto the indicated media to visualize the total number of cells (left) and the two-hybrid interaction (right). (B and C) The indicated Dbf4 tyrosine mutants were assayed for a two-hybrid interaction with the Rad53 FHA1 (B) and FHA2 (C) domains. Although Y127A and Y204A mutants eliminate the binding of both FHA domains, there is no loss of binding by substituting the structurally similar but non-phosphorylatable amino acid, phenylalanine (Y127F and Y204F). (D) Two hybrid interaction data of the Dbf4 N-terminus (66-227) with all remaining FHA domains in the yeast genome, spotted as in (A). Dma1 (pJK135, 137-302aa), Dma2 (pJK137, 246-408aa), Dun1 (pJK275, 1-160aa), Far10 (pJK277, 61-227aa), Fhl1 (pJK279, 253-400aa), Fkh1 (pJK281, 41-185aa), Fkh2 (pJK287, 1-254aa), Mek1 (pJK283, 1-152aa), Pml1 (pJK289, 54-204), Xrs2 (pJK285, 1-125aa).

Figure 14. (cont'd)

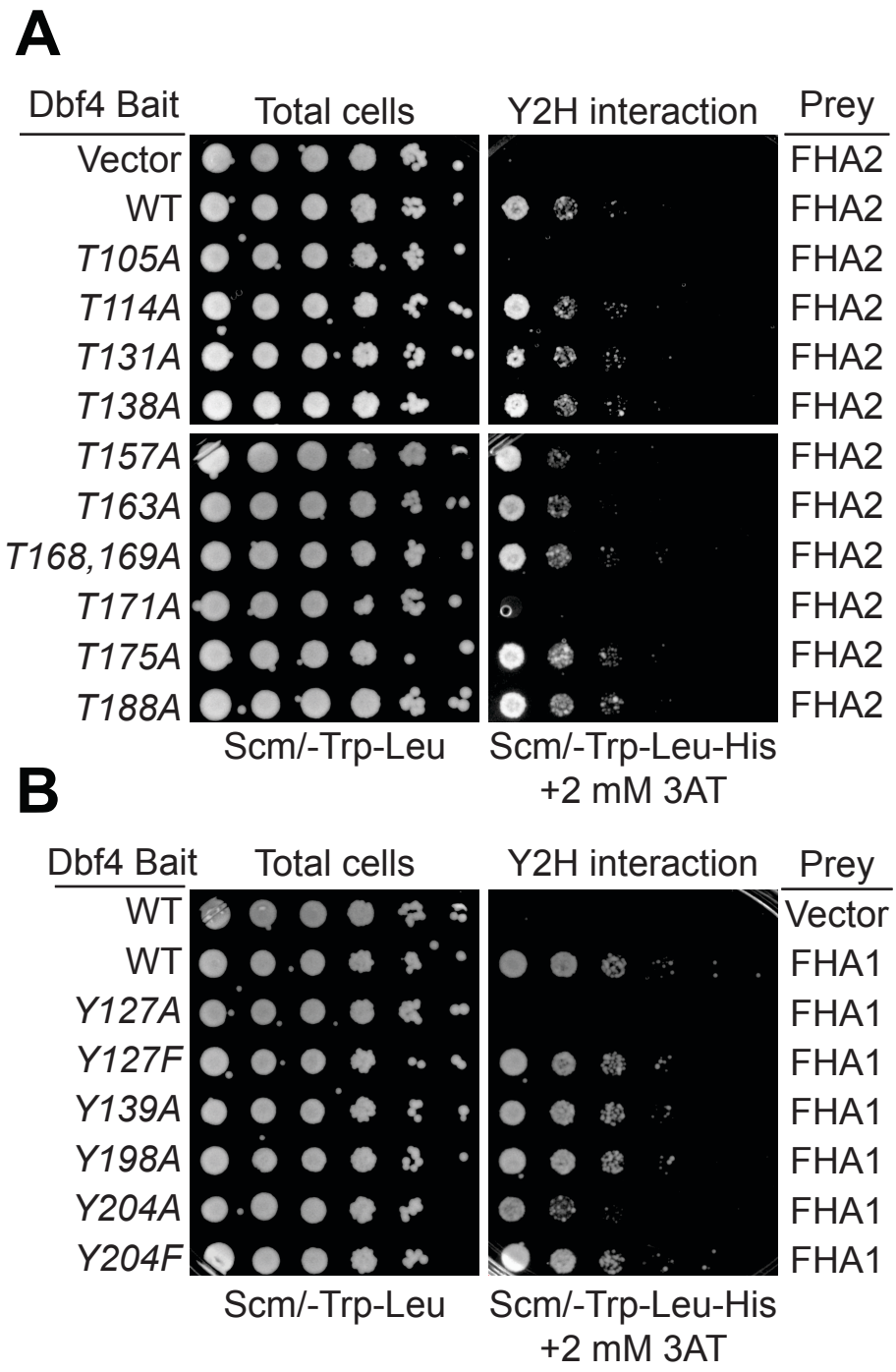
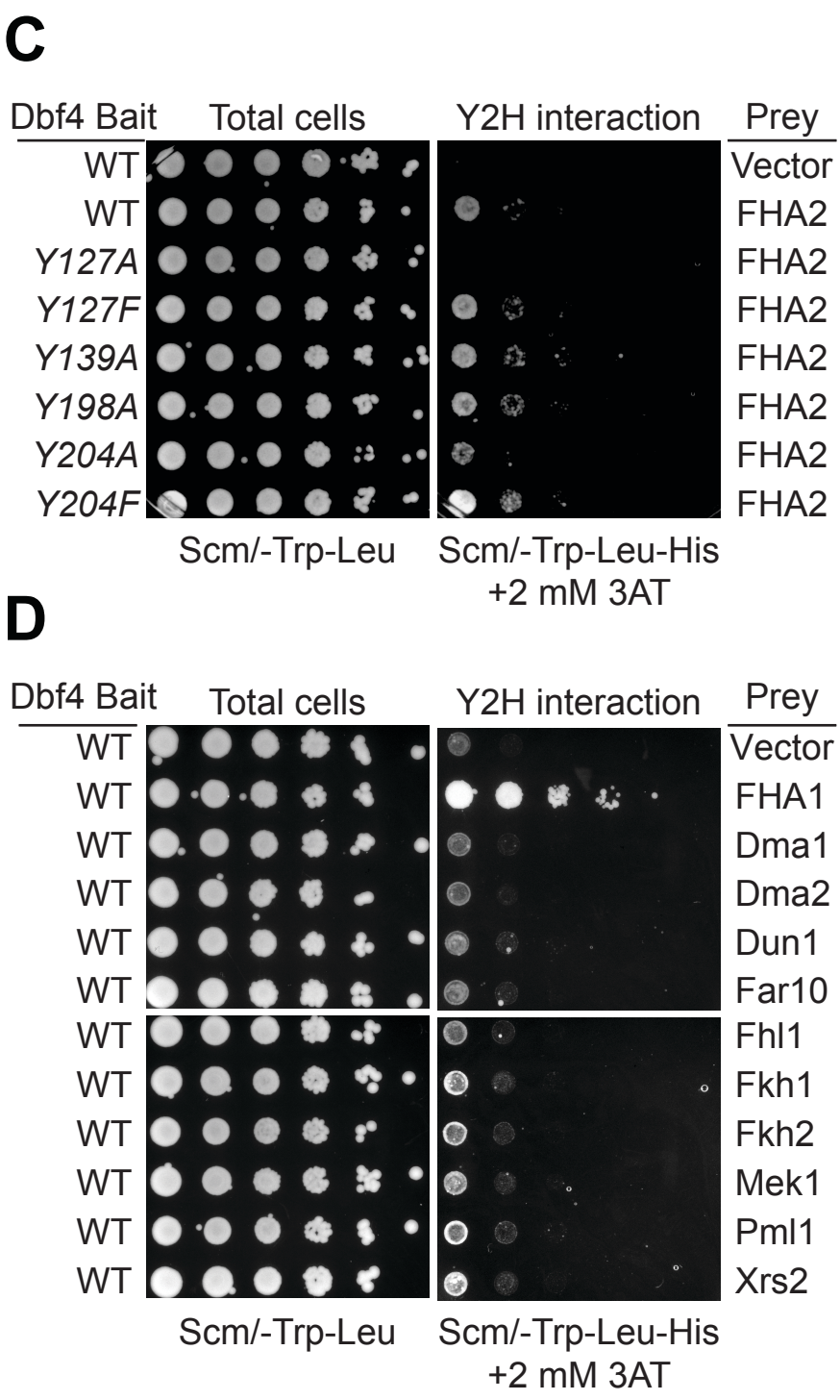


Figure 14. (cont'd)



Both Rad53 FHA domains are required to interact with the Dbf4 N-terminus

Although residues 66-227 are sufficient for the interaction with full-length Rad53, residues 110-227 are not (Figure 13B). So, the same residues required for binding Rad53 within full length Dbf4 are also required in this shorter N-terminal Dbf4 construct. Interestingly, both the FHA1 and FHA2 domains are required for Rad53 binding to the Dbf4 N-terminus (Figure 13B) or to full length Dbf4 (not shown). FHA domains are phospho-threonine specific protein binding modules and recognition of the pT residue requires a conserved arginine residue (Durocher et al. 2000; Byeon et al. 2001). Alanine substitutions of the corresponding arginine residues in the FHA1 and FHA2 domains (R70A and R605A, respectively) abolished the Dbf4 interaction (Figure 13B). These results not only indicate that Rad53 binding to the Dbf4 N terminus relies on both FHA domains, but also suggest that the Rad53-Dbf4 interaction is phosphorylation-dependent.

To identify the FHA binding sites in Dbf4, we first verified that the FHA1 (Figure 13C) and FHA2 (Figure 14A) domains could bind Dbf4 residues 66-227 independently. We then tested a series of deletion constructs within the 66-227 region for their ability to bind FHA1 and FHA2. Although Dbf4 constructs as short as 100-227 retained FHA binding, deletions beyond residue 100 completely lost FHA1 (and FHA2) binding. This indicates that a Dbf4 sequence following residue 100 is required for the FHA domain interactions. Although residues 66-109 preceding the BRCT domain are required for the Rad53 interaction (Figure 13A), residues 66-109 were not sufficient for the interaction with the FHA1 domain (Figure 13C). As a control, Dbf4 residues 66-109

were sufficient to interact with the Cdc5 Polo-box domain (PBD) (Figure 13C, bottom) as shown previously (Miller et al. 2009). Finally, the Dbf4-FHA domain interaction also required the Dbf4 BRCT domain comprising residues ~115-224. Any C-terminal deletion that affected the BRCT domain or point mutants in conserved BRCT residues (G159L, A160L, F166L, and W202A) disrupted the Dbf4-FHA domain interaction. To summarize, Dbf4 residues 100-227 comprise the minimal region required to bind Rad53 by a two-hybrid assay and mutation of residues within the BRCT domain or immediately preceding it abolish that interaction (Figure 13D).

Rad53 FHA domains recognize a T-x-x-E-L motif in the Dbf4 N-terminus

In orientated peptide library screens the Rad53 FHA1 and FHA2 domains were shown to selectively bind phospho-threonine (Durocher et al. 2000; Byeon et al. 2001). Therefore, we mutated each threonine to alanine within Dbf4 residues 100-227, i.e. the minimal Rad53 binding region we defined (Figure 14A and Figure 15A). We found that the T105A or T171A substitutions strongly impaired the Dbf4-FHA domain interaction. The surrounding sequences of these two threonines ($\underline{T}^{105}$-P-K-$\underline{E}$ and $\underline{T}^{171}$-I-V-$\underline{I}$) strongly resemble the binding consensus for FHA1 (pT-x-x-D) and FHA2 domains (pT-x-x-V/L), respectively (Durocher et al. 2000; Byeon et al. 2001). However, a recent crystal structure of the Dbf4 BRCT domain (Matthews et al. 2012), showed that the T^{171}-I-V-I sequence forms part of the hydrophobic core of the BRCT domain and is not solvent accessible (T171 is only partially buried). So although the T^{171}-I-V-I motif conforms to a typical FHA2 binding sequence (pT-x-x-V/L), this motif is buried and is therefore unlikely to interact with the FHA2 domain directly. However, T105 maps just prior to an alpha

helix adjacent to the BRCT domain and is solvent accessible. Based on two-hybrid data (described below), we suggest that the Rad53 FHA domains directly recognize T105.

We next determined the amino acids required for Rad53 binding between residues 100-114 using a series of point mutants. In addition to T105, we found that mutation of V104, E108, L109 and W112 disrupted FHA1 and FHA2 domain binding as summarized (Figure 15B; two-hybrid data in Figure 16). The V104A substitution disrupted the interaction but V104L had only a modest effect, suggesting a structural role or hydrophobic contact for this residue. The E108A mutation strongly impaired FHA binding and E108K abolished FHA binding. However, a conservative E108D mutation retained FHA binding, suggesting that glutamate and aspartate are interchangeable at the +3 position following T105. As expected for an FHA binding consensus site, the Dbf4 residues P106 and K107 at the +1 and +2 positions to T105 were not important for binding, strongly suggesting that T^{105}-x-x-E is a *bona fide* FHA1 binding site. Our site-direct mutagenesis studies also found that several hydrophobic residues nearby T105 are important. The loss of interaction caused by the W112A mutation can be rescued by substituting F, a bulky hydrophobic residue, suggesting that W112 plays a structural role for FHA domain binding. Indeed, W112 falls within an alpha helix preceding the BRCT domain and makes hydrophobic contacts with the BRCT domain (Matthews et al. 2012). However, L109 may be directly involved in FHA binding, since it is adjacent to E108 and neither the L109A nor L109V mutants interact with the FHA domains (Figure 15B).

Figure 15. The Rad53 FHA domains require a T^{105}-x-x-E-L motif in the Dbf4 N terminus for interaction

(A) An alanine scan of all Dbf4 threonines within the minimal Rad53 binding region (residues 100-227) using two-hybrid assays. (B) Summary of Dbf4 mutants within residues 100-114 for their effect on the interaction of FHA1 and FHA2 domains, respectively. The growth assays are shown in Figure 16.

Figure 15. (cont'd)

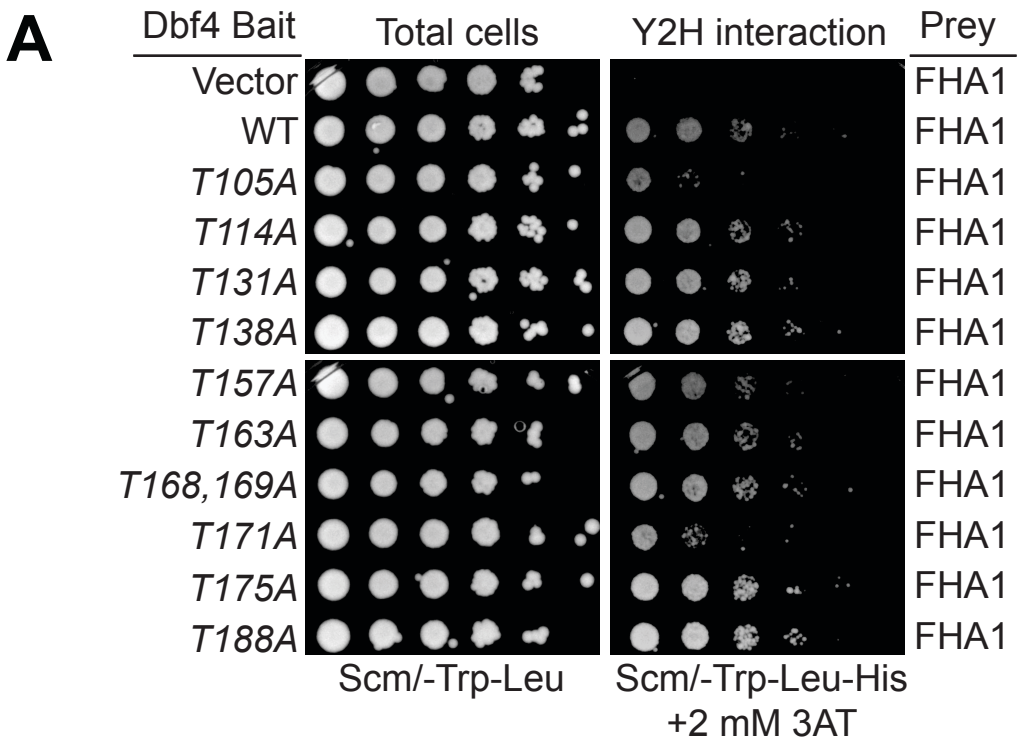


Figure 15. (cont'd)

B

					*			*	*							
100	101	102	103	104	**105**	106	107	**108**	**109**	110	111	112	113	114	**FHA1 interaction**	**FHA2 interaction**
V	**E**	**P**	**R**	**V**	**T**	**P**	**K**	**E**	**L**	**L**	**E**	**W**	**Q**	**T**	+ + + +	+ +
A															+ + + +	+ +
	A														+ + + +	+ +
	K														+ + + +	+ +
		A													+ + + +	+ +
			A												+ + + +	+ +
			E												+ + + +	+ +
				A											+	-
				L											+ + + +	+ +
					A										+	-
						A									+ + + +	+ +
							A								+ + + +	+ + + +
							E								+ + + +	+ + + +
								A							+	-
								K							-	-
								D							+ + + +	+ +
									A						+	-
									V						+	-
										A					+ + + +	+ +
											A				+ + + +	+ +
												A			-	-
												F			+ + + +	+ +
													A		+ + + +	+ +
														A	+ + + +	+ +
V	**E**	**P**	**R**	**V**	**T**	**P**	**K**	**E**	**L**	**L**	**E**	**W**	**Q**	**T**	+ + + +	+ +
100	101	102	103	104	**105**	106	107	**108**	**109**	110	111	112	113	114	**FHA1 interaction**	**FHA2 interaction**

Figure 16. Dbf4 residues V104, T105, E108, L109, and W112 are required for the binding the Rad53 FHA domains

The indicated substitutions within residues 100-114 of the Dbf4 N-terminal (66-227) bait plasmid were assayed for a two-hybrid interaction with the Rad53 FHA1 (panel A) and FHA2 domains (panel B). Spotting as in Figure 15.

Figure 16. (cont'd)

A

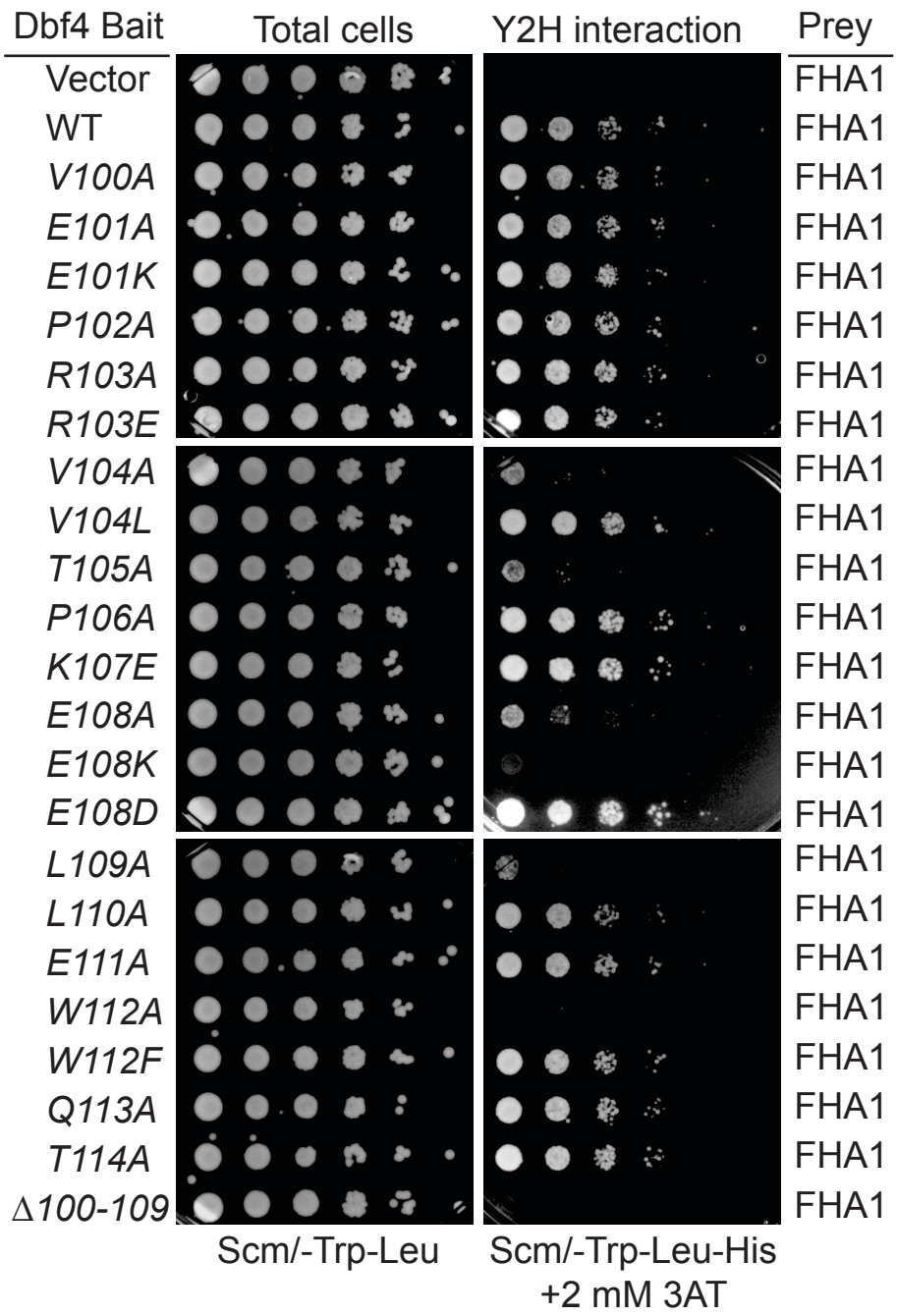


Figure 16. (cont'd)

B

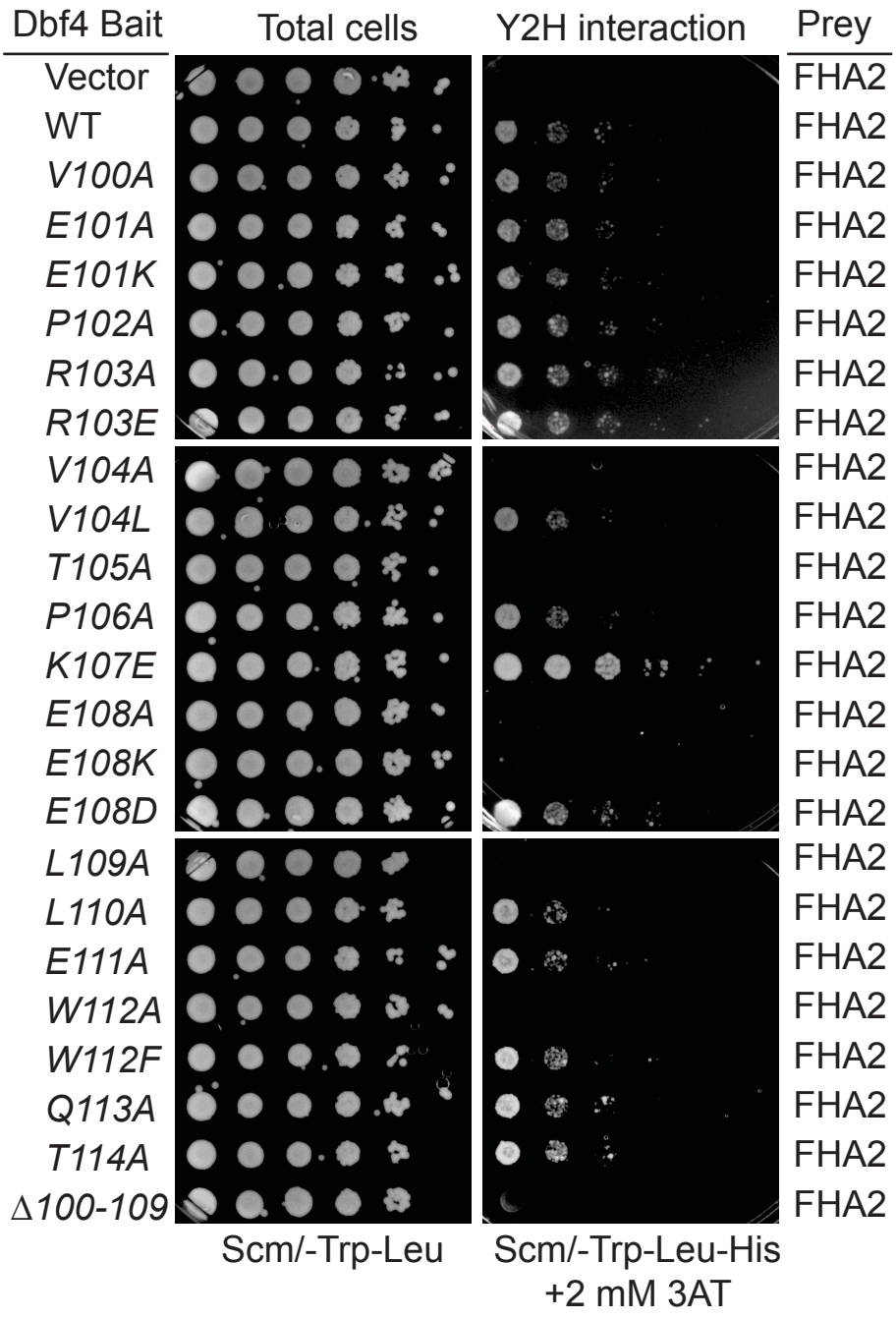


Figure 17. The Rad53 FHA1 domain directly binds to a T105 phosphorylated Dbf4 peptide

(A) Biotinylated Dbf4 peptides (residues 98-113) were tested for interaction with the purified 6His-FHA1 domain using the AlphaScreen Assay. Data represents the average of three independent experiments ± SEM. (B) Purified 6His-FHA2 domain does not interact with the pThr105 Dbf4 peptide, but does selectively bind a Rad9 phosphorylated peptide. (C) The Dbf4-FHA1 domain interaction was competed by non-biotinylated, T105-phosphorylated Dbf4 peptide (pThr105), a peptide containing the optimal FHA1 binding sequence (pT105-E108D), but not by the T105 (non-phosphorylated) Dbf4 peptide. (D) Summary of peptide sequences and the IC50 values determined by competition assays.

Figure 17. (cont'd)

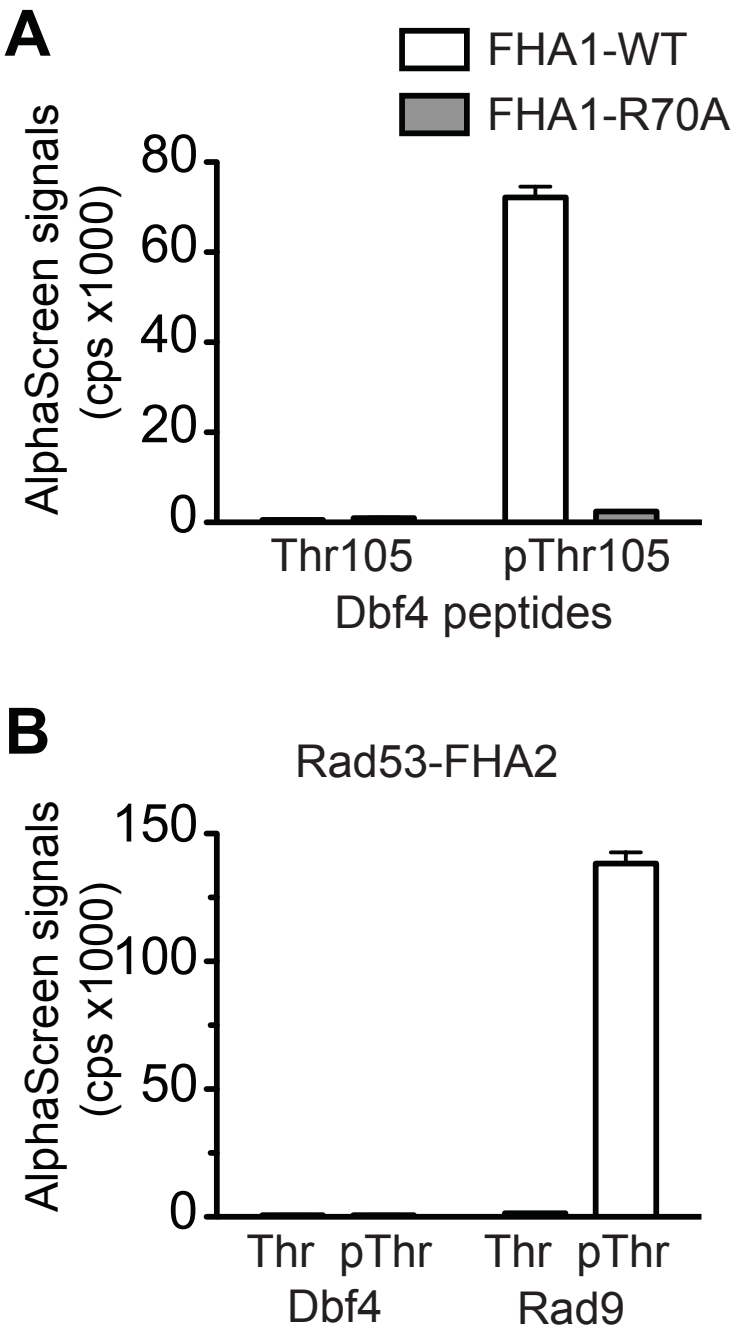


Figure 17. (cont'd)

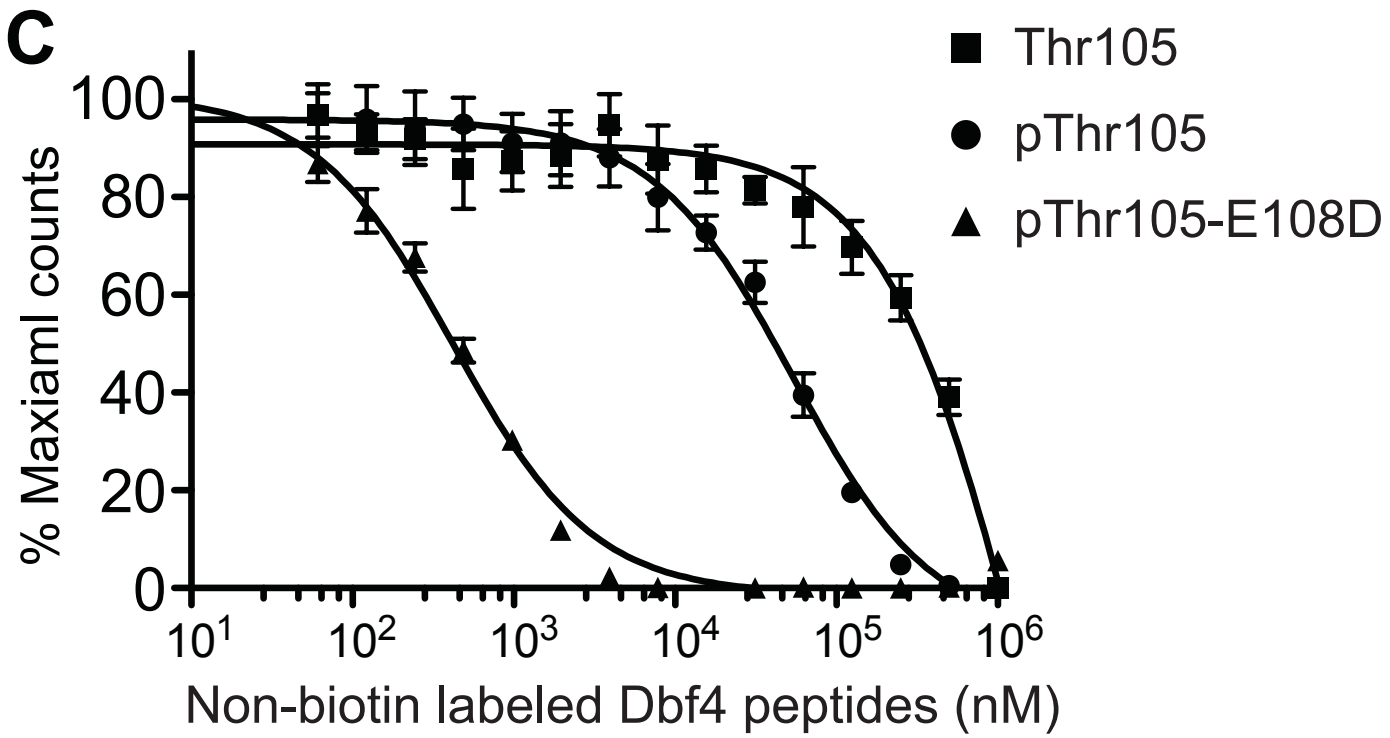


Figure 17. (cont'd)

D

Peptide	Sequence	IC50 (μM)
Dbf4 (98-113aa)	KNV EPR VTP KEL LEW Q	>1500
pDbf4 (pThr105)	KNV EPR V(**pT**)P KEL LEW Q	53.639
pDbf4-V104A	KNV EPR **A**(**pT**)P KEL LEW Q	176.664
pDBf4-E108A	KNV EPR V(**pT**)P K**A**L LEW Q	422.522
pDbf4-E108D	KNV EPR V(**pT**)P K**D**L LEW Q	2.285
pDbf4-L109A	KNV EPR V(**pT**)P KE**A** LEW Q	334.297
Rad9	IMS EVE LTQ ELP EVE	
pRad9	IMS EVE L(**pT**)Q ELP EVE	

The Rad53 FHA2 domain binds peptides with a different binding consensus from the FHA1 domain (Liao et al. 1999; Wang et al. 2000; Byeon et al. 2001). However, using a two-hybrid assay we found that the FHA2 domain interacts with Dbf4 using the same residues as the FHA1 domain, albeit more weakly than the FHA1-Dbf4 interaction. Significant exceptions are that the K107A or K107E substitutions (at the +2 position) substantially enhance FHA2 binding but do not affect FHA1 binding (Figure 15B). Taken together, these results suggest that both the Rad53 FHA1 and FHA2 domains recognize the Dbf4 sequence, T^{105}-x-x-E-L.

The Dbf4-FHA1 domain interaction is phospho-threonine dependent

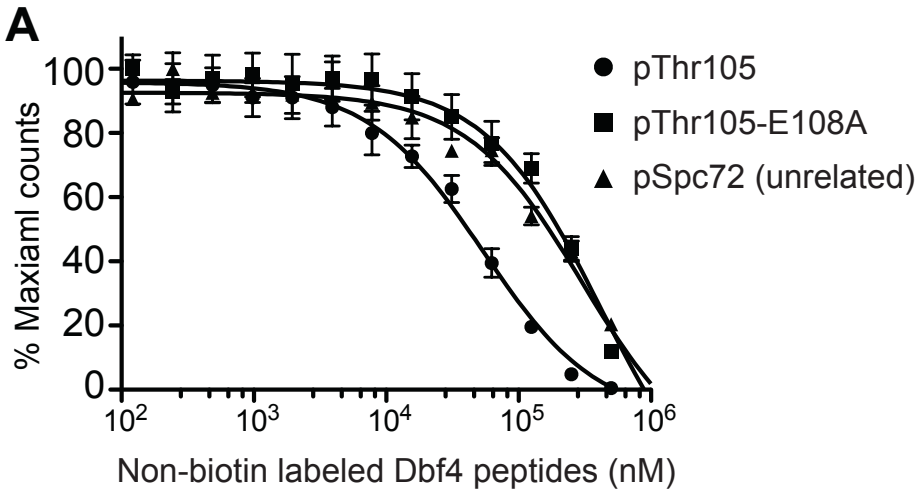
To investigate whether the Dbf4-FHA1 domain interaction required phosphorylation of Dbf4 residue T105, we purified the FHA1 domain and tested its ability to bind synthetic Dbf4 peptides using the AlphaScreen proximity assay (Ullman et al. 1994). The FHA1 domain bound to the biotinylated Dbf4 peptides containing residues 98-113 but only if T105 was phosphorylated (Figure 17A). In addition, mutation of the conserved R70 to A in FHA1 abolished the interaction with the Dbf4 pT105 peptide. This data indicates that the Dbf4-FHA1 domain interaction requires T105 phosphorylation. In contrast, although the FHA2 domain bound efficiently to an optimal Rad9 phosphorylated peptide (Byeon et al. 2001) it was unable to bind the same pT105 Dbf4 peptide (Figure 17B). FHA domains bind to pT plus adjacent residues but also make further extensive substrate contacts outside the pT binding loop (Mahajan et al. 2008). Since neither FHA domain bound to Dbf4 residues 66-109 in the two-hybrid assay unless the BRCT domain was

included, FHA1 and FHA2 binding to Dbf4 also requires the BRCT domain and includes perhaps distinct FHA-BRCT contacts.

To test whether additional residues discovered in the two-hybrid screen were important for the FHA1-Dbf4 peptide interaction, we used non-biotinylated peptides to compete FHA1::biotin-pT105 peptide binding. As expected, the FHA1::biotin-pT105 interaction was competed by an identical pT105 peptide but not by a non-phosphorylated T105 peptide or by an unrelated phospho-peptide (Figure 17C and Figure 18A). The FHA1-pT105 peptide competed with an IC50 of 50-60 μM, indicating a moderate FHA1 binding affinity to this peptide. In the yeast two-hybrid assays, we found that E108 and the hydrophobic residues immediately adjacent to the pT^{105}-x-x-E motif were critical for the FHA1 interaction. In agreement with that data, a pT^{105}-x-x A peptide was significantly impaired in its ability to compete the FHA1::biotin-pT105 peptide interaction (Figure 18A). Similarly, alanine substitutions of V104 or L109 within otherwise identical pT105 peptides reduced the ability to compete the FHA1::biotin-pT05 peptide interaction (Figure 18B). Finally, the E108D mutation, which did not affect the Rad53-Dbf4 interaction in the two-hybrid assay and matches the optimal binding sequence for the FHA1 domain, competed the interaction but with a much higher binding affinity (1-5 μM) as shown in Figure 17C. Based on the two-hybrid and biochemical assays, the Rad53 FHA1 domain selectively binds a pT-x-x-E sequence, which closely conforms to an FHA1 binding consensus sequence.

Figure 18. Dbf4 residues V104, E108, and L109 are critical for the specific binding of Rad53 FHA domains

(A) The Dbf4 biotinylated peptide pThr105-FHA1 interaction was competed by the non-biotinylated T105-phosphorylated Dbf4 peptides (pThr105), but not by the same Dbf4 peptide with an E108A substitution, or by an unrelated phospho-serine peptide (pSpc72). (B) The pThr105-V104A and pThr105-L109A peptides were also defective in competing the biotinylated pThr105-FHA1 interaction.



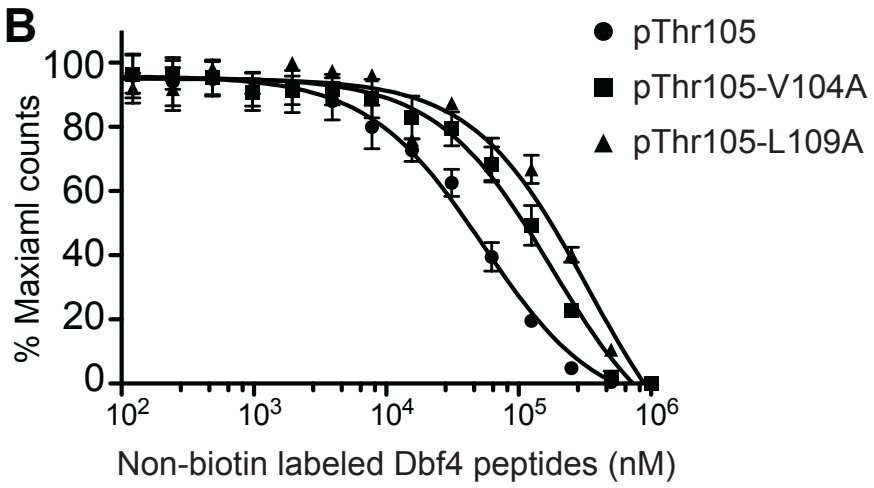
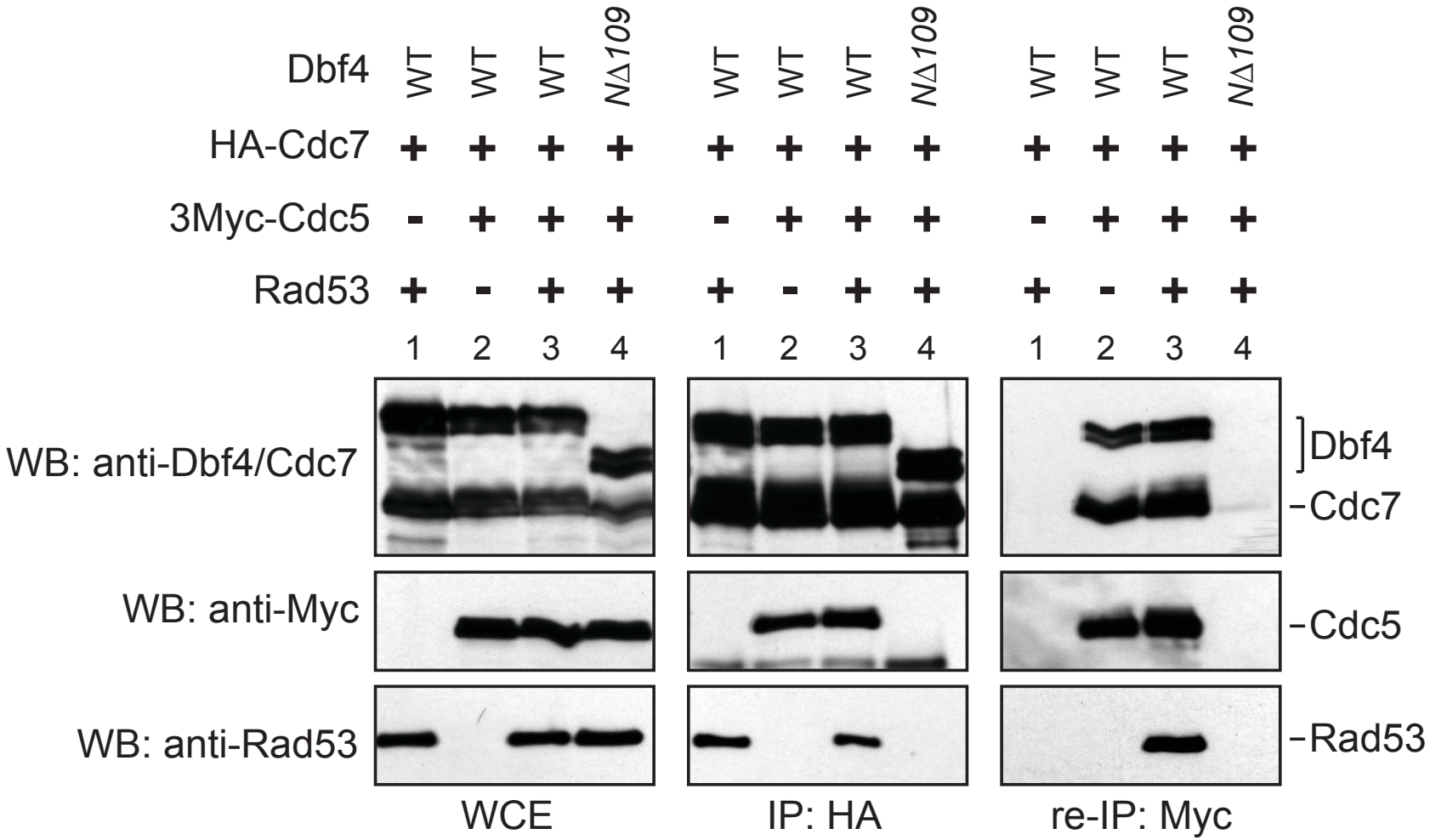


Figure 19. DDK, Rad53 and Cdc5 form a ternary protein complex

HA-Cdc7-Dbf4 complexes were immunoprecipitated from baculovirus-infected Sf9 cells using 12CA5 antibodies and examined for co-immunoprecipitation of Rad53 and 3Myc-Cdc5 by Western blotting. Rad53 and Cdc5 were co-immunoprecipitated by wild-type HA-Cdc7-Dbf4 but not by the HA-Cdc7-Dbf4(NΔ109) mutant (middle). Following 12CA5 immunoprecipitation, proteins were eluted from the beads using HA peptide and subjected for another round of immunoprecipitation by 9E10 antibodies. Rad53 was co-immunoprecipitated with 3Myc-Cdc5 and wild type DDK but not if Dbf4-NΔ109 was expressed (right).

Figure 19. (cont'd)



Dbf4 mediates the association of Cdc7, Rad53, and Cdc5 kinases

Although Dbf4 is well known for its essential role in binding and activating Cdc7 to initiate DNA replication, we recently proposed that Dbf4 also functions as a molecular scaffold for the Cdc7 and Cdc5 kinases. Dbf4 residues 83-88 directly interact with the Polo-box domain of the Cdc5 kinase and functionally inhibit Cdc5 in the mitotic exit network (MEN) (Miller et al. 2009; Chen and Weinreich 2010). Now that we have defined a distinct binding site for the Rad53 kinase in the Dbf4 N-terminus, in close proximity to the Cdc5 binding site, we wondered whether Dbf4 formed a ternary complex with the Rad53, Cdc5, and Cdc7 kinases.

To examine the DDK interaction with Cdc5 and Rad53 we employed a baculovirus system to express Rad53, Cdc5, Cdc7, and various Dbf4 derivatives in Sf9 cells. Consistent with previous reports (Miller et al. 2009; Chen and Weinreich 2010), Cdc5 was co-immunoprecipitated (co-IPd) with wild type HA-Cdc7-Dbf4 but not with the HA-Cdc7-Dbf4(NΔ109) truncation derivative (Figure 19, middle panel). Similarly, Rad53 bound to wild type HA-Cdc7-Dbf4, but not to HA-Cdc7-Dbf4(NΔ109). These results indicate that the association of Rad53 and Cdc5 with DDK depends on the first 109 residues of Dbf4, which contains the Cdc5 binding site (residues 83-88) and a Rad53 binding site (residues 104-109).

The co-immunoprecipitation results suggest two different possibilities. Either DDK exists in two distinct protein complexes (DDK-Rad53 and DDK-Cdc5) or alternatively, DDK can bind to Cdc5 and Rad53 simultaneously. To clarify this, we asked whether

Rad53 binds the DDK-Cdc5 complex by performing a sequential co-IP. We expressed all four proteins in Sf9 cells and IPd DDK using the HA tag on the Cdc7 subunit. This procedure immunoprecipitates proteins bound to DDK, which will include DDK-(Myc-Cdc5), DDK-Rad53 and presumptive DDK-(Myc-Cdc5)-Rad53 complexes. We then eluted the bound proteins using 1 mM HA peptide and performed a second round of immunoprecipitation using 9E10 monoclonal antibodies to IP just the DDK-(Myc-Cdc5) complexes. Rad53 was present in the second IP (Figure 19, right panel) indicating that Rad53 forms a ternary complex with Cdc5 and DDK. Together these results demonstrate that the Dbf4 N -terminus acts as a docking site for both Rad53 and Cdc5, and that both kinases can simultaneously associate with DDK.

A Rad53 checkpoint defect together with loss of specific Dbf4 N-terminal residues results in synthetic lethality

DDK and *rad53* mutants show a series of complex genetic interactions. For instance, hypomorphic *cdc7* and *dbf4* mutants are synthetically lethal with *rad53* hypomorphic mutants (Desany et al. 1998; Dohrmann et al. 1999; Dohrmann and Sclafani 2006; Gabrielse et al. 2006). However, *cdc7-1* is also synthetically lethal with the *rad53-31* mutant, which is checkpoint proficient (Dohrmann et al. 1999). We previously reported that the *dbf4-NΔ109* mutant was synthetically lethal with the *rad53-1* hypomorphic mutant (Gabrielse et al. 2006). This is interesting since the *dbf4-NΔ109* mutant exhibits an apparently normal S-phase, is not defective for activating early or late replication origins, and is not sensitive to genotoxic agents (Gabrielse et al. 2006). However, the Dbf4-NΔ109 protein is defective for binding Cdc5 (Miller et al. 2009; Chen and

Weinreich 2010) and Rad53 (this study). Therefore, we tested whether the synthetic lethality between *dbf4-NΔ109* and *rad53-1* was due to the loss of the Dbf4-Cdc5 or Dbf4-Rad53 interactions. We first sequenced the *rad53-1* gene (Weinert et al. 1994) and found a single *G653E* point mutation, which is identical to that reported for the *rad53-11* allele (Dohrmann and Sclafani 2006). G653 falls within a loop between the b6 and b7 strands of the FHA2 domain and is adjacent to the conserved N655 residue, which plays an important role in substrate recognition (Figure 20A) (Byeon et al. 2001). The *rad53-1* (G653E) or N655A full-length Rad53 mutants were unable to bind the Dbf4 N-terminus in the two-hybrid assay (Figure 20B), highlighting the importance of the FHA2 domain for the Dbf4 interaction. Both mutants were expressed similarly to the wild type (data not shown). Similarly we found that the *rad53-R70A* mutant, which cannot interact with Dbf4, was synthetically sick or lethal with *dbf4-NΔ109* but obviously not with *DBF4* (Figure 20C). Not surprisingly, we also observed synthetic lethality between *dbf4-NΔ109* and the *rad53-K227A* (kinase dead) allele (Figure 20D). Since the *rad53-R70A* and *rad53-G653E* (*rad53-1*) mutants are defective for interacting with DDK to begin with, the synthetic lethality with *dbf4-NΔ109* cannot be due to the further loss of just the Rad53 binding site on Dbf4. The synthetic lethality is likely caused by compromised Rad53 function coupled with loss of a Rad53-*independent* function of Dbf4 present in the N-terminal 109 residues. We know that this function is not the ability to bind Cdc5, since a *dbf4-Δ82-88* mutant, which is completely defective for binding Cdc5 (Miller et al. 2009; Chen and Weinreich 2010), is not synthetically lethal with *rad53-1* (Figure 21A). Furthermore, a *rad53Δ sml1Δ* strain is not synthetically lethal with the *dbf4-Δ82-88* allele (defective for Cdc5 binding) or the *dbf4-Δ100-109* allele

(defective for Rad53 binding) but is synthetically lethal with the *dbf4-NΔ109* allele (Figure 21B). Deletion analysis of the region between residues 65 and 109 indicates that loss of sequences between residues 82-109 causes synthetic lethality with *rad53Δ sml1Δ* (Figure 21), which includes both the Cdc5 and Rad53 binding sites. This genetic data strongly suggest (remarkably) that yet another function of the Dbf4 N-terminus cooperates with Rad53 to ensure cell viability.

The Dbf4-Rad53 physical interaction is required to inhibit late origin firing during replication checkpoint activation

In response to replication fork arrest, Rad53 phosphorylates both Dbf4 and Sld3 to inhibit late origin firing (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010). The critical Rad53 phosphorylation sites on Dbf4 map toward the C-terminus between motifs M and C. A *dbf4-4A* mutant that changes 4 serine and threonine Rad53 phosphorylation sites to alanine is sufficient to allow late origin activation when combined with an *sld3-38A* mutant, containing alanine mutations in 38 Rad53 phosphorylation sites (Zegerman and Diffley 2010). We hypothesized that Rad53 phosphorylation of Dbf4 depends on its physical interaction with the Dbf4 N-terminus. To test this, we examined whether the combination of a *dbf4-NΔ109* mutant (defective for Rad53 binding) and the *sld3-38A* mutant, which cannot be phosphorylated by Rad53, would allow late origin firing in the presence of HU. Yeast cells were synchronized in G1 phase using mating pheromone and then released into S phase in the presence of 0.2 M HU to stall replication forks from early origins. At different time points following release from the G1 arrest, replication intermediates (RI) near ARSs were separated on

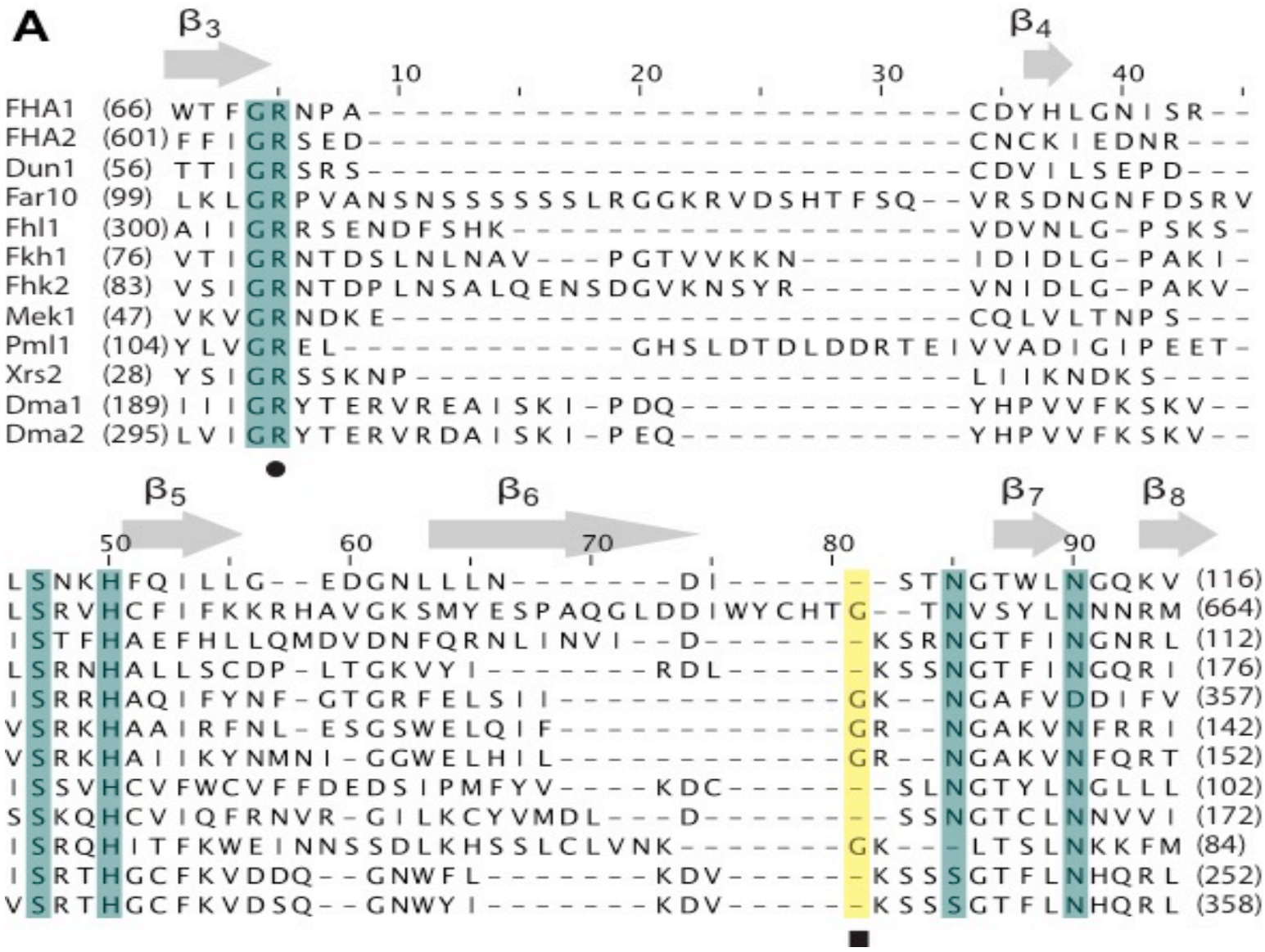
alkaline gels and detected by Southern blotting with ARS-specific probes to measure replication origin activity. As a control, Rad53 was activated (evidenced by the phosphorylation-dependent mobility shift) in both wild type and mutant cells following HU treatment (Figure 22A), indicating that neither the *dbf4* nor the *sld3* mutations affect Rad53 checkpoint activation.

The early origin, *ARS305*, was active in the wild type, *dbf4-NΔ109*, *dbf4-4A sld3-38A,* and *dbf4-NΔ109 sld3-38A* mutant strains indicating that induction of the replication checkpoint does not interfere with early origin firing in these cells. Although Rad53 activation inhibited the firing of late origins *ARS501* and *ARS603* in the wild type and the single mutants as expected (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010; Duch et al. 2011), replication intermediates were detected at late origins in both the *sld3-38A dbf4-4A* and *sld3-38A dbf4-NΔ109* double mutants to a similar extent (Figure 22C and D). Thus, the *dbf4-NΔ109* mutant defective for Dbf4-Rad53 binding is similarly defective in preventing late origin firing in HU as the *dbf4-4A* phosphorylation site allele. This result is consistent with the previous observation that the *dbf4-NΔ109* mutant significantly impaired Rad53-mediated Dbf4 hyper-phosphorylation in HU (Gabrielse et al. 2006). These data strongly suggest that Rad53 must stably interact with Dbf4 through its N-terminal binding site to phosphorylate Dbf4 and inhibit late origin firing in response to HU.

Figure 20. *dbf4-NΔ109* is synthetically lethal with *rad53-R70A*, *rad53-K227A*, and *rad53-G653E*

(A) Sequence alignment of all FHA domain-containing proteins in *Saccharomyces cerevisiae*. Absolutely conserved residues are highlighted in aquamarine. The Rad53 R70 and R605 residues are marked below with a black circle. The *rad53-1* mutant was sequenced and found to contain a single point mutation (G653E) within the ß 6-7 loop of the FHA2 domain (highlighted yellow, black square). (B) The *rad53-G653E* and *rad53-N655A* mutants do not interact with Dbf4 in yeast two-hybrid assays. (C and D) Representative tetrads from diploid strains of genotype *DBF4*/*dbf4-NΔ109 RAD53/rad53-R70A* and *DBF4*/*dbf4-NΔ109 RAD53/rad53-K227A* were sporulated and dissected onto YPD plates. Recombinant genotypes are indicated. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 20. (cont'd)



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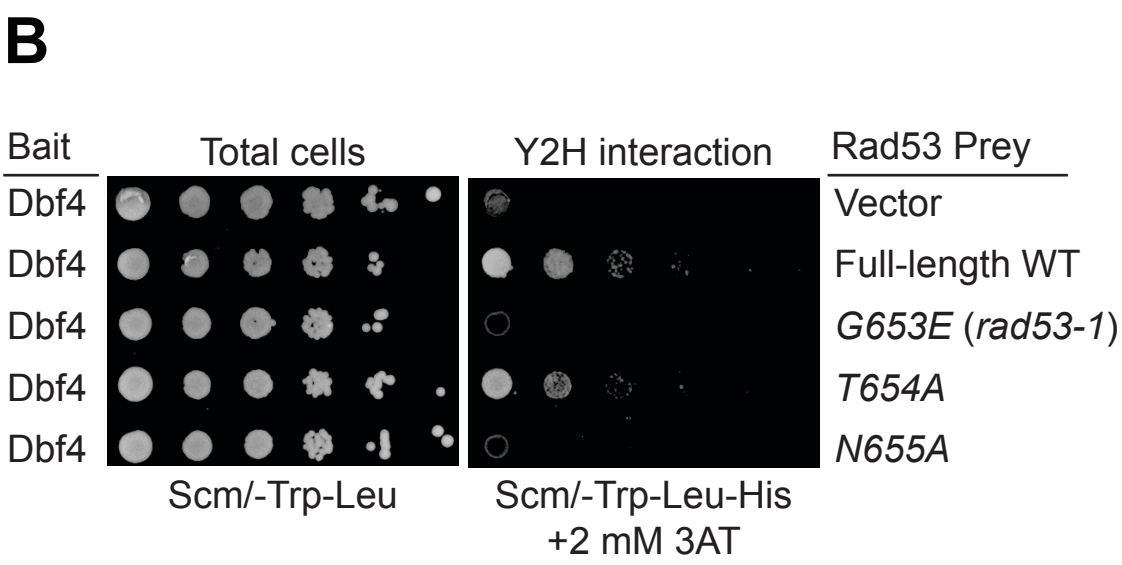


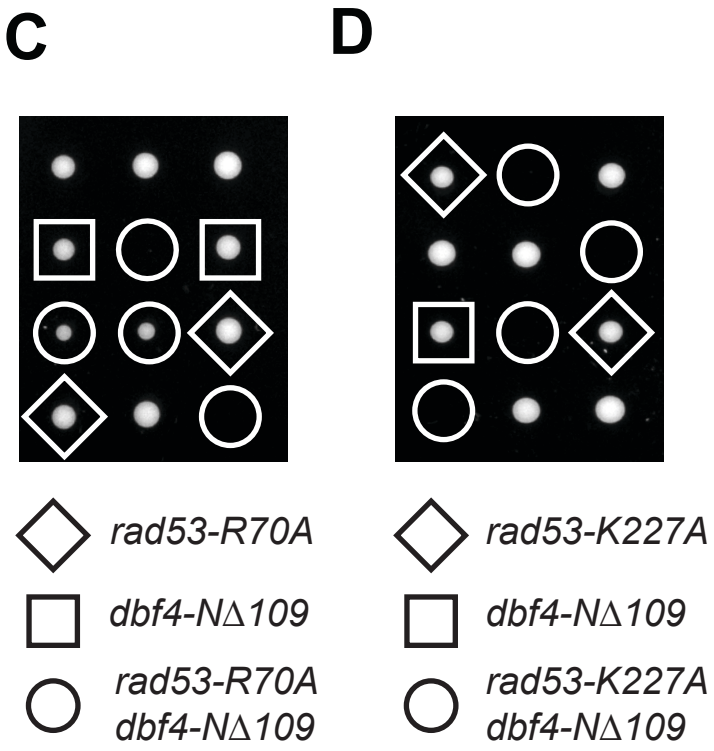


Figure 21. The synthetic lethality between *dbf4-NΔ109* and *rad53-1* or *rad53Δ* is not due solely to either loss of Cdc5 interaction or increased Dbf4 stability but requires sequences between residues 82-109

Wild type and various *dbf4* mutants were cloned in low-copy number (*ARS/CEN/LEU2*) vectors, driven by the *DBF4* endogenous promoter. Plasmids were transformed into M1589 (*rad53-1 dbf4Δ::kanMX6 [pDBF4-URA3]*) or M3581 (*rad53Δ::TRP1 sml1Δ::HIS3 dbf4Δ::kanMX6 [pDBF4-URA3]*) and the wild-type *DBF4-URA3* plasmids were selected against on FOA. Cells that could not grow on FOA plates were scored as having a synthetic lethal interaction. The NΔ65 deletion causes increased Dbf4 stability by deleting sequences important for ubiquitin-mediated proteolysis. The Δ82-88 deletion prevents Cdc5 binding to Dbf4, while the Δ100-109 deletion prevents Rad53 binding to Dbf4.

Figure 21. (cont'd)

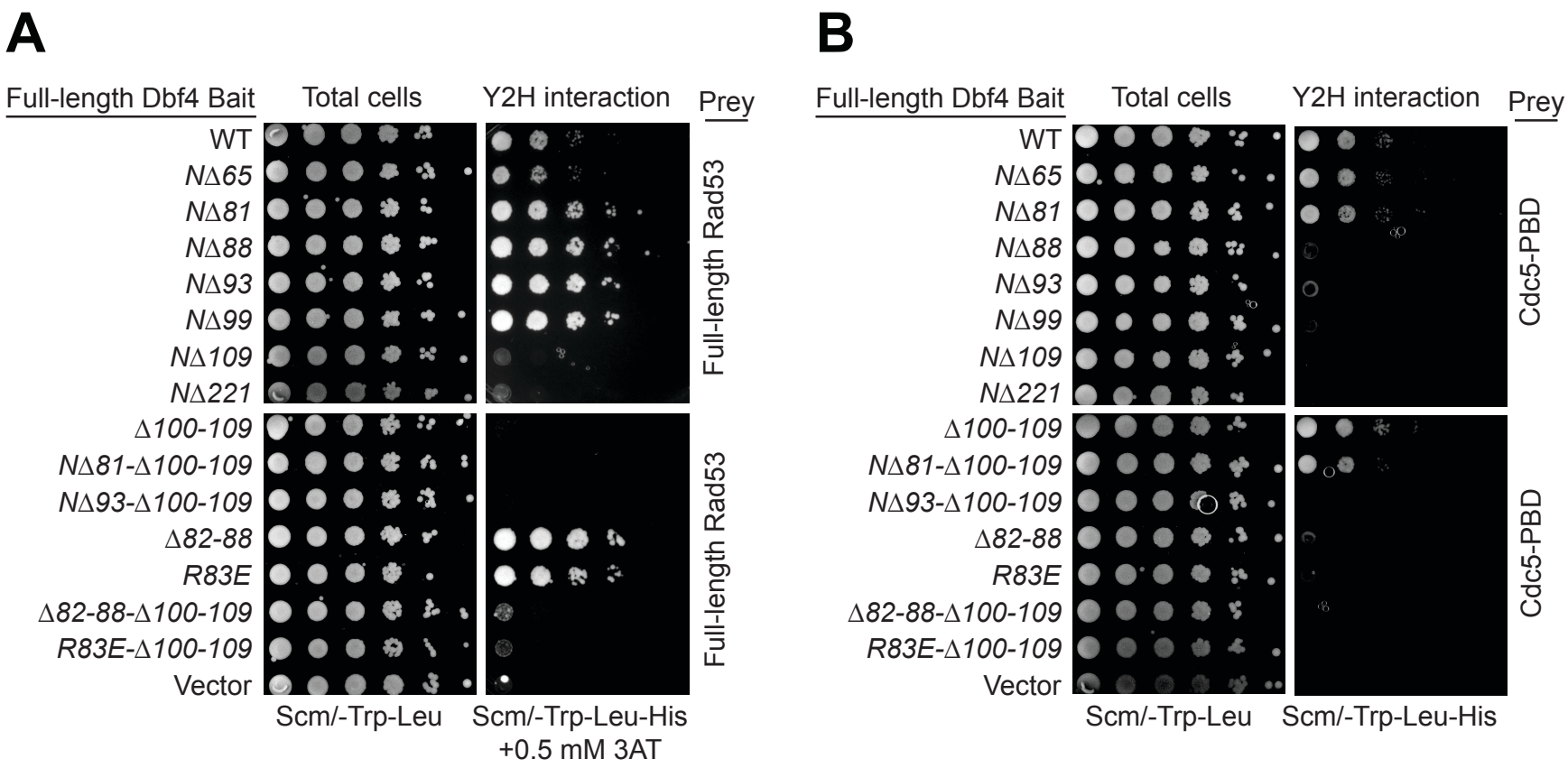
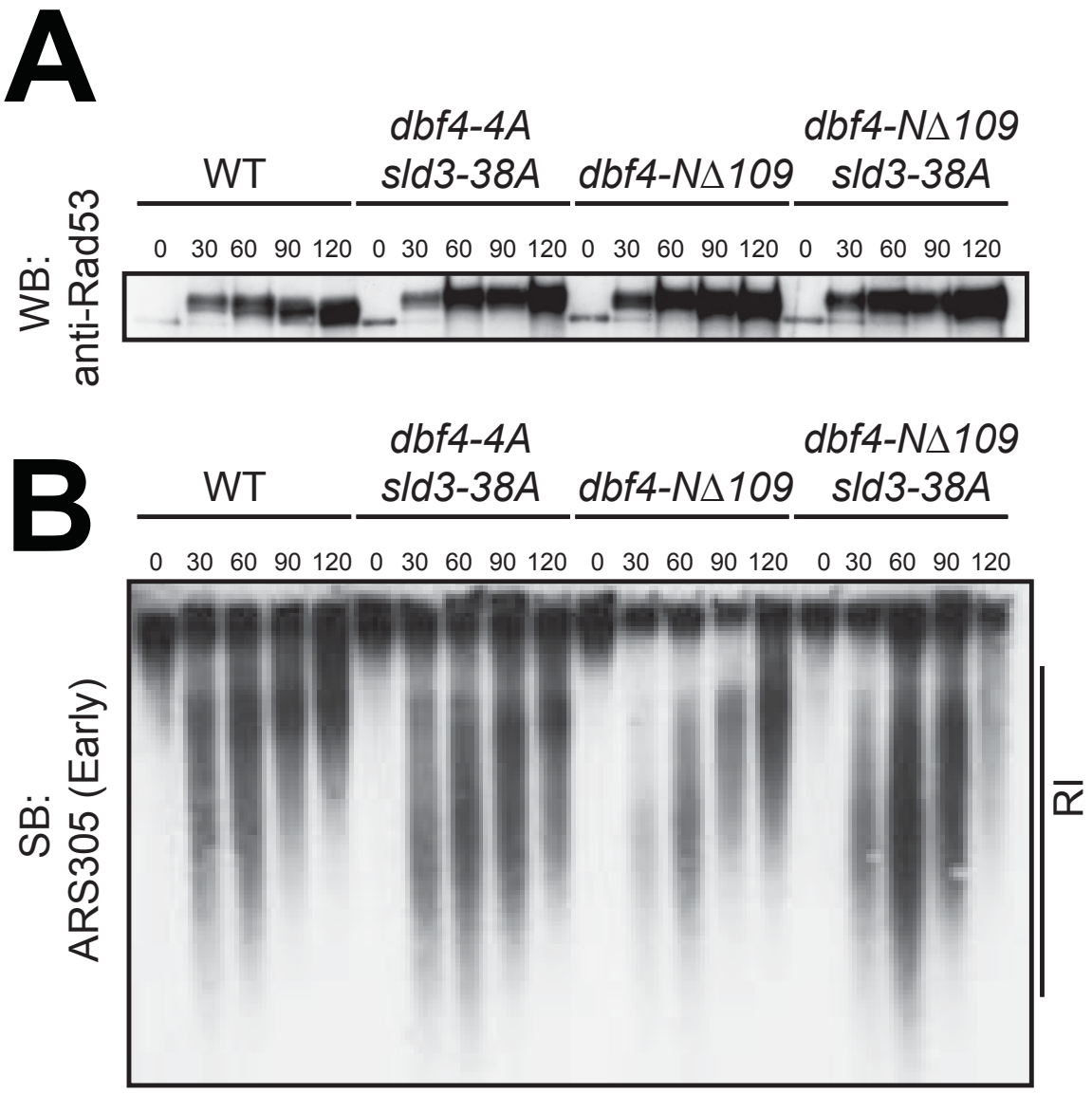


Figure 22. The *dbf4-N∆109 sld3-38A* double mutant allows late origin firing in the presence of HU

(A) Wild type and mutant cells were synchronized in G1 phase by alpha-factor and released into S phase into medium containing 0.2 mM HU for the indicated times. Total protein extracts were examined by Western blotting for Rad53 to assess Rad53 activation (upper band). (B-D) Replication intermediates (RI) were separated by alkaline gel electrophoresis and detected by Southern blotting to measure the activity of early (*ARS305*) and late (*ARS501* and *ARS603*) origins. Flow cytometry assays and budding index are shown in (E) and (F).

Figure 22. (cont'd)



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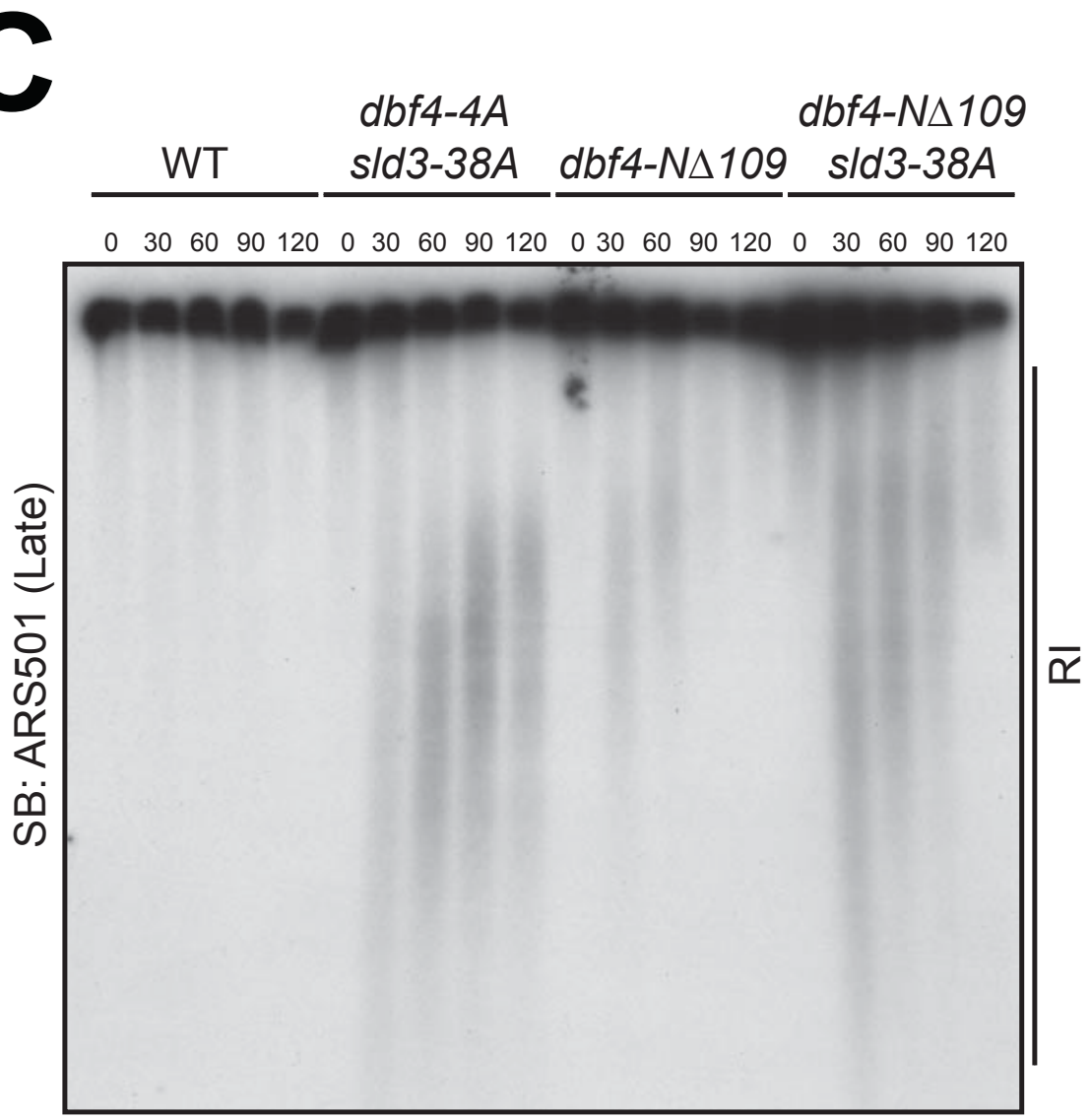

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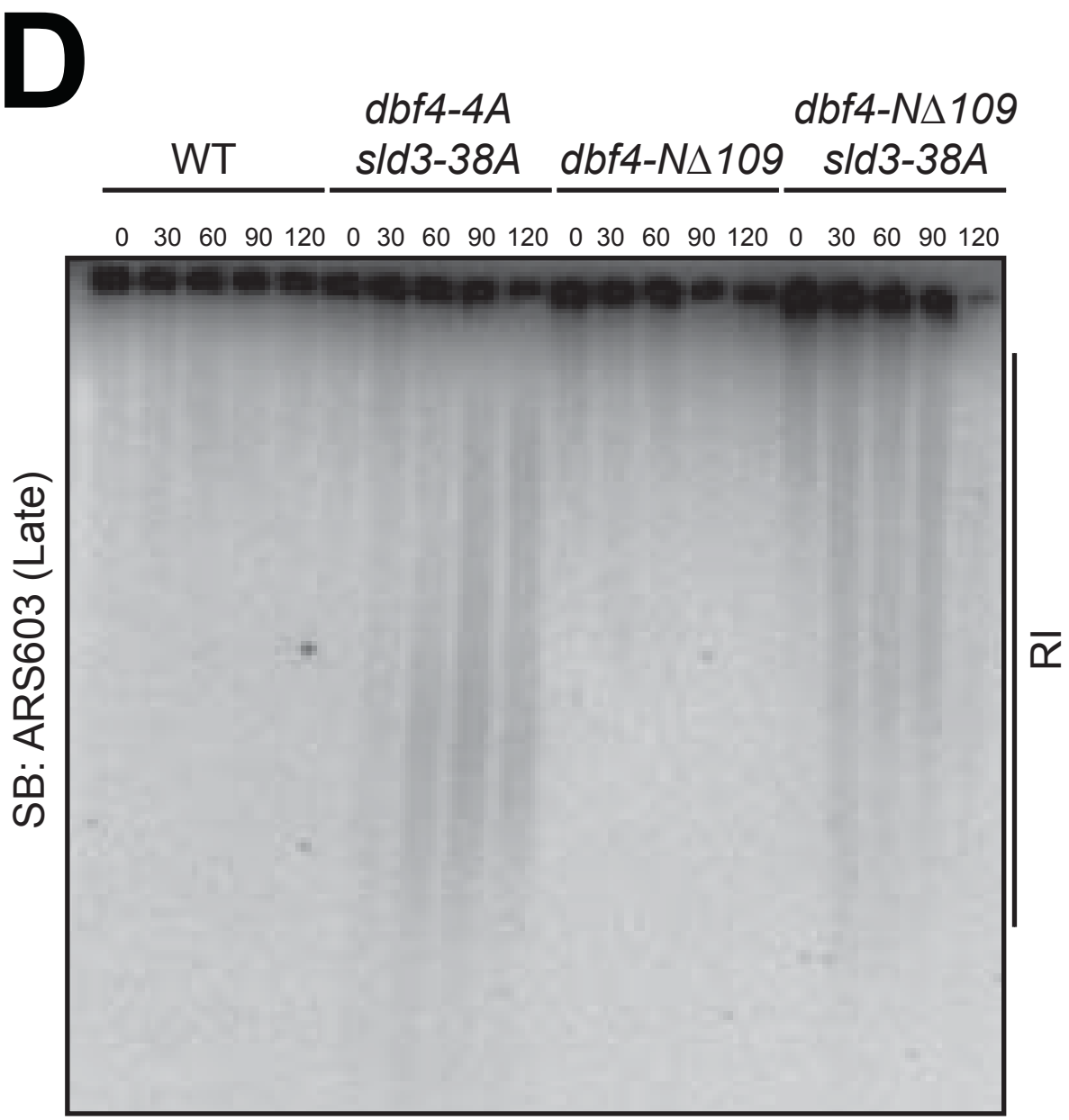

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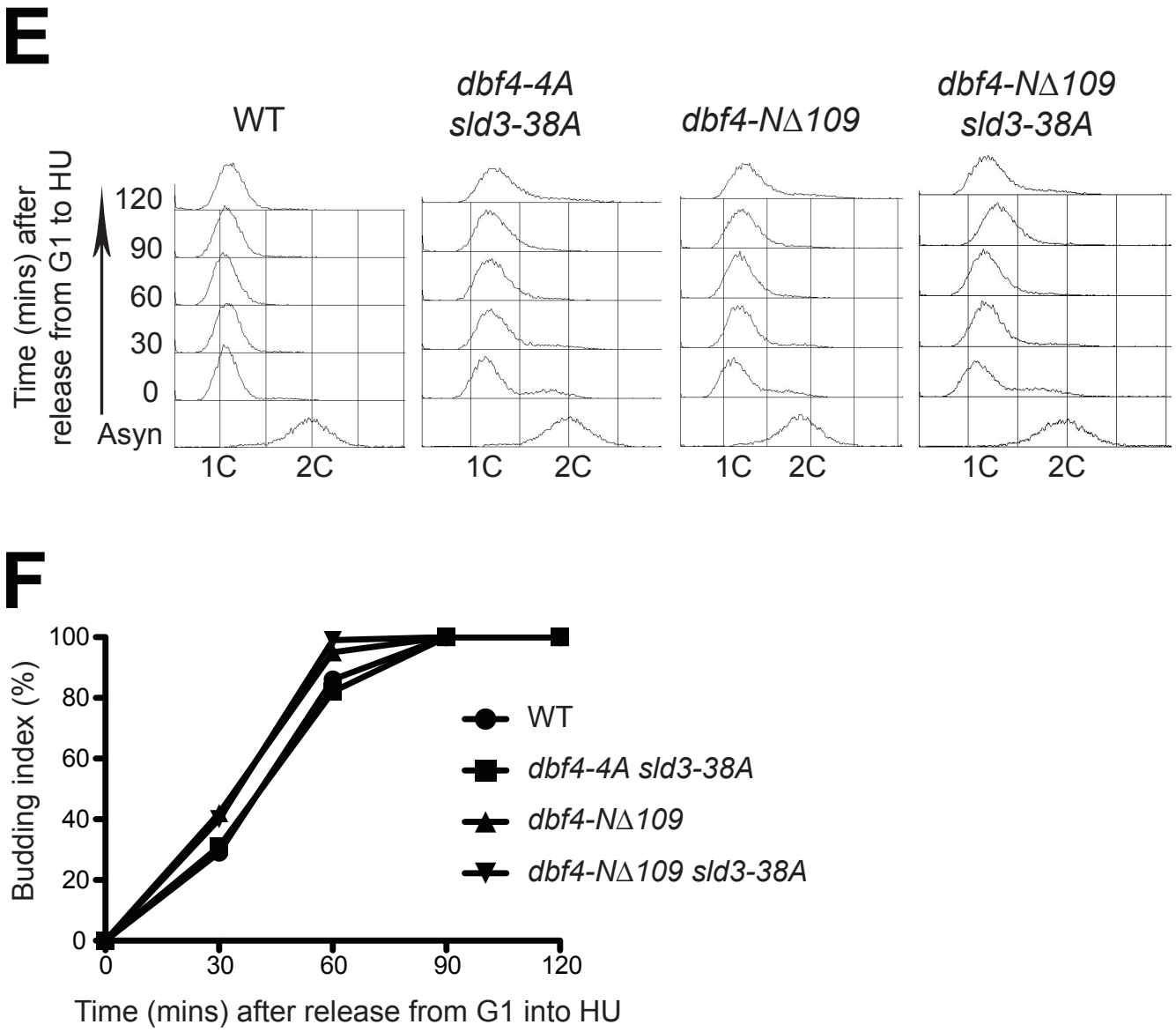


DISCUSSION

Rad53 interacts with Dbf4 using a phospho-threonine dependent mechanism

Multiple groups have reported genetic and physical interactions between *S. cerevisiae* Dbf4 and Rad53 (Dohrmann et al. 1999; Weinreich and Stillman 1999; Kihara et al. 2000; Duncker et al. 2002; Gabrielse et al. 2006; Matthews et al. 2012), and *S. pombe* Dfp1 and Cds1 (Takeda et al. 2001; Fung et al. 2002). Furthermore, in response to DNA damage, human and Xenopus DDK are downstream targets of ATR (Costanzo et al. 2003; Lee et al. 2012). The Dbf4-Rad53 interaction is conserved in yeasts and a DDK-ATR interaction in vertebrates because it likely promotes genome stability. Here we have mapped a Rad53 binding site in the Dbf4 N-terminus and have shown that a Rad53-Dbf4 physical interaction is critical for regulating late replication origin firing. The minimal Rad53 binding region corresponds to Dbf4 residues 100-227, which comprise the Dbf4 BRCT domain (~118-224) and residues immediately N-terminal to this domain. Mutations in either conserved BRCT residues or residues 100-109 abrogated Rad53 binding. This indicates that the BRCT domain and the region preceding it are both required for Rad53 binding. Surprisingly, and despite their different consensus peptide binding sites, both Rad53 FHA domains interacted with this 100-109 region independently and apparently using the same Dbf4 residues (see below). Furthermore, since mutations that impair phospho-threonine substrate recognition in either Rad53 FHA domain blocked the interaction with Dbf4, this further suggested that the Rad53 interaction with Dbf4 is mediated by phosphorylation and is multivalent.

Very recently a crystal structure of the Dbf4 BRCT domain (that included residues 98 to 221) was described (Matthews et al. 2012). These authors also showed that Rad53 interacted with the Dbf4 BRCT domain plus the preceding alpha helix using two-hybrid assays, however none of threonines contained within the structure was shown to directly interact with Rad53. So a phosphorylation-independent interaction between Dbf4 and Rad53 was proposed. In contrast, our studies showed that residues T105, E108, L109, W112, and R209 (not shown) mediated the Dbf4-Rad53 interaction. The structure of the Dbf4 N-terminus allows us to rationalize this data (data not shown). T105 is solvent exposed and occurs within a sequence (T-x-x-E) that closely matches a FHA1 binding site. The E108 residue has the same spatial orientation as T105, and L109 is directly adjacent to E108. The W112 residue packs against L214 present at the C-terminus of the BRCT a3 helix. This hydrophobic interaction presumably helps stabilize the a0-a3 orientation and would explain why a W112A mutation disrupts the Dbf4-Rad53 interaction but W112F does not. Finally, R209 on the a3 helix is solvent exposed and is suitably oriented to interact with an FHA domain bound to a0 or, alternatively, to mediate BRCT-BRCT domain interactions. In a tandem BRCT-BRCT dimer the a2 helix from one monomer packs against the a1 and a3 helices from the second monomer (Glover et al. 2004). Mutation of R209 but not K212 (which is oriented orthogonally to R209, away from a0) abolished the Dbf4-FHA1 two-hybrid interaction (JK and MW, unpublished). Since the purified Rad53 FHA1 domain bound only to T105 phosphorylated Dbf4 peptides, together these data strongly suggest a phospho-threonine dependent binding mechanism between Dbf4 and the Rad53 FHA1

in which the pT^{105}-x-x-E-L motif in Dbf4 mediates a direct interaction with the Rad53 FHA domains.

The FHA1 binding site in Dbf4 is pT-x-x-E, instead of the preferred pT-x-x-D. A pT-x-x-D Dbf4 peptide (that substituted D108 for E) bound to the FHA1 domain significantly better than the wild type but gave a similar two-hybrid interaction with Rad53. The use of a lower affinity pT-x-x-E interaction may reflect a selection for some biological property needed for the Rad53-Dbf4 interaction. Alternatively, a high-affinity binding site might not be required since the Dbf4 BRCT domain also contributes to Rad53 binding as shown by our study and also an earlier study (Duncker et al. 2002). In summary, we have identified pT^{105}-x-x-E as a Dbf4 motif that binds to the Rad53 FHA1 domain. Although the Rad53 FHA2 domain binds to the same sequence in the two-hybrid assay (Figure 15), it does not bind to the pT-x-x-E peptide *in vitro* and this sequence does not match the optimal FHA2 binding site consensus. Although the FHA2 domain might bind to Dbf4 indirectly in the two-hybrid assay, the FHA2 interaction still occurs in a strain deleted for Rad53 (data not shown) and so, it is not mediated by endogenous Rad53. Interestingly, no other FHA domain in yeast can bind to this Dbf4 sequence (Figure 14D), suggesting again that the FHA2 interaction is biologically relevant. Since a previous study also demonstrated an interaction between FHA2 and Dbf4 using GST-pull downs from yeast (Duncker et al. 2002), we suggest that the FHA2 domain interaction with Dbf4 is stabilized *in vivo* by additional contacts within the BRCT domain or by the FHA1-pT105 interaction.

Models for Rad53 binding to Dbf4

We propose two models to explain how Rad53 interacts with Dbf4. Either Rad53 uses each FHA domain to bind two separate sites within Dbf4 or both Rad53 FHA domains bind to the same pT-x-x-E sequence, but on different Dbf4 subunits within a Dbf4 multimer.

FHA1 and FHA2 domains bind to different sites in Dbf4

Our data showed that the FHA1 or FHA2 domain alone could bind the Dbf4 N-terminus (Figure 15). Furthermore, mutation of either FHA domain alone within the context of full length Rad53 abrogated the Rad53-Dbf4 interaction (Figures 1 and 5) indicating that both FHA domains are required for the Dbf4 interaction. Although we have shown that both Rad53 FHA domains require the same T-x-x-E sequence for binding, the BRCT domain may contain another surface for the interaction with full-length Rad53. It is possible that the Rad53 FHA1 domain binds to pT^{105}-x-x-E and that the FHA2 domain binds to another phosphorylated residue in the BRCT domain. This seems unlikely however, since mutation of every other threonine (Figure 15) or tyrosine residue (Figure 14) in the BRCT domain had no effect on FHA1 or FHA2 binding, with the exception of T171 discussed above. Although FHA domains are well known as phospho-threonine binding modules, work from Tsai's group demonstrated that the Rad53 FHA2 domain can bind both phospho-threonine and phospho-tyrosine containing peptides (Liao et al. 1999; Wang et al. 2000; Byeon et al. 2001). It is still possible that the FHA2 domain binds weakly to a non-consensus site within the BRCT domain and this is not readily detected by our two-hybrid assay. As stated above, it is formally possible that FHA2

binds to a bridging protein in the 2-hybrid assay that also recognizes T-x-x-E, but the putative bridging protein is not Rad53 itself. Two FHA domains binding to separate sites would likely promote a higher binding affinity between Rad53 and Dbf4. This may explain why we detected a moderate binding affinity to pT-x-x-E versus pT-x-x-D peptides in our *in vitro* FHA1 binding assays.

Rad53 FHA domains bind to a Dbf4 dimer using the same sequence

Given that the Dbf4 residues T105 and E108 were critical to binding both FHA1 and FHA2 domains in the two-hybrid assay, we prefer a second model in which the Dbf4 N-termini form a dimer using the BRCT domains, and then this Dbf4 dimer provides two pT^{105}-x-x-E^{108} sites for the binding of the FHA1 and FHA2 domains separately. DDK or Dbf4 oligomerization has been suggested previously (Shellman et al. 1998; Matthews et al. 2012). Furthermore, tandem BRCT domains are present in many proteins where they form dimers that also function as phospho-recognition motifs (Caldecott 2003; Rodriguez and Songyang 2008). In addition, inter-molecular dimerization between BRCT domains has been described for the DNA repair proteins XRCC1 and Ligase III (Cuneo et al. 2011). In support of this model, we saw an interaction between Dbf4 N-termini using the yeast two-hybrid assay and substitutions of conserved residues in the Dbf4 BRCT domain disrupted the Dbf4-Dbf4 two-hybrid interaction (Figure 23). Although Dbf4-Dbf4 two-hybrid signal was significant, it was a relatively weak interaction compared with the Dbf4-Rad53 interaction. However, the association with Rad53, Cdc7, or other proteins may stabilize Dbf4 dimerization. Arguing against this model is the lack of biochemical data supporting an interaction between the Rad53

FHA2 domain and the pT^{105}-x-x-E peptide, however as stated above, FHA2 likely makes additional contacts with the BRCT domain.

Dbf4-Rad53 binding is critical for regulation of late origin activation

The DNA replication checkpoint is important for controlling the fidelity of DNA synthesis. Upon sensing DNA damage or replication fork stalling, Rad53 activation directly phosphorylates Dbf4 and Sld3, and consequently inhibits late origin firing (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010). A *dbf4-4A sld3-38A* double mutant in the Dbf4 and Sld3 Rad53 phosphorylation sites bypasses the replication checkpoint to allow unchecked S phase progression during replication stress. Similarly, we demonstrated that a *dbf4-NΔ109* mutant defective in the Dbf4-Rad53 interaction coupled with the *sld3-38A* mutant allows late origin firing in the presence of HU (Figure 22). This indicates that the Rad53-mediated Dbf4 phosphorylation during replication checkpoint likely depends on the physical interaction between Dbf4 and Rad53. We showed previously that deletion of the Dbf4 N-terminal 109 residues largely blocked Rad53-dependent phosphorylation in HU assayed by a Dbf4 mobility shift (Gabrielse et al. 2006). Our study also suggests that the Dbf4-Rad53 interaction is promoted by phosphorylation of Dbf4 residue T105.

We propose that the regulation of DDK in the replication checkpoint depends on two phosphorylation events: the first is the phosphorylation of Dbf4 residue T105 by an unknown kinase, which promotes the Rad53-DDK interaction. The second is the subsequent Rad53-mediated phosphorylation of Dbf4 at the critical sites between motifs

M and C. Since Rad53 cannot bind Dbf4-NΔ109 and this leads to a substantial defect in Rad53 phosphorylation of Dbf4, our data implies that stable binding of Rad53 to its targets may be needed for efficient phosphorylation. This is similar for instance to DDK itself, which is targeted to Mcm4 through an N-terminal sequence (Sheu and Stillman 2010). We note that T105 phosphorylation is likely not essential for the Rad53-Dbf4 physical interaction since a Dbf4 quadruple mutant protein (S84A S92A T95A T105A) still underwent a Rad53-dependent shift in HU (Gabrielse et al. 2006). However, since Rad53 interacts with Dbf4 using multiple residues and perhaps two different binding sites, a single point mutation is unlikely to eliminate binding.

Role for a DDK-Rad53-Cdc5 complex?

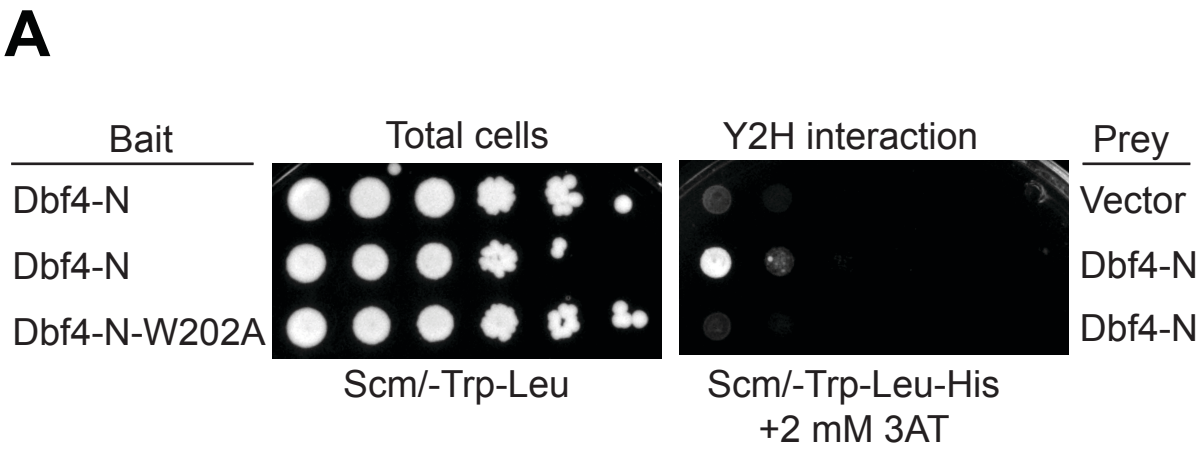
We demonstrated that three essential kinases, Rad53, Cdc7 and Cdc5 can form a complex with Dbf4 following co-expression in insect cells. This ternary interaction depends on the N-terminal 109 residues of Dbf4 in agreement with the yeast two-hybrid studies. Although we showed that the Dbf4 interaction with Cdc5 inhibited Cdc5 activation of the MEN pathway (Miller et al. 2009), Cdc5 may have additional functions in a DDK-Rad53-Cdc5 complex. Recent studies show that Cdc5 attenuates Rad53 activation to allow checkpoint adaptation (Donnianni et al. 2010; Vidanes et al. 2010). It is possible that Cdc5 phosphorylates Rad53 with the ternary complex to attenuate its activity. Also, a complex regulation may underlie the DDK interaction with Rad53 and Cdc5. Our two-hybrid results showed that the loss of the Dbf4-Cdc5 interaction (either by deleting residues 82-88, or point mutations within the Cdc5 binding site) promoted a stronger Dbf4-Rad53 two-hybrid interaction (Figure 22). In contrast, Rad53

phosphorylates Dbf4 on residue S84 following exposure to HU (Duch et al. 2011). Serine 84 falls within the Cdc5 binding site and we previously demonstrated that phosphorylation of S84 peptide prevented binding to Cdc5 (Chen and Weinreich 2010). This suggests that activated Rad53 might prevent Cdc5 binding to Dbf4 through S84 phosphorylation. Since Cdc5 is expressed late in S phase in contrast to DDK and Rad53, it may only bind DDK after replication is completed. Whether Cdc5 associates with DDK singularly or within a DDK-Cdc5-Rad53 ternary complex in response to replication checkpoint activation (or at other cell cycle transitions) remains to be determined.

Figure 23. Evidence for a Dbf4-Dbf4 N-terminal interaction

(A-B) Dbf4 N-terminal residues 66-227 were cloned in two-hybrid bait and prey plasmids separately to examine Dbf4 dimerization. Two-hybrid interactions were quantitated by spotting assays on selective media (panel A) or by β-galactosidase assays (panel B). A W202A substitution in the Dbf4 BRCT domain abolished the Dbf4-Dbf4 interaction. (C) The expression of representative Dbf4 mutants in two-hybrid assays is shown by Western blotting against the c-Myc epitope tag on the Gal4BD (DNA Binding Domain) fusions. Whole cell extracts prepared by TCA extraction method were equally loaded onto each lane (Ponceau S staining, left). Gal4BD fused Dbf4 were detected by anti-Myc antibody (9E10), followed by anti-mouse second antibody (right).

Figure 23. (cont'd)



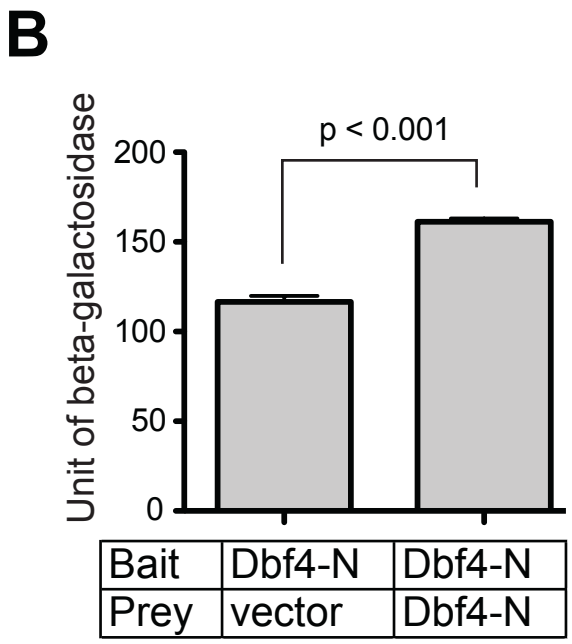


Figure 23. (cont'd)

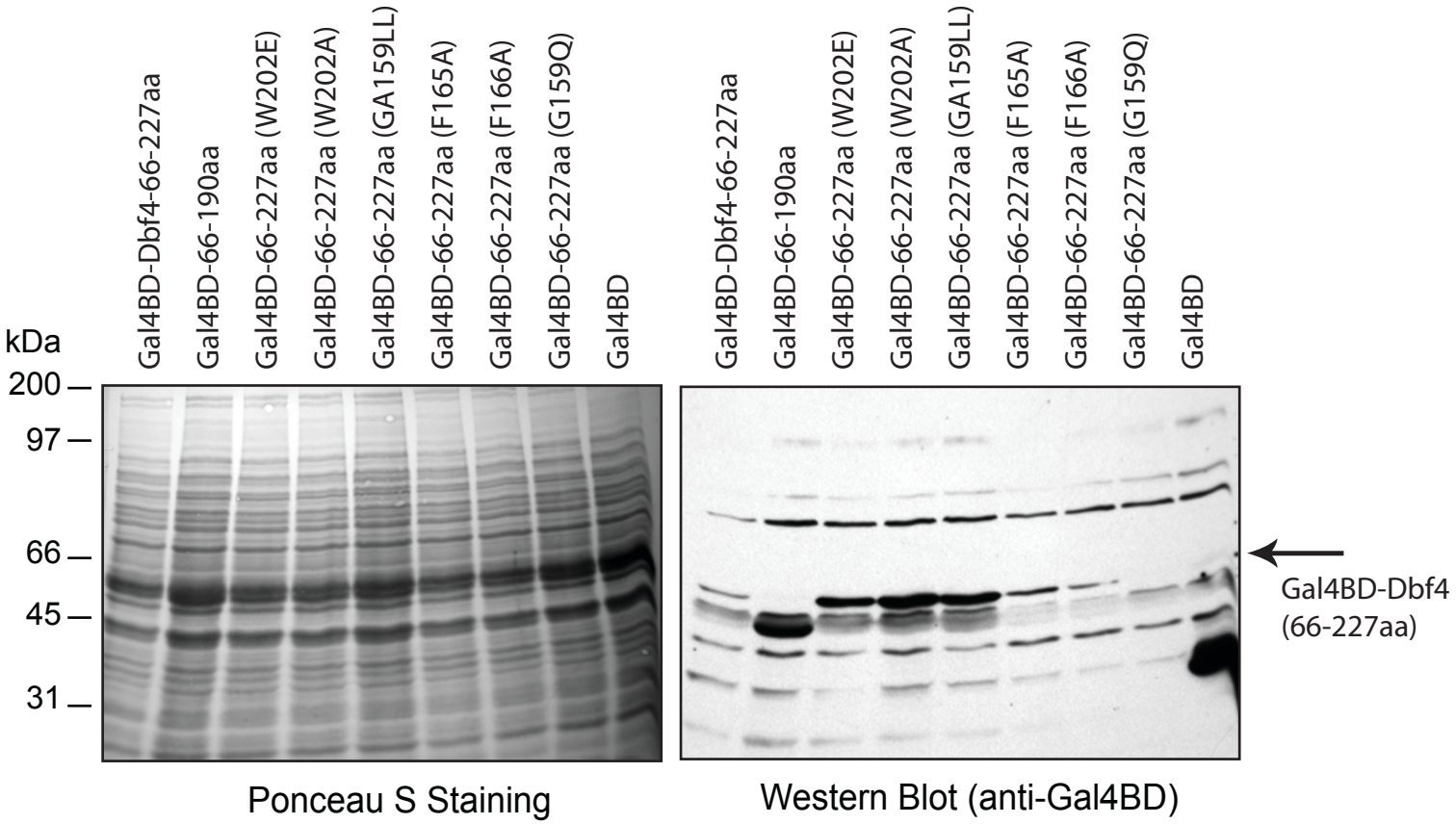
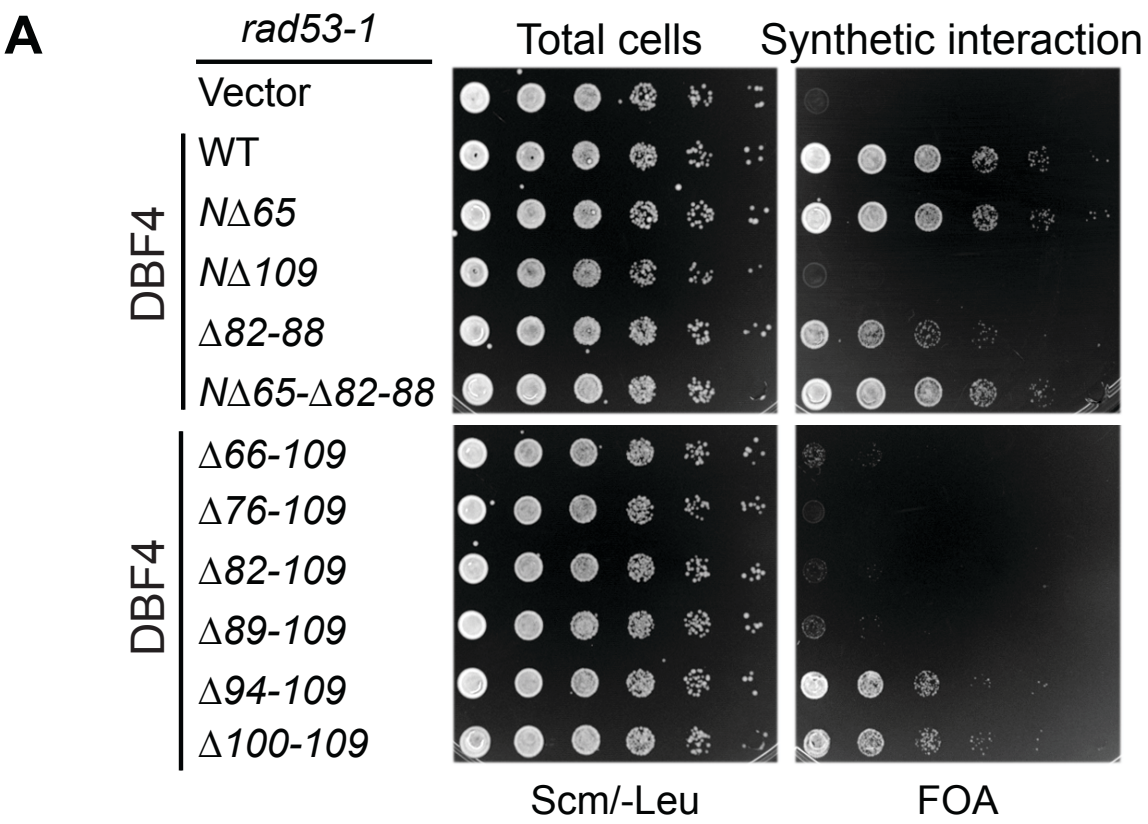


Figure 24. Sequences between Dbf4 residues 65-88 act to inhibit the Rad53 interaction

(A) A series of full-length Dbf4 deletions was assayed by two-hybrid for interaction with full length Rad53 (panel A) or the Cdc5 Polo-box domain (PBD) (panel B). The *dbf4-∆100-109* deletion causes a loss of Rad53 binding, but still allows interaction with the Cdc5-PBD. In particular, the *dbf4-R83E* and *dbf4-∆82-88* mutants that cannot bind the PBD domain show increased interaction with Rad53 compared with wild type Dbf4, suggesting that Cdc5 binding can inhibit Rad53 binding to Dbf4.

Figure 24. (cont'd)



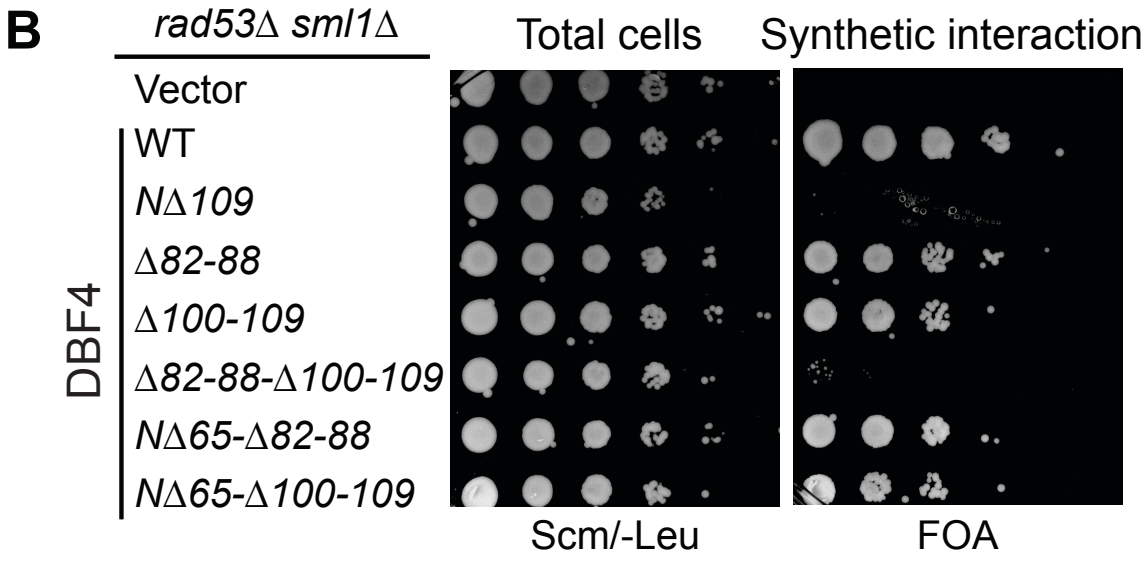
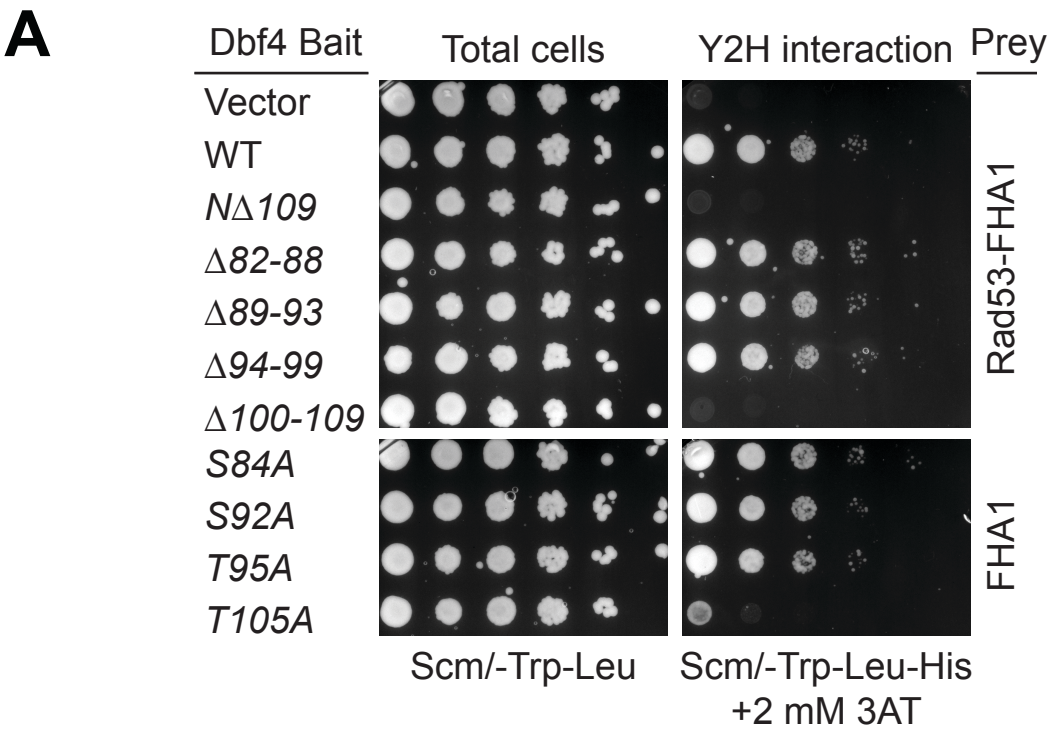
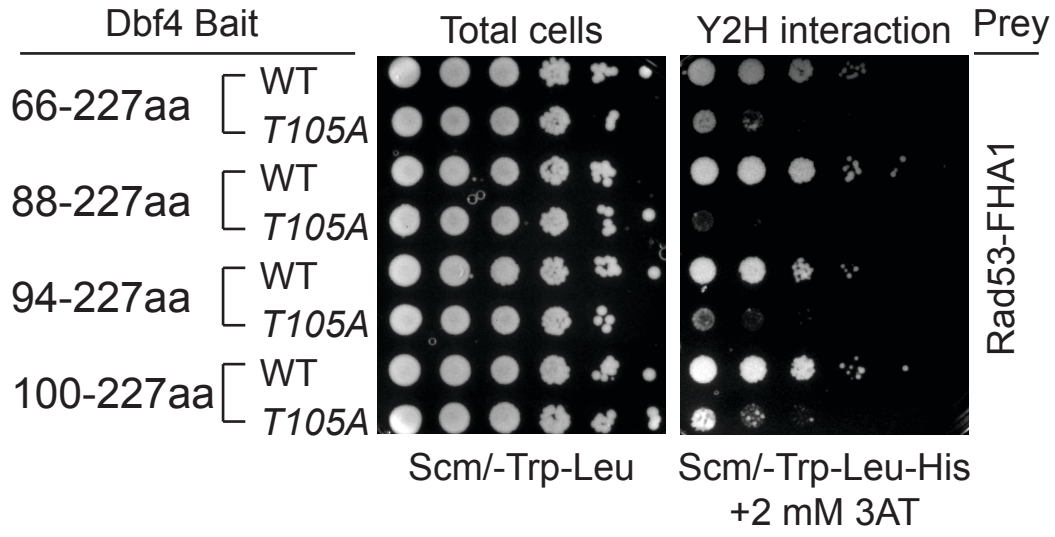


Figure 25. Dbf4 T105 residue is critical for the Dbf4-FHA1 domain interaction

(A) The *dbf4-∆100-109*, *dbf4-T105A* and *dbf4-N∆109* mutants cause a loss of FHA1 domain binding in two-hybrid assays. The *dbf4-S84A, -S92A*, and *-T95A* mutants did not show any effect on FHA1 domain binding. (B) Substitution of T105A on various Dbf4 truncations consistently caused a loss of interaction with the FHA1 domain.

Figure 25. (cont'd)





MATERIALS AND METHODS

Construction of Yeast Strains, Plasmids, and Baculoviruses

Plasmids and yeast strains used in this study are listed in Tables 1 and 2. PJ69–4a cells (*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met::GAL7-lacZ*) were used for two-hybrid experiments. All other strains were derivatives of W303-1A (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3*). The natMX4 cassette flanked with *DBF4* target sequences was PCR amplified from p4339 with primers (5'-CTA TCA ACG GCA ATG TTA TTG AAT CAC TTT CTC ATT CAC CCT TGT ACA TGG AGG CCC AGA ATA CC-3') and (5'- ATG CAA TTG ATA ATA TAT GGA CGA GTA AAT AAG AGT TAA GTC AAT CAG TAT AGC GAC CAG CAT TC-3') (Goldstein and McCusker 1999), and transformed into M1261 (*dbf4-NΔ109*). clonNAT (Werner Bioagents) resistant transformants were confirmed with natMX4 marker and then backcrossed to W303. The *epitope-tagged RAD53* strains were made by the method of Longtine et al (Longtine et al. 1998). Deletions and point mutations within *DBF4* and *RAD53* were generated by site-directed mutagenesis using the QuikChange system (Stratagene). PCR-amplified EcoRI-PstI fragments containing the full-length *RAD53* coding sequence (1 to 821), FHA1 domain (1 to 300), FHA2 domain (483 to 821) and *DBF4* coding sequence (66 to 227) were cloned into the same sites of pGAD-C1 (Clontech) to give the Gal4 activation domain fusions. Rad53 residues 2-164 were cloned on a BamHI-XhoI into pET24a-GST for expression of His_6-GST-FHA1 domain. Construction of baculovirus plasmids encoding wild type Dbf4, Dbf4-NΔ109, HA-Cdc7, and 3Myc-Cdc5 was previously described (Gabrielse et al. 2006). An NcoI-PstI fragment containing the full-length *RAD53* coding sequence (1 to 821) was cloned

in the baculovirus transfer vector, pAcSG2. High-titer baculoviruses were generated by transfection of Sf9 cells using the BaculoGold kit (BD Biosciences) followed by plaque purification and virus amplification.

Growth Conditions, Cell Cycle Synchronization, and Replication intermediate assays

Yeast cells were cultured in YPD and Synthetic complete medium (Scm). Cells were synchronized in G_1 phase with 5 μg/ml α-factor for 3 hours and released into 0.2M hydroxyurea for the indicated times. The alkaline gel electrophoresis and probes for the replication origins (ARS305, ARS501, ARS603) were previously described (Mantiero et al. 2011). DNA content was analyzed by flow cytometry as previously published (Mantiero et al. 2011).

Two-hybrid Analysis

Various *DBF4* bait constructs containing Gal4 DNA binding domain were transformed with Gal4 activation domain prey plasmids in PJ69–4a and selected on SCM plates lacking tryptophan and leucine. These were spotted at 10-fold serial dilutions on the same plates and also on plates also lacking histidine but containing 2 mm 3-aminotriazole and cultured for 2–3 days at 30 °C. o-nitrophenyl-β-D-galactoside (ONPG) (Sigma) β-galactosidase assay was previously described (J. H. Miller (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press).

Immunoprecipitation from Sf9 Cells and Western Blotting

Sf9 cells were co-infected with HA-Cdc7, 3Myc-Cdc5, Rad53, and Dbf4 mutants as previously described (Chen and Weinreich 2010). Whole cell extracts and IPs were probed with polyclonal antibodies against Cdc7 (1:4000) and Dbf4 (1:1000). Rad53 and 3Myc-Cdc5 were detected with yC-19 (Santa Cruz Biotechnology) and 9E10 antibodies respectively.

Protein Purification and Peptide Binding Assays

His_6-GST-FHA1 and His_6-GST-FHA2 domains were induced in BL21(DE3) cells for 3 h at 30 °C using 0.5 mM isopropyl 1-thio-β-d-galactopyranoside. Protein purification and the AlphaScreen luminescence proximity assay (PerkinElmer Life Sciences) were previously described (Chen and Weinreich 2010). All peptides used in this study are listed in Table 6.

Table 4. Plasmids

Plasmid	Description	Source
p4339	pCRII-TOPO::natRMX4	Goldstein and McCusker, 1999
pAcSG2		BD Biosciences
pCG10	pRS415-*DBF4*$_{N\Delta 109}$	Gabrielse et al., 2006
pCG40	pAcSG2-*DBF4*$_{N\Delta 109}$	Miller et al., 2009
pCG44	pAcSG2-*DBF4*$_{N\Delta 221}$	Gabrielse et al., 2006
pCG52	pGBKT7-*DBF4*$_{66-227}$	Miller et al., 2009
pCG53	pYJ204-*DBF4*$_{N\Delta 65}$	Miller et al., 2009
pCG60	pCG52$_{ADH1\ promoter-\Delta(-732)-(-802)}$	Miller et al., 2009
pCG63	pCG60 W202E	This study
pCG64	pCG60 W202A	This study
pCG74	pYJ204-*DBF4*$_{N\Delta 109}$	Miller et al., 2009
pCG75	pYJ204-*DBF4*$_{N\Delta 221}$	Miller et al., 2009
pCG91	pAcSG2-*DBF4*$_{N\Delta 65}$	Gabrielse et al., 2006
pCG101	pCG60 GA159,160LL	This study
pCG108	pCG60 F165A	This study
pCG110	pCG60 F166A	This study
pCG146	pCG60 G159Q	This study
pCG265	pGAD-C1-*CDC7*$_{1-507}$	Harkins et al., 2009
pCM16	pAcSG2-3myc-*CDC5*$_{65-705}$	Miller et al., 2009
pCM21	pCG60-*DBF4*$_{66-109}$	Miller et al., 2009
pET24a-GST		Chen and Weinreich, 2010
pGAD-C1		James et al. 1996
pGAD-Cdc5.3	pGAD-C1-*CDC5*$_{421-705}$	Miller et al., 2009
pGBKT7		Clontech
pJK18	pCG60 T171E	This study
pJK20	pCG60 E108A	This study
pJK22	pCG60 T171S	This study
pJK25	pCG60 V100A	This study
pJK26	pCG60 R103A	This study
pJK27	pCG60 V104A	This study
pJK29	pCG60 P106A	This study
pJK31	pCG60 L109A	This study
pJK33	pCG60 K107A	This study
pJK34	pCG60 T105A E108A	This study
pJK36	pCG60 E108K	This study
pJK37	pCG60 T171A	This study
pJK39	pCG60 E101A	This study
pJK41	pCG60 P102A	This study
pJK45	pYJ204-*DBF4*$_{N\Delta 81}$	This study
pJK47	pYJ204-*DBF4*$_{N\Delta 93}$	This study

Table 4. (cont'd)

pJK48	pYJ204-*DBF4*$_{N\Delta 99}$	This study
pJK49	pCG60 T105S	This study
pJK51	pCG60 K107E	This study
pJK53	pCG60 T131A	This study
pJK55	pCG60 L110A	This study
pJK57	pCG60 E111A	This study
pJK59	pCG60 W112A	This study
pJK61	pCG60 T114A	This study
pJK67	pCG60-*DBF4*$_{\Delta 94-99}$	This study
pJK76	pYJ204-*DBF4*$_{N\Delta 88}$	This study
pJK82	pCG60 V104L	This study
pJK83	pCG60 L109V	This study
pJK85	pCG60 W112F	This study
pJK86	pCG60 T188A	This study
pJK89	pCG60 T157A	This study
pJK91	pCG60 T163A	This study
pJK93	pCG60 TT168,169AA	This study
pJK95	pCG60 T175A	This study
pJK97	pYJ319 G653E	This study
pJK99	pYJ319 T654A	This study
pJK101	pYJ319 N655A	This study
pJK103	pYJ380 G653E	This study
pJK105	pYJ380 T654A	This study
pJK107	pYJ380 N655A	This study
pJK108	pCG60 Y127A	This study
pJK110	pCG60 Y139A	This study
pJK112	pCG60 Y198A	This study
pJK114	pCG60 Y204A	This study
pJK121	pCG60 Y127S	This study
pJK122	pCG60 Y127T	This study
pJK124	pCG60 I130A	This study
pJK125	pCG60 T171V	This study
pJK126	pCG60 Y204F	This study
pJK128	pCG60 Y127F	This study
pJK135	pGAD-C1-*DMA1*$_{137-302}$	This study
pJK137	pGAD-C1-*DMA2*$_{246-408}$	This study
pJK149	pCG60 T95A	Chen and Weinreich, 2010
pJK169	pET24a-GST-*RAD53*$_{2-164}$	This study
pJK170	pET24a-GST-*RAD53*$_{2-175}$	This study
pJK171	pET24a-GST-*RAD53*$_{2-279}$	This study
pJK179	pCG60-*DBF4*$_{N\Delta 87}$ T105A	This study

Table 4. (cont'd)

pJK181	pCG60-*DBF4*$_{N\Delta 99}$ T105A	This study
pJK185	pCG60-*DBF4*$_{N\Delta 93}$ T105A	This study
pJK269	pET24a-GST-*RAD53*$_{2\text{-}164}$ R70A	This study
pJK275	pGAD-C1-*DUN1*$_{1\text{-}160}$	This study
pJK277	pGAD-C1-*FAR10*$_{61\text{-}227}$	This study
pJK279	pGAD-C1-*FHL1*$_{253\text{-}400}$	This study
pJK281	pGAD-C1-*FKH1*$_{41\text{-}185}$	This study
pJK283	pGAD-C1-*MEK1*$_{1\text{-}152}$	This study
pJK285	pGAD-C1-*XRS2*$_{1\text{-}125}$	This study
pJK287	pGAD-C1-*FKH2*$_{1\text{-}254}$	This study
pJK289	pGAD-C1-*PML1*$_{54\text{-}204}$	This study
pJK380	pET24a-GST-*RAD53*$_{483\text{-}821}$	This study
pJK382	pET24a-GST-*RAD53*$_{549\text{-}730}$	This study
pJK410	pYJ380 R605A	This study
pJK420	pET24a-GST-*RAD53*$_{523\text{-}821}$	This study
pMW1	pAcPK30-*DBF4*$_{1\text{-}704}$	Gabrielse et al., 2006
pMW47	pAcSG2-HAHIS6-*CDC7*$_{1\text{-}507}$	Gabrielse et al., 2006
pMW489	pRS415-*DBF4*$_{1\text{-}704}$	Gabrielse et al., 2006
pMW490	pRS416-*DBF4*$_{1\text{-}704}$	Gabrielse et al., 2006
pMW526	pRS415-*DBF4*$_{N\Delta 65}$	Gabrielse et al., 2006
pRS415	*LEU2 ARS-CEN*	Sikorski and Hieter, 1989
pRS416	*URA3 ARS-CEN*	Sikorski and Hieter, 1989
pYJ3	pCG60-*DBF4*$_{\Delta 67\text{-}81}$	Chen and Weinreich, 2010
pYJ4	pCG60-*DBF4*$_{\Delta 67\text{-}88}$	Chen and Weinreich, 2010
pYJ5	pCG60-*DBF4*$_{\Delta 67\text{-}93}$	Chen and Weinreich, 2010
pYJ6	pCG60-*DBF4*$_{\Delta 67\text{-}99}$	Chen and Weinreich, 2010
pYJ7	pCG60-*DBF4*$_{\Delta 67\text{-}103}$	Chen and Weinreich, 2010
pYJ8	pCG60-*DBF4*$_{\Delta 67\text{-}107}$	Chen and Weinreich, 2010
pYJ9	pCG60-*DBF4*$_{N\Delta 109}$	Chen and Weinreich, 2010
pYJ16	pCG60 S84A	Chen and Weinreich, 2010
pYJ30	pCG60 R83E	Chen and Weinreich, 2010
pYJ38	pCG60-*DBF4*$_{\Delta 82\text{-}88}$	Miller et al., 2009
pYJ74	pMW489-*DBF4*$_{\Delta 82\text{-}88}$	Chen and Weinreich, 2010
pYJ167	pCG60 S92A	Chen and Weinreich, 2010
pYJ182	pAcSG2-*DBF4*$_{\Delta 82\text{-}88}$	Chen and Weinreich, 2010
pYJ193	pMW489-*DBF4*$_{\Delta 76\text{-}109}$	This study
pYJ195	pMW489-*DBF4*$_{\Delta 82\text{-}109}$	This study
pYJ198	pMW489-*DBF4*$_{\Delta 66\text{-}109}$	This study
pYJ201	pMW489-*DBF4*$_{N\Delta 65\text{-}\Delta 82\text{-}88}$	Chen and Weinreich, 2010
pYJ204	pGBKT7-*DBF4*$_{1\text{-}704}$	Miller et al., 2009
pYJ206	pYJ204-*DBF4*$_{\Delta 82\text{-}88}$	Miller et al., 2009

Table 4. (cont'd)

pYJ218	pMW489-*DBF4*$_{\Delta 89\text{-}109}$	This study
pYJ219	pMW489-*DBF4*$_{\Delta 100\text{-}109}$	This study
pYJ222	pMW489-*DBF4*$_{\Delta 94\text{-}109}$	This study
pYJ308	pGAD-C1-*RAD53*$_{1\text{-}300}$	This study
pYJ319	pGAD-C1-*RAD53*$_{1\text{-}821}$	This study
pYJ326	pCG60-*DBF4*$_{\Delta 89\text{-}93}$	Chen and Weinreich, 2010
pYJ332	pCG60-*DBF4*$_{\Delta 100\text{-}109}$	This study
pYJ336	pCG60 T105A	This study
pYJ340	pMW489-*DBF4*$_{\Delta 82\text{-}88\text{-}\Delta 100\text{-}109}$	This study
pYJ355	pYJ308 R70A	This study
pYJ368	pCG60-*DBF4*$_{66\text{-}190}$	This study
pYJ372	pCG60-*DBF4*$_{66\text{-}150}$	This study
pYJ380	pGAD-C1-*RAD53*$_{483\text{-}821}$	This study
pYJ384	pYJ319 R70A	This study
pYJ388	pYJ319 R605A	This study
pYJ392	pCG60 T105E	This study
pYJ394	pCG60 T105D	This study
pYJ422	pAcSG2-*DBF4*$_{\Delta 100\text{-}109}$	This study
pYJ424	pAcSG2-*DBF4*$_{\Delta 82\text{-}88\text{-}\Delta 100\text{-}109}$	This study
pYJ426	pMW489-*DBF4*$_{N\Delta 65\text{-}\Delta 100\text{-}109}$	This study
pYJ428	pAcSG2-*RAD53*$_{1\text{-}821}$	This study
pYJ461	pYJ204 R83E	This study
pYJ462	pYJ204-*DBF4*$_{\Delta 100\text{-}109}$ R83E	This study
pYJ464	pYJ204-*DBF4*$_{\Delta 100\text{-}109}$	This study
pYJ466	pYJ204-*DBF4*$_{\Delta 82\text{-}88\text{-}\Delta 100\text{-}109}$	This study
pYJ489	pCG60 E101K	This study
pYJ491	pCG60 R103E	This study
pYJ493	pCG60 Q113A	This study
pYJ494	pYJ204-*DBF4*$_{N\Delta 81\text{-}\Delta 100\text{-}109}$	This study
pYJ497	pYJ204-*DBF4*$_{N\Delta 93\text{-}\Delta 100\text{-}109}$	This study
pYJ507	pCG60 E108D	This study
pYJ512	pCG60 T138A	This study
pYJ535	pGAD-C1-*DBF4*$_{66\text{-}227}$	This study

Table 5. Yeast Strains

Stain	Genotype	Source
PJ69-4A	*MAT**a** trp1-901 leu2-3, -112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al., 1996
W303-1A	*MAT**a** ade2-1, ura3-1 his3-11, -15 trp1-1 leu2-3, -112 can1-100*	Thomas and Rothstein, 1989
y57	W303 *MAT**a** rad53-R70A sml1Δ::HIS3*	Pike et al., 2004
y59	W303 *MAT**a** rad53-K227A sml1Δ::HIS3*	Pike et al., 2004
y205	W303 *MAT**a** rad53-R605A sml1Δ::HIS3*	Pike et al., 2004
y1853	W303 *MAT**a** sml1Δ::URA3 sld3-38A-10his-13MYC::kanMX4*	Zegerman and Diffley, 2010
y2573	W303 *MAT**a** dbf4Δ::TRP1 his3::PDBF4-dbf4 4A::HIS3 sld3-38A-10his-13MYC::kanMX4*	Zegerman and Diffley, 2010
M517	W303 *MAT**a** rad53-1*	Cabrielse at al., 2006
M895	W303 *MAT**a** dbf4Δ::kanMX6* [pMW490; pRS416-*DBF4 URA3*]	Cabrielse at al., 2006
M1261	W303 *MAT**a** dbf4-NΔ109*	Cabrielse at al., 2006
M1589	W303 *MAT**a** rad53-1 dbf4Δ::kanMX6* [pMW490; pRS416-*DBF4 URA3*]	Cabrielse at al., 2006
M1800	W303 *MAT1 dbf4-NΔ109-kanMX6*	Miller et al., 2009
M3581	W303 *MAT**a** rad53Δ::TRP1 sml1Δ::HIS3 dbf4Δ::kanMX6* [pMW490; pRS416-*DBF4 URA3*]	This study
M3831	W303 *MAT**a** RAD53-3MYC-TRP1*	This study
M3890	W303 *MAT**a** dbf4-NΔ109-natMX4*	This study
M3905	W303 *MAT**a** dbf4-NΔ109-natMX4 sld3-38A-10his-13MYC::kanMX4*	This study
M3913	W303 *MAT**a** dbf4-NΔ109-kanMX6 sml1::HIS3*	This study
M3920	W303 *MAT**α** RAD53-3MYC-TRP1 dbf4-NΔ109-kanMX6 sml1Δ::HIS3*	This study

Table 6. Peptides

Peptide name	Peptide sequence	Length	MW
Biotin-Dbf4 (98-113)	Biotin- KNV EPR VTP KEL LEW Q	Biotin + 17	2192.9
Biotin-pDbf4	Biotin- KNV EPR V(pT)P KEL LEW Q	Biotin + 17	2273.2
Dbf4 (98-113)	KNV EPR VTP KEL LEW Q	17	1966.4
pDbf4 (pThr105)	KNV EPR V(pT)P KEL LEW Q	17	2047.5
pDbf4-V104A	KNV EPR A(pT)P KEL LEW Q	17	2019.8
pDbf4-E108A	KNV EPR V(pT)P KAL LEW Q	17	1989.9
pDbf4-E108D	KNV EPR V(pT)P KDL LEW Q	17	2032.7
pDbf4-L109A	KNV EPR V(pT)P KEA LEW Q	17	2005
Biotin-Rad9	Biotin- IMS EVE LTQ ELP EVE	15	1972.28
Biotin-pRad9	Biotin- IMS EVE L(pT)Q ELP EVE	15	2052.26
pSpc72	EEF LSL AQS (pS)PA GSQ LES RD	20	2231.3

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CHAPTER 4

FUNCTIONAL CHARACTERIZATION OF THE DBF4 N-TERMINUS BY A GENOME-WIDE SYNTHETIC LETHALITY SCREEN

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FUNCTIONAL CHARACTERIZATION OF THE DBF4 N-TERMINUS BY A GENOME-WIDE SYNTHETIC LETHALITY SCREEN

ABSTRACT

The Dbf4-dependent Cdc7 kinase (DDK) is a conserved two-subunit kinase required for eukaryotic DNA replication. We recently reported that Dbf4 interacts with Cdc5 (yeast Polo-like kinase) and Rad53 (the ortholog of mammalian Chk2 kinase), and proposed that Dbf4 serves a molecular scaffold to assemble a ternary complex (Dbf4-Cdc7-Cdc5-Rad53) that coordinates chromosome segregation and checkpoint signaling pathways in post-replicative cell-cycle regulation. Since *dbf4* mutants unable to interact either with Cdc5 or Rad53 exhibit normal cell-cycle progression and grow well under various genotoxic stresses, we suggest that other pathways act in parallel with Dbf4 function. We report here the results of a genome-wide screen for genes that are synthetically lethal or sick in combination with a *dbf4* N-terminal deletion, which is defective in binding both Cdc5 and Rad53. This genetic interaction network showed that Dbf4 is involved in multiple surveillance mechanisms that control genome stability (Sgs1, Top3, and Rmi1), DNA replication or damage checkpoint signaling (Rad17, Mec3, Rad24, Rad9, Rad54, Pol32, and Bmh1), and chromosome segregation (Csm1, Ctf18, Ctf8, Dcc1, and the HIR complex). These data not only provide insight into the role of Dbf4 in the convergence of checkpoint signaling and mitotic regulation, but also contribute to a comprehensive understanding of Dbf4 function in cell-cycle regulation.

INTRODUCTION

In many signaling networks, scaffold proteins are known to recruit pathway components and facilitate the specificity of signal transduction (Burack and Shaw 2000; Ubersax and Ferrell 2007). Although binding partners of a scaffold protein can be determined using biochemical approaches that discover protein-protein interactions, little is known about the biological outcome of the scaffold-modulated assembly. The signals that transmit though a scaffold protein can be identified by genetic mapping of scaffold mutants, which provides a global view of the functional relationships between genes and pathways (Boone et al. 2007; Dixon et al. 2009).

Dbf4 is an essential regulator of the S-phase kinase Cdc7 (also known as Dbf4-dependent kinase, DDK), which directly phosphorylates and activates the DNA helicase Mcm2-7 for DNA synthesis (Sclafani 2000; Bell and Dutta 2002). Work in the budding yeast *Saccharomyces cerevisiae* has shown that an Mcm mutation, *mcm5-bob1*, bypasses the deletion of the essential genes *DBF4* and *CDC7*, suggesting that Mcm2-7 is the main physiological substrate of DDK (Hardy et al. 1997; Johnston et al. 1999; Sclafani et al. 2002). Structural studies of an archaeal Mcm complex containing an analogous mutation indicate that the genetic suppression is the result of a conformational change that probably mimics the activation of Mcm2-7 helicase (Hoang et al. 2007). Also, DDK phosphorylation has been shown to be required to recruit Cdc45 and the GINS complex to the Mcm2-7 helicase (Owens et al. 1997; Zou and Stillman 2000;

Masai et al. 2006; Yabuuchi et al. 2006). Cdc45, Mcm2-7, and GINS form an active replicative helicase that plays a major role in the initiation of DNA synthesis (Weinreich and Stillman 1999; Gambus et al. 2006; Moyer et al. 2006; Sheu and Stillman 2006; Francis et al. 2009).

Dbf4 orthologs have been identified in *Schizosaccharomyces pombe* (*dfp1+*), *Drosophila melanogaster* (*chiffon*), *Xenopus laevis* (*XDBF4*), mice (*MmDbf4*), and humans (*HsDbf4/ASK*) (Brown and Kelly 1998; Jiang et al. 1999; Kumagai et al. 1999; Landis and Tower 1999; Lepke et al. 1999; Johnston et al. 2000; Furukohri et al. 2003). Multiple sequence alignment of Dbf4 proteins across species revealed three conserved motifs, termed motif-N, -M, and -C (Masai and Arai 2000). It is generally thought that Dbf4 motifs-M and -C bind to and activate Cdc7 kinase (Jones et al. ; Harkins et al. 2009), while the Dbf4 N-terminus (residues 1-296) is dispensable for the essential function of Dbf4 in DNA replication (Duncker et al. 2002; Gabrielse et al. 2006; Miller et al. 2009; Chen and Weinreich 2010). Recently, we characterized two distinct binding motifs within the Dbf4 N-terminus that interact independently with the mitotic Polo-like kinase Cdc5 and the checkpoint kinase Rad53 (Miller et al. 2009; Chen and Weinreich 2010; Chen et al. 2012). Importantly, Cdc5, Rad53 and Cdc7 can form a stable complex with Dbf4 (Chen et al. 2012). *CDC5* is the single *Polo* ortholog in budding yeast and plays multiple essential roles in mitotic and meiotic regulation (Sunkel and Glover 1988; Barr et al. 2004; Archambault and Glover 2009). Genetic evidence suggests that Dbf4 inhibits Cdc5 through a direct

interaction to prevent premature exit from mitosis (Miller et al. 2009; Chen and Weinreich 2010). Intriguingly, this Cdc5 inhibition depends on the association between Cdc7 and Dbf4, suggesting that Dbf4 serves as a scaffold for Cdc7 to mediate the Cdc5 inhibition in mitotic exit.

When replication forks stall, the active Rad53 kinase binds to the Dbf4 N-terminus in a phosphorylation-dependent manner and phosphorylates the C-terminus of Dbf4, subsequently inhibiting late origin firing (Duch et al. 2010; Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010; Chen et al. 2012). Although *dbf4* mutants that cannot bind either Cdc5 or Rad53 exhibit wild-type growth and normal S-phase progression (Gabrielse et al. 2006; Miller et al. 2009; Chen and Weinreich 2010; Chen et al. 2012), N-terminal deletions of *DBF4* are lethal when Rad53 function is compromised, suggesting that Rad53, Cdc5, or both cooperate with DDK to perform an essential cell cycle function (Gabrielse et al. 2006; Chen et al. 2012). However, little is known about how Dbf4 acts in replication fork stability and post-replicative cell-cycle regulation. To identify this, we have performed a genome-wide synthetic lethal screen using the *dbf4-NΔ109* mutant, which is defective in binding both Rad53 and Cdc5.

Synthetic genetic interactions are usually identified when a second-site mutation suppresses or enhances the original mutant phenotype. In particular, synthetic lethality occurs when two mutations are separately viable but their combination results in lethality or a reduced fitness that is more severe than that of the

individual single mutations. In principle, when two genes show a synthetic lethal interaction, it often reflects the gene products operating in parallel pathways or participating in the same protein complex (Hartman et al. 2001). With its genetic tractability and short generation time, *S. cerevisiae* has become a powerful model for large-scale mapping of synthetic genetic interactions. A yeast library that collected non-essential deletions has been developed for a Synthetic Genetic Array (SGA) (Giaever et al. 2002; Tong et al. 2004; Huang and Kolodner 2005; Pan et al. 2006b; Boone et al. 2007).

We set up a genome-wide SGA screen using the *dbf4-NΔ109* mutant to query nearly 4300 deletion mutants (representing 82% of the genes in budding yeast) for synthetic genetic interactions. The genetic interactions that we uncovered represent a small number of functional categories, including control of genome stability (the Top3- Sgs1-Rmi1 complex), DNA damage or checkpoint signaling (the 9-1-1 complex, Rad54, Rad9, and Bmh1), and chromosome segregation (the CTF and HIR complexes and Csm1). We also described a two-hybrid interaction between Dbf4 and Bmh1 that maps to site overlapping the Rad53 binding site on Dbf4. These results strongly suggest that Dbf4 N-terminus plays a parallel role in the Rad53-mediated checkpoint activation, as well as highlighting that Dbf4 coordinates multiple checkpoint responses through the interactions with various cell-cycle regulators.

RESULTS

An SGA screen for the dbf4-NΔ109 mutant

Our earlier reports indicated that the Dbf4 N-terminus (residues 1-109) interacts with two essential kinases, Cdc5 and Rad53 (Gabrielse et al. 2006; Miller et al. 2009; Chen and Weinreich 2010; Chen et al. 2012). The *dbf4-NΔ109* allele not only suppresses the temperature sensitivity of the *cdc5-1* mutant, but also shows a synthetic lethal interaction with *rad53-1*, a hypomorphic mutant of the *RAD53*. Despite advanced studies in molecular interactions, the biological function of the Dbf4-associated complexes is poorly understood, because N-terminal deletions of *dbf4* do not show any significant phenotype, even with various genotoxic treatments. This may be due to a redundant pathway that acts in parallel with Dbf4 function. Thus, we performed an SGA analysis using the *dbf4-NΔ109* mutant, which abolishes the interaction with Cdc5 and Rad53, as the query strain to probe the specific function of the Dbf4 N-terminus on a genome-wide scale.

We first constructed the *dbf4-NΔ109* mutant and integrated a nourseothricin resistance marker (*natMX4*) in its 3' UTR. This query mutant was crossed with the 4,293 viable yeast deletion mutants on the SGA. The yeast mating-type alpha (MAT**α**) strain carrying *dbf4-NΔ109::natMX4* was systematically crossed onto the SGA, on which all the deletion mutants were in the mating-type a (MAT**a**) (Tong et al. 2004). Strains showing resistance to both nourseothricin and geneticin were selected as diploid strains. The resulting heterozygous diploids were transferred to a medium with reduced carbon and nitrogen to induce

sporulation and the formation of haploid spore progeny. Spores were further transferred to a synthetic medium that specifically selects for germination of MAT**a** through the engineered mating-type reporter (*MFA1pr-HIS3*) (Pan et al. 2004). Two recessive markers, *can1*Δ and *lyp1*Δ, which confer drug resistance to canavanine and thialysine, were also used as haploid-selectable markers (Baryshnikova et al. 2010). The MAT**a** meiotic progeny were then transferred onto medium contained both nourseothricin and geneticin for the selection of double mutants. A synthetic lethal or synthetic sick interaction was identified when the colony size of double-mutant progeny was smaller than that of wild-type controls. For the purpose of computer-based scoring, the *adjusted calibrated p-values* were calculated by comparing the measurements between the mutants and wild-type controls (Tong et al. 2004). Following normalization, statistical significance was indicated by the values of *t-statistics*.

Table 7 lists the validated synthetic lethal and synthetic sick interactions with *dbf4-N*Δ*109* from our SGA screen. The genetic interaction profile of *dbf4-N*Δ*109* partially overlapped with the SGA profiles of the *dbf4-1* and *cdc7-1* hypomorphic mutants (Tong et al. 2004) (Table 8 and data not shown). Genes involved in maintaining chromosome stability (*TOP3, SGS1,* and *YLR235C*), in the CTF complex (*CTF18, CTF8,* and *DCC1*), in the 9-1-1 complex and checkpoint signaling (*RAD17, MEC3*, and *BMH1*), and in chromatin structure and chromosome segregation (*HIR1, HIR3,* and *HPC2*) were found in the three screens. We also identified genes that specifically interact with the *dbf4-N*Δ*109*

allele, including *RMI1, POL32, RAD54, HIR2, ASF1, CSM1, BUB3*, and a handful of transcriptional regulators (*LSM7, CDC73, SRB2, MED1*, and *CKB2*). Numerous candidates had sequence similarity to human genes or analogous functions, so we focused further analysis and interpretation on those genes.

Table 7. Synthetic lethality or sickness with *dbf4-NΔ109*

Rank	*ORF*	*Gene*	*Gene Function*
1	YKL139W	*CTK1*	Transcriptional regulator
2	YLR235C	*TOP3**	Top3-Sgs1-Rmi1 complex, recombination, genome stability
3	YPL024W	*RMI1*	Top3-Sgs1-Rmi1 complex, recombination, genome stability
4	YNL147W	*LSM7*	Transcriptional regulator
5	YGR270W	*YTA7*	Transcriptional regulator
6	YMR237W	*BCH1*	Intracellular trafficking
7	YLR418C	*CDC73*	Transcriptional regulator
8	YBR215W	*HPC2*	HIR complex, heterochromatin formation and kinetochore assembly
9	YOR368W	*RAD17*	9-1-1 clamp complex, DNA damage checkpoint
10	YMR190C	*SGS1*	Top3-Sgs1-Rmi1 complex, recombination, genome stability
11	YOR026W	*BUB3*	Spindle checkpoint
12	YPL213W	*LEA1*	Transcriptional regulator
13	YPL178W	*CBC2*	Transcriptional regulator
14	YLR338W	*OPI9*	Cytoskeletal organization
15	YPL079W	*RPL21B*	Biosynthesis
16	YOR039W	*CKB2*	Transcriptional regulator
17	YCR077C	*PAT1*	Transcriptional regulator
18	YHR191C	*CTF8*	CTF complex, sister chromatid cohesion
19	YHR041C	*SRB2*	Transcriptional regulator
20	YOR297C	*TIM18*	Intracellular trafficking
21	YMR205C	*PFK2*	Glycolysis
22	YCR086W	*CSM1*	Chromosome segregation
23	YJR043C	*POL32*	DNA replication
24	YPR070W	*MED1*	Transcriptional regulator
25	YER173W	*RAD24*	9-1-1 clamp complex, DNA damage checkpoint

Table 7. (cont'd)

34	YMR078C	*CTF18*	CTF complex, sister chromatid cohesion
36	YLR234W	*TOP3*	Top3-Sgs1-Rmi1 complex, recombination, genome stability
39	YOR038C	*HIR2*	HIR complex, heterochromatin formation and kinetochore assembly
43	YGL163C	*RAD54*	DNA double-strand break repair
45	YJL115W	*ASF1*	HIR complex, heterochromatin formation and kinetochore assembly
47	YBL008W	*HIR1*	HIR complex, heterochromatin formation and kinetochore assembly
48	YJR140C	*HIR3*	HIR complex, heterochromatin formation and kinetochore assembly
49	YCL016C	*DCC1*	CTF complex, sister chromatid cohesion
70	YDR363W	*ESC2*	Chromatin silencing
93	YER177W	*BMH1*	14-3-3 homolog

* open reading frame partially overlaps the gene *TOP3*

Table 8. Summary of common hits in the SGA screens

dbf4-NΔ109	*dbf4-1**	*cdc7-1**	*Gene Function*
YLR235C	*YLR235C*	*YLR235C*	Top3-Sgs1-Rmi1 complex
RAD17	*RAD17*	*RAD17*	9-1-1 clamp complex
CTF8	*CTF8*	*CTF8*	CTF complex
RAD24	*RAD24*	*RAD24*	9-1-1 clamp complex
TOP3	*TOP3*	*TOP3*	Top3-Sgs1-Rmi1 complex
RAD54	*RAD54*	*RAD54*	DNA repair
HIR1	*HIR1*	*HIR1*	HIR complex
HIR3	*HIR3*	*HIR3*	HIR complex
DCC1	*DCC1*	*DCC1*	CTF complex
ESC2	*ESC2*	*ESC2*	Chromatin silencing
RMI1	*RMI1*		Top3-Sgs1-Rmi1 complex
HPC2	*HPC2*		HIR complex
BMH1	*BMH1*		14-3-3 homolog
SGS1		*SGS1*	Top3-Sgs1-Rmi1 complex
CKB2		*CKB2*	Transcriptional regulator
CTF18		*CTF18*	CTF complex
	CHK1	*CHK1*	DNA repair
	IRA2	*IRA2*	Signal transduction
	STI1	*STI1*	Protein folding
	RAD9	*RAD9*	DNA repair
	RTT107	*RTT107*	DNA repair
	TIF1	*TIF1*	Glycolysis
	MEC3	*MEC3*	9-1-1 clamp complex

* Tong et al., Science (2004)

Dbf4 has strong synthetic interactions with the Top3-Sgs1-Rmi1 complex

Three of our top ten hits mapped to the Top3-Sgs1-Rmi1 complex, which is related to maintenance of genomic integrity (summarized in Table 7). *Top3* is a type IA topoisomerase that resolves the catenation of chromosomes in replication forks (Mankouri and Hickson 2007; Suski and Marians 2008; Cejka et al. 2012). Deletion of the *TOP3* gene results in elevated levels of recombination at repetitive sequences, which subsequently leads to abnormal chromosomal translocation and rearrangement, an increased rate of sister chromatid exchanges and chromosome loss, and slow growth (Wallis et al. 1989; Myung et al. 2001). The DNA helicase Sgs1, one of the RecQ family of DNA helicases, was identified as a slow growth suppressor of the *top3*-null mutant (Gangloff et al. 1994). The physical interaction between Top3 and Sgs1 is evolutionarily conserved from yeast to humans (Harmon et al. 1999; Bennett et al. 2000; Wu et al. 2000; Harmon et al. 2003). Three of five RecQ homologs in the human genome are associated with rare genetic diseases: Bloom (*BLM*), Werner (*WRN*), and Rothmund-Thomson (*RecQ4*) syndromes, which were characterized by genomic instability, predisposition to cancer, and premature aging. The budding yeast gene *SGS1* is most homologous to human *BLM* (Chu and Hickson 2009).

Two independent genetic screens identified Rmi1 as a third member of the Top3-Sgs1 complex (Chang et al. 2005; Mullen et al. 2005). Rmi1 physically associates with Top3 and Sgs1, and is required for the Top3-Sgs1 function of ressolving Holliday junctions to complete recombination (Cejka et al. 2010a;

Cejka et al. 2010b; Hickson and Mankouri 2011; Cejka et al. 2012). To validate the genetic interaction, which was performed in yeast strain BY4741 (a derivative in the S288C background), we integrated *dbf4-NΔ109* and *rmi1*Δ into a commonly used yeast strain, W303, to retest the synthetic effects. W303 cells harboring a deletion of *RMI1* grew slowly, whereas *dbf4-NΔ109* mutants underwent normal cell cycle progression. Tetrad analysis by crossing *rmi1*Δ to *dbf4-NΔ109* showed that the double mutants were synthetically lethal (Figure 26A and Table 9). Similarly, we placed the *sgs1*Δ allele in the W303 background and confirmed that *dbf4-NΔ109* was synthetically sick with *sgs1*Δ (Figure 26B).

YLR235C is a dubious open reading frame, which overlaps the C-terminal portion of the *TOP3* gene, likely resulting in a *top3* hypomorphic mutant. *YLR235C*Δ exhibits a strong synthetic effect with *dbf4-NΔ109* in the S288C background. Attempts at introducing a *YLR235C* null mutation into W303 caused severe defects in growth that were similar to those of the *top3*Δ mutant. Therefore, we used a W303 *top3* null mutation (*top3-2)* in which the growth defects are suppressed by the *sgs1-3* allele (Lu et al. 1996). The *top3-2 sgs1-3* strain was crossed to the *dbf4-NΔ109* strain for tetrad analysis. The phenotypes of the resulting colonies indicated that the *top3-sgs1-dbf4* triple mutants were synthetic lethal or sick, even though the growth of the *top3-sgs1* double mutants resembled that of the wild-type strain (Figure 26C). Taken together, we found strong genetic interactions with *dbf4-NΔ109* and of all components of the Top3-Sgs1-Rmi1 complex in both the S288C and W303 backgrounds.

To identify signaling pathways that might be regulated by these genetic interactions, we compared the *dbf4-NΔ109* data with previous SGA profiles that probed individually for *top3Δ, sgs1Δ, or rmi1Δ*. A small group of gene functions were significantly enriched (data not shown), particularly those directly involved in DNA metabolism and genomic maintenance (the Ctf18-Ctf8-Dcc1 complex, Asf1, Pol32, Csm1, Pat1, Rad24, Rad53, and Rad54). These results indicate that there is an extensive crosstalk in which Dbf4 and the Top3-Sgs1-Rmi1 complex share redundant mechanisms to control genome integrity.

Figure 26. The Dbf4 N-terminus genetically interacts with the Top3-Sgs1-Rmi1 complex

dbf4-NΔ109 is synthetically lethal or synthetically sick with *rmi1Δ*, *sgs1Δ*, and *top3-2 sgs1-3* in the W303 background. Representative tetrads from diploid strains of genotype (A) *DBF4*/*dbf4-NΔ109 RMI1/rmi1Δ,* (B) *DBF4*/*dbf4-NΔ109 SGS1/sgs1Δ,* and (C) *DBF4*/*dbf4-NΔ109 TOP3/top3-2 SGS1/sgs1-3* were sporulated and dissected onto YPD plates. Recombinant genotypes are indicated. Detailed GO annotations are summarized in Table 9.

Figure 26. (cont'd)

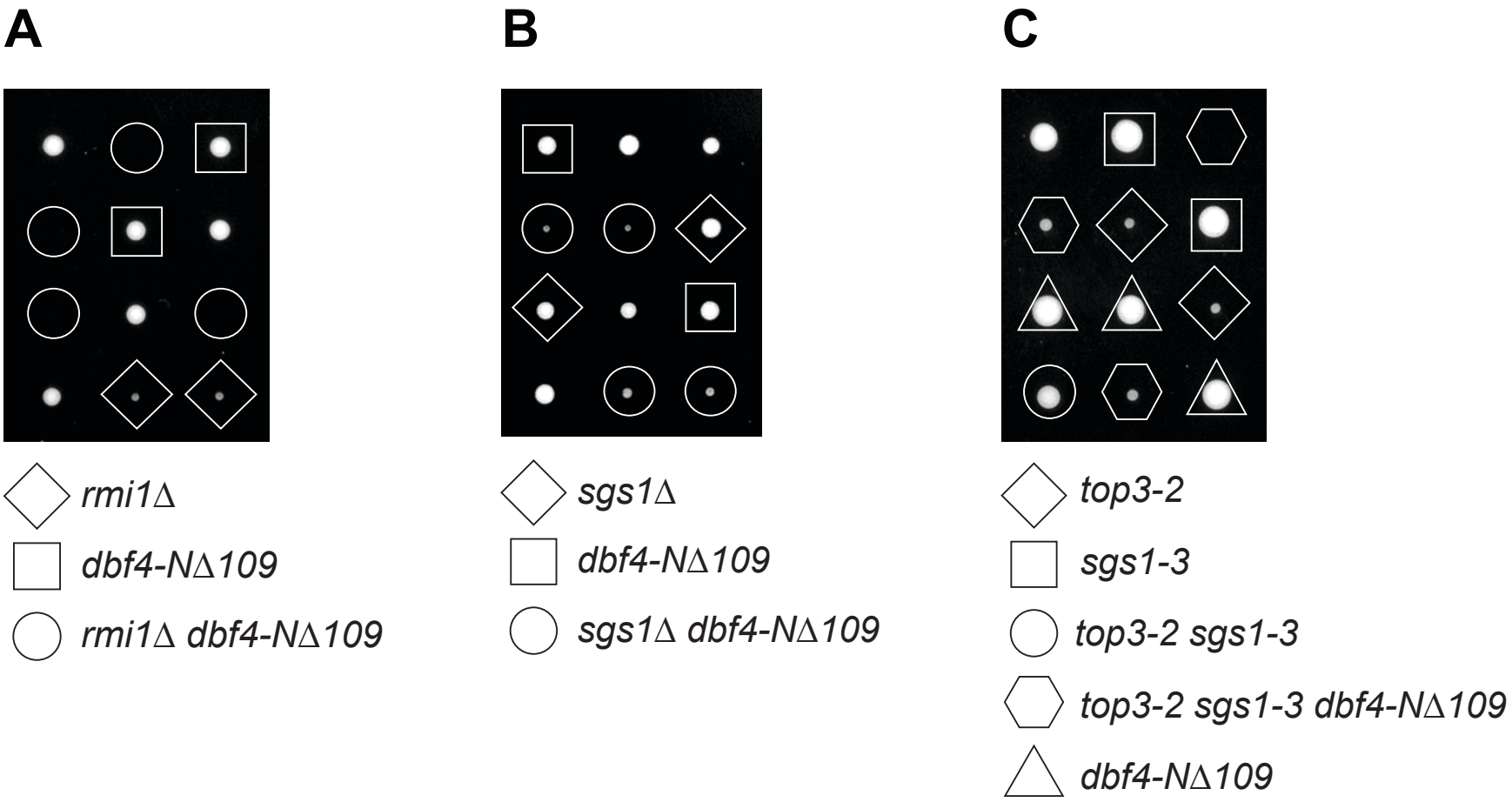


Table 9. Synthetic genetic interaction between *dbf4-NΔ109* and the BLM complex in W303

BLM complex (Top3-Sgs1-Rmi1)			
TOP3	*SGS1*	*RMI1*	*YLR235C*
YLR234W	YMR190C	YPL024W	
Synthetic lethal/sick	Synthetic sick	Synthetic lethal	N/D
Topoisomerase	Slow Growth Suppressor	RecQ Mediated genome Instability	
DNA Topoisomerase III, conserved protein that functions in a complex with Sgs1 and Rmi1 to relax single-stranded negatively-supercoiled DNA, involved in telomere stability and regulation of mitotic recombination.	Nucleolar DNA helicase of the RecQ family, involved in genome integrity maintenance; regulates chromosome synapsis and meiotic joint crossover formation, similar to human BLM and WRN proteins implicated in Bloom and Werner syndromes.	Subunit of the Top3-Sgs1 complex, stimulates superhelical relaxing and ssDNA binding activities of Top3, involved in response to DNA damage, null mutants display increased rates of recombination and delayed S phase.	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data, partially overlaps the verified gene *TOP3*.

Genetic and functional interactions between Dbf4 and the CTF complex

In the 50 highest scoring candidates from our SGA screen, we found *CTF18*, *CTF8*, and *DCC1*, which represent all of the non-essential components in the CTF (chromosome transmission fidelity) complex (Hanna et al. 2001; Mayer et al. 2001; Naiki et al. 2001). The CTF complex is an alternative RFC (replication factor C) and shares four common subunits (Rfc2, Rfc3, Rfc4, and Rfc5) with Rfc1-5 (the canonical RFC complex). Rfc1 is replaced by Rad24, Ctf18-Ctf8-Dcc1 or Elg1 to give three alternative RFC-like complexes involved in various DNA metabolism (Green et al. 2000; Majka and Burgers 2004; Aroya and Kupiec 2005; Kim et al. 2005; Shiomi et al. 2007). The Ctf18-Ctf8-Dcc1-RFC complex (termed Ctf18-RFC) is required for proper cohesion of sister chromatids in DNA replication, and mutations in these genes cause chromosome mis-segregation (Wang et al. 2000; Edwards et al. 2003; Lengronne et al. 2006). Recent studies indicate that Ctf18-RFC also participates in a Rad53 checkpoint that guards fork stability during replication stress (Pan et al. 2006a; Crabbe et al. 2010).

The *ctf18*, *ctf8* and *dcc1* null alleles were introduced separately into the W303 strain to validate their synthetic genetic interactions with *dbf4-NΔ109*. The *ctf dbf4* double mutants had slower growth phenotypes in tetrad analyses (data not shown) and they exhibited striking temperature-sensitive phenotypes (Figure 27A). The double mutants also showed increased sensitivity to hydroxyurea (HU), methyl methanesulfonate (MMS) or benomyl (Figure 27B-D). The synergistic combination of genetic alternation and chemical treatment suggests a complex

genetic network between Dbf4 and the CTF complex. Comparison between the SGA results of *dbf4-NΔ109* and CTF null mutants identified a subgroup of genes that are closely related to checkpoint activation (*TOP3, SGS1, RAD17, RAD24, RAD53, RAD54* and *POL32*) and the regulation of chromosome dynamics (*ASF1, CSM1,* and *BUB3*) (data not shown). Overall, these results suggest that Dbf4 is involved in an additional level of control for achieving the coordination between checkpoint signal transduction and chromosome segregation. The genetic and functional interactions between Dbf4 and the CTF complex are likely due to a combined defect in Rad53-dependent checkpoint signaling during stresses.

It has been previously shown that Dbf4 and Cdc7 function as key regulators for homologous chromosome segregation in meiosis I (Valentin et al. 2006; Matos et al. 2008; Wan et al. 2008; Katis et al. 2010; Lo et al. 2012). DDK collaborates with the Cdc5 kinase to establish the monopolin complex (Mam1-Lrs4-Csm1), which allows kinetochores to specifically attach to spindles from the same spindle pole body (equivalent to the centrosome in higher eukaryotes) (Clyne et al. 2003; Lee and Amon 2003; Marston 2009). Given that *dbf4-NΔ109* is synthetically sick with *csm1Δ* in both S288C and W303 (Table 7 and Figure 28A), this suggests that the Dbf4-Cdc5 interaction and Csm1 also work together to accomplish successful chromosome segregation. Similarly, proper control of chromosome segregation also depends on the centromeric cohesion that may be promoted by the Ctf18-RFC complex.

Table 10. Synthetic genetic interaction between *dbf4-NΔ109* and the CTF complex in W303

CTF8	CTF18	DCC1
YHR191C	YMR078C	YPL194W
temperature sensitive	temperature sensitive	temperature sensitive
Chromosome Transmission Fidelity	Chromosome Transmission Fidelity	DNA Damage Checkpoint
Subunit of a complex with Ctf18 that shares some subunits with Replication Factor C and is required for sister-chromatid.	Subunit of a complex with Ctf8 that shares some subunits with Replication Factor C and is required for sister-chromatid cohesion, have overlapping functions with Rad24p in the DNA damage replication checkpoint.	DNA damage checkpoint protein, part of a PCNA-like complex required for DNA damage response.

Figure 27. *dbf4 ctf* double mutants exhibit synthetic defects in growth upon environmental stresses

Serial dilution of log-phase cells of indicated genotypes were spotted on YPD medium (A) at various temperatures and on the YPD medium that contained (B) HU, (C) MMS, or (D) benomyl.

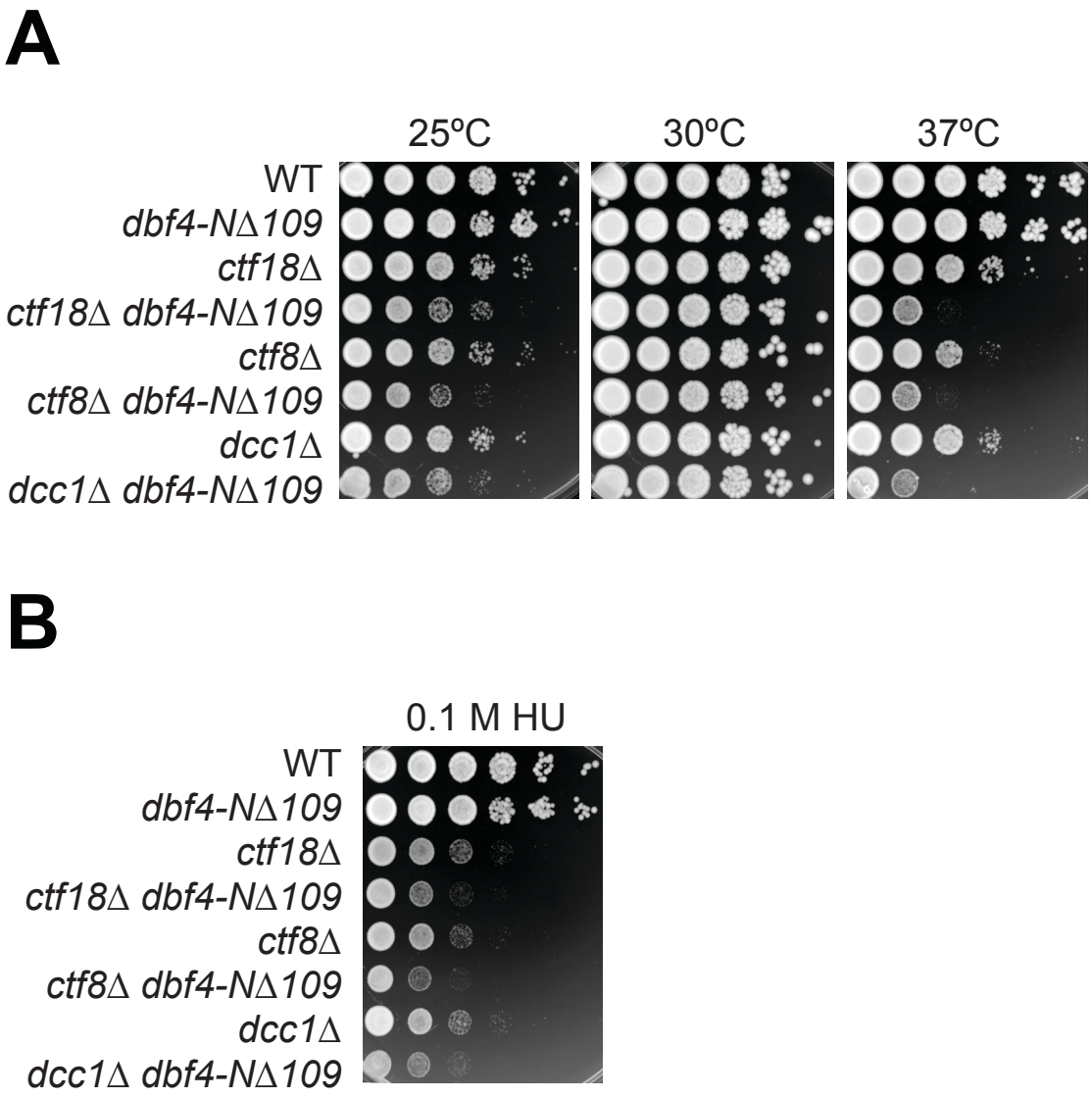


Figure 27. (cont'd)

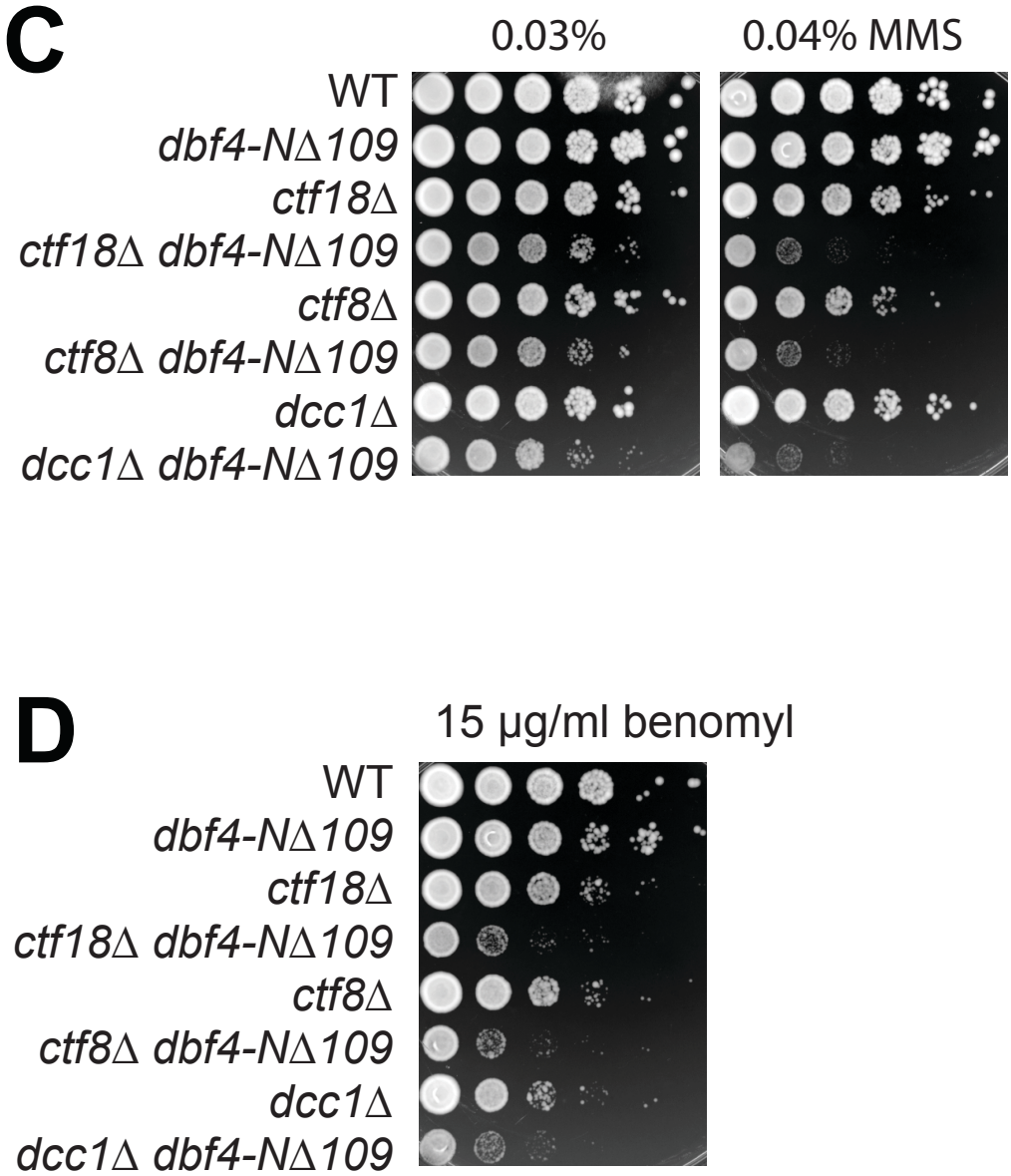


Figure 28. The Dbf4 N-terminus genetically interacts with Csm1, Pol32, and Rad54

dbf4-NΔ109 is synthetically sick with *csm1Δ*, *pol32Δ*, and *rad54Δ* in the W303 background. Representative tetrads from diploid strains of genotype (A) *DBF4*/*dbf4-NΔ109 CSM1/csm1Δ,* (B) *DBF4*/*dbf4-NΔ109 POL32/pol32Δ,* and (C) *DBF4*/*dbf4-NΔ109 RAD54/rad54* were sporulated and dissected onto YPD plates. Recombinant genotypes are indicated. Detailed GO annotations are summarized in Table 11.

Figure 28. (cont'd)

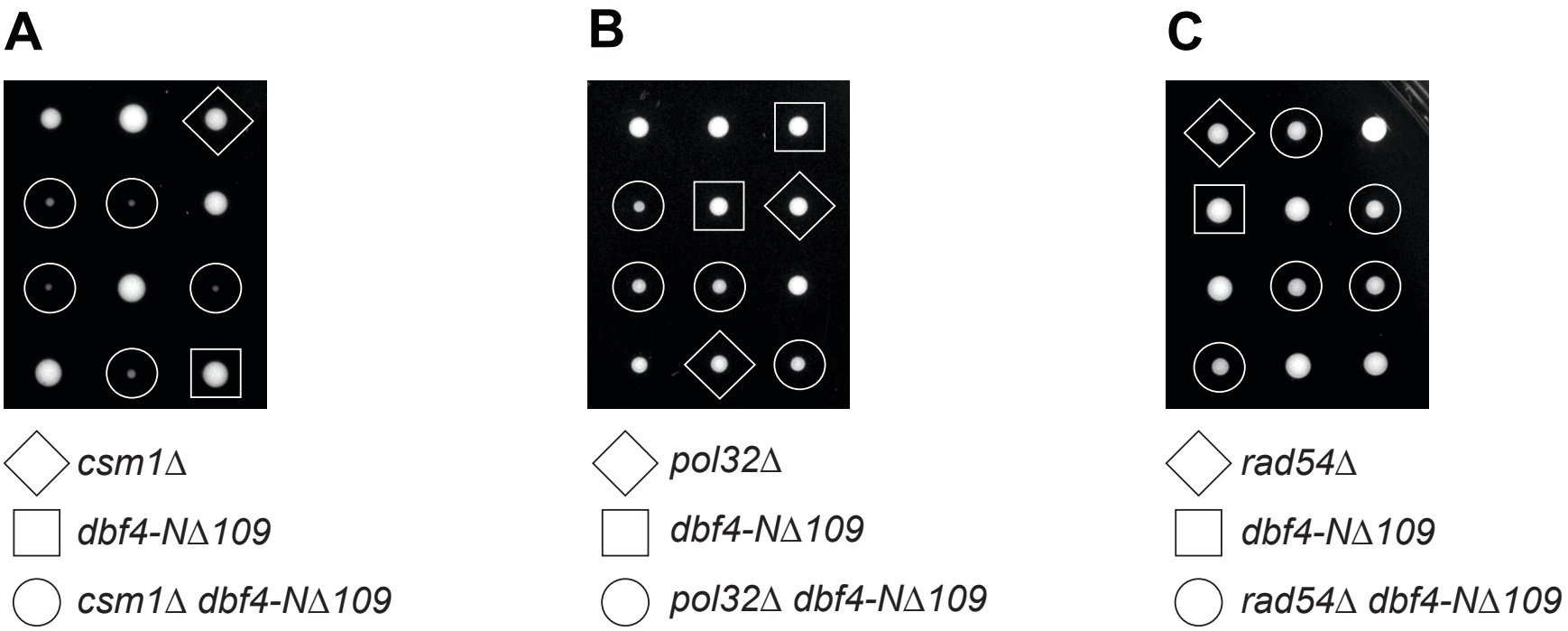


Table 11. Validation of the *dbf4-NΔ109* SGA results in W303

DNA metabolism		
CSM1	POL32	RAD54
YCR086W	YJR043C	YGL163C
Synthetic sick	Synthetic sick	Synthetic sick
Chromosome Segregation in Meiosis	POLymerase	RADiation sensitive
Nucleolar protein that forms a complex with Lrs4 and then Mam1 at kinetochores during meiosis I to mediate accurate homolog segregation, required for condensin recruitment to the replication fork barrier site and rDNA repeat segregation.	Third subunit of DNA polymerase delta, involved in chromosomal DNA replication, required for error-prone DNA synthesis in the presence of DNA damage and processivity.	DNA-dependent ATPase, stimulates strand exchange by modifying the topology of double-stranded DNA, involved in the recombinational repair of DNA double-strand breaks.

DDK participates in DNA damage checkpoint response

The fission yeast Dbf4-dependent kinase (Hsk1) has been recently shown to phosphorylate the PCNA-like 9-1-1 clamp (Ddc1-Rad17-Mec3; named Rad9-Rad1-Hus1 in *S. pombe* and humans) in response to DNA damage (Furuya et al. 2010). It is thought that the heterotrimeric complex of 9-1-1, with its clamp loader Rad24-RFC, acts as a DNA damage sensor, which binds Dbp11 (the homolog of TopBP1) to recruit the checkpoint kinase Mec1 (functional homolog of ATR in mammals) during checkpoint activation (Majka et al. 2006; Labib and De Piccoli 2011). The subsequent signal transduction relies on Rad9 (*S. cerevisiae*) as an adaptor to activate the checkpoint effector kinases Rad53 and Chk1, which are integral transducers of cellular responses to genotoxic stress (Branzei and Foiani 2009).

We have previously shown that the N-terminal deletions of *dbf4* mutants are synthetically lethal with *mec1-1*, *rad53-1*, *rad53*Δ *sml1*Δ, or *chk1*Δ (Duncker et al. 2002; Gabrielse et al. 2006; Chen et al. 2012). Furthermore, *mec3*Δ*, chk1*Δ*,* and *rad9*Δ have separately been shown to be synthetically sick with *dbf4-1* or *cdc7-1* (Tong et al. 2004). Here, we found that *rad24*Δ and *rad17*Δ were synthetically sick with *dbf4-N*Δ*109* in the S288C background (summarized in Table 7). These data confirmed the genetic interaction of *DBF4* with the DNA damage checkpoint pathway. However, components of the 9-1-1 clamp or Rad24 did not exhibit synthetic growth phenotype with *dbf4-N*Δ*109* in W303 strains.

Dbf4 genetically interacts with the HIR complex and RNA modulators

In budding yeast, the HIR (histone regulatory) and CAF-1 (chromatin assembly factor 1) complexes are known as histone chaperones and are involved in nucleosome deposition and chromatin-mediated transcriptional silencing (Osley and Lycan 1987; Smith and Stillman 1989; Xu et al. 1992; Kaufman et al. 1997; Dimova et al. 1999). It is generally thought that CAF-1 participates in the replication-coupled nucleosome assembly via a direct interaction with PCNA (proliferating cell nuclear antigen), while the HIR complex mainly regulates the histone dynamics outside of S phase (Green et al. 2005). *HIR1, HIR2, HIR3*, and *HPC2* encode the subunits of the HIR complex; *CAC1, CAC2*, and *CAC3/MSI1* encode the subunits of the CAF-1 complex. The growth of *S. cerevisiae* cells is not affected when either *HIR* or *CAC* genes are mutated, but *hir*Δ *cac*Δ double-mutants exhibit slow growth, suggesting that two complexes functionally overlap (Kaufman et al. 1998; Qian et al. 1998). Both complexes interact with a conserved H3/H4-binding protein Asf1 (anti-silencing function), which is found in a complex with the checkpoint kinase Rad53 (Emili et al. 2001; Hu et al. 2001; Sharp et al. 2005; Jiao et al. 2012). A compelling model is that a Rad53-mediated checkpoint response prevents the deposition of newly synthesized histones in the cells upon DNA damage and replication stress and thus contributes to genome integrity (Hu et al. 2001; Singh et al. 2009).

In our SGA screen, *hpc2*Δ*, asf1*Δ*, hir1*Δ*, hir2*Δ*,* and *hir3*Δ were among the top 50 candidates that were synthetically sick with *dbf4-N*Δ*109* (summarized in Table 7).

In particular, Δ*hpc2, hir1*Δ and *hir3*Δ were found earlier to be synthetically sick with *dbf4-1* or *cdc7-1*. None of the CAF-1 components was identified in our SGA screen, indicating that Dbf4 is specifically relevant to HIR-associated histone homeostasis, and separable from the role of CAF-1 within S phase.

In addition to acting as histone chaperones, the HIR-Asf1 complex is also involved in gene silencing, the repression of histone genes, nucleosome disassembly, and aspects of transcriptional regulation (Sharp et al. 2002; Prochasson et al. 2005; Amin et al. 2012; Eriksson et al. 2012; Zunder and Rine 2012). Whether and how Dbf4 participates in these mechanisms has not been investigated yet, but it is intriguing to find that one third of the top hits in the *dbf4-NΔ109* SGA screen are known to be involved in transcriptional regulation. Although it is likely that these genetic interactions come from indirect effects, it is noteworthy that most synthetic effects with RNA metabolism genes were confirmed in the W303 background (summarized in Table 12). In particular, the deletion of the *LSM7*, *CDC73*, *SBR2*, *CKB2,* and *MED1* genes showed significant synthetic effects with *dbf4-NΔ109* (Figure 29).

Dbf4 physically and genetically interacts with the yeast 14-3-3 proteins

We recently showed that the checkpoint kinase Rad53 directly binds to the Dbf4 N-terminus using both FHA1 and FHA2 domains (Chen et al. 2012). Rad53 also interacts with Bmh1 and Bmh2, which are homologs of the 14-3-3 protein family and can form homo- or heterodimers to regulate diverse biological processes,

including signal transduction in G1/S and G2/M checkpoints, DNA replication, genome stability, apoptosis, cytoskeleton organization, and malignant transformation (Lottersberger et al. 2003; Usui and Petrini 2007; Grandin and Charbonneau 2008; Freeman and Morrison 2012; Gardino and Yaffe 2012). At least seven isoforms of 14-3-3 are present in humans, but *BMH1* and *BMH2* are the only two *14-3-3* genes in budding yeast. We found that Bmh1 and Bmh2 associate with Dbf4 in yeast two-hybrid assays (Figure 30). Surprisingly, both yeast 14-3-3 proteins bind to Dbf4 by recognizing a sequence overlapping the Rad53 binding site, suggesting that Rad53 recruits Bmh1 and Bmh2 to associate with Dbf4.

Interestingly, *bmh1*Δ is one of the candidates showing a synthetic sick interaction with *dbf4-N*Δ*109* in the SGA screen, but *bmh2*Δ is not. We observed similar results in the W303 background by tetrad analyses. As shown in Figure 31, the *bmh1*Δ *dbf4-N*Δ*109* double mutant is synthetically sick and synergistically sensitive to benomyl treatment, whereas *bmh2*Δ *dbf4-N*Δ*109* double mutants had no such growth phenotype (data not shown). Since *dbf4-N*Δ*109* is synthetically lethal with *rad53-1*, we tested whether *bmh1*Δ had synthetic effects with *rad53-1*. As expected, *bmh1*Δ *rad53-1* double mutants had more severe growth defects than did the *bmh1*Δ *dbf4-N*Δ*109* mutants (Figure 31B). These genetic data suggest that the functional link between Dbf4 and Bmh1 relies on the participation of Rad53, which probably promotes the interaction between Dbf4 and Bmh1.

Figure 29. The Dbf4 N-terminus is involved in transcriptional regulation

dbf4-NΔ109 displays synthetic effects with *lsm7*Δ, *cdc73*Δ, *srb2*Δ*, ckb2*Δ*,* and *med1*Δ in the W303 background. Representative tetrads from diploid strains of genotype (A) *DBF4*/*dbf4-NΔ109 LSM7/lsm7*Δ*,* (B) *DBF4*/*dbf4-NΔ109 CDC73/cdc73*Δ*,* (C) *DBF4*/*dbf4-NΔ109 SRB2/srb2*Δ*,* (D) *DBF4*/*dbf4-NΔ109 CKB2/ckb2*Δ*,* and (E) *DBF4*/*dbf4-NΔ109 MED1/med1* were sporulated and dissected onto YPD plates. Recombinant genotypes are indicated. Detailed GO annotations are summarized in Table 12. (F) Serial dilution of log-phase cells of indicated genotypes were spotted on YPD medium at various temperatures.

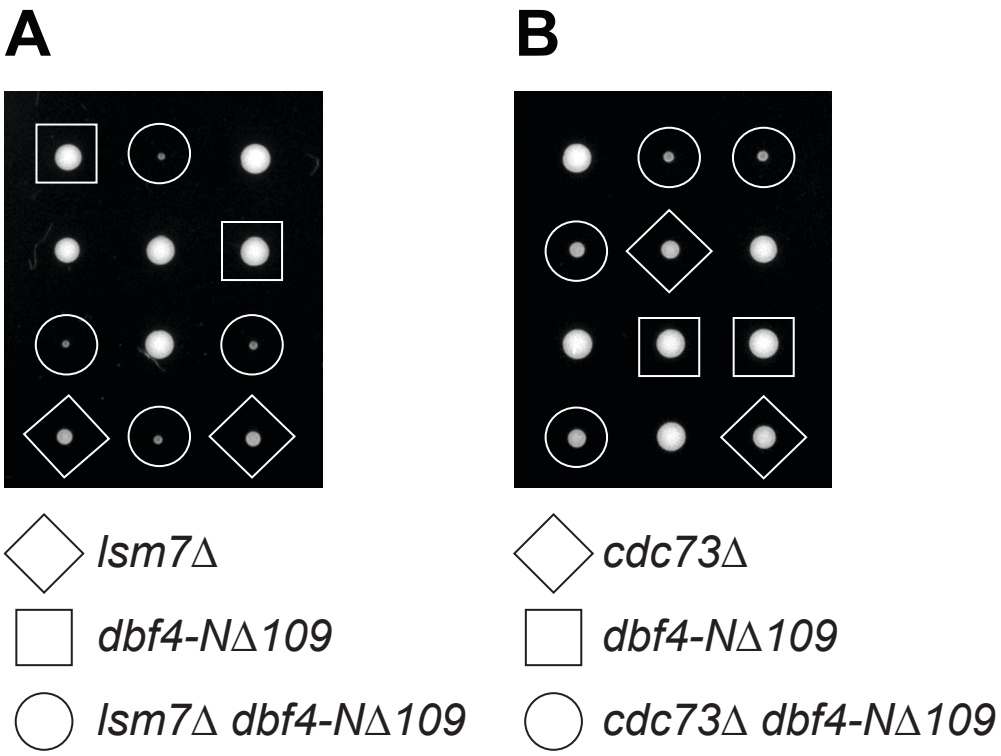
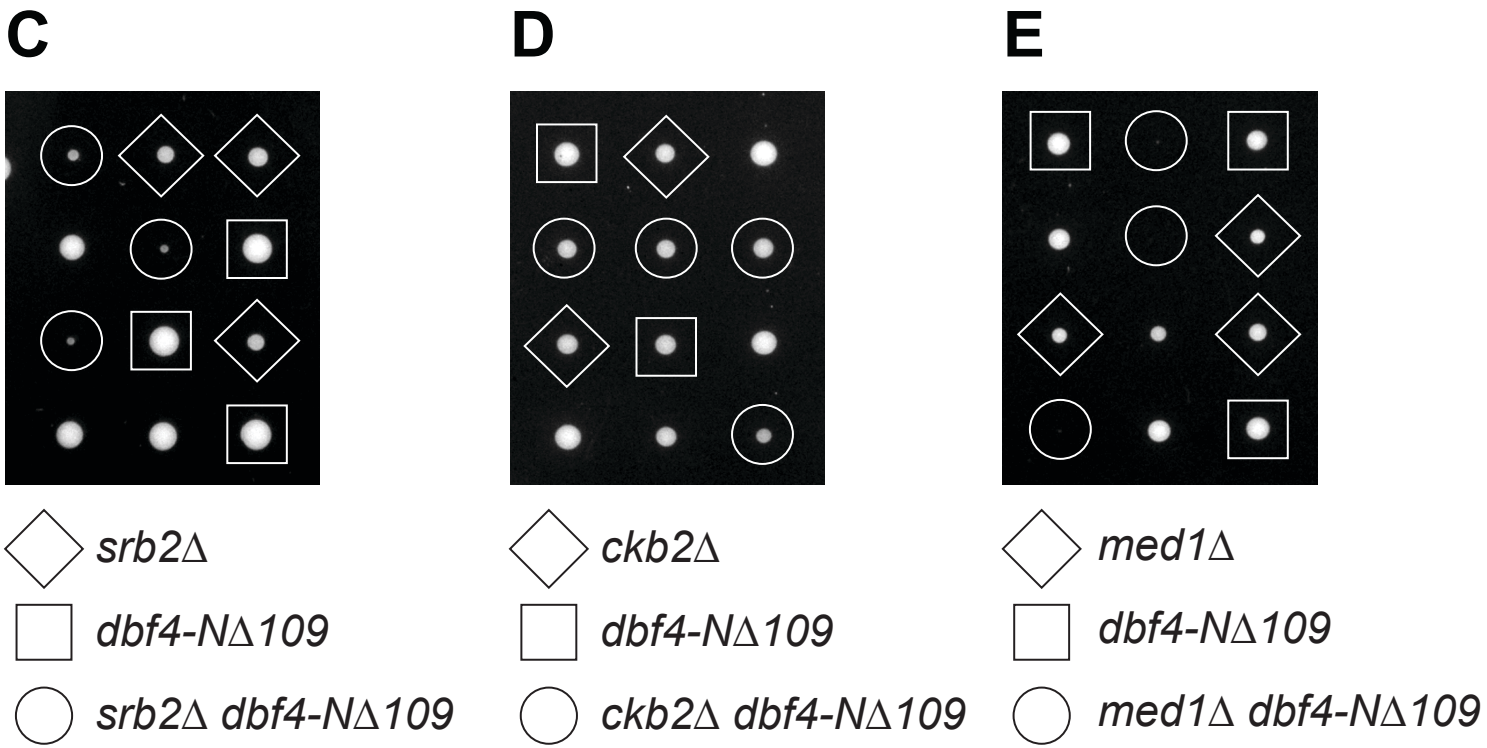


Figure 29. (cont'd)



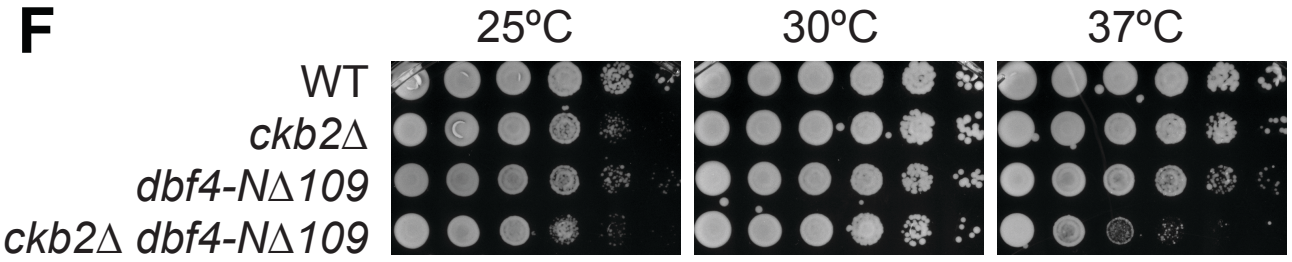


Table 12. Synthetic genetic interaction between *dbf4-NΔ109* and transcriptional regulators

RNA metabolism (transcriptional regulation)		
LSM7	CDC73	SRB2
YNL147W	YLR418C	YHR041C
Synthetic sick	Synthetic sick	Synthetic sick
Like SM	Cell Division Cycle	Suppressor of RNA polymerase B
Lsm (Like Sm) protein, part of heteroheptameric complexes (Lsm2-7), involved in mRNA decay and processing of tRNA, snoRNA, and rRNA.	Component of the Paf1 complex, binds to and modulates the activity of RNA polymerases I and II, required for gene expression, histone modification, and telomere maintenance.	Subunit of the RNA polymerase II mediator complex, associates with core polymerase subunits to form the RNA polymerase II holoenzyme, involved in telomere maintenance.

Table 12. (cont'd)

RNA metabolism (transcriptional regulation)	
CKB2	MED1
YOR039W	YPR070W
Temperature sensitive	Synthetic lethal
Casein Kinase Beta' subunit	MEDiator complex
Beta' regulatory subunit of casein kinase 2 (CK2), a Ser/Thr protein kinase with roles in cell growth and proliferation, CK2, comprised of CKA1, CKA2, CKB1 and CKB2, has many substrates including transcription factors and all RNA polymerase.	Subunit of the RNA polymerase II mediator complex, associates with core polymerase subunits to form the RNA polymerase II holoenzyme.

Figure 30. Mapping the interaction between Dbf4 and yeast 14-3-3 protein

(A-B) N-terminal Dbf4 deletion or point mutants were tested for a two-hybrid interaction with the Bmh1 and Bmh2, separately. 10-fold serial dilutions of saturated cultures were spotted onto SCM-Trp-Leu plates to visualize total cells and Scm/-Trp-Leu-His + 2 mM 3AT plates, to score the two-hybrid interaction. (C) Schematic of the features in Dbf4 N-terminus are shown, including motifs N, M and C and Cdc5 (Polo) and Rad53 binding sites, along with a summary of the two-hybrid data.

Figure 30. (cont'd)

A

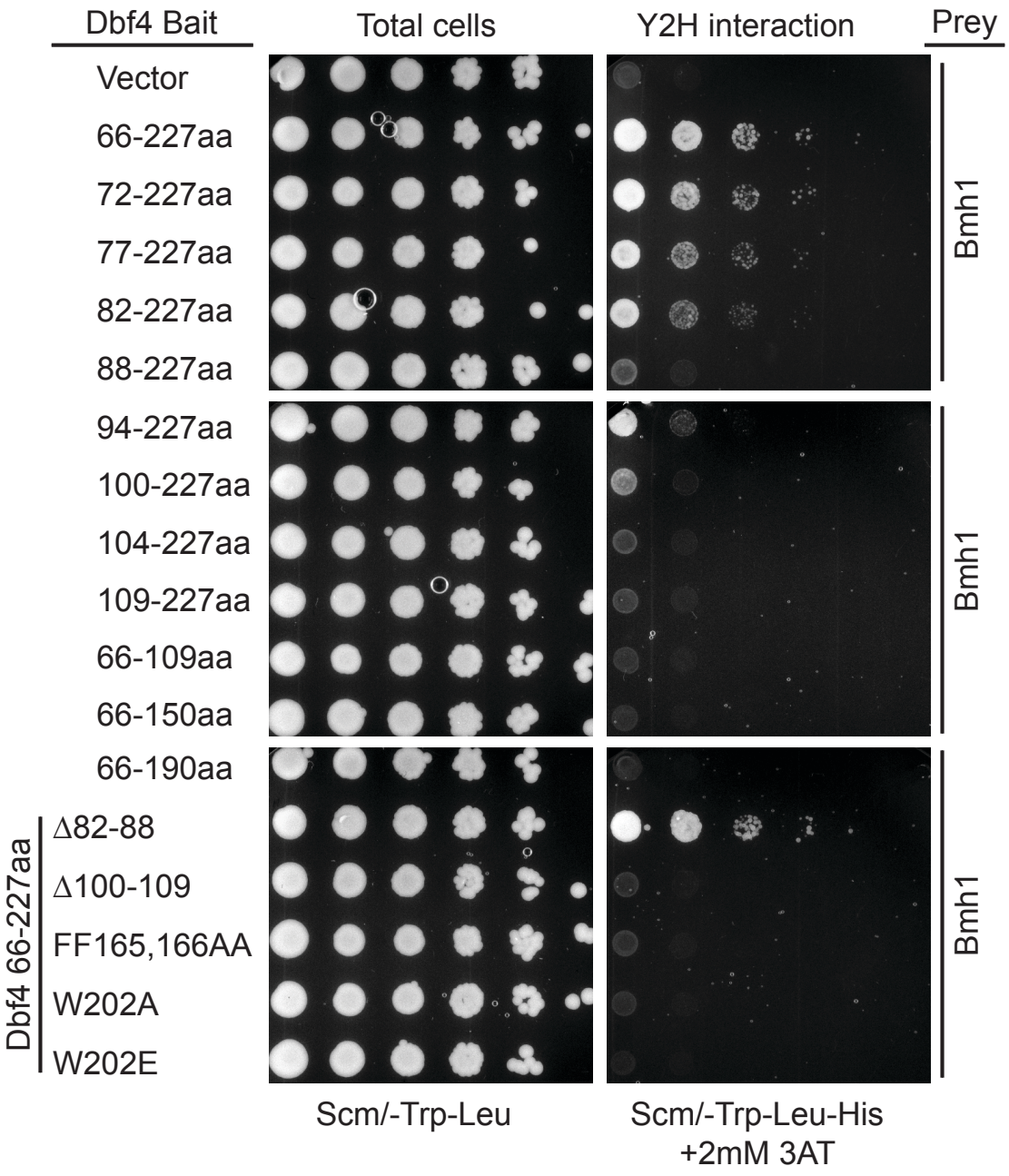


Figure 30. (cont'd)

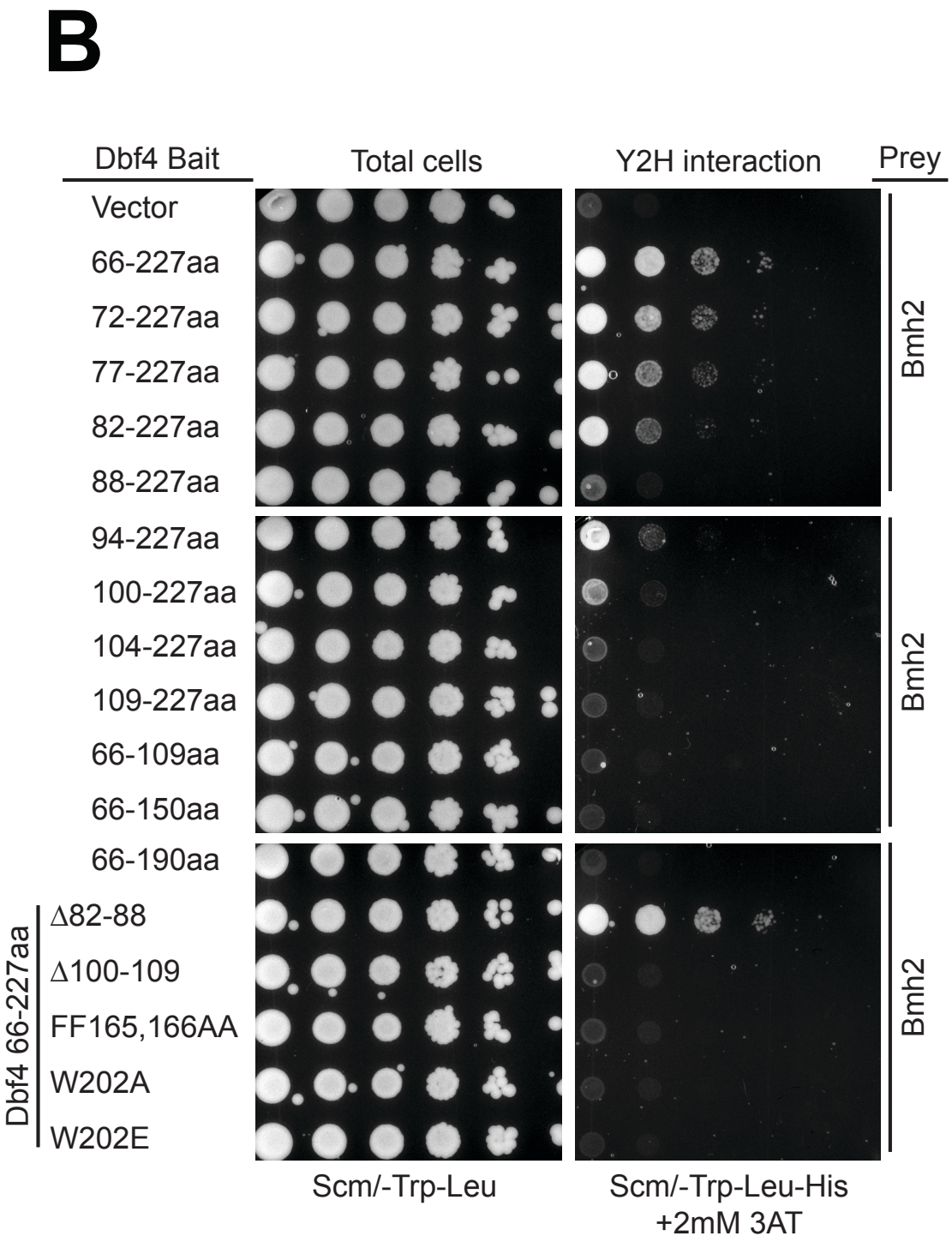


Figure 30. (cont'd)

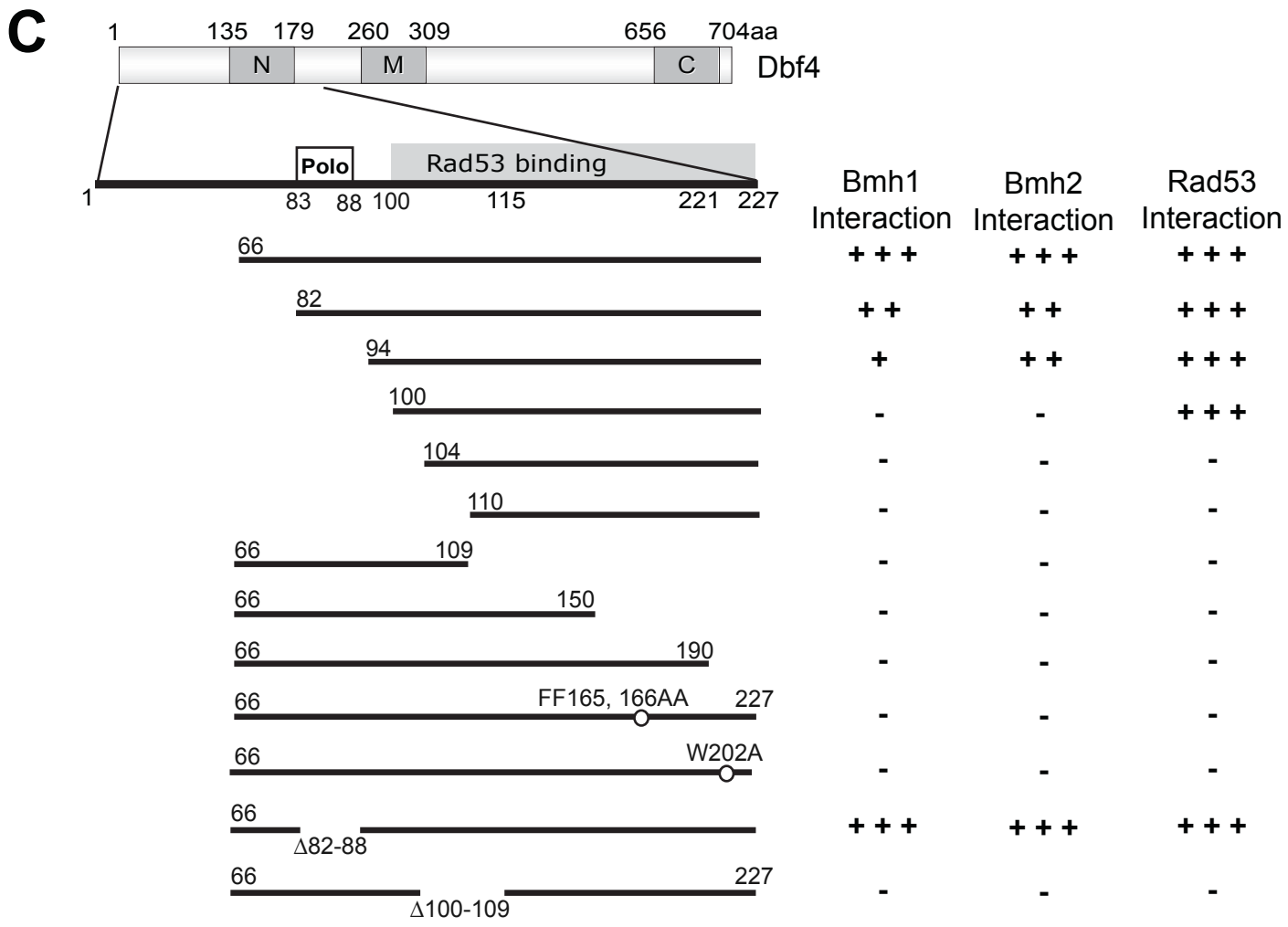


Figure 31. The Dbf4 N-terminus genetically interacts with Bmh1

bmh1 is synthetically sick with *dbf4-NΔ109* and synthetically lethal with *rad53-1* in the W303 background. Representative tetrads from diploid strains of genotype (A) *DBF4*/*dbf4-NΔ109 BMH1/bmh1Δ* and (B) *RAD53*/*rad53-1 BMH1/bmh1Δ* were sporulated and dissected onto YPD plates. Recombinant genotypes are indicated. (C) Serial dilution of log-phase cells of indicated genotypes were spotted on the YPD medium that contained benomyl.

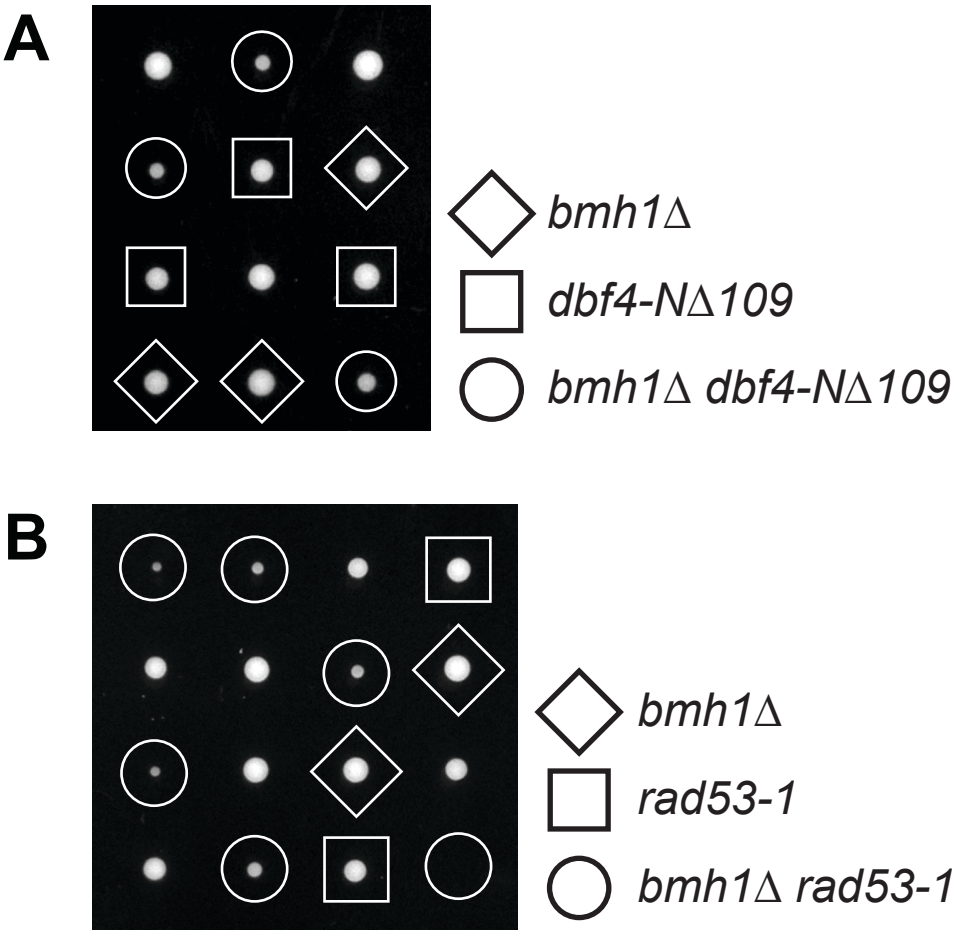
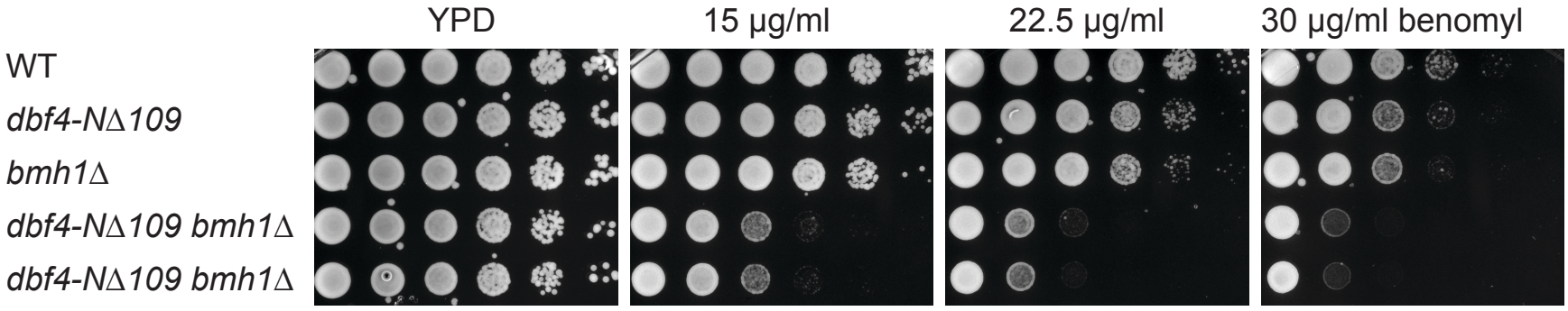


Figure 31. (cont'd)

C



DISCUSSION

Functional characterization of the Dbf4 N-terminus

Dbf4 is well known as a regulatory subunit of the Cdc7 kinase (Johnston et al. 2000; Sclafani 2000). Motif-M and motif-C of Dbf4 are required for the essential function of Cdc7 in DNA replication, but the Dbf4 motif-N is dispensable for yeast viability (Masai and Arai 2000; Gabrielse et al. 2006; Harkins et al. 2009; Jones et al. 2010). In recent years, it has been thought that the Dbf4 N-terminus has separate roles in post-replicative cell-cycle regulation. A series of biochemical and genetic studies identified various binding partners in the Dbf4 N-terminus, including Orc2 and Orc3 (origin recognition complex) (Duncker et al. 2002), Cdc5 (Polo-like kinase) (Miller et al. 2009; Chen and Weinreich 2010), and Rad53 (checkpoint kinase) (Duncker et al. 2002; Chen et al. 2012; Matthews et al. 2012). Among these, the molecular basis of the Dbf4-Cdc5 and Dbf4-Rad53 interactions was extensively studied, but the biological relevance of these physical interactions is not completely understood. Cdc5, Rad53, and Cdc7 simultaneously complex with Dbf4 (Chen et al. 2012), and the ternary complex is likely involved in different surveillance mechanisms in DNA replication checkpoint, G2/M checkpoint adaptation, and spindle position checkpoint. We proposed that Dbf4 serves as a molecular scaffold and that the rewiring of different checkpoint signal transmissions depends on the dynamic complex formation of Dbf4.

Since the N-terminal first 109 residues of Dbf4 are required for the Cdc5 and Rad53 interactions (Miller et al. 2009; Chen and Weinreich 2010), a synthetic

lethal screen using *dbf4-NΔ109* as a bait was proposed to uncover genes involved in Rad53 or Cdc5 signaling. In fact, the *dbf4-NΔ109* SGA analysis identified a group of genes that control genome integrity, chromosome segregation, and DNA damage response, in which Cdc5 or Rad53 are known to play important roles. These data suggest that the role of Dbf4 N-terminus is complemented by either Cdc5 or Rad53-related pathways, and cells can tolerate the *dbf4-NΔ109* mutation when Cdc5 or Rad53 function is unperturbed. Such an observation is consistent with recent findings that *dbf4-NΔ109* is synthetically lethal with *rad53-1* and that the *cdc5-1* temperature-sensitive mutant loses viability by introducing a *dbf4* N-terminal deletion (Gabrielse et al. 2006; Miller et al. 2009; Chen and Weinreich 2010; Chen et al. 2012).

Earlier SGA screens with *dbf4-1* and *cdc7-1* revealed that DDK genetically interacts with the Top3-Sgs1-Rmi1 complex (Tong et al. 2004), suggesting that DDK is involved in preserving the fidelity of genome inheritance. Unlike the *dbf4-1* and *cdc7-1* mutants, cells harboring the *dbf4-NΔ109* allele show no effect in DNA synthesis (Gabrielse et al. 2006). Our present work showed that the *dbf4-NΔ109* mutant displays reduced fitness in combination with any mutant in the Top3-Sgs1-Rmi1 complex, strongly suggesting that these synthetic genetic interactions are not due to a defect in DNA replication. Because the Dbf4 1-109 residues are not required for the essential function of Cdc7 in S phase, these results also imply that, in addition to the initiation of DNA synthesis, the Cdc7 kinase has a distinct function linked to the Dbf4 N-terminus. This idea is

consistent with our recent finding that Cdc7 is crucial for Dbf4-regulated Cdc5 inhibition during mitotic exit (Miller et al. 2009; Chen and Weinreich 2010).

With *dbf4-NΔ109* as a query in the SGA screen, we isolated multiple genes in the 9-1-1, CTF, and HIR complexes. These interacting genes not only reflected novel roles of Dbf4 in chromatin dynamics, but also provided insights into the molecular mechanism. To follow identification of each potential candidate, we validated the SGA results in the W303 background. Twenty of the 34 top-scoring hits showed synthetic effects in W303. *rmi1*Δ, *sgs1*Δ, and *top3*Δ consistently exhibited strong phenotypes with *dbf4-NΔ109*. Though the *ctf dbf4* double-mutants had mild growth defects under normal growth conditions, they showed increased sensitivity to various genotoxic stresses. It is striking that none of genes in the HIR-Asf1 or 9-1-1 complexes showed synthetic effects with *dbf4-NΔ109* in the W303 background, even though many of them were already identified in previous SGA studies by using the *dbf4-1* and *cdc7-1* alleles. Further studies on the divergence between different genetic backgrounds are clearly warranted.

It's all about Rad53 activation

The genome-wide synthetic lethal screen enables us to take an unbiased approach to studying the biological significance of the *dbf4-NΔ109* allele. Many candidate genes and pathways are involved in the mechanism of Rad53 activation, suggesting that Rad53 is the central node in the genetic and biochemical networks of *DBF4*.

DNA damage response and checkpoint signaling

The Top3-Sgs1-Rmi1 and 9-1-1 complexes play important roles in recognizing and processing DNA breaks (Harrison and Haber 2006). In *S. cerevisiae*, DNA double-strand break (DSB) repair is initiated by end resection. The conserved Mre11-Rad50-Xrs2 (MRX) complex, together with Sae2, recruits the Dna2 nuclease, Exo1 exonuclease, and Top3-Sgs1-Rmi1 helicase complexes to the break sites and removes oligonucleotides from the 5′ strand. The resulting ssDNA 3′ overhang is then coated by RPA (replication protein A). This ssDNA-RPA intermediate interacts with the 9-1-1 complex, leading to Tel1/Mec1 (the ATM/ATR kinase homolog in mammals) recruitment and subsequent activation of Rad53 checkpoint kinase. Therefore, a loss of function of the Top3-Sgs1-Rmi1 or 9-1-1 complex can limit Rad53 activation. We previously found that checkpoint-compromised *rad53* mutants (*rad53-1, rad53-11,* and *rad53*Δ *sml1*Δ) were synthetically lethal with *dbf4-N*Δ*109* (Gabrielse et al. 2006; Chen et al. 2012). Similarly, deleting genes in the Top3-Sgs1-Rmi1 or 9-1-1 complexes caused synthetic lethal or sick phenotypes with *dbf4-N*Δ*109* in the SGA screen, suggesting that the synthetic genetic interaction may be mediated by a combined defect in Rad53 activation. Furthermore, *mec1-1* (the principal kinase for Rad53 activation) was synthetically lethal with *dbf4* N-terminal deletions (Gabrielse et al. 2006), and *chk1*Δ (partially redundant with Rad53 function) and *rad9*Δ (an adaptor for Rad53 activation in response to DNA damage) were synthetically sick with *dbf4-1* or *cdc7-1* (Tong et al. 2004), indicating that these synthetic genetic

interactions reflect complex and partially overlapped mechanisms in the Rad53-dependent checkpoint signaling. In support of this notion, previous studies found that deletion of *CTF18, CTF8* or *DCC1,* which were synthetically sick with *dbf4-NΔ109* in the SGA screen, caused defects in the Rad53 activation in response to folk stalling (Pan et al. 2006a; Crabbe et al. 2010). Accordingly, the Rad53 kinase responds to genotoxic stress by inducing transcription of genes that modulate cell cycle progression (Bastos de Oliveira et al. 2012; Travesa et al. 2012). The synthetic effects between *dbf4-NΔ109* and several transcriptional regulators may be analogous to a scenario that is conferred by the *dbf4-NΔ109 rad53* double mutants; nonetheless, we do not rule out the possibility that Dbf4 coordinates transcriptional responses to replication stresses or that the synthetic interactions with *dbf4-NΔ109* are indirect.

The yeast 14-3-3 proteins, Bmh1 and Bmh2, contribute to a robust activation of checkpoints upon DNA damage and replication stress, as well as two distinct spindle checkpoints (Lottersberger et al. 2003; Usui and Petrini 2007; Grandin and Charbonneau 2008). *BMH1* and *BMH2* were found as high-copy suppressors of the *rad53-AT* mutant, which is deficient in Rad53 autophosphorylation and activation (Usui and Petrini 2007). The genetic interaction led to the identification of a physical association between Rad53 and Bmh1 (or Bmh2) and to the model that Bmh1 facilitates the Rad53 activation by a direct interaction. Similarly, we not only showed that *dbf4-NΔ109* is synthetic sick with *bmh1Δ* in the SGA screen, but also showed that the *dbf4-NΔ109* mutant

eliminates the Dbf4-Bmh1 interaction in yeast two-hybrid assays. These results suggest that the Dbf4 N-terminus functions in parallel with Bmh1 and Rad53 to promote genome integrity. In this context, the *dbf4-NΔ109 bmh1Δ* double mutant synthetically affects the Rad53 activation and thus shows reduced fitness and additive benomyl sensitivity.

Maintenance of replication-fork integrity

ssDNA-RPA intermediates are also generated by stalled replication forks with uncoupled DNA polymerases and helicases (Branzei and Foiani 2009). The surveillance mechanism of the DNA replication checkpoint (also known as the S-phase checkpoint) regulates the activity of replication origins and prevents the collapse of replisomes when S-phase progression is postponed during replication stress. Rad53 directly phosphorylates Dbf4 and Sld3, which are required for activating the Mcm2-7 helicase, and consequently inhibits late origin firing (Duch et al. 2010; Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010). We recently showed that the *dbf4-NΔ109 sld3* double mutant, which is defective in the Dbf4-Rad53 interaction and the Rad53-mediated Sld3 phosphorylation, bypasses the replication checkpoint to allow late origin firing in the presence of HU (Chen et al. 2012). Although these results imply that the Rad53 kinase binds to and phosphorylates Dbf4 to prevent late origin firing, the *dbf4-NΔ109* mutation alone does not interfere with early origin firing or display any genotoxic sensitivity (Gabrielse et al. 2006).

In the SGA analysis, the *dbf4-NΔ109* allele was found to be synthetically lethal or sick with the null mutants of the Top3-Sgs1-Rmi1 complex, which is known to control the stability of replication forks and the recovery from checkpoint arrest (Hegnauer et al. ; Cobb et al. 2005; Hegnauer et al. 2012; Yang et al. 2012). Intriguingly, the Rad53 kinase also plays a crucial role in preserving fork integrity (Labib and De Piccoli 2011). Aberrant replication intermediates and reversed forks are generally found in *rad53* mutants. Though the mechanism of fork stabilization is not clear, recent studies showed that the Sgs1 subunit has separable roles in the Rad53 activation (Hegnauer et al. 2012). A defect in Sgs1-related Rad53 activation might contribute to the synthetic effect in the *dbf4 sgs1* double mutants. Arguing against a redundant role for Dbf4 in Rad53 activation, Mrc1 (Claspin in mammals), which functions together with Sgs1 in the Rad53 activation (Labib and De Piccoli, 2011), was not isolated in our SGA screen. However, either Rad24 or Rad9 can act in parallel to the Mrc1 function (Bjergbaek et al. 2005), and both of them were identified in *dbf4* SGA studies. In addition, deletion of the *POL32* gene (Figure 28B), the smallest subunit of DNA polymerase delta also targeted by the replication checkpoint, exhibits synergistic fitness defects with *dbf4-NΔ109*. We thus conclude that the Rad53 activation for replication stalling crucially relies on the complex crosstalk of Rad24, Rad9, Sgs1, and Dbf4 signaling pathways.

Rad53-mediated histone homeostasis

Like Bmh1 and Bmh2, the histone chaperone Asf1 was found to genetically and physically interact with the Rad53 checkpoint kinase (Jiao et al. 2012). Rad53 has a crucial role in controlling histone levels and that this regulation is independent from classic Rad53 activation upon DNA damage or replication stress (Hu et al. 2001; Singh et al. 2009). Because histone deposition is coordinated with DNA replication during S phase, imbalanced histone synthesis causes cytotoxic effects, such as genomic instability and chromosome mis-segregation. The direct interaction between Rad53 and Asf1 is thought to link the surveillance mechanism to histone metabolism. In addition, a recent study showed that DDK phosphorylation on histone H3-Thr45 is responsive to the replication stress (Baker et al. 2010). The finding that *dbf4-NΔ109* is synthetically sick with the *asf1* null mutant suggests that Dbf4 is operating in concert with the Rad53-Asf1 complex to regulate histone dynamics during DNA replication. Interestingly, only the components of the HIR complex, and not the CAF-1 complex, are synthetic sick with *dbf4-NΔ109*. These results provide an exciting insight that Dbf4 specifically participates in a pathway that responds to Asf1-HIR involved histone regulation and Rad53 activation.

MATERIALS AND METHODS

Plasmids, Yeast strains, and media

Yeast strains and primers used for strain construction are listed in Tables 13 and 14. PJ69–4a cells (*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ*

LYS2::GAL1-HIS3 GAL2-ADE2 met::GAL7-lacZ) were used for two-hybrid experiments. All other strains were derivatives of W303-1A (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3*). Strains with the deletion of non-essential genes were made by the method of Longtine (Longtine et al. 1998). The natMX4 cassette flanked with *DBF4* target sequences was PCR amplified from p4339 with primers (5'-CTA TCA ACG GCA ATG TTA TTG AAT CAC TTT CTC ATT CAC CCT TGT ACA TGG AGG CCC AGA ATA CC-3') and (5'- ATG CAA TTG ATA ATA TAT GGA CGA GTA AAT AAG AGT TAA GTC AAT CAG TAT AGC GAC CAG CAT TC-3') (Goldstein and McCusker 1999), and transformed into M1261 (W303 *dbf4-N*∆*109*). clonNAT (Werner Bioagents) resistant transformants were confirmed with natMX4 marker and then backcrossed to W303. The *dbf4-*∆*N109-natMX4* allele was PCR amplified from the genomic preparation of M3120 (W303 dbf4-∆N109-natMX4) by primers Dbf4-genomic5F/R, and then integrated into M3052 (Y5565). Yeast deletion strains derived from BY4741 (*MATa his3*∆*1 leu2*∆*0 met15*∆*0 ura3*∆*0*) and generated by the *S. cerevisiae* deletion consortium were maintained in an ordered array on agar plates at a density of 1536 strains (384 unique strains arrayed in quadruplets) per plate and manipulated robotically with a colony arrayer (Bio-Rad) (Tong et al. 2001).

For the synthetic genetic screening, yeast sporulation was performed using medium 2% agar, 1% potassium acetate, 0.1% yeast extract and 0.05% glucose, supplemented with uracil, histidine and leucine. Filter-sterilized solutions of L-canavanine (50 mg/l; Sigma), G418 (200 mg/l; Invitrogen Life Technologies) and

clonNAT (100 mg/l; Werner Bioagents) were added to cooled media where indicated. In cases where synthetic complete medium (Scm) was supplemented with clonNAT or G418, the ammonium sulfate was replaced with monosodium glutamate and the medium termed Scm/MSG (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.1% monosodium glutamic acid, 0.2% amino acid add back, 2% glucose and 2% agar). Benomyl (Sigma) was added directly to plates immediately before pouring (final 0.2% DMSO (v/v)).

Plasmids used in this study are listed in Table 15. Deletions and point mutations within *DBF4* were generated by site-directed mutagenesis using the QuikChange system (Stratagene). *BMH1* (2 to 267) and *BMH2* (2 to 273) were PCR amplified from genomic DNA and cloned into the *EcoR1-PstI* sites of pGAD-C1 to give the Gal_{AD}-Bmh1 and Gal_{AD}-Bmh2 fusions.

Synthetic lethal screen and data analysis

Genome-wide synthetic lethal screens were performed using synthetic genetic array (SGA) analysis as described previously (Parsons et al. 2004; Tong et al. 2004). Colonies of double-mutant progeny were photographed by using a high-resolution digital imaging system developed from S&P Robotics, Inc. The colony sizes were compared to a reference set of wild-type controls. A synthetic lethal or sick interaction is determined when the colony size of double-mutant progeny is smaller than that of wild-type controls.

Positive hits were sorted by Gene Ontology (GO) to annotate their molecular function and biological process. The programs FunSpec and FunAssociate were used to assist functional annotations (Robinson et al. 2002; Berriz et al. 2003). Genes not falling into any category were designated as unknown function. References for all genes in this study can be found at the Saccharomyces Genome Database (SGD; (http://www.yeastgenome.org), the Yeast Proteome Database (YPD; http://www.proteome.com) and the Comprehensive Yeast Genome Database (CYGD) at MIPS (http://mips.gsf.de). All genetic interaction data is available at the General Repository for Interaction Datasets (GRID; http://biodata.mshri.on.ca/grid).

Two-hybrid Analysis

Various *DBF4* bait constructs containing Gal4 DNA binding domain were transformed with Gal4 activation domain prey plasmids in PJ69-4a and selected on Scm plates lacking tryptophan and leucine. These were spotted at ten-fold serial dilutions on the same plates and also on plates also lacking histidine but containing 2 mM 3-aminotriazole (3AT) at 30°C and cultured for 2-3 days.

Table 13. Yeast strains

Stain	Genotype	Source
PJ69-4A	*MAT**a** trp1-901 leu2-3, -112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al., 1996
W303-1A	*MAT**a** ade2-1, ura3-1 his3-11, -15 trp1-1 leu2-3, -112 can1-100*	Thomas and Rothstein, 1989
M1261	*W303 MAT**a** dbf4-NΔ109*	Cabrielse et al., 2006
M1800	*W303 MAT**α** dbf4-NΔ109-kanMX6*	Miller et al., 2009
M3446	*W303 MAT**a** hir1Δ::HIS3*	Sharp et al., 2005
M3447	*W303 MAT**a** hir2Δ::URA3*	Sharp et al., 2005
M3448	*W303 MAT**a** hir3Δ::HIS3*	Sharp et al., 2005
M3449	*W303 MAT**a** asf1Δ::TRP1*	Sharp et al., 2005
M3496	*W303 MAT**a** hir1Δ::HIS3 dbf4-NΔ109::kanMX6*	This study
M3497	*W303 MAT**a** hir2Δ::URA3 dbf4-NΔ109::kanMX6*	This study
M3498	*W303 MAT**a** hir3Δ::HIS3 dbf4-NΔ109::kanMX6*	This study
M3499	*W303 MAT**a** asf1Δ::kanMX4 dbf4-NΔ109::kanMX6*	This study
M3561	*W303 MAT**a** ctf18Δ::HIS3*	This study
M3562	*W303 MAT**a** ctf8Δ::HIS3*	This study
M3563	*W303 MAT**a** dcc1Δ::HIS3*	This study
M3593	*W303 MAT**a** ctf18Δ::HIS3 dbf4-NΔ109::kanMX6*	This study
M3597	*W303 MAT**a** ctf8Δ::HIS3 dbf4-NΔ109::kanMX6*	This study
M3599	*W303 MAT**a** dcc1Δ::HIS3 dbf4-NΔ109::kanMX6*	This study
M3890	*W303 MAT**a** dbf4-NΔ109-natMX4*	This study
M3943	*W303 MAT**α** top3-2::HIS3 sgs1-3::TRP1*	Mullen et al., 1999
M4004	*W303 MAT**a** bmh1Δ::kanMX4*	This study
M4007	*W303 MAT**a** bmh2Δ::kanMX4*	This study
M4154	*W303 MAT**a** bmh1Δ::kanMX4 dbf4-NΔ109::kanMX6*	This study

Table 13. (cont'd)

M4171	*W303 MAT**a** bmh1Δ::kanMX4 rad53-1*	This study
M4198	*W303 MAT**a** rmi1Δ::kanMX4*	This study
M4202	*W303 MAT**a** sgs1Δ::kanMX4*	This study
M4206	*W303 MAT**a** rad17Δ::kanMX4*	This study
M4210	*W303 MAT**a** rad24Δ::kanMX4*	This study
M4214	*W303 MAT**a** rad17Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4222	*W303 MAT**a** rad24Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4230	*W303 MAT**a** sgs1Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4284	*W303 MAT**a** bch1Δ::kanMX4*	This study
M4288	*W303 MAT**a** cdc73Δ::kanMX4*	This study
M4292	*W303 MAT**a** ctk1Δ::kanMX4*	This study
M4296	*W303 MAT**a** lsm7Δ::kanMX4*	This study
M4300	*W303 MAT**a** bch1Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4308	*W303 MAT**a** cdc73Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4316	*W303 MAT**a** ctk1Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4320	*W303 MAT**a** lsm7Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4336	*W303 MAT**a** rad54Δ::kanMX4*	This study
M4340	*W303 MAT**a** srb2Δ::kanMX4*	This study
M4344	*W303 MAT**a** yta7Δ::kanMX4*	This study
M4348	*W303 MAT**a** rad54Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4352	*W303 MAT**a** srb2Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4356	*W303 MAT**a** yta7Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4380	*W303 MAT**a** bub3Δ::kanMX4*	This study
M4384	*W303 MAT**a** ckb2Δ::kanMX4*	This study
M4388	*W303 MAT**a** tim18Δ::kanMX4*	This study
M4392	*W303 MAT**a** bub3Δ::kanMX4 dbf4-NΔ109::natMX4*	This study

Table 13. (cont'd)

M4396	*W303 MAT**a** ckb2Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4400	*W303 MAT**a** tim18Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4404	*W303 MAT**a** rpl21bΔ::kanMX4*	This study
M4408	*W303 MAT**a** opi9Δ::kanMX4*	This study
M4412	*W303 MAT**a** sgs1-3::TRP1*	This study
M4416	*W303 MAT**a** top3-2::HIS3*	This study
M4420	*W303 MAT**a** sgs1-3::TRP1 dbf4-NΔ109::natMX4*	This study
M4422	*W303 MAT**a** top3-2::HIS3 sgs1-3::TRP1 dbf4-NΔ109::natMX4*	This study
M4424	*W303 MAT**a** rpl21bΔ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4428	*W303 MAT**a** opi9Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4432	*W303 MAT**a** csm1Δ::kanMX4*	This study
M4436	*W303 MAT**a** hpc2Δ::kanMX4*	This study
M4440	*W303 MAT**a** csm1Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4444	*W303 MAT**a** hpc2Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4494	*W303 MAT**a** med1Δ::kanMX4*	This study
M4498	*W303 MAT**a** med1Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M4501	*W303 MAT**a** pol32Δ::kanMX4*	This study
M4505	*W303 MAT**a** pol32Δ::kanMX4 dbf4-NΔ109::natMX4*	This study
M517	*W303 MAT**a** rad53-1*	Cabrielse et al., 2006
Y5565	*MAT**α** can1Δ::MFA1pr-HIS3 mfα1Δ::MFα1pr-LEU2 lyp1Δ ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*	Boone Lab
M3130	*MAT**α** can1Δ::MFA1pr-HIS3 mfα1Δ::MFα1pr-LEU2 lyp1Δ ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 dbf4-NΔ109-natMX4*	This study
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems

Table 13. (cont'd)

M3429	*BY4741 MAT**a** bub3Δ::kanMX4*	Open Biosystems
M3431	*BY4741 MAT**a** hir2Δ::kanMX4*	Open Biosystems
M3432	*BY4741 MAT**a** asf1Δ::kanMX4*	Open Biosystems
M3433	*BY4741 MAT**a** sgs1Δ::kanMX4*	Open Biosystems
M3435	*BY4741 MAT**a** tim18Δ::kanMX4*	Open Biosystems
M3437	*BY4741 MAT**a** top3Δ::kanMX4*	Open Biosystems
M3438	*BY4741 MAT**a** ctf8Δ::kanMX4*	Open Biosystems
M3439	*BY4741 MAT**a** pol32Δ::kanMX4*	Open Biosystems
M3440	*BY4741 MAT**a** rad17Δ::kanMX4*	Open Biosystems
M3441	*BY4741 MAT**a** csm1Δ::kanMX4*	Open Biosystems
M3445	*BY4741 MAT**a** dcc1Δ::kanMX4*	Open Biosystems
M3450	*BY4741 MAT**a** lsm7Δ::kanMX4*	Open Biosystems
M3451	*BY4741 MAT**a** rad54Δ::kanMX4*	Open Biosystems
M3454	*BY4741 MAT**a** rmi1Δ::kanMX4*	Open Biosystems
M3455	*BY4741 MAT**a** ctk1Δ::kanMX4*	Open Biosystems
M3458	*BY4741 MAT**a** rad24Δ::kanMX4*	Open Biosystems
M3903	*BY4741 MAT**a** bmh1Δ::kanMX4*	Open Biosystems
M3904	*BY4741 MAT**a** bmh2Δ::kanMX4*	Open Biosystems
M4185	*BY4741 MAT**a** bch1Δ::kanMX4*	Open Biosystems
M4186	*BY4741 MAT**a** ylr235cΔ::kanMX4*	Open Biosystems
M4187	*BY4741 MAT**a** cdc73Δ::kanMX4*	Open Biosystems
M4188	*BY4741 MAT**a** rpl21bΔ::kanMX4*	Open Biosystems
M4189	*BY4741 MAT**a** ckb2Δ::kanMX4*	Open Biosystems
M4191	*BY4741 MAT**a** cbc2Δ::kanMX4*	Open Biosystems
M4192	*BY4741 MAT**a** hpc2Δ::kanMX4*	Open Biosystems
M4193	*BY4741 MAT**a** yta7Δ::kanMX4*	Open Biosystems

Table 13. (cont'd)

M4194	*BY4741 MAT**a** med1Δ::kanMX4*	Open Biosystems
M4195	*BY4741 MAT**a** srb2Δ::kanMX4*	Open Biosystems
M4196	*BY4741 MAT**a** opi9Δ::kanMX4*	Open Biosystems
M4258	*BY4741 MAT**a** lea1Δ::kanMX4*	Open Biosystems
M4259	*BY4741 MAT**a** pat1Δ::kanMX4*	Open Biosystems
M4260	*BY4741 MAT**a** pfk2Δ::kanMX4*	Open Biosystems

Table 14. Primers

Primer Name	Sequence (5'-3')
BCH1-kanMX-F	GACCCAAAGTCTATGTGAATG
BCH1-kanMX-R	GATATTTGAGTAAAGCTGATC
BMH1-EcoRI-F	GATCGAATTCATGTCAACCAGTCGTG
BMH1-PstI-R	GCTCTGCAGTTACTTTGGTGCTTCAC
BMH1-kanMX-F	CGGTGGCAAATAGCTTCCTC
BMH1-kanMX-R	GAAGCTAAAGTTGCTTCTCGC
BMH2-EcoRI-F	GATCGAATTCATGTCCCAAACTCGTG
BMH2-PstI-R	GCTCTGCAGTTATTTGGTTGGTTCAC
BMH2-kanMX-F	GTCGGTCGAAAGGGGCAAATG
BMH2-kanMX-R	GAAAATTACTACTCAATTACTC
BUB3-kanMX-F	GTCACCAGAAAACTCCAGTG
BUB3-kanMX-R	GAGCTCTATCGCTTTATCGT
CDC73-kanMX-F	GCGATGTAAAGTATAAAGTG
CDC73-kanMX-R	CTTATGGAGGTATTACAAAATTG
CKB2-kanMX-F	GTATATTGTTTTATGAAGAC
CKB2-kanMX-R	CCAATAATTCGTGGGTAACC
CSM1-kanMX-F	CAATTTTACGAATTATTTAC
CSM1-kanMX-R	GGGCAACAAGAAGCAGAAGC
CTK1-kanMX-F	GTGAAGCTCTATTTTTTTCG
CTK1-kanMX-R	GTTGGTTGATAGGTAGTTAC
Dbf4-genomic5F	CCAAATCCGTCCCACTAATAGTTTC
Dbf4-genomic5R	CTTAGCCAAATCCTCCACCAAG
DBF4-natR-F	CTATCAACGGCAATGTTATTGAATCACTTTCTCATTCACCCTTGTACATGGAGGCCCAGAATACC
DBF4-natR-R	ATGCAATTGATAATATATGGACGAGTAAATAAGAGTTAAGTCAATCAGTATAGCGACCAGCATTC

Table 14. (cont'd)

HPC2-kanMX-2F	CCCGCTGTTTCCCTCTCCCTC
HPC2-kanMX-R	GTGGATAAAAACGAATCTC
LSM7-kanMX-F	CTGTACGGACCAATTCACTC
LSM7-kanMX-R	GGAATCAGTAAATAATTAAG
MED1-kanMX-F	GAAAAAATTTTTTTTCTCAAGC
MED1-kanMX-R	CCTCCTACCTACCTATCTAC
OPI9-kanMX-F	GGTAGTGGTGGTGGAGGCGG
OPI9-kanMX-R	CGGTTTGTCCGCTACATTGC
POL32-kanMX-F	GAAACCGAGCGGCGCTAAGC
POL32-kanMX-R	GGGATGACGCTGATGAAAAAAG
RAD17-kanMX-F	CTACAAGATGGTACTGGATG
RAD17-kanMX-R	CATTGATCAAGGTTGCTGATG
RAD24-kanMX-F	CCTTCGTTTCATGCTCAG
RAD24-kanMX-R	CGTTAGACAAAGCTTGAAG
RAD54-kanMX-F	GCAAAGGGGAAGACCCTTCCG
RAD54-kanMX-R	CTTGCCATAATCTTTTTTGGC
RMI1-kanMX-F	GTCCTCTTGACAGGTCCGGC
RMI1-kanMX-R	GTTTAGTATCTGGTCCGAGTG
RPL21B-kanMX-F	CTGCAGCAACGATGCTTTTTC
RPL21B-kanMX-R	CAGACATTGATGTTTTAAATAC
SGS1-kanMX-F	GAAGCTTCTCTCCACATGTCC
SGS1-kanMX-R	CTGTAGAAGAAATTGCGAACG
SRB2-kanMX-F	GTGCGTTATCTACTGGGAG
SRB2-kanMX-R	CTACACCAGGAACCCCGCCC
TIM18-kanMX-F	CATATATGTTTCGAAGAAATC
TIM18-kanMX-R	CATCATTAAAGAAACAAAAAGC
YLR235C-kanMX-F	GAATTGTATCTCACATATATACC
YLR235C-kanMX-R	CAGGTCTCGTAGTCCTAGAGAG

Table 14. (cont'd)

YTA7-kanMX-F	GTTGAGGCATTAGCCGCTG
YTA7-kanMX-R	GATGAATCAGCAGAGTATTC

Table 15. Plasmids

Plasmid	Description	Source
p4339	pCRII-TOPO::natRMX4	Goldstein and McCusker, 1999
pCG53	pGBKT7-*DBF4*$_{66-227}$	Miller et al., 2009
pCG60	pCG53$_{ADH1\ promoter-\Delta(-732)-(-802)}$	Miller et al., 2009
pCG63	pCG60 W202E	This study
pCG64	pCG60 W202A	This study
pCG101	pCG60 GA159,160LL	This study
pCM21	pCG60-*DBF4*$_{66-109}$	Miller et al., 2009
pGAD-C1		James et al. 1996
pGAD-Cdc5.3	pGAD-C1-*CDC5*$_{421-705}$	Miller et al., 2009
pGBKT7		Clontech
pJK117	pGAD-C1-*BMH1*$_{2-267}$	This study
pJK119	pGAD-C1-*BMH2*$_{2-273}$	This study
pYJ1	pCG60-*DBF4*$_{N\Delta71}$	Chen and Weinreich, 2010
pYJ2	pCG60-*DBF4*$_{N\Delta77}$	Chen and Weinreich, 2010
pYJ3	pCG60-*DBF4*$_{N\Delta81}$	Chen and Weinreich, 2010
pYJ4	pCG60-*DBF4*$_{N\Delta87}$	Chen and Weinreich, 2010
pYJ5	pCG60-*DBF4*$_{N\Delta93}$	Chen and Weinreich, 2010
pYJ6	pCG60-*DBF4*$_{N\Delta99}$	Chen and Weinreich, 2010
pYJ7	pCG60-*DBF4*$_{N\Delta103}$	Chen and Weinreich, 2010
pYJ8	pCG60-*DBF4*$_{N\Delta107}$	Chen and Weinreich, 2010
pYJ9	pCG60-*DBF4*$_{N\Delta109}$	Chen and Weinreich, 2010
pYJ30	pCG60 R83E	Chen and Weinreich, 2010
pYJ38	pCG60-*DBF4*$_{\Delta82-88}$	Miller et al., 2009
pYJ308	pGAD-C1-*RAD53*$_{1-300}$	This study
pYJ332	pCG60-*DBF4*$_{\Delta100-109}$	This study
pYJ368	pCG60-*DBF4*$_{66-190}$	This study
pYJ372	pCG60-*DBF4*$_{66-150}$	This study

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CHAPTER 5

CONCLUSIONS AND OUTLOOK

This chapter was taken partly from the manuscript currently in preparation:

Dbf4, a multifaceted cell-cycle regulator

Ying-Chou Chen and Michael Weinreich

CONCLUSIONS AND OUTLOOK

RECENT INSIGHTS INTO DDK

Dbf4 is a regulator of chromosome segregation

Until recently, our knowledge about the role of the Dbf4-Cdc7 kinase in cell cycle regulation has expanded from the initiation of DNA replication to the complex control of chromosome segregation in mitosis and meiosis. Our studies have highlighted the direct interaction between Dbf4 and Cdc5 and indicated that Dbf4 inhibits Cdc5 function during mitotic exit (Miller et al. 2009; Chen and Weinreich 2010). The C-terminal truncation of *dbf4* mutants that are defective in Cdc7 binding bypassed the Dbf4-mediated Cdc5 inhibition, suggesting that Dbf4 functions as a scaffold for Cdc7 to regulate Cdc5. Indeed, the molecular basis for the Dbf4-Cdc5 interaction was subsequently characterized as a non-canonical binding mechanism between the Dbf4 N-terminus and Cdc5 Polo-box domain (PBD), suggesting a specific role of Dbf4, together with Cdc7, in Cdc5-related cell cycle regulation.

FEAR, MEN, and SPoC

Among multiple roles of Cdc5 in mitosis, it has been shown that Dbf4 is functionally linked to the FEAR and MEN pathways. The segregation of highly repetitive ribosomal DNA (rDNA) on chromosome III of budding yeast depends on the activation of Cdc14 phosphatase via the FEAR pathway (D'Amours and Amon 2004). Recent studies found that stabilization of Dbf4 by removing the N-terminal D-boxes (*dbf4-N∆65*) led to the delay of rDNA segregation and that this *dbf4* mutant was synthetically lethal with the

cdc5-1 hypomorphic mutant (Sullivan et al. 2008; Miller et al. 2009; Chen and Weinreich 2010). More interestingly, *dbf4* mutants (*dbf4-NΔ109* or *-Δ82-88*) that abolish the Dbf4-Cdc5 interaction suppressed the temperature sensitive phenotype of *cdc5-1* (Miller et al. 2009; Chen and Weinreich 2010), implying that elevated levels of Dbf4 inhibit Cdc5 function in Cdc14 activation in the FEAR pathway. It should be noted, however, that neither *dbf4-NΔ109* nor *dbf4-Δ82-88* allows Cdc5 to induce premature Cdc14 activation (Miller et al. 2009). Because Cdc5 also activates Cdc14 though the MEN pathway, we examined the genetic interactions between *dbf4-NΔ109* and other MEN mutants and found that *dbf4-NΔ109* suppresses the temperature sensitivity of *dbf2-1* (one of essential kinases in the MEN signal transduction), implying a closer functional connection between Dbf4 and the MEN pathway (Miller et al. 2009). Apart from *cdc5-1* and *dbf2-1*, none of the other genes in the MEN pathway has a genetic interaction with *DBF4*. In addition, the *dbf4-Δ82-88* and *-R83E* mutants, which specifically lose the Cdc5 interaction, cannot suppress the temperature sensitivity of *dbf2-1* (unpublished data, Weinreich lab), suggesting that the Dbf4 N-terminus may also participate in the MEN pathway though a Cdc5-independent mechanism.

Correct orientation of the mitotic spindle is vital for faithful chromosome segregation (Bloecher et al. 2000; Pereira et al. 2000). In *S. cerevisiae*, a surveillance mechanism known as the spindle position checkpoint (SPoC) modulates MEN signaling to restrain mitotic exit when the spindles are misaligned (Fraschini et al. 2008). In normal mitotic exit, Cdc5 antagonizes a two-subunit GAP (GTPase activating protein, comprising Bub2 and Bfa1), which inhibits the Tem1 kinase and subsequently prevents the kinase

cascades in the MEN pathway (Hu et al. 2001; Lee et al. 2001; Hu and Elledge 2002; Ro et al. 2002; Geymonat et al. 2003). Therefore, when the SPoC gets activated, it is known that Cdc5 is down-regulated to allow the Bub2/Bfa1-mediated inhibition of Tem1. We first proposed that Dbf4 inhibits Cdc5 in the MEN pathway and that the defect of the *dbf4-NΔ109* mutant in the SPoC activation was due to loss of the Dbf4-Cdc5 interaction (Miller et al. 2009). However, the *dbf4-Δ82-88* mutant has an intact SPoC (unpublished data, Weinreich lab). This suggests that instead of inhibiting Cdc5 by a direct interaction, Dbf4 is likely responsible through a distinct mechanism in SPoC activation.

Meiotic recombination and mono-orientation

Studies in yeast meiosis indicate that inactivation of the Cdc7 kinase using temperature-sensitive or analog-sensitive mutants results in pleiotropic effects on chromosome dynamics (Valentin et al. 2006; Wan et al. 2006; Matos et al. 2008; Wan et al. 2008). These mutants undergo DNA replication, but are arrested in prophase I with defects in meiotic recombination. This is probably because that DDK activity is required for the phosphorylation of Mer2, which facilitates the Spo11-mediated double-strand break (DSB) formation and interhomolog crossovers (also known as chiasmata) (Sasanuma et al. 2008; Wan et al. 2008). Because this recombination mechanism is crucial for accurate segregation of homologous chromosomes in meiosis I, it is also thought that the compromised DDK function triggers a checkpoint to cause the arrest before the first meiotic division. Furthermore, DDK is required for the transcriptional expression of *NDT80* (Lo et al. 2008; Lo et al. 2012), which is a meiosis-specific transcriptional activator that regulates genes involved in exit from prophase I and the following meiotic

progression (Hollingsworth 2008). In the absence of Cdc7 kinase activity, prophase I arrest is bypassed by overexpressing *NDT80*. Taken together, these findings suggest multiple roles of DDK in setting up meiotic chromosome segregation.

The Dbf4-dependent kinase also contributes to the mono-orientation of sister kinetochores (also known as syntely) in the first division of meiosis (Matos et al. 2008). Syntelic kinetochore attachment and monopolar chromosome segregation depend on the maintenance of centromeric cohesion and the assembly of the monopolin complex (Lee and Orr-Weaver 2001; Dudas et al. 2011). It is known that the casein kinase Hrr25 and DDK phosphorylation of Rec8 (Petronczki et al. 2006; Katis et al. 2010), which is the meiosis-specific cohesin subunit, are necessary for cleavage by separase Esp1 (Marston and Amon 2004), whereas the cleavage of the mitotic cohesin Scc1 relies on the phosphorylation of Cdc5 (Alexandru et al. 2001; Barr et al. 2004; Archambault and Glover 2009). It has long been known that the centromeric Rec8 is protected from cleavage by shugoshin until meiosis II, which ensures that sister kinetochores remain associated and segregated to the same pole (Watanabe and Kitajima 2005). Recent evidence unraveled that the Hrr25 and DDK phosphorylation of Rec8 is counteracted by the shugoshin-associated PP2A phosphatase (Rts1 in *S. cerevisiae*) in meiosis I (Ishiguro et al. 2010). Further, the Hrr25 kinase, the meiosis-specific protein Mam1, and two nucleolar proteins (Lrs4 and Csm1) form the monopolin complex. Cdc5 and DDK are both required for the translocation of Lrs4 and Csm1 from nucleoli to sister kinetochores, where they interact with Mam1 (Toth et al. 2000; Rabitsch et al. 2003; Petronczki et al. 2006; Dudas et al. 2011; Corbett and Harrison 2012). Indeed, DDK and

Cdc5 have been thought to complex together and execute a dual phosphorylation on Lrs4 for subcellular trafficking (Clyne et al. 2003; Lee and Amon 2003; Lo et al. 2008; Matos et al. 2008). Interestingly, genetic analyses indicate that cells harboring a *dbf4-NΔ109* allele, which abolishes the Dbf4-Cdc5 interaction, are synthetically sick with *csm1Δ* (Chen et al. 2012a), suggesting that DDK and Cdc5 also play in a collaborative way to assemble Csm1 in the monopolin complex.

Sister-chromatid cohesion

In budding yeast, the establishment of sister-chromatid cohesion strictly occurs in S phase when cohesin subunit Smc3 is acetylated by Eco1 (Ivanov et al. 2002; Rolef Ben-Shahar et al. 2008; Terret et al. 2009). Recent studies suggest that DDK plays a crucial role in Eco1 regulation, which allows proper mitotic chromosome segregation (unpublished result, Morgan Lab, UCSF). Since cohesion establishment is highly linked to S-phase progression, independent genetic screens have identified that various genes within the complex of sister-chromatid cohesion are involved in DNA synthesis and DNA replication checkpoint, including *MRC1, TOF1, CSM3, CTF4*, and *CTF18* (Mayer et al. 2004; Xu et al. 2007). Among these, Ctf18 associates with Ctf8 and Dcc1 to form an alternative RFC (replication factor C) complex, which not only is required to maintain the cohesion of sister chromatids (Hanna et al. 2001; Mayer et al. 2001; Pan et al. 2006) but also regulates late origin firing during replication stress (Crabbe et al. 2010). We recently identified that the *ctf18*, *ctf8* and *dcc1* null mutants are synthetically sick with *dbf4-NΔ109* and display additive sensitivity to DNA-damaging agents and spindle

poison (Chen et al. 2012a). These results suggest that Dbf4 has a parallel role with Ctf18-RFC in an interplay between DNA replication and chromosome segregation.

Dbf4 relays the checkpoint signal

DNA replication checkpoint

In the current model of DNA replication checkpoint, Dbf4 has been considered as a substrate downstream of the Rad53 kinase in response to replication fork arrest (Labib and De Piccoli 2011). It has been known that activated Rad53 phosphorylates multiple sites in the Dbf4 C-terminus and consequently inhibits the Cdc7 kinase from promoting late origin firing (Gabrielse et al. 2006; Yabuuchi et al. 2006; Duch et al. 2010; Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010). Given an N-terminal deletion to Dbf4 residue 109, the Rad53-mediated Dbf4 hyperphosphorylation is significantly impaired, suggesting that these residues play a crucial role in the Rad53-Dbf4 interaction. By studying the molecular interaction between Dbf4 and Rad53, we recently found that Rad53 FHA domains directly bind to a Dbf4 T105-x-x-E-L motif in a phosphorylation-dependent manner, and we proposed that Rad53 interacts with Dbf4 dimers or multimers (Chen et al. 2012b). Moreover, loss of the Rad53-Dbf4 physical interaction prevents Rad53 phosphorylation of Dbf4, which allows late origin firing in the presence of HU. These data indicate that Dbf4 not only functions as a scaffold to conduct the Rad53-mediated Cdc7 inhibition, but also participates in relaying the signal through its self-assembly and sequential phosphorylation events.

Although it is thought that the Dbf4-Rad53 interaction relies on the phosphorylation of Dbf4 residue Thr105 for FHA domain binding (Chen et al. 2012b), the kinases involved are largely unknown. Recent evidence has shown that a majority of yeast kinases specifically recognize the residues flanking at the +1, -2, and -3 positions to their target serine or threonine, such as proline at the +1 position or arginine at the -2 or -3 position (Mok et al. 2010). The latter arginine-directed kinases account for 35 of 61 analyzed yeast kinases. Mutagenesis studies showed that neither Dbf4 Arg103 nor Pro106 is necessary for the Rad53 interaction, suggesting that they do not contribute to the phosphorylation of Thr105. In contrast, there are a limited number of acidophilic kinases in mammals that are able to selectively phosphorylate the FHA domain-recognizing sequence, Thr-x-x-Glu/Asp, including Polo-like kinase 1 (Cdc5 in *S. cerevisiae*), CK2 (Cka1), and GSK3 (Mck1, Mrk1, and Rim11) (Fiol et al. 1988; Songyang et al. 1996; Johnson et al. 2007). However, the relative importance of these kinases in replication checkpoint will require further studies.

In addition to regulating the firing of late origins, Dbf4 has also been implicated in preserving replisome stability when replication forks stall. Synthetic lethal screens (SGAs, synthetic genetic assays) found that the function of Dbf4 (or Cdc7) is partially redundant with the Top3-Sgs1-Rmi1 complex, which is critical for maintaining genomic integrity by preventing the accumulation of aberrant replication or recombination intermediates (Tong et al. 2004; Chang et al. 2005; Pan et al. 2006). The DNA helicase Sgs1 (BLM in humans) has multiple roles in checkpoint signal transduction, including the binding and activation of the Rad53 checkpoint kinase (Myung et al. 2001;

Bjergbaek et al. 2005; Cobb et al. 2005; Hegnauer et al. 2012). Similarly, Rmi1 is required for normal fork progression and stalled fork recovery (Yang et al. 2012). The synthetic lethal or sick interaction observed between the *dbf4-NΔ109* allele and the null mutants of the Top3-Sgs1-Rmi1 complex indicates that the N-terminal fragment of Dbf4 functions in cooperation with the Top3-Sgs1-Rmi1 complex in response to replication perturbations (Chen et al. 2012a). Indeed, *dbf4-NΔ109* is unable to interact with Rad53 and is synthetically lethal with the *rad53* kinase-defective mutant, *rad53-1* (equivalent to the *rad53-11* mutant) (Gabrielse et al. 2006; Chen et al. 2012b), suggesting that the Dbf4 associates with activated Rad53 at stalled replication forks to promote checkpoint responses. In support of this idea, it has been shown recently that human Dbf4 and Chk2 (Rad53 in budding yeast) are both direct targets of ATM and ATR kinases (Mec1 and Tel1) in activating the DNA replication checkpoint (Lee et al. 2012).

DNA damage checkpoint

One recent study in fission yeast showed that Hsk1 (Dbf4-dependent kinase) phosphorylates the PCNA-like 9-1-1 clamp (composed of Rad9, Rad1, and Hus1; known as Ddc1-Rad17-Mec3 in *S. cerevisiae*) in response to DNA damage (Furuya et al. 2010). The heterotrimeric complex, together with its clamp loader (Rad24 in budding yeast), plays crucial roles in recognizing DNA damage and recruiting DNA repair enzymes. The 9-1-1 complex also serves as a platform for ATR- and ATM-mediated checkpoint activation via binding to replication protein A coated single-stranded DNA (ssDNA-RPA) (Harper and Elledge 2007). DDK-mediated phosphorylation facilitates the disassociation of the 9-1-1 clamp from ssDNA-RPA intermediates and is required for

subsequent DNA repair. Even though a two-hybrid interaction between Dbf4 and the 9-1-1 clamp or clamp loader Rad24 has not been detected so far (unpublished data, Weinreich lab), it is possible that the budding yeast DDK and the 9-1-1 clamp associate by co-localizing to DNA damage loci. Consistently, it has been recently shown that DDK phosphorylation on histone H3-Thr45 is critical for the DNA damage responses in the S phase (Baker et al. 2010). In addition, deleting genes of the 9-1-1 complex produces synthetic sickness with *dbf4-NΔ109* in the SGA screens (Chen et al. 2012a), suggesting a synergistic role between Dbf4 and 9-1-1 in the response to DNA damage. Synthetic genetic interactions were also observed between *dbf4* (or *cdc7*) and several genes (*mec1, rad53, chk1*, and *rad9*) required for DNA damage checkpoint activation (Tong et al. 2004; Gabrielse et al. 2006; Chen et al. 2012b), suggesting the possibility that DDK indirectly modulates a signaling pathway downstream the 9-1-1 function. Together with DNA replication machinery components (Pol32, Ctf4, and the Ctf18-RFC complex), DNA repair genes (Rad52, Rad54, and Sgs1), and the yeast 14-3-3 protein (Bmh1), these defined pathways form the genetic network of *DBF4* in DNA damage responses (Chen et al. 2012a).

Checkpoint adaptation

It was been proposed that Dbf4 functions as a molecular scaffold to coordinate three essential kinases, Rad53, Cdc7, and Cdc5, in cell cycle regulation (Miller et al. 2009; Chen and Weinreich 2010; Chen et al. 2012b). It has become clear that the association of Rad53-Cdc7-Dbf4 participates in checkpoint activation in response to genotoxicity or replication stress, whereas the Cdc5-Cdc7-Dbf4 interaction is involved in various mitotic

and meiotic controls. However, the biological relevance of such a ternary complex (Rad53-Cdc5-Cdc7-Dbf4) remains to be demonstrated. Recent studies in checkpoint adaptation in budding yeast and higher eukaryotes provide insights into the role of Dbf4 in the convergence of checkpoint signaling and mitotic regulation.

Checkpoint adaptation refers to a mechanism by which cells are unable to repair DNA lesions ultimately escape checkpoint arrest and enter mitosis (Sandell and Zakian 1993). Yeast genetic screens have identified a number of genes that are required for checkpoint adaptation, including the casein kinase II Cka1, the phosphatases Ptc2 and Ptc3, the helicase Srs2, and the Polo-like kinase Cdc5 (Pellicioli et al. 2001; Vaze et al. 2002; Leroy et al. 2003). Recent evidence indicates that Cdc5 counteracts checkpoint activation by inhibiting the Mec1 and Rad53-mediated signaling pathway (Donnianni et al. 2010; Schleker et al. 2010; Vidanes et al. 2010). It has been shown that the *cdc5* kinase-defective mutant loses the ability to adapt to irreparable DSBs, and overexpression of Cdc5 accelerates checkpoint adaptation. Similar molecular mechanisms were observed by studying *CDC5* homologues in *Xenopus* (Plx1) and mammals (Plks) (Yoo et al. 2004; Syljuasen et al. 2006; van Vugt et al. 2010). In particular, Plk1, Plk3, and Plk4 not only bind to Chk2 but also phosphorylate Chk2 (Bahassi el et al. 2002; Tsvetkov et al. 2003; Tsvetkov et al. 2005; Petrinac et al. 2009), likely leading to G2/M checkpoint termination and cell cycle re-entry.

We found that the budding yeast Dbf4 simultaneously interacts with Cdc5 and Rad53 as measured by co-immunoprecipitation assays from in insect lysates (Chen et al. 2012b).

Interestingly, yeast two-hybrid results have shown that loss of the residues required for the Dbf4-Cdc5 interaction promoted the interaction between Dbf4 and Rad53. This result suggests that the DNA replication or damage checkpoint signaling mediated by the Dbf4-Rad53 interaction is blocked by Cdc5 binding. In contrast, the *dbf4* mutant that disrupts the Rad53-Dbf4 interaction (*dbf4-Δ100-109*) did not affect the Dbf4-Cdc5 interaction, suggesting that the Cdc5-Dbf4 interaction is relatively stable than the Rad53-Dbf4 interaction. Unexpectedly, one recent report indicated that Rad53 phosphorylates the Dbf4 residue Ser84 within the Cdc5 binding site in Dbf4 (residues 83-88) after HU treatment (Duch et al. 2010). This phosphorylation on Dbf4 residue Ser84 impairs the binding affinity to Cdc5 *in vitro* (Chen and Weinreich 2010), suggesting that Rad53 may prevent the recruitment of Cdc5 and early checkpoint adaptation by an inhibitory phosphorylation in the Dbf4 N-terminus.

CONCLUDING REMARKS

Although the essential role of Dbf4 in DNA replication has been studied intensively, not much is known about how Dbf4 links the replication machinery to checkpoint response and post-replicative cell-cycle regulation. The identification of the molecular interactions and genetic networks of *DBF4* has advanced our understanding of its novel functions. These studies have given rise to a prevailing view of Dbf4 serving as a scaffold to coordinate DNA synthesis, checkpoint pathways, and chromosome segregation via the direct interactions with the Cdc7, Rad53 and Cdc5 kinases. Upon replication stresses or DNA damage, Dbf4 acts in parallel with the Top3-Sgs1-Rmi1, 9-1-1, and Ctf18-RFC complexes to converge multiple checkpoint signals for the Rad53 activation. It is known

that Rad53 directly interacts with and phosphorylates Dbf4, and then inhibits Cdc7 in late origin firing. Dbf4 may also function as a central regulator by tethering other checkpoint components or cell-cycle regulators in response to the S-phase perturbation. In particular, a profound understanding of the interaction between Dbf4 and Cdc5 has provided insights into the roles of Dbf4 in chromosome segregation, checkpoint adaptation, and various meiotic processes.

Despite these progress, further challenges remain. For example, the molecular mechanism by which Dbf4 inhibits Cdc5 in the mitotic exit network or spindle position checkpoint is not completely understood. The role of the Cdc7 kinase in the Dbf4-mediated Cdc5 inhibition is also not known. Additionally, the recent discovery that Dbf4 can simultaneously associate with Cdc7, Rad53, and Cdc5 raises the question of how the ternary complex is temporally and spatially assembled *in vivo*. Given that these genes are evolutionarily conserved, it is tempting to speculate that they play similar roles in checkpoint response and cell cycle regulation in higher eukaryotes. Even though genome-wide synthetic lethal screens were begun to provide a global view of Dbf4 function, much work will be required to evaluate the biological outcome of such synthetic genetic interactions. The discussion here has focused on the physiological functions of Dbf4; however, it is equally important to address the potential roles of the Dbf4-interacting partners, such as Cdc5, in DNA replication and checkpoint signaling cascades. Certainly, much remains to be learned about how different cell-cycle regulatory pathways are intertwined through the Dbf4 interactions, and elucidation of

these links will provide insights into the mechanisms leading to genomic stability, which is a prominent hallmark of cancer susceptibility in humans.

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