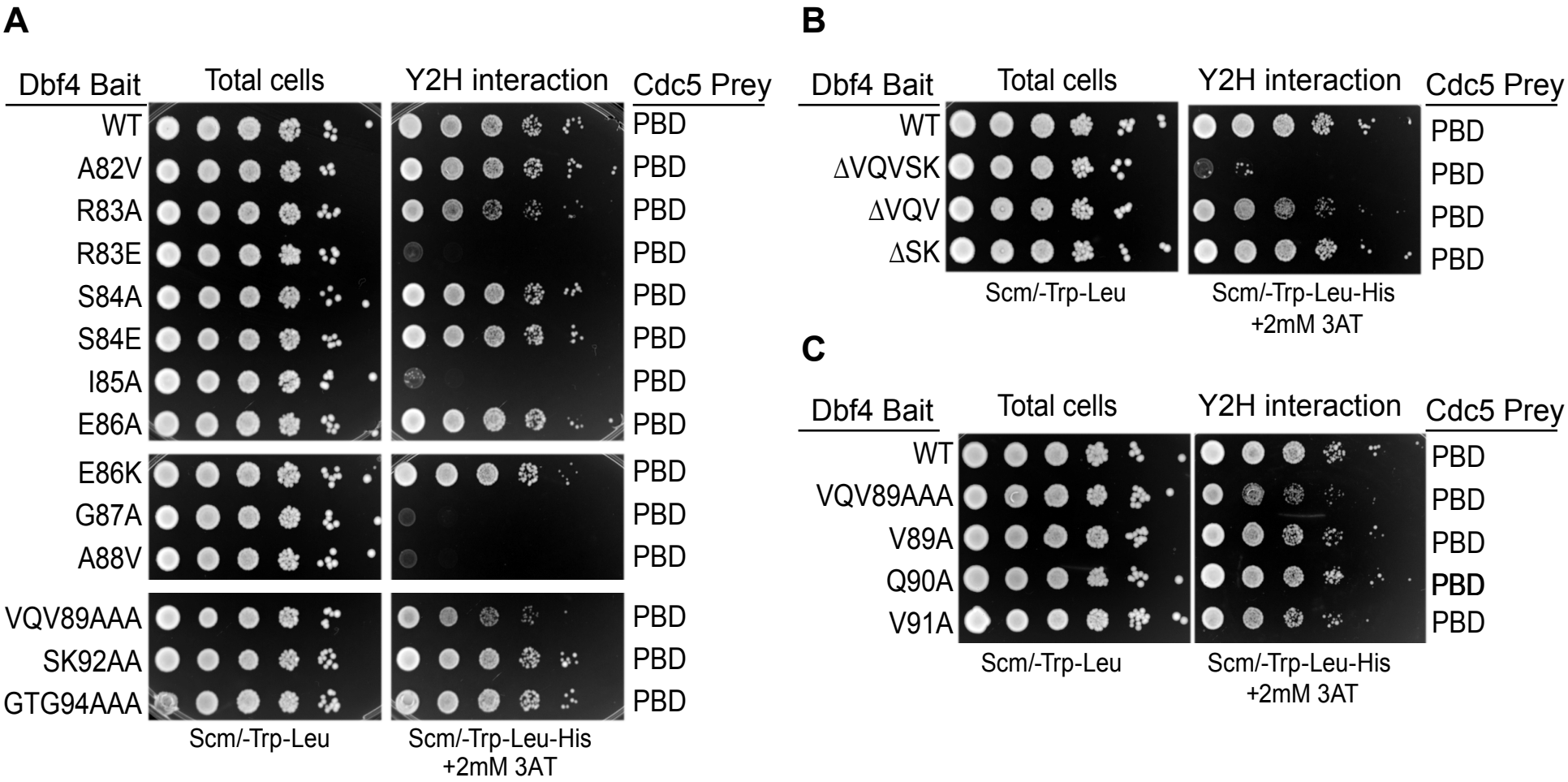
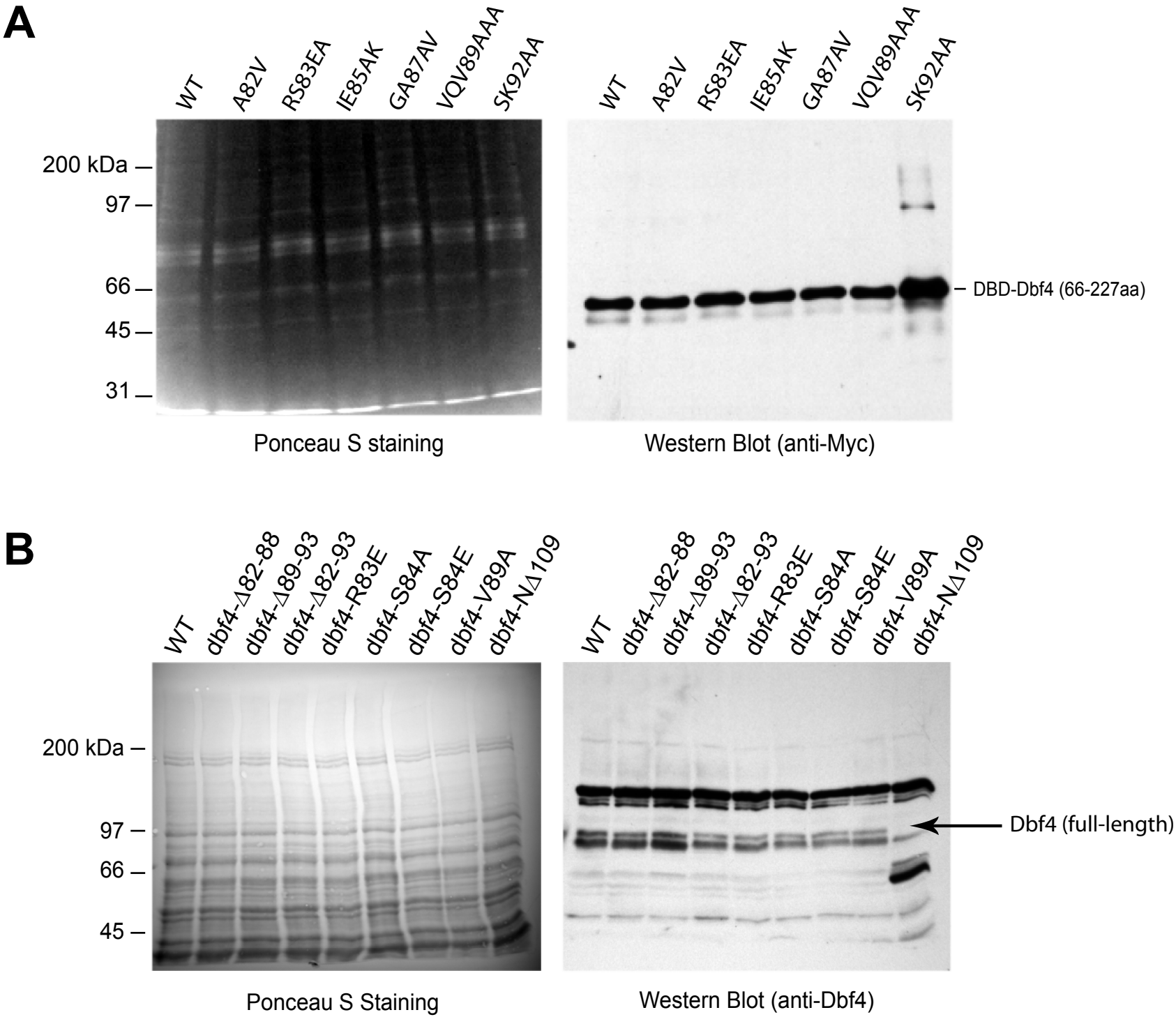
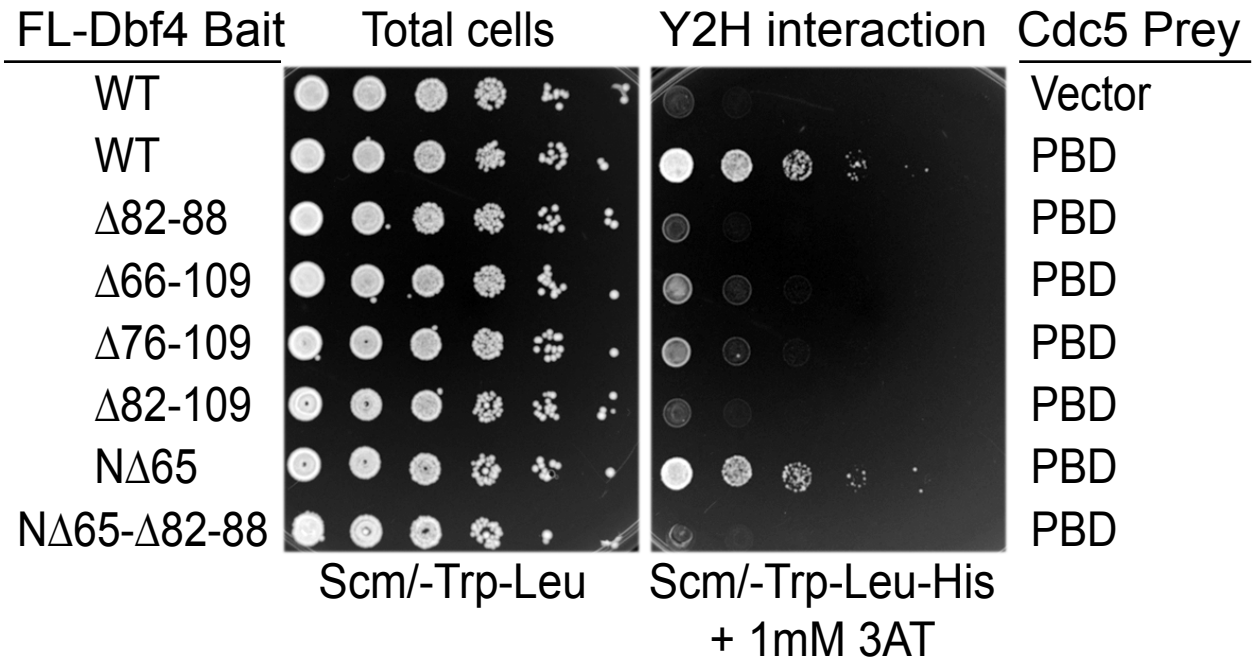
Suppl Figure 1



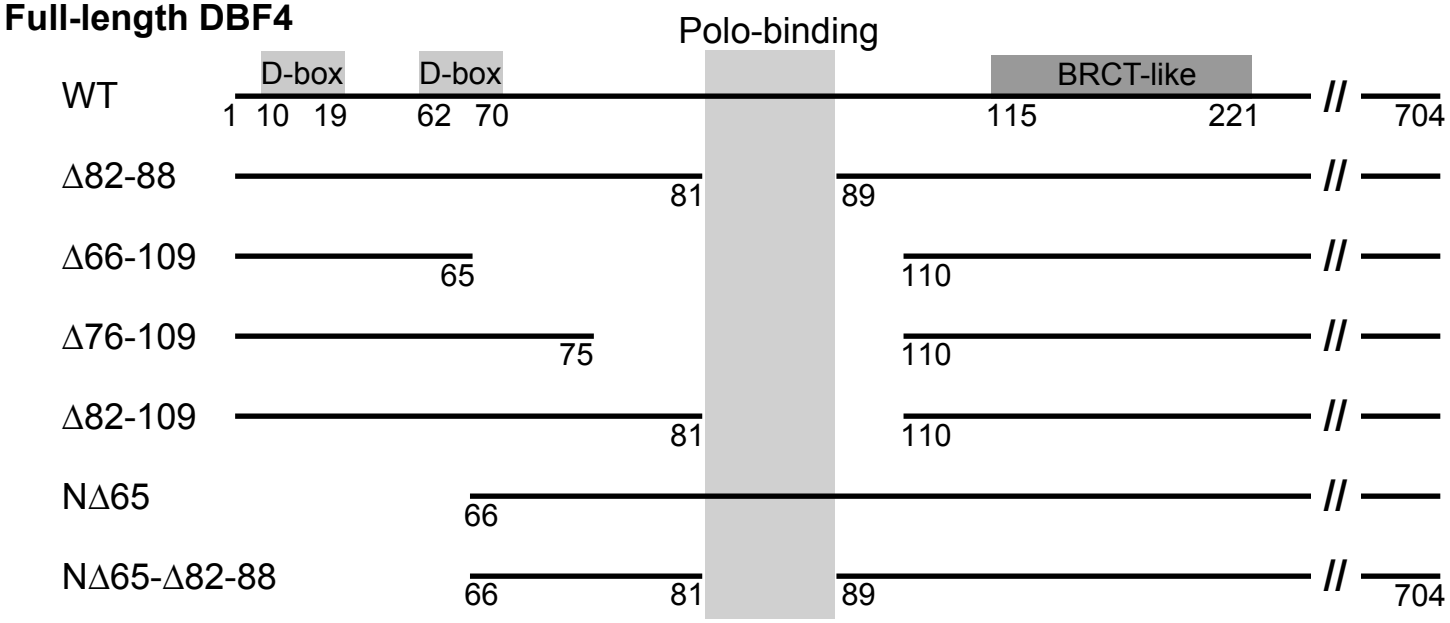
Suppl Figure 2

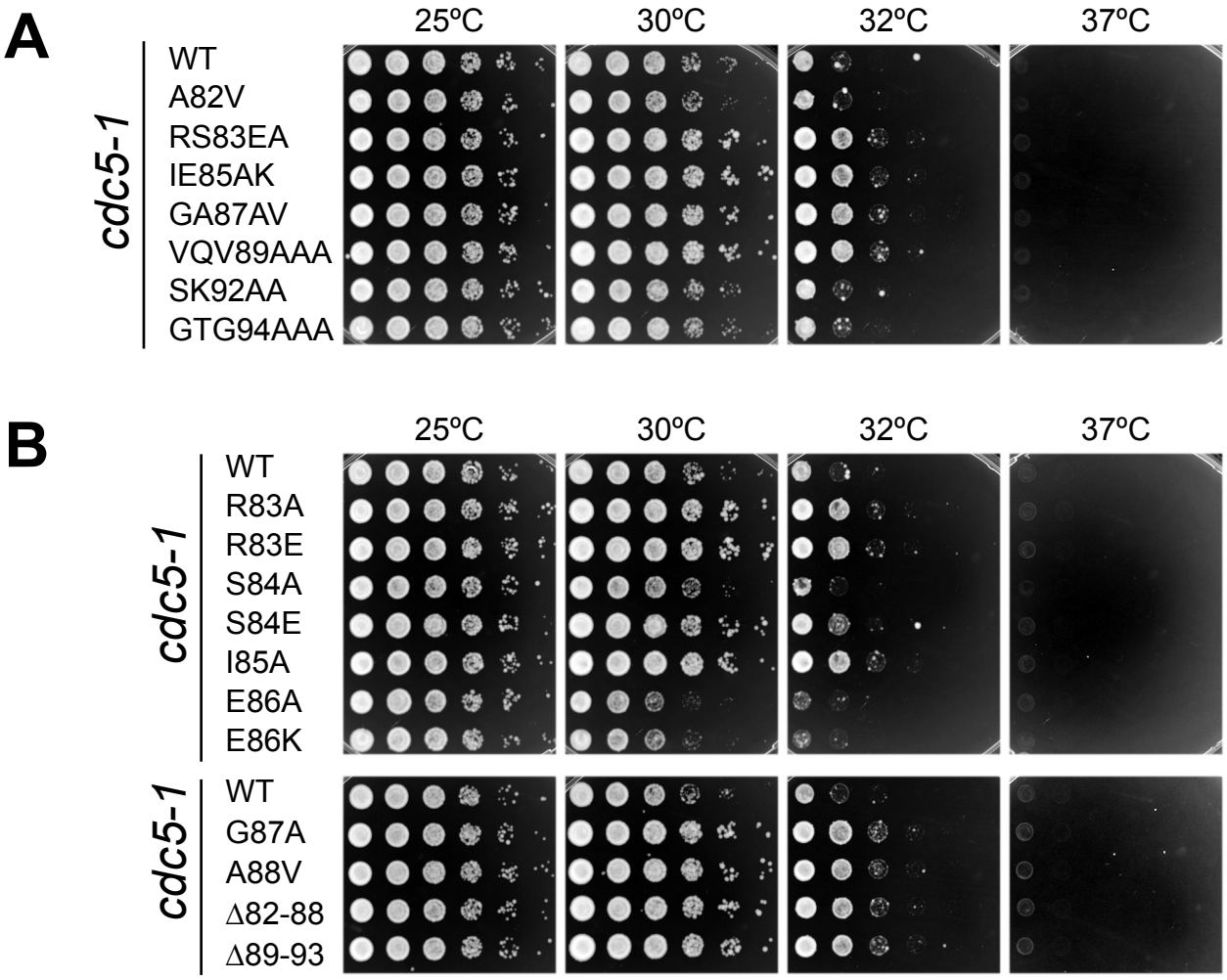


A


FL-Dbf4 Bait
Total cells
Y2H interaction
Cdc5 Prey
WT
WT
Δ82-88
Δ66-109
Δ76-109
Δ82-109
NΔ65
NΔ65-Δ82-88
Vector
PBD
PBD
PBD
PBD
PBD
PBD
PBD
Scm/-Trp-Leu
Scm/-Trp-Leu-His
+ 1mM 3AT

B


Full-length DBF4
Polo-binding
D-box
D-box
BRCT-like
WT
1 10 19
62 70
115
221
704
Δ82-88
81
89
Δ66-109
65
110
Δ76-109
75
110
Δ82-109
81
110
NΔ65
66
NΔ65-Δ82-88
66
81
89
704


A
25ºC
30ºC
32ºC
37ºC
cdc5-1
WT
A82V
RS83EA
IE85AK
GA87AV
VQV89AAA
SK92AA
GTG94AAA
B
25ºC
30ºC
32ºC
37ºC
cdc5-1
WT
R83A
R83E
S84A
S84E
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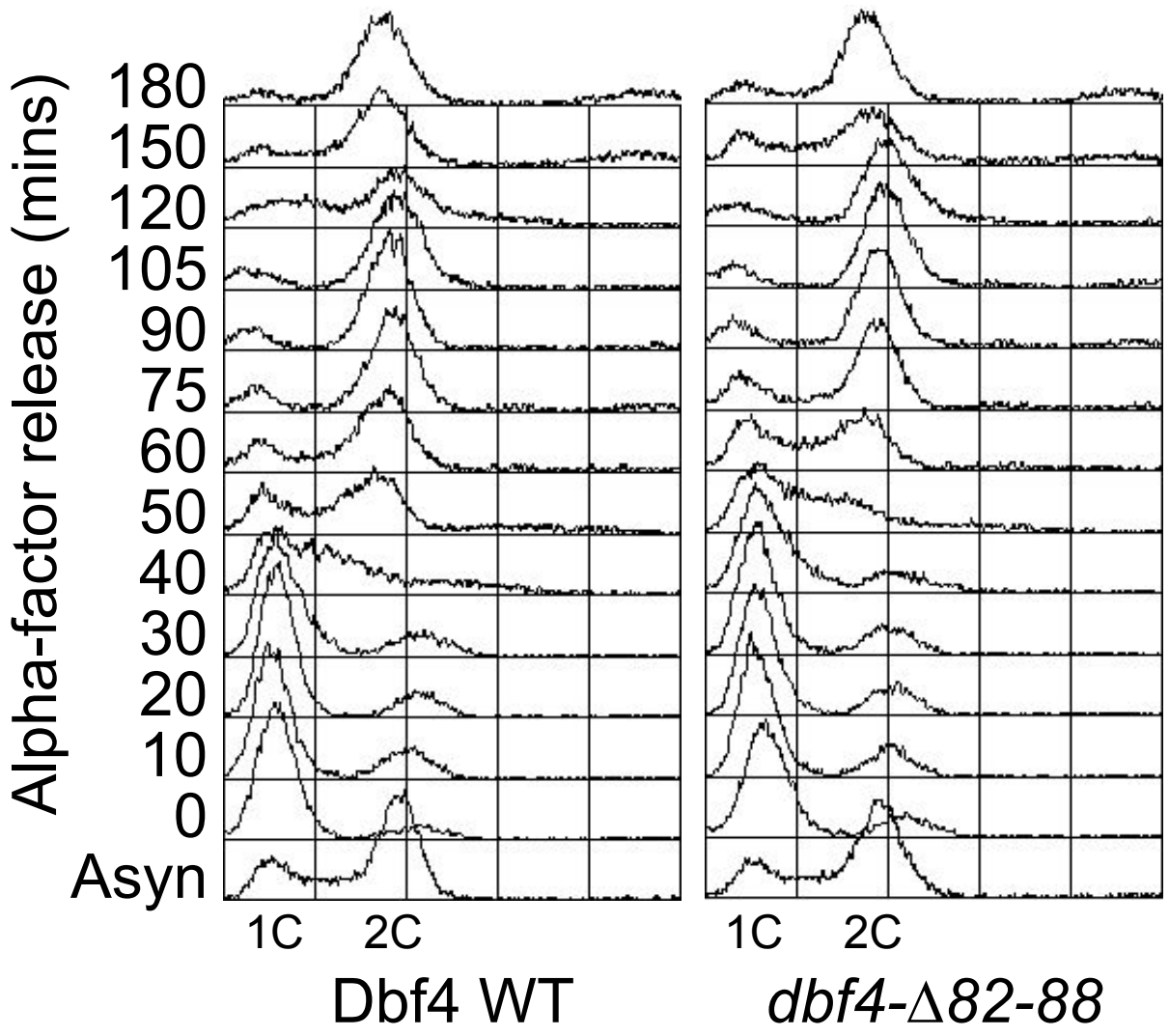
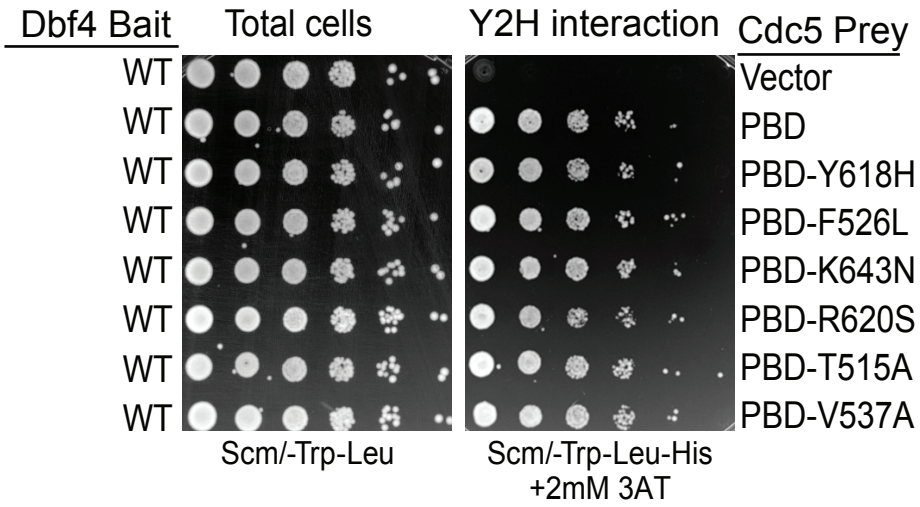
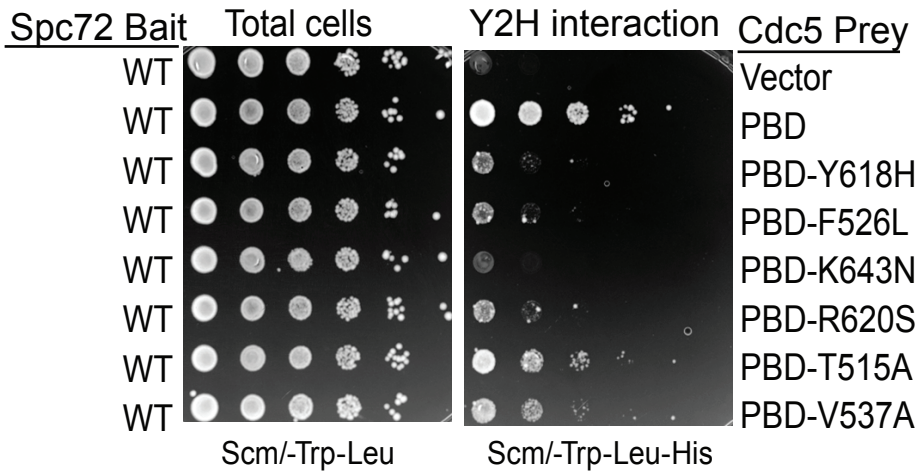
Δ82-88
Δ89-93


Alpha-factor release (mins)
180
150
120
105
90
75
60
50
40
30
20
10
0
Asyn
1C
2C
1C
2C
Dbf4 WT
dbf4-Δ82-88

A



B



C

			Y2H interaction with Dbf4	Y2H interaction with Spc72	
Cdc5	(hsPlk1)		+ + + +	+ + + +	
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

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. 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.

Supplementary Figure 2. 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*).

Supplementary Figure 3. 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.

Supplementary Figure 4. 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 30°C, consistent with the increased interaction between the Dbf4 E86K peptide and the PBD (Figure 2E).

Supplementary Figure 5. The *dbf4-Δ82-88* mutant exhibits normal cell cycle progression. Wild-type W303-1A (M138) and *dbf4-Δ82-88* (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.

Supplementary Figure 6. 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.

Suppl 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
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
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
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

Suppl 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
M3112	W303 *MAT**a** cdc5-1 dbf4-Δ82-88-kanMX6*	Miller et al., 2009
M3114	W303 *MAT**α** cdc5-1 dbf4-Δ82-88-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** cdc5Δ::kanMX6* [pMW535; pRS415-*CDC5 LEU2*]	This study
M3377	W303 *MAT**a** cdc5Δ::kanMX6* [pMW541; pRS415-*cdc5-H641A-K643M LEU2*]	This study
M3378	W303 *MAT**a** cdc5Δ::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

Suppl 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