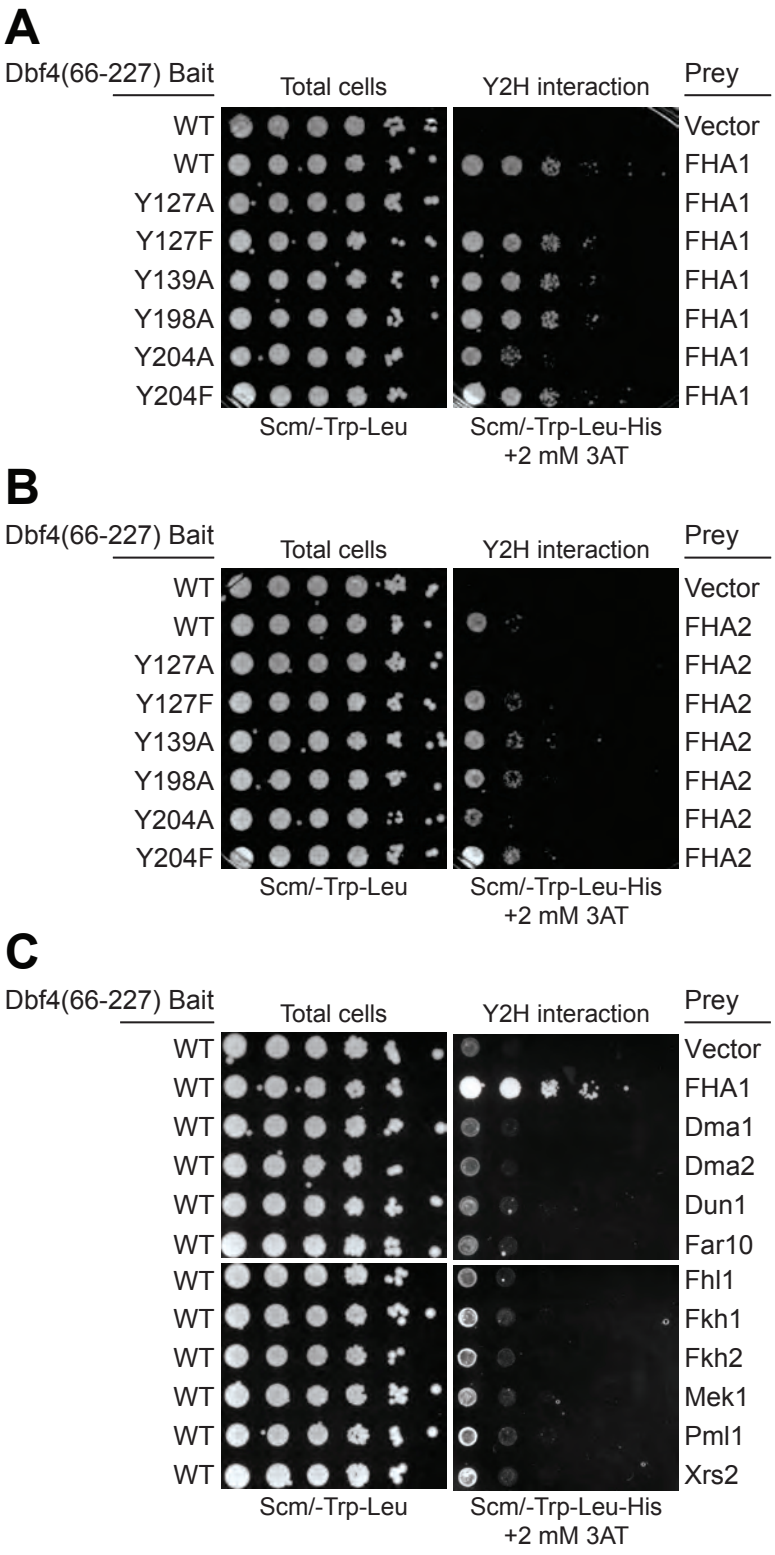

A
Dbf4(66-227) Bait
Total cells
Y2H interaction
Prey
WT
WT
Y127A
Y127F
Y139A
Y198A
Y204A
Y204F
Vector
FHA1
FHA1
FHA1
FHA1
FHA1
FHA1
FHA1
Scm/-Trp-Leu
Scm/-Trp-Leu-His
+2 mM 3AT
B
Dbf4(66-227) Bait
Total cells
Y2H interaction
Prey
WT
WT
Y127A
Y127F
Y139A
Y198A
Y204A
Y204F
Vector
FHA2
FHA2
FHA2
FHA2
FHA2
FHA2
FHA2
Scm/-Trp-Leu
Scm/-Trp-Leu-His
+2 mM 3AT
C
Dbf4(66-227) Bait
Total cells
Y2H interaction
Prey
WT
WT
WT
WT
WT
WT
WT
WT
WT
WT
WT
WT
Vector
FHA1
Dma1
Dma2
Dun1
Far10
Fhl1
Fkh1
Fkh2
Mek1
Pml1
Xrs2
Scm/-Trp-Leu
Scm/-Trp-Leu-His
+2 mM 3AT

Figure S1 Analysis of FHA domain-Dbf4 interactions including a screen of all Y residues in Dbf4 residues 100-227. (A and B) The indicated Dbf4 tyrosine mutants were assayed for a two-hybrid interaction with the Rad53 FHA1 (A) and FHA2 (B) 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). (C) Two hybrid interaction data of the Dbf4 N-terminus (66-227) with all remaining FHA domains in the yeast genome. Dma1 (pJK135, 137-302aa) (DUROCHER and JACKSON 2002), Dma2 (pJK137, 246-408aa) (DUROCHER and JACKSON 2002), Dun1 (pJK275, 1-160aa) (HAMMET *et al.* 2000), Far10 (pJK277, 61-227aa) (DUROCHER and JACKSON 2002), Fhl1 (pJK279, 253-400aa) (WADE *et al.* 2004), Fkh1 (pJK281, 41-185aa) (DUROCHER and JACKSON 2002), Fkh2 (pJK287, 1-254aa) (DARIEVA *et al.* 2003), Mek1 (pJK283, 1-152aa) (DUROCHER and JACKSON 2002), Pml1 (pJK289, 54-204) (BROOKS *et al.* 2009), Xrs2 (pJK285, 1-125aa) (PALMBOS *et al.* 2008).

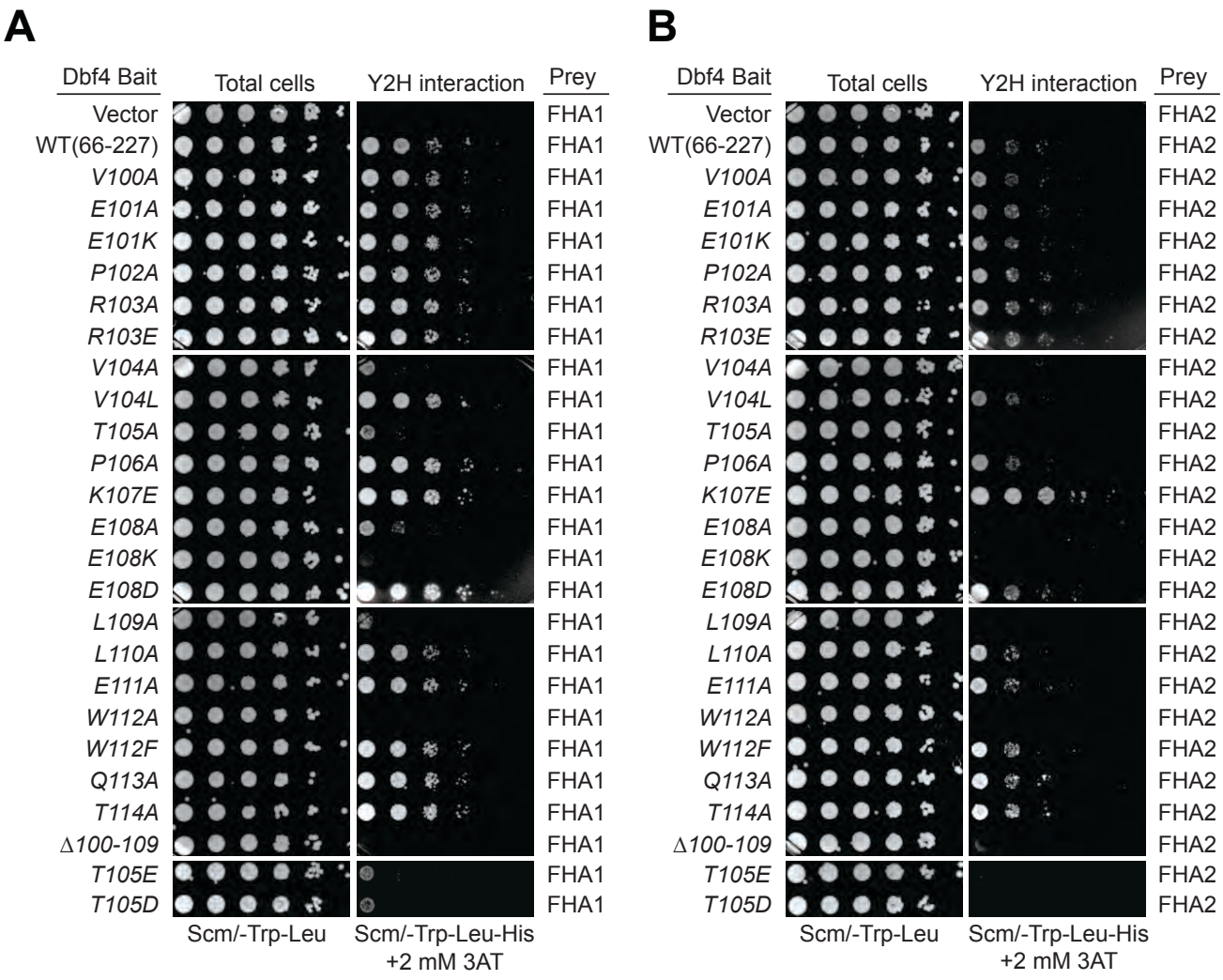


Figure S2 Dbf4 residues V104, T105, E108, L109, and W112 were required for binding the Rad53 FHA domains. The indicated substitutions within residues 100-114 of the Dbf4 Nterminal (66-227) bait plasmid were assayed for a two-hybrid interaction with the Rad53 FHA1 (panel A) and FHA2 domains (panel B). Spotting as in Figure S1.

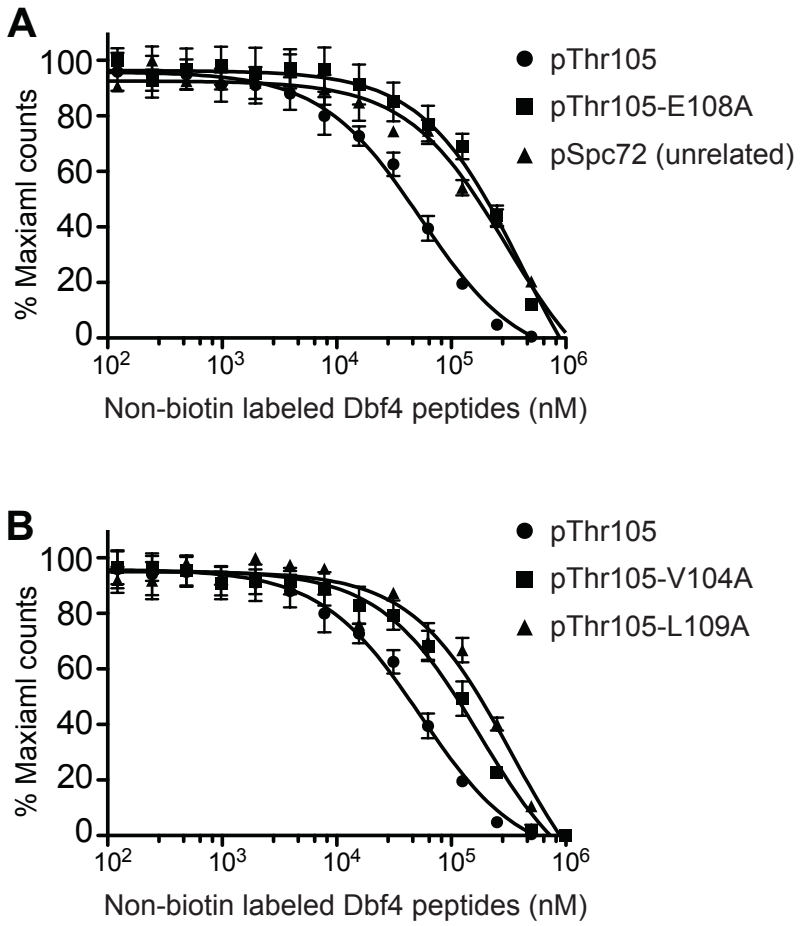
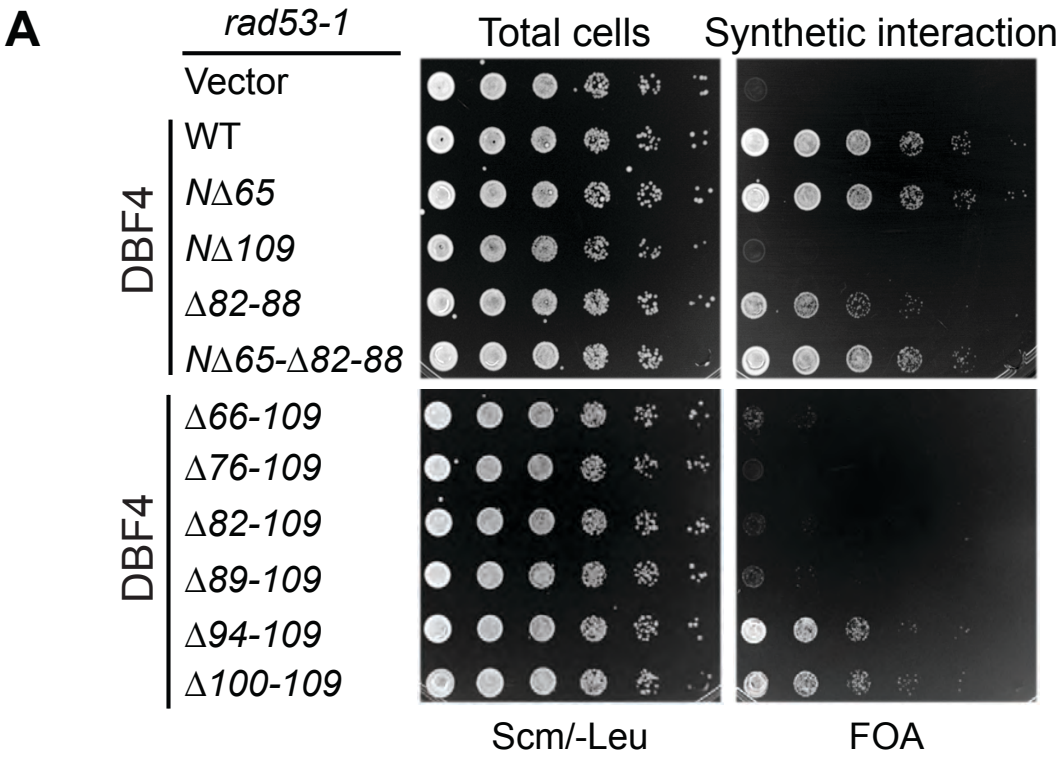


Figure S3 Dbf4 residues V104, E108, and L109 were critical for binding the Rad53 FHA domains. (A) The Dbf4 biotinylated peptide pThr105-FHA1 interaction was competed by the non-biotinylated T105-phosphorylated Dbf4 peptides (pThr105), but not by the same Dbf4 peptide with an E108A substitution, or by an unrelated phospho-serine peptide (pSpc72). (B) The pThr105-V104A and pThr105-L109A peptides were also defective in competing the biotinylated pThr105-FHA1 interaction.


A
rad53-1
Total cells
Synthetic interaction
Vector
DBF4
WT
NΔ65
NΔ109
Δ82-88
NΔ65-Δ82-88
DBF4
Δ66-109
Δ76-109
Δ82-109
Δ89-109
Δ94-109
Δ100-109
Scm/-Leu
FOA

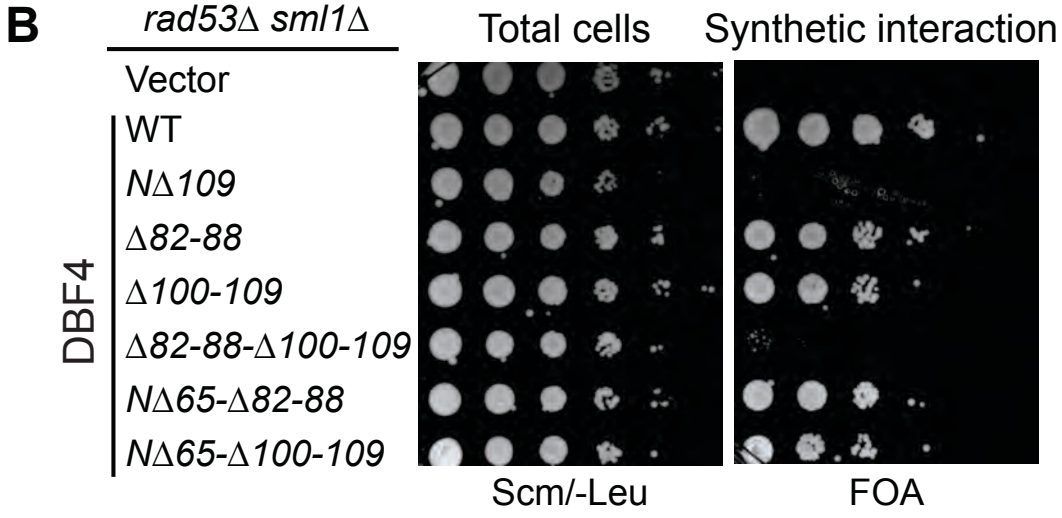

B
rad53Δ sml1Δ
Total cells
Synthetic interaction
Vector
DBF4
WT
NΔ109
Δ82-88
Δ100-109
Δ82-88-Δ100-109
NΔ65-Δ82-88
NΔ65-Δ100-109
Scm/-Leu
FOA

Figure S4 The synthetic lethality between *dbf4-NΔ109* and *rad53-1* or *rad53Δ* was not due 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 prevented the Cdc5 interaction with Dbf4, while the Δ100-109 deletion prevented the interaction with Rad53 (see Figure S6).

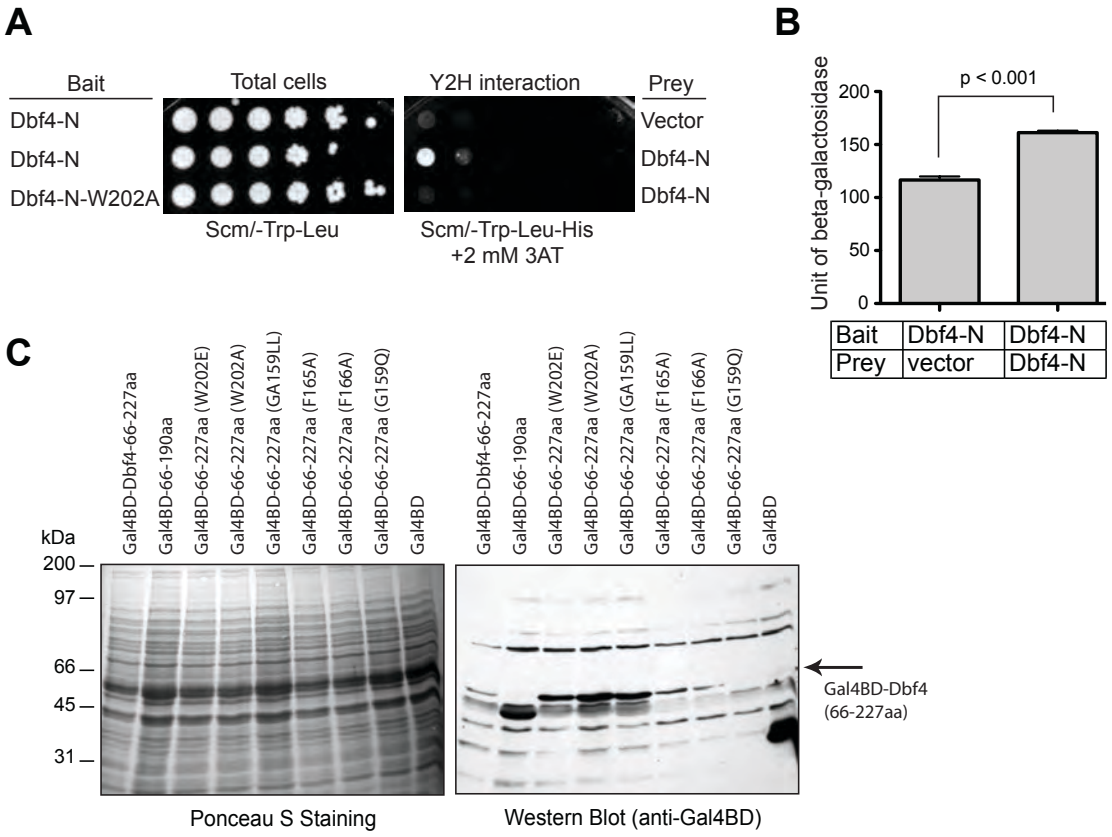


Figure S5 Evidence for a Dbf4-Dbf4 N-terminal interaction. (A-B) Dbf4 N-terminal residues 66-227 were cloned in two-hybrid bait and prey plasmids separately to examine Dbf4 dimerization. Two-hybrid interactions were quantitated by spotting assays on selective media (panel A) or by β-galactosidase assays (panel B). (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).

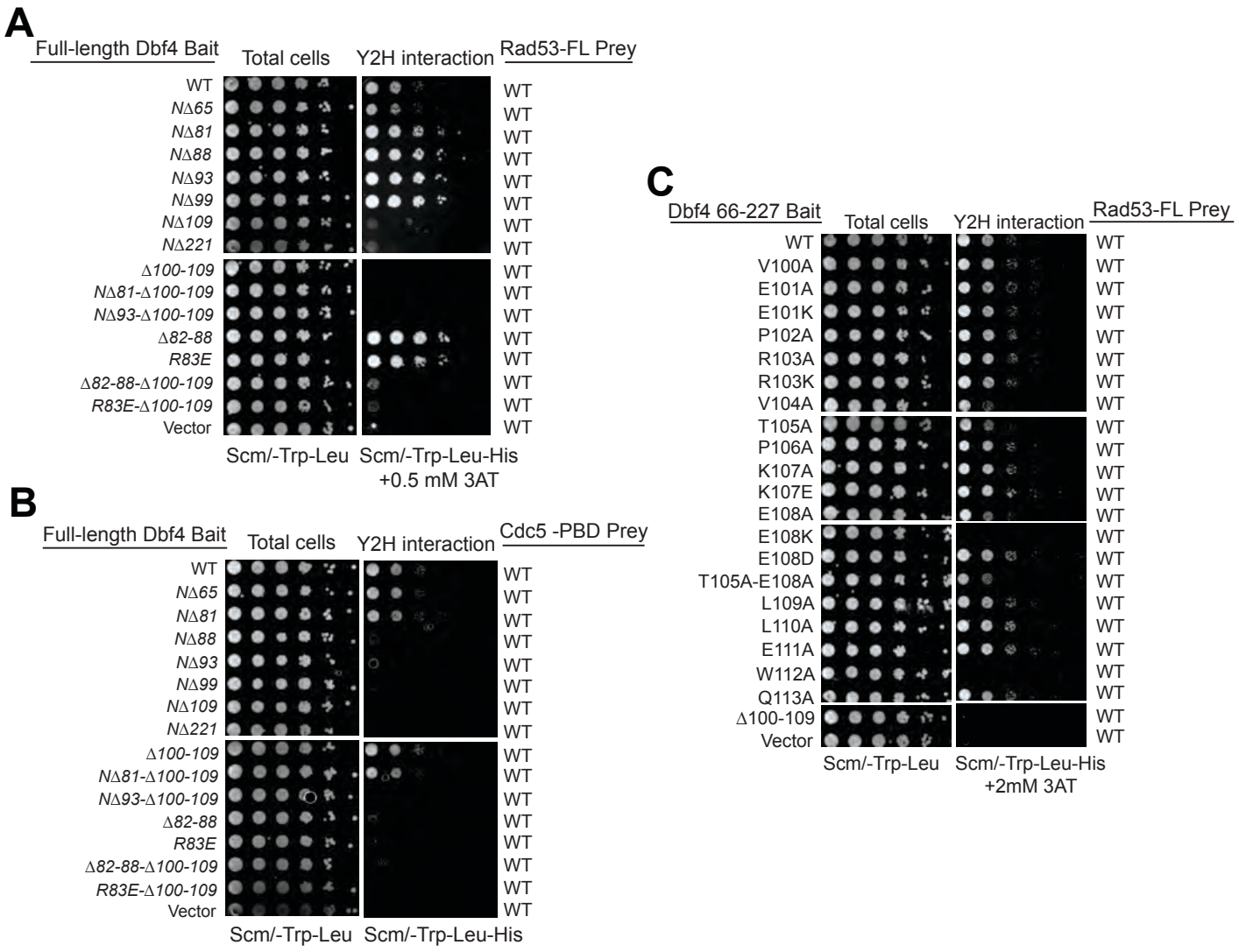


Figure S6 Dbf4 sequences important for binding full length Rad53 and the Cdc5 PBD. (A) A series of deletion in full-length Dbf4 was assayed by two-hybrid for interaction with full length Rad53 (panel A) or with the Cdc5 Polo-box domain (PBD) (panel B). The *dbf4-Δ100-109* deletion caused a loss of Rad53 binding, but still allowed interaction with the Cdc5-PBD. The *dbf4-Δ82-88* deletion caused loss of Cdc5 binding but not Rad53. An N-terminal deletion through residue 81 (NΔ81) or disruption of the Cdc5 binding site (Δ82-88 and R83E) caused increased Rad53 binding compared to full length Dbf4. (C) Dbf4 point mutations were assayed for their two-hybrid interaction against full length Rad53. The Δ100-109 deletion caused a loss of the two-hybrid signal similar to the vector control. The V104A, T105A, E108A mutations resulted in a diminished Rad53 interaction.

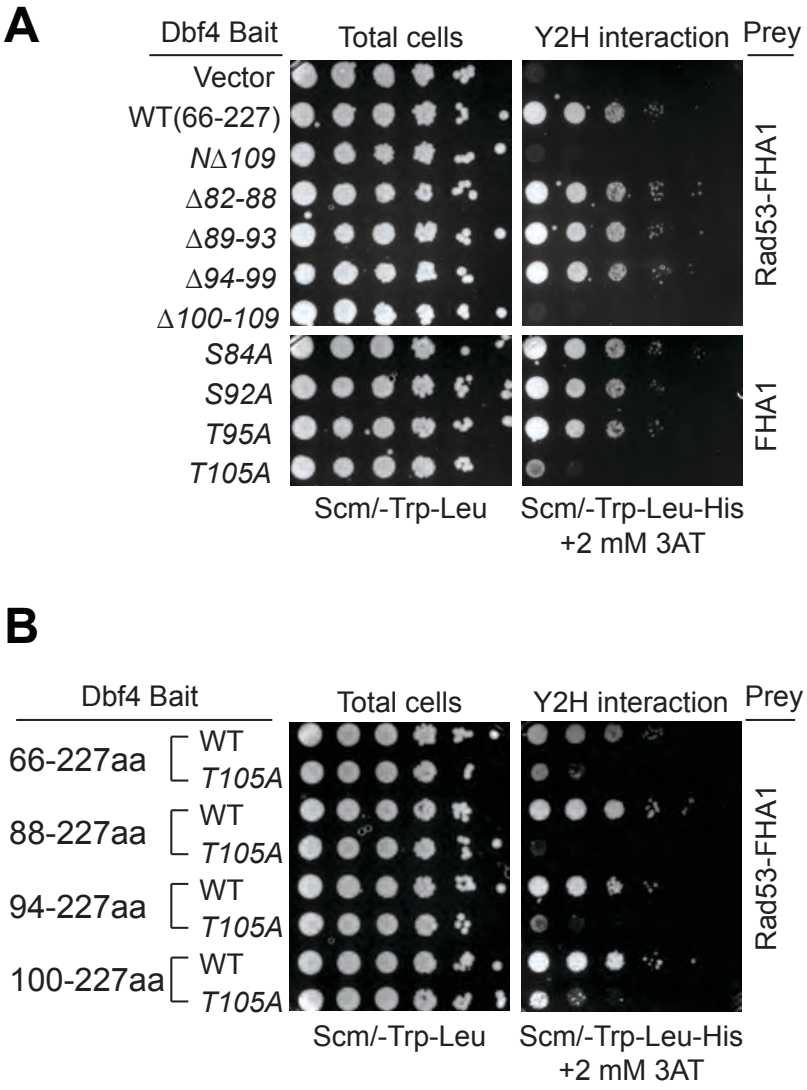


Figure S7 Dbf4 T105 residue was critical for the Dbf4-FHA1 domain interaction. (A) The *dbf4-Δ100-109*, *dbf4-T105A* and *dbf4-NΔ109* mutants caused a loss of FHA1 domain binding in two-hybrid assays. The *dbf4-S84A, -S92A*, and *-T95A* mutants did not show any effect on FHA1 domain binding. (B) Substitution of T105A within various Dbf4 truncations consistently caused a loss of interaction with the FHA1 domain.

Table S1 Plasmids used in this study

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
pGAD-YOR.3	pGAD-C3-*MSA169-530*	This study
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* $_{NΔ81}$	This study
pJK47	pYJ204-*DBF4* $_{NΔ93}$	This study
pJK48	pYJ204-*DBF4* $_{NΔ99}$	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* $_{Δ94-99}$	This study
pJK76	pYJ204-*DBF4* $_{NΔ88}$	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* $_{N\Delta87}$ T105A	This study
pJK181	pCG60-*DBF4* $_{N\Delta99}$ T105A	This study
pJK185	pCG60-*DBF4* $_{N\Delta93}$ T105A	This study
pJK269	pET24a-GST-*RAD53* $_{2-164}$ R70A	This study
pJK275	pGAD-C1-*DUN1* $_{1-160}$	This study
pJK277	pGAD-C1-*FAR10* $_{61-227}$	This study
pJK279	pGAD-C1-*FHL1* $_{253-400}$	This study
pJK281	pGAD-C1-*FKH1* $_{41-185}$	This study
pJK283	pGAD-C1-*MEK1* $_{1-152}$	This study
pJK285	pGAD-C1-*XRS2* $_{1-125}$	This study
pJK287	pGAD-C1-*FKH2* $_{1-254}$	This study
pJK289	pGAD-C1-*PML1* $_{54-204}$	This study
pJK380	pET24a-GST-*RAD53* $_{483-821}$	This study
pJK382	pET24a-GST-*RAD53* $_{549-730}$	This study
pJK410	pYJ380 R605A	This study
pJK420	pET24a-GST-*RAD53* $_{523-821}$	This study
pJK468	pCG60 R209E	This study
pJK469	pCG60 K212E	This study
pJK487	pCG60 K206E	This study
pJK542	pRS415-DBF4$_{N\Delta94}$	This study
pJK544	pCG60-DBF4$_{N\Delta94}$	This study
pMW1	pAcPK30-*DBF4* $_{1-704}$	Gabrielse et al., 2006
pMW47	pAcSG2-HAHIS6-*CDC7* $_{1-507}$	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* $_{N\Delta65}$	Gabrielse et al., 2006
pRS415	*LEU2 ARS-CEN*	Sikorski and Hieter, 1989
pRS416	*URA3 ARS-CEN*	Sikorski and Hieter, 1989
pYJ3	pCG60-*DBF4* $_{\Delta67-81}$	Chen and Weinreich, 2010
pYJ4	pCG60-*DBF4* $_{\Delta67-88}$	Chen and Weinreich, 2010
pYJ5	pCG60-*DBF4* $_{\Delta67-93}$	Chen and Weinreich, 2010
pYJ6	pCG60-*DBF4* $_{\Delta67-99}$	Chen and Weinreich, 2010
pYJ7	pCG60-*DBF4* $_{\Delta67-103}$	Chen and Weinreich, 2010
pYJ8	pCG60-*DBF4* $_{\Delta67-107}$	Chen and Weinreich, 2010
pYJ9	pCG60-*DBF4* $_{N\Delta109}$	Chen and Weinreich, 2010
pYJ16	pCG60 S84A	Chen and Weinreich, 2010
pYJ30	pCG60 R83E	Chen and Weinreich, 2010
pYJ38	pCG60-*DBF4* $_{\Delta82-88}$	Miller et al., 2009
pYJ74	pMW489-*DBF4* $_{\Delta82-88}$	Chen and Weinreich, 2010
pYJ167	pCG60 S92A	Chen and Weinreich, 2010
pYJ182	pAcSG2-*DBF4* $_{\Delta82-88}$	Chen and Weinreich, 2010
pYJ193	pMW489-*DBF4* $_{\Delta76-109}$	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
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 S2 Yeast strains used in this study

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 rad5-535*	Thomas and Rothstein, 1989
y57	W303 *MAT* **a** *rad53-R70A sml1Δ::HIS3 RAD5*	Pike et al., 2004
y59	W303 *MAT* **a** *rad53-K227A sml1Δ::HIS3 RAD5*	Pike et al., 2004
y205	W303 *MAT* **a** *rad53-R605A sml1Δ::HIS3 RAD5*	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*	Gabrielse at al., 2006
M895	W303 *MAT* **a** *dbf4Δ::kanMX6* [pMW490; pRS416-*DBF4 URA3*]	Gabrielse at al., 2006
M927	W303 *MAT* **a** *dbf4Δ::kanMX4 3HA-CDC7-TRP1* [pMW490; pRS416-*DBF4-URA3*]	Gabrielse at al., 2006
M932	W303 *MAT* **a** *dbf4Δ::kanMX4 3HA-CDC7-TRP1* [pMW489; pRS415-*DBF4-LEU2*]	Gabrielse at al., 2006
M936	W303 *MAT* **a** *dbf4Δ::kanMX4 3HA-CDC7-TRP1* [pCG10; pRS415-DBF4-*NΔ109-LEU2*]	Gabrielse at al., 2006
M2864	W303 *MAT* **a** *dbf4Δ::kanMX4 3HA-CDC7-TRP1* [pCG10; pRS415-DBF4-*NΔ94-LEU2*]	This study
M1261	W303 *MAT* **a** *dbf4-NΔ109*	Gabrielse at al., 2006
M1589	W303 *MAT* **a** *rad53-1 dbf4Δ::kanMX6* [pMW490; pRS416-*DBF4 URA3*]	Gabrielse 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 S3 Peptides used in this study

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	IMS EVE LTQ ELP EVE	15	1972.28
Biotin-pRad9	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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