Supplementary information:

Mechanism of Regulation of "Chromosome- Kissing" Induced by Fob1 and its Physiological Significance

Malay Choudhury [1*], Shamsu Zaman[1*], James C. Jiang[2], S Michal Jazwinski[2] and Deepak Bastia[1#]

1, Department of Biochemistry and Molecular Biology

Medical University of South Carolina

Charleston, SC 29425.

2, Tulane Center for Aging and Department of Medicine

Tulane University School of Medicine

New Orleans, LA 70112

*These authors contributed equally to the project

Corresponding author; phone: 843-792-0491; email: bastia@musc.edu

Supplementary methods

4C analysis

Method or protocols were mainly followed from published articles by (Splinter et al. 2004), (Miele and Dekker, 2009) (Singh et al. 2010) with some modifications.

Four strains of *Saccharomyces cerevisiae* were used, i.e., LPY11, LPY11fobΔ, LPY11fobE373V, and LPY11rad52Δ. All these strains were transformed with pBBHyg Ter plasmid (see main text).

Transformed yeast cells were grown in YPD containing Hygromycin (200 µg/ml). Overnight cultures of 50 ml yeast cells were grown at 30^0C. The next day cells were diluted to an OD_{600} = 0.2 in 500 ml. Cultures were incubated at 30^0C till OD reached 0.7. Formaldehyde was added (1% final volume) for 10 min. with constant stirring, quenched with 125 mM glycine for 15 min at room temperature. Cells were harvested by centrifugation at 1500xg for 5 min. Control cells without HCOH treatment were also harvested and processed in parallel. Cells were resuspended in 50 ml of spheroplast buffer (0.4M sorbital, 0.4M KCl, 40mM Potassium Phosphate buffer, pH7.2 and 0.5mM MgCl2) with 30 mM DTT and were centrifuged at 1500xg for 10 min. Pellet was resuspended in 50 ml of spheroplast buffer with 1 mM DTT. Fixed cells were converted to spheroplast with 250 µl of 10 mg/ml Zymolyase for 1 hr at 30^0C with very gentle rotation. In between cells were checked under the microscope for spheroplast formation.

Spheroplasts were collected by centrifugation at 4^0C for 5 min at 1500xg. Pellet was washed twice with 50 ml of spheroplast buffer. Pellet was resuspended and washed in 50 ml restriction enzyme (RE) buffer. Final pellet was resuspended in 8 ml RE buffer (50mM Tris.HCl pH 7.9, 100 mM MgCl2, 1mM DTT and 100 µg/ml BSA) + 80 µl of 10% SDS , 440 µl of 20% Triton X-100 were added and divided into 17 tubes. Tubes were incubated at 65^0C for 20 min followed by 37^0C for 1 hr with shaking.

Lysates were digested with Eco RI and Afl III enzymes (30 units/ chromatin samples) overnight at 37^0C with constant mixing. Next day morning more (10 Units each tube) enzymes were added for continuing digestion. DNA was sheared by sonication using a number of short pulses (10 sec) with pauses (30 sec) while controlling the temperature by keeping the suspension over ice. DNA was fragmentated to <1000 bp. This step was checked by gel electrophoresis in control DNA/pilot study.

Enzymes were inactivated by adding 90µl of 10% SDS, 1% Triton X-100 and incubated at 80^0C for 10 min, 65^0C for 20 min followed by 1 hr at 37^0C. Digested and sheared DNA was pooled and diluted into ligation buffer (10x, 500 mM Tris.HCl, pH 7.5, 100 mM MgCl2, 100 mM DTT, 10xBSA (10 mg/ml) and 100 mM ATP), incubated for 1 hr at 37^0C. T4 ligase (200 units/Tube) was added and ligation was carried out at 4^0C for 4 days with continuous slow rotation. Every 12 hrs fresh ATP was added to the ligation mix at 2 mM concentration. After completion of ligation for reversal of cross linking, proteinase K (0.5%, 20mg/ml) was added and the sample was incubated overnight at 65^0C. Next day, additional

proteinase K (0.5%, 20mg/ml) was added and incubation was continued for 2 hrs at 55^0C. The suspension was cooled down to room temperature, 3M Na-acetate, pH 5.2 and isopropanol were added and it was kept at -80^0C for 2 hrs to precipitate DNA. Samples were centrifuged at 5000xg. DNA pellet was dissolved in 500 µl of TE and after addition of 10 µl RNAse A (10 mg/ml) sample was incubated at 37^0C overnight. DNA was extracted 3-times with phenol/chloroform. DNA was precipitated with isopropanol and washed 3-times with 70% ethanol. The DNA pellet was dissolved in 50 µl of TE. To detect ligated product, PCR was conducted with two pairs of primer, i.e. F1- F3 and F2- F4. PCR conditions were 95^0C -5 min, (95^0C /10sec, 54^0C /10sec, 72^0C /10sec) 20 cycles.

Construction of strains for RLS determination

The BamHI/SalI fragments from plasmid pGAD424-F373, pGAD424-F373ΔC, pGAD424-FT504A-S519A and pGAD424-FT504D-S519D were subcloned into the BamHI/SalI site of the yeast intergrative plasmid pRS406, which contains a URA3 selectable marker (Sikorski and Hieter, 1989). Newly-constructed plasmids containing the relevant point mutations in Fob1 were linearized with the restriction enzyme AflIII, which cuts only once in the middle of the FOB1 coding region. This linearized plasmid was used to transform yeast strain YPK9 (Kirchman et al. 1999). Transformants were selected on uracil- plates. Then, the transformants were grown in YPD liquid medium overnight to allow the pRS406-URA3 sequence to pop out. 100 µl of culture were streaked on 5'FOA plates to select clones lacking the URA3

gene, and mutants were verified by DNA sequencing (Scherer and Davis, 1979). This included the FOB1 coding region plus 220 bp upstream and downstream. For mutants containing ΔC, a knock-out/knock-in method was employed. The *FOB1* gene was knocked out with replacement by the *URA3* gene. The resulting strain was transformed with a PCR fragment consisting of 50 bp of FOB1 5'-flanking region—the fob1 mutant---50 bp of FOB1 3'-flanking region. Knock-in mutants were selected on 5'FOA plates and their sequence was verified, as described above. The strains containing the T504A-S519A and T504D-S519D substitutions in Fob1 were generated using the same knock-out/knock-in strategy as above. Strains were verified by DNA sequencing, as above.

Supplementary references

Kirchman PA, Kim S, Lai CY, Jazwinski SM. 1999. Interorganelle signaling is a determinant of longevity in Saccharomyces cerevisiae. *Genetics* **152:** 179-190.

Miele A, Dekker J. 2009. Mapping cis- and trans- chromatin interaction networks using chromosome conformation capture (3C*). Methods Mol Biol* **464:** 105-121.

Scherer S, Davis RW. 1979. Replacement of chromosome segments with altered DNA sequences constructed in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **76:** 4951-4955.

Sikorski RS, Hieter P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122:** 19-27.

Singh S, Sabatinos S, Forsburg SL, Bastia D. 2010. Regulation of replication termination by Reb1 protein-mediated action at a distance. *Cell* **142:** 868-878.

Splinter E, Grosveld F, De Laat W. 2004. 3C technology: analyzing the spatial organization of genomic loci in vivo. *Methods in Enzymology* **375:** 493-507.

Table 1. Yeast Strains and plasmids

***S. cerevisiae* strains**	**Genotype**	**Source**
W303	MATa (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*)	R. Rothstein
LPY11	W303a *sir2*Δ:*HIS3*	L. Pillus
Lfob1	LPY11 *fob1*Δ:G418	Mohanty et al.,(2009)
Lrad52	LPY11 *rad52*Δ:G418	Mohanty et al.,(2009)
MC11	Lfob1*fob1E373V*: Phleomycin	This study
MC12	Lfob1*fob1E373V*Δ*C*: Phleomycin	This study
MC13	Lfob1*fob1S504A,S519A*: Phleomycin	This study
MC14	Lfob1*fob1S504D,S519D*: Phleomycin	This study
PJ69-4A	MATa *trp1-Δ901 leu2-3,112 901 ura3-52 his3-*Δ*200 gal4*Δ *gal8*Δ *GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*	(James et al., 1996)
YPK9	*MATa ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura 52*	(Kirchman et al., 1999)
Plasmid		
pGAD424		Clontech
pGBT9		Clontech
pGAD424F0b1		This study
pGAD424fob1M213L		This study
pGAD424fob1E373V		This study
pGAD424fob1S504A,519A		This study
pGAD424fob1S504D,519D		This study
pGBT9fOB1E373V		This study
pGAD424fob1T322I		This study
pGBT9Fob1		This study
pGBT9fob1S504A,S519A		This study
pGBT9fob1S504D,S519D		This study
pGAD424NFob1(1-1287)		This study
pGBT9CFob1(1288-1701)		This study

pGBT9Cfob1T452S,P530A	This study
pGBT9Cfob1Q550E,G561S	This study
pGBT9Cfob1K454T	This study
pMALCFob1	This study
pMALCF0b1 K454T	This study

Table 2. Oligonucleotides

A. Primers for Site Directed Mutagenesis of fob1

M213L-F 5'GGAATATAAACGTCCTGACTTGTACGATAAACTAC3'

M213L-R 5'GTAGTTTATCGTACAAGTCAGGACGTTTATATTCC3'

E373V-F 5'TGCAAGTACTACTTAGTGTAGTTCCAGGTCACAATG3'

E373V-R 5'GCATTGTGACCTGGAACTACACTAAGTAGTACTTGC3'

K454T-F 5**'**ATCTAACTGAAACAAACTATATCGATGAGTATG3'

K454T-R 5'ATACTCATCGATATAGTTTGTTTCAGTTAGATCAAATTG3'

T504A-F 5**'**CCAATGCAGGGAGCAGAAGAGCCTGAAAAAG3'

T504A-R 5**'**TTTTTGAGGCTCTTCTGCTCCCTGCATTGGGGC3'

S519A-F 5'AAAGTGACGGTGCAGCACAAGTAGATCAAAG3'

S519A-R 5CTTTGATCTACTTGTGCTGCACCGTCAC3'

T504D-F 5'AGCCCCAATGCAGGGAGATGAAGAGCCTGAAAAAG3'

T504D-R 5'CCTTTTTCAGGCTCTTCATCTCCCTGCATTGGGGCTG3'

S519D-F 5'GAAAGTGACGGTGCAGACCAAGTAGATCAAAGTG3'

S519D-R 5'CACTTTGATCTACTTGGTCTGCACCGTCACTTTC3'

B. Primers for fob1 cloning in 2 hybrid vectors

FOBAM-F 5**'**GCGGCGGATCCGGATGACGAAACCGCGTTACAATGACG3'

FOBSAL-R 5**'**GCGGTCGACCAATTCCATTGATGTGCCAAAGTCTCTTG3'

NFOBSAL 5'GCGGTCGACTCCATTATTGTTACAGCTATTATCCGC3'

CFOBBAM 5'GCGGGATCCGGATGATTCTTCATAATAACAATATAGG3'

C. Primers for fob 1 cloning in expression vector

FOBBAM-F 5'CACACGGATCCACATATGACGAAACCGCGTTACAATGACGTGTTG3'

FOBBAM-R 5'CACACGGATCCTACAATTCATTGATGTGCCAAAGTCTCTTG3'

FOBMBP-F 5'GGCGGATCCATGACGAAACCGCGTTACAATG3'

CFOBMBP-F 5'CGGGATCCATGATTCTTCATAATAACAATATAG3'

D. Primers for rDNA probe preparation and used in 2D gel probe

RDN1350 5’CTGAACATGTCTGGACCCTGCCCTC3’

RDN2800 5’AGGCGTCCTTGTGGCGTCGCTGAAC3’

E. Primers for fob1 Knock In/Knock out

FOB1F44+22

5’CAATTTAACGATTGTGTGAGTGTGAATTTGTGCTGAGGATAACAATGAC GAAACCGCGTTACAATGACG3’

FOBCRELOX-F 5’GAGATCAAACAAGAGACTTTGGCACATCAATGGAATTGTAACAGCTGA AGCTTCGTACGCTGCAG3’

FOB1CRELOX-R 5’GCGTACGAAGCTTCAGCTGTTACAATTCCATTGATGTGTGCCAAAGTCT CTTGTTTGATCTC3’

NFOBCRELOX-F 5’GAAGCGGATAATAGCTGTAACAATAATGGATAACAGCTGAAGCTTCGT ACGCTGCAG3’

NFOB-CRELOX-R

5’GCGTACGAAGCTTCAGCTGTTATCCATTATTGTTACAGCTATTATCCGCT TCATTAGC3’

FOB+41TO1CRELOX-R

5’CACCTATGGTGACTCCTCCTTTCATTCTATCCTACATATTAGCATAGGCC ACTAGTGGATCTGATAT3’

F. Primers for 4C

F1 5’GCACTGGCTATTCATCTTGCACTTTTCCTC3’

F2 5’GGAAAAGTGCAAGATGAATAGCCAGTG3’

F3 5’CGATGAGGATGATAGTGTGTAAAGAGTG3’

F4 5’GGTACACTCTTACACACTATCATCCTCATCG3’

Table 3. Statistical analyses of RLS

Experiment	strain	mRLS	maxRLS	p-values		
I	wt	19.8	36	*	*	
	fob1Δ	29.8	52	0.0001	*	
	fob1^{E373V}	27.2	50	0.0001	0.29	
II	wt	20.7	30	*	*	
	fob1^{E373V}	27.7	39	0.001	*	
	fob1$^{E373V\Delta C}$	28.7	45	0.001	0.17	
III	wt	18.7	29	*	*	*
	fob1Δ	29.9	44	0.0001	*	*
	fob1$^{T504A, S519A}$	22.6	35	0.008	0.001	*
	fob1$^{T504D, S519D}$	18.6	31	0.66	0.0001	0.002

Supplementary Figure legends

Fig.S1. Biochemical activities of N-Fob1 in comparison with full length Fob1. A, 2D gels showing that N-Fob1 and Fob1 arrest replication forks to comparable extents where as ∆fob1, as expected fails to arrest forks at Ter sites; **B**, silencing of the mUra3 reporter occurs to similar extents in cells expressing Fob1 and NFob1. The results show that amino acid sequence of C-Fob1 is dispensable for the aforementioned biological activities of N-Fob1.

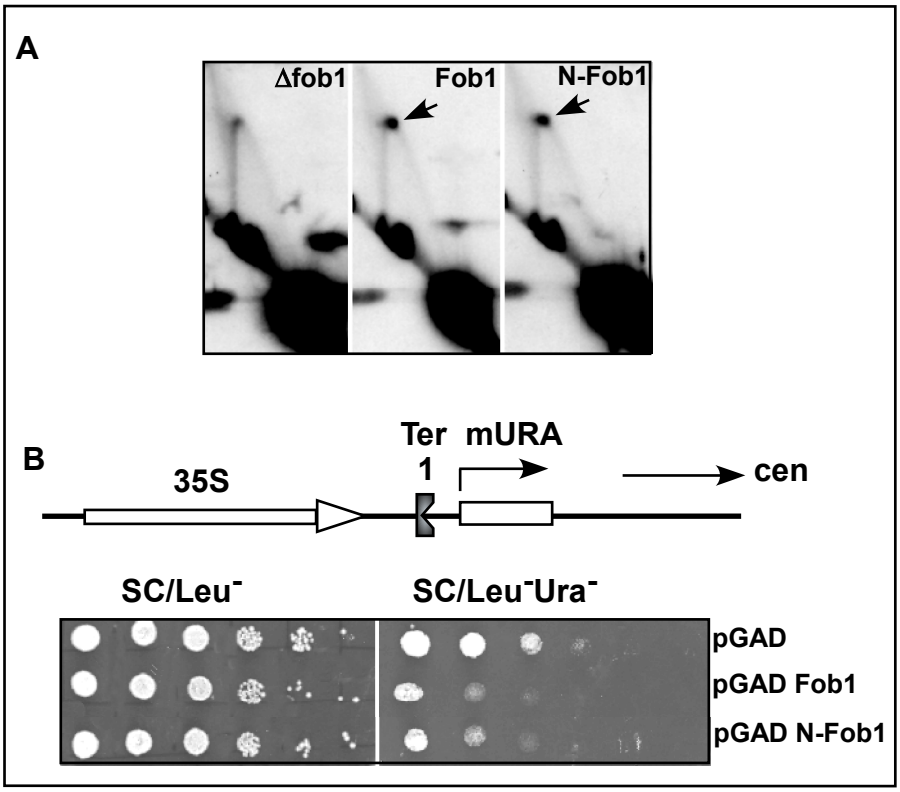
Fig.S2. C-Fob1 inhibits the protein-protein interactions between Fob1 and RPA2. A, Y2H analysis showing that whereas RPA2 interacts very poorly with full length Fob1, it interacts strongly with NFob1;**B,I,** phosphorimager-generated data showing the interactions of 0.1-0.3 pmoles of labeled WT C-Fob1 and m3-C-fob1 with 1 pmole each of matrix-bound N-Fob1, **ii,** quantification of binding data; **C**, the activities of the lacZ reporter present in the Y2H indicator cells confirm the results shown in A; **D**, Y2H analysis showing that the double A substituted fob1 protein binds poorly to RPA2 and the binding is partially restores by the double D substitutions.

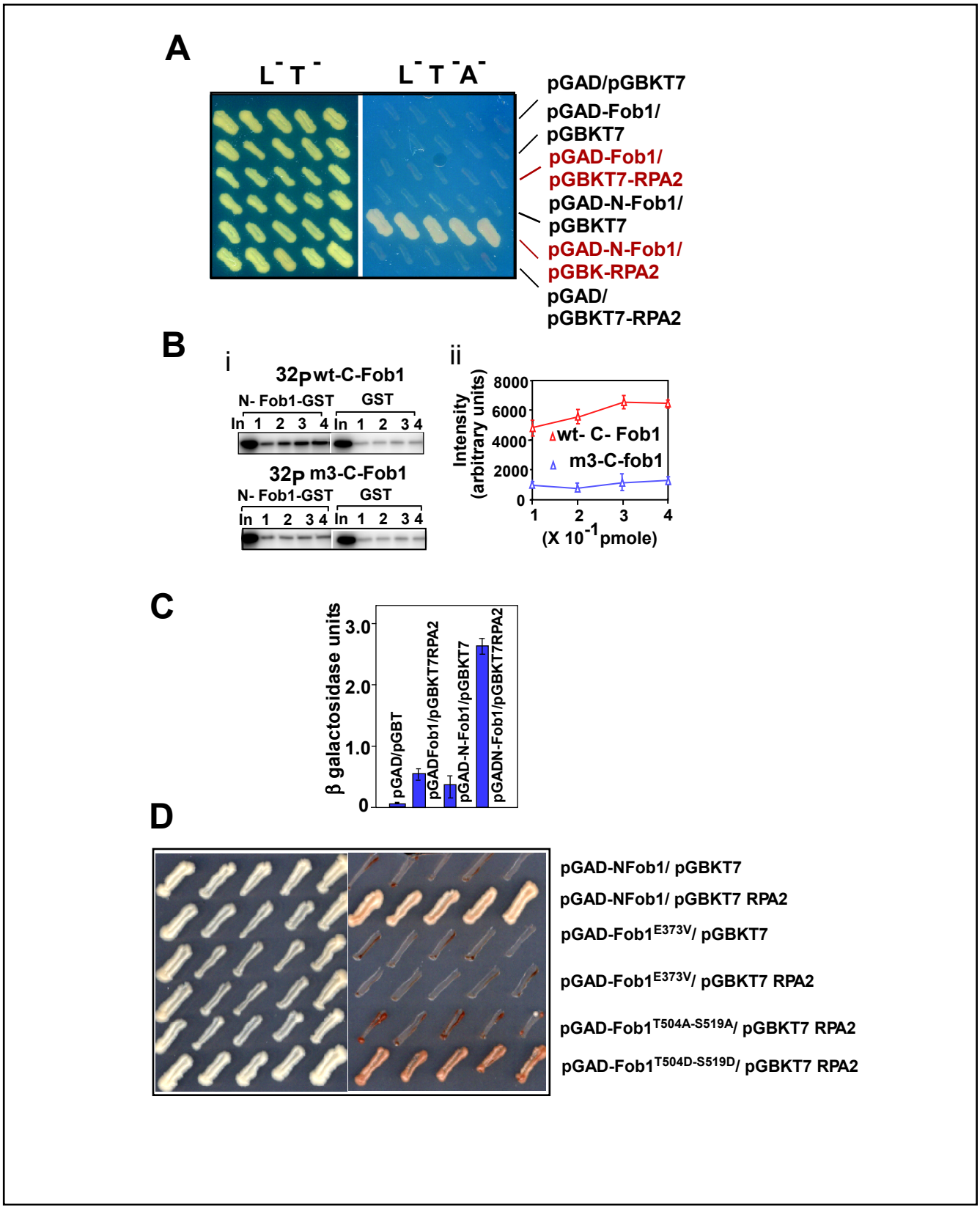
Fig.S3. C-Fob1K454T that interacts very poorly in *trans* with N-Fob1 (Fig.2), has the interaction ameliorated in *cis*. A, Y2H analysis showing that fob1K454T interacts somewhat more than wtFob1 with than the latter with itself, fob1K454T similarly interacts slightly better with wt N-Fob1in comparison with the interaction of N-Fob1 with itself; **B,** as confirmed by the activity of the b galactosidase reporter of the yeast 2 hybrid indicator strain.

Fig.S4. **Various measurements showing that a deletion of the inhibitory C-terminus overcomes the loss of Fob1 tetramerization in the E373V fob1 mutant, significantly overcomes integrative recombination between a plasmid-borne Ter and chromosomal Ter (Fig.4C) but fails to reduce the magnitude of life span extension by the E373V mutation; A,** Y2H data showing that whereas fob1 E373V fails to interact with itself (row IV) deletion of its C-terminal domain at least partly restores this interaction; **B**, β-galactosidase

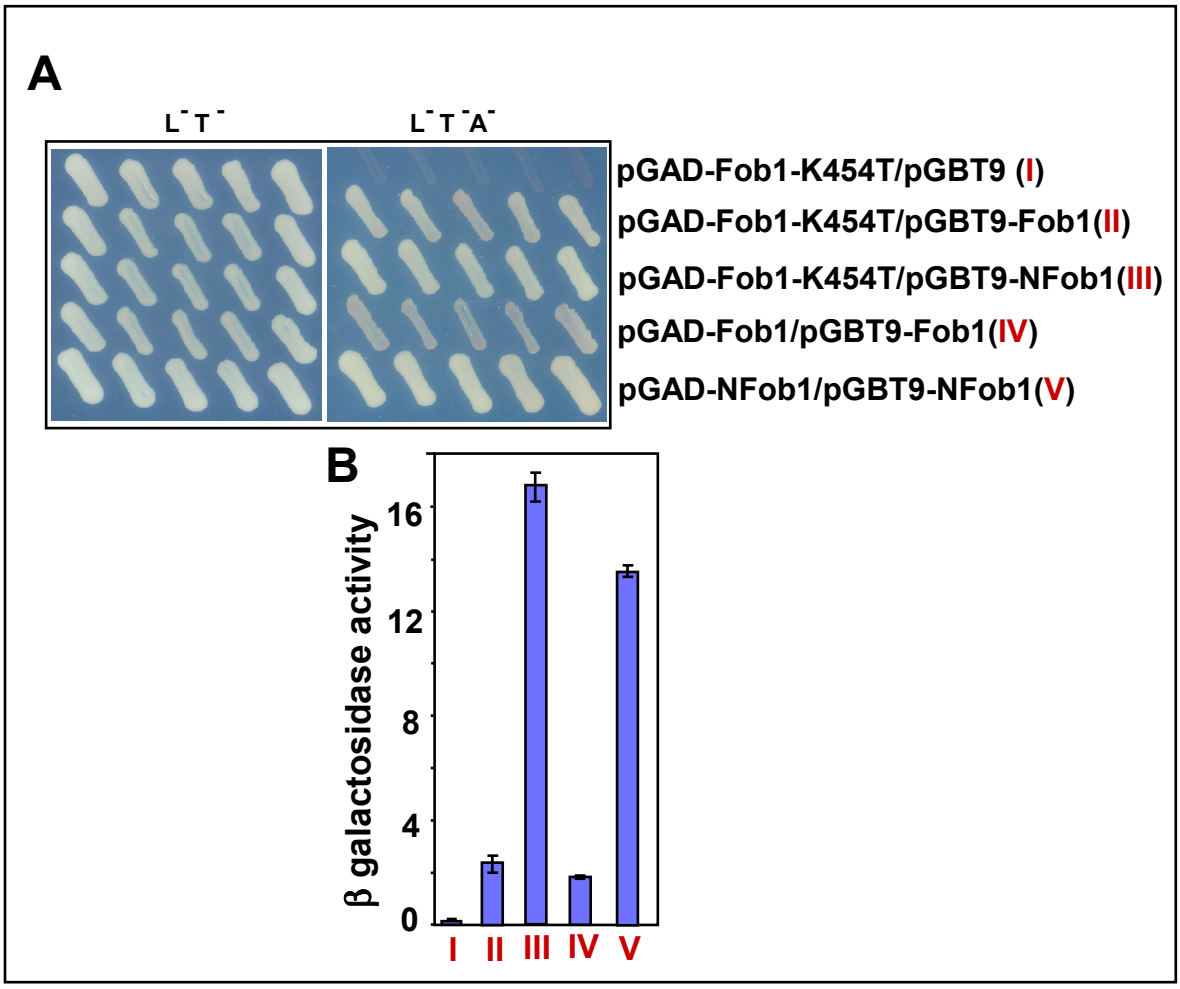
assays confirming the data shown in A; C, integrative recombination assay showing that deletion of the C-terminus, partially restores that ability of fob1E373VΔC to integrate in the plasmid integration assay (Fig.4); **D**, RLS analyses showing that 4 separate isolates of fob1E373VΔC have the same life span as fob1 E373V.

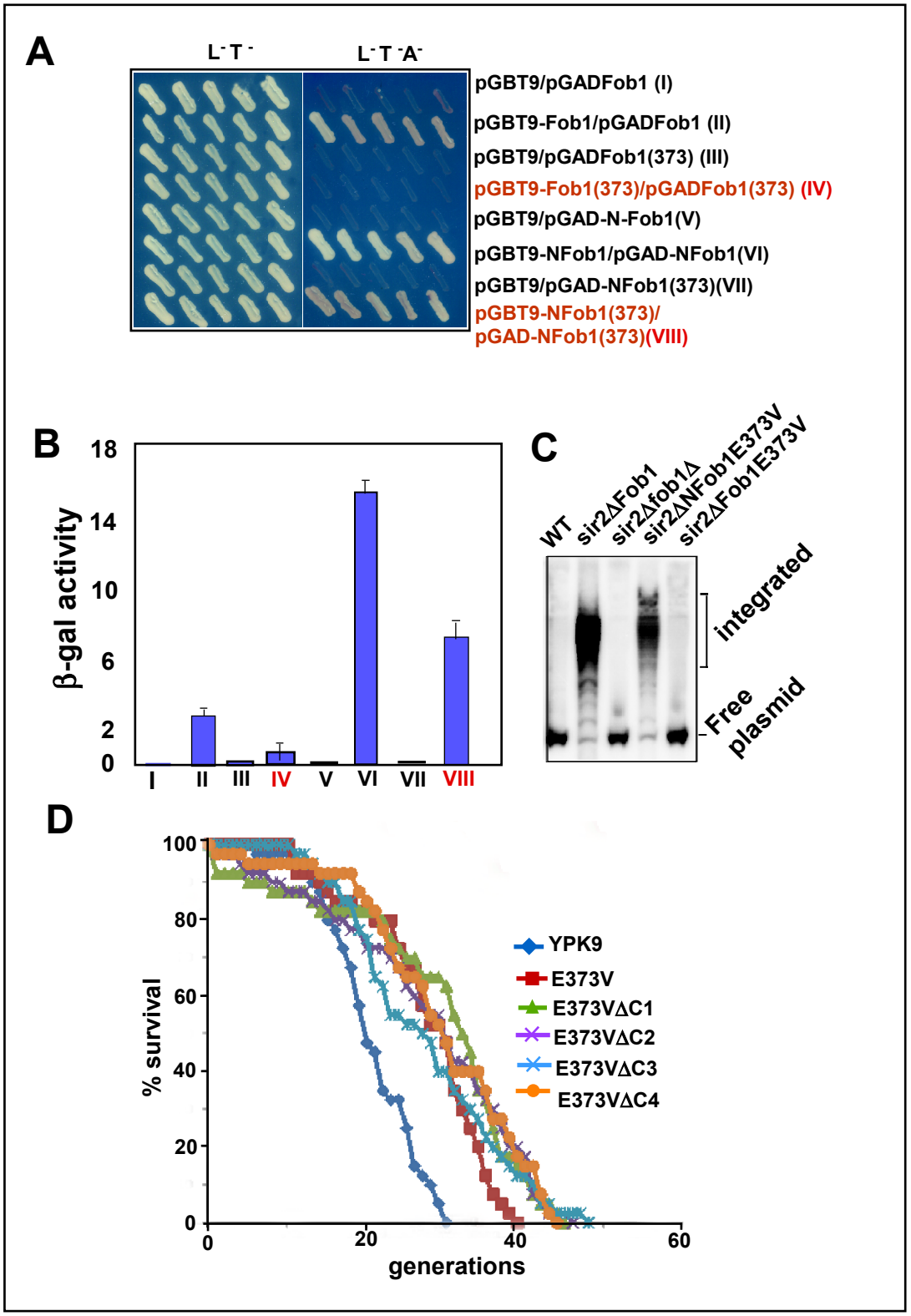
Choudhuri et al. Fig.S1




A
L- T-
L- T- A-
pGAD/pGBKT7
pGAD-Fob1/ pGBKT7
pGAD-Fob1/ pGBKT7-RPA2
pGAD-N-Fob1/ pGBKT7
pGAD-N-Fob1/ pGBK-RPA2
pGAD/ pGBKT7-RPA2
B
i
32P wt-C-Fob1
N- Fob1-GST
GST
In 1 2 3 4 In 1 2 3 4
32P m3-C-Fob1
N- Fob1-GST
GST
In 1 2 3 4 In 1 2 3 4
ii
Intensity (arbitrary units)
8000
6000
4000
2000
0
wt- C- Fob1
m3-C-fob1
1 2 3 4
(X 10-1 pmole)
C
β galactosidase units
3.0
2.0
1.0
0
pGAD/pGBT
pGADFob1/pGBKT7RPA2
pGAD-N-Fob1/pGBKT7
pGADN-Fob1/pGBKT7RPA2
D
pGAD-NFob1/ pGBKT7
pGAD-NFob1/ pGBKT7 RPA2
pGAD-Fob1E373V/ pGBKT7
pGAD-Fob1E373V/ pGBKT7 RPA2
pGAD-Fob1T504A-S519A/ pGBKT7 RPA2
pGAD-Fob1T504D-S519D/ pGBKT7 RPA2

Choudhuri et al. Fig.S3




A
L- T -
L- T -A-
pGBT9/pGADFob1 (I)
pGBT9-Fob1/pGADFob1 (II)
pGBT9/pGADFob1(373) (III)
pGBT9-Fob1(373)/pGADFob1(373) (IV)
pGBT9/pGAD-N-Fob1(V)
pGBT9-NFob1/pGAD-NFob1(VI)
pGBT9/pGAD-NFob1(373)(VII)
pGBT9-NFob1(373)/
pGAD-NFob1(373)(VIII)
B
β-gal activity
18
14
10
6
2
0
I
II
III
IV
V
VI
VII
VIII
C
WT
sir2ΔFob1
sir2Δfob1Δ
sir2ΔNFob1E373V
sir2ΔFob1E373V
integrated
Free
plasmid
D
% survival
100
80
60
40
20
0
0
20
40
60
generations
YPK9
E373V
E373VΔC1
E373VΔC2
E373VΔC3
E373VΔC4