Inhibition of homologous recombination by a cohesin-associated clamp complex recruited to the rDNA recombination enhancer

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Supplemental Discussion

Associations with Top1 and nuclear envelope proteins

Our purifications provide links between rDNA silencing factors and several other nuclear proteins that may regulate rDNA structure or subnuclear localization. The association of Topoisomerase I (TopI) with Fob1 provides support for a direct role for Top1 in silencing, as first suggested by genetic experiments (Christman et al. 1988; Bryk et al. 1997; Smith et al. 1999), and its Fob1-dependent recruitment to the NTS1 region (Vogelauer et al. 1998; Di Felice et al. 2005). Furthermore, purifications of both Sir2 and the Lrs4/Csm1 complex also yield karyopherins, suggesting a possible mechanism for subnuclear localization of silent chromatin. Finally, rDNA-specific silencing factors Tof2 and Lrs4/Csm1 interact with two putative inner nuclear envelope proteins, Src1 and Ydl089w (Huh et al. 2003), raising the possibility that the NTS1 region of rDNA may also play a role in tethering rDNA to and positioning the nucleolus along the nuclear periphery. Notably, Src1 and Ydl089w contain putative transmembrane domains as well as LEM domains, which, are frequently found in metazoan inner nuclear envelope proteins and interact with BAF, a highly conserved metazoan protein which has roles in chromatin and nuclear organization (Bengtsson and Wilson 2004; Segura-Totten and Wilson 2004). A possible role for nuclear envelope proteins in the regulation of rDNA remains to be determined.

Supplemental Materials and Methods

Yeast strains and plasmids

The endogenous copies of the *NET1*, *FOB1, TOF2, LRS4, and CSM1* genes were deleted or modified with the C-terminal TAP, MYC13, HA3, or GFP epitope tags as described (Longtine et al. 1998; Rigaut et al. 1999; Huang and Moazed 2003). The *TOF2* gene was modified with the C-terminal HA3 tag by integration of plasmid pDM240.

The m*URA3* gene contains the *TRP1* promoter followed by the *URA3* open reading frame (Smith and Boeke 1997). Plasmids for integrating the NTS1 and NTS2 *mURA3* reporters and reporter yeast strains have been previously described (Huang and Moazed 2003). All transformations were performed with the lithium acetate method (Guthrie and Fink 1991), and proper integration was confirmed by PCR. Telomeric silencing strains were a gift from A. Rudner.

pDM240 was constructed by using primers DM267 (CGG GGT ACC TTG CCA ATG CTG GGA AAC) and DM268 (GAT GCG GCC GCC CTG GTC GTC TTC ATC ACT) to amplify a 0.5 kb *Asp*718-*Eag*I fragment of *TOF2* from genomic DNA. This fragment was ligated to the Yplac111d vector to generate pDM240, which was cut with *Msc*I to integrate at *TOF2*. pDM749 (*pCEN-TOF2-HIS3*) was constructed by ligation of a ~2.8 kb *Xho*I-*Eag*I PCR product containing the *TOF2* gene into pRS313 (*pCEN-HIS3*). The *TOF2* gene was amplified from genomic DNA using primers JH362 (TAC ctc gag TTT CCG GGA AAA CAT GTC) and JH363 (ATT cgg ccg ATA TGG TTG AGA GAT CCC).

Purification and identification of native complexes

Cells were grown at 30°C to late log phase (optical density at 600 nm of ~4.0) in YEP media containing 4% glucose. Cells were harvested, washed once with water, and frozen in liquid nitrogen. Approximately 8-15 g of frozen cells was combined with an equal volume of 2X ice cold buffer L (12 mM Na_2HPO_4, 8 mM $NaH_2PO_4 \cdot H_2O$, 0.2% NP-40, 300 mM NaCl, 4 mM EDTA, 2 mM EGTA, 100 mM NaF, 0.2 mM Na_3VO_4, 40 mM β-mercaptoethanol, 2 mM PMSF, 4 mM benzamidine, and 2 mM each of leupeptin, bestatin, and pepstatin). All subsequent steps were performed at 4°C unless stated otherwise.

An equal volume of cold glass beads was added to the cells, and the mixture was bead-beat for ten pulses of 10 sec each in a small chamber bead-beater (BioSpec Products Inc.). The extract was centrifuged at 30,000 g for 25 min, and the supernatant was incubated with 300 µl of a 50% slurry of pre-washed IgG-sepharose beads (GE) for 2 to 3 hrs. Beads were transferred to a Poly-Prep chromatography column (BioRad) and washed three times with 10 ml each of buffer W (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40, and 1mM DTT), followed with one wash with 10 ml of TEV-C buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 5% glycerol, and 1mM DTT). Beads were washed with 200 µl of TEV-C buffer containing 5 µg/ml HIS6-TEV protease purified from E. coli, followed by an overnight incubation with 1 ml of TEV-C buffer containing 5 µg/ml TEV protease.

After cleavage, eluate was transferred to a new PolyPrep column and combined with two 1 ml washes of the IgG-sepharose beads with TEV-C buffer. To the TEV cleavage eluate and washes, 6 ml of binding buffer CAM-B (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% NP-40, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 5% glycerol, and 10 mM β-mercaptoethanol), 9 µl of 1M $CaCl_2$, and 250 µl of a 50% slurry of pre-washed calmodulin-sepharose beads (GE) was added and incubated on a nutator for 2 to 3 hr. The beads were

washed three times each with 1.5 ml of CAM-B buffer and eluted as five 250 µl fractions with elution buffer CAM-E (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.02% NP-40, 1 mM magnesium acetate, 1 mM imidazole, 10 mM EGTA, 5% glycerol, and 10 mM β-mercaptoethanol). Ten percent of the peak fraction was run on a 10-20% SDS-PAGE gradient gel and silver stained. Half of the peak fraction was precipitated in 20% TCA on ice for 20 min and centrifuged at maximum speed at 4°C for 20 min. The pellet was washed with cold (-20°C) acetone, centrifuged at 4°C for 30 min, and air-dried. Mixture mass spectrometry analysis was then performed as described.

Silencing assays

rDNA silencing assays were performed as described (Huang and Moazed 2003). Cells lacking *LRS4*, *CSM1*, or *TOP1* were plated in parallel with wild-type cells but were photographed later due to slower growth compared to wild-type cells. We observed that *tof2Δ* cells consistently formed smaller colonies on –URA medium compared with wild-type or *sir2Δ* cells. Telomeric silencing was assayed by plating cells onto synthetic complete (SC) or SC supplemented with 0.8g/L 5-FOA.

Immunofluorescence microscopy

Immunofluorescence assays were performed essentially as described (Guthrie and Fink 1991). Images were collected and processed using a Nikon Eclipse 80i upright microscope and MetaMorph (Version 6.0) software at the Nikon Imaging Center at Harvard Medical School. Five milliliter cultures were grown in liquid YEPD at 30°C to an optical density at 660 nm (OD_{660}) of 0.5, fixed by adding 0.7 ml of 37% formaldehyde for 1 hr. Cells were washed twice

with water and resuspended in 1 ml SP buffer (1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.0). Cells were spheroplasted for 15-30 min in 1 µl β-mercaptoethanol and 20 µl of lyticase (1mg/ml in 1M sorbitol) per 0.5 ml of cells. Cells were washed with 1 ml of SP and resuspended in 0.5-1 ml of SP. Fifteen microliters of cell suspension was adhered to each well of pre-coated 10-well slides (Polysciences, Inc.) for 5 min. Wells were aspirated, washed three times with PBS, and dried for 10 min at room temperature. Slides were pre-coated with polylysine by rinsing with water and drying, followed by incubation with 15 µl of polylysine (1 mg/ml) per well for 10 min at room temperature. After aspiration of excess polylysine, slides were dried, rinsed with water, and incubated at 37°C for 15 min.

Wells were blocked and permeabilized with 15 µl of blocking buffer (PBS with 1% BSA and 0.1% Triton X-100) for 1 hr, followed by four washes with PBS. Primary antibodies in antibody binding buffer (1% BSA in PBS) were spun for 15 min at 13K and incubated with cells for 1 hr at room temperature or overnight at 4°C (15 µl per well), followed by three washes with PBS. Mouse anti-HA (HA11, BabCO) and rabbit anti-GFP (gift from A. Rudner) were used at 1:1000 and 1:5000 dilutions, respectively. Cells were incubated with secondary antibodies for 1-2 hrs in the dark. FITC-conjugated goat anti-rabbit or Texas Red Rhodamine-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch Labs) were used at 1:500 dilutions in antibody binding buffer. Wells were washed three times with PBS and twice with water, followed by incubation with 15 µl DAPI (1 ng/ml) for 5 min at room temperature. Wells were washed once with water and covered by a coverslip after addition of mounting media and sealed with clear nail polish.

ChIP assays

ChIP assays were performed essentially as previously described (Huang and Moazed 2003). Relative fold enrichment was determined by calculating the ratio of rDNA to *CUP1* enrichment in the immunoprecipitated material (IP) and comparing this to the ratio of rDNA to *CUP1* enrichment in the whole-cell extract material (WCE). This is represented in the following calculation: [rDNA(IP)/*CUP1*(IP)/ rDNA(WCE)/*CUP1*(WCE)]. *CUP1* is a repetitive, non-silenced locus that serves as a negative control and a control for PCR efficiency. In Figure 3, the amount of *CUP1* sequences in the immunoprecipitated material was below the linear range of *RDN1* quantification, and the *CUP1* value used was an average of *CUP1* values from all of the multiplex PCR reactions for each yeast strain within a single experiment.

Unequal sister chromatid exchange assays

Assays were performed as previously described (Kaeberlein et al. 1999). Cells were grown to an OD_{600} of 0.4-0.8, sonicated briefly to prevent aggregation, and plated at a density of ~400 cells per SC plate. Cells were incubated at 30°C for 2-5 days and transferred to 4°C for 1-3 days to enhance color development. The unequal sister chromatid crossover rate was calculated by dividing the number of half-red/half-white colonies by the total number of colonies. Red colonies were excluded from all calculations. At least 12,000 colonies total from 3-5 independent isolates were examined for each genotype except for *csm1Δ sir2Δ*, for which at least 8,500 colonies were counted.

Immunoprecipitation assays

Assays were performed essentially as described (Straight et al. 1999). Fifty-milliliter cultures of yeast cells were grown to an optical density at 600 nm (OD_{600}) of 1.5-1.8. Cells were

harvested, washed once with cold TBS (20 mM Tris-HCl at pH 7.6 and 150 mM NaCl), and frozen at –80°C. Cell pellets were resuspended in 400 µl of lysis buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM EDTA, 2 mM benzamidine, 1 mM PMSF, and 1 µg/ml each of pepstatin, leupeptin, and bestatin), and bead-beat with glass beads (beads and Mini Beadbeater, Biospec Products) twice for 30 sec. Lysates were centrifuged at 13,000 rpm for 5 and 15 min. Clarified extract was incubated with 1 µg of rabbit anti-GFP antibody (gift from A. Rudner), mouse anti-HA (HA11, BabCO), or mouse anti-Myc (9E10) at 4°C for 2 h. Thirty microliters of a 50% slurry of pre-washed Protein A Sepharose beads (GE) was added and incubated for an additional for 2 h. Beads were washed once with 1 ml of lysis buffer, twice with 1 ml wash buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, and 1 mM EDTA), and resuspended in 2X SDS sample buffer. One percent of input whole-cell extract or 25% of bound fractions was run on 4-12% gradient gels (NuPage, Invitrogen) or 8% SDS-PAGE gels and blotted to nitrocellulose membranes for Western analysis. Membranes were probed using 1:5000 dilutions of rabbit anti-Sir2, mouse anti-Myc (9E10), and mouse anti-HA (HA.11) and 1:10,000 dilution of mouse anti-Act1 antibodies in TBS with 0.1% Tween-20 and 5% milk.

Whole cell protein analysis

Seven hundred microliters of a saturated culture was harvested by centrifugation, resuspended in 150 µl of 1.5X SDS sample buffer supplemented with 2 mM PMSF and 5 mM benzamidine, and bead-beat with glass beads (beads and Mini Beadbeater, Biospec Products) twice for 90 sec. Lysates were centrifuged briefly at 13,000 rpm and heated at 95°C for 5 min. Five microliters of sample was run on an 8% SDS-PAGE gel and blotted to nitrocellulose for Western analysis. Sir2 and Act1 proteins were detected using 1:5000 and 1:10,000 dilutions of

rabbit anti-Sir2 and mouse anti-Act1 (Chemicon International) antibodies, respectively, in TBS with 0.1% Tween-20 and 5% milk.

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Supplemental Table 1. Mock Purification Results

#unique peptides	ORF name	Common name	Mol weight
31	SSB1	YDL229W	66485
30	SSA1	YAL005C	69642
25	MYO1	YHR023W	223483
19	DED1	YOR204W	65422
18	RPS1A	YLR441C	28660
17	CDC19	YAL038W	54412
15	ATP3	YBR039W	30620
15	TEF2	YBR118W	49912
15	PAB1	YER165W	64204
14	RPS7B	YNL096C	21543
14	PDC1	YLR044C	61382
13	UBP3	YER151C	14627
13	RPS3	YNL178W	26378
13	RPL26B	YGR034W	101915
12	TDH3	YGR192C	15766
12	RPS19B	YNL302C	35612
12	SSE1	YPL106C	77235
11	RPS14A	YCR031C	14445
11	HSP60	YLR259C	58249
10	RPL31A	YDL075W	12830
10	RPL4A	YBR031W	21529
10	RPS5	YJR123W	24937
10	PGK1	YCR012W	39022
10	EFT2	YDR385W	44636
10	RPS7A	YOR096W	62865
10	CNA1	YLR433C	93145
10	YMR031C	YMR031C	93200
9	RPL25	YOL127W	15643
9	SSA2	YLL024C	26336
9	ILV2	YMR108W	43773
9	VMA4	YOR332W	64757
9	SPT5	YML010W	69342
9	TUF1	YOR187W	82532
9	RPG1	YBR079C	110198
9	YGL245W	YGL245W	115480
8	ENO2	YHR174W	15761
8	HSC82	YMR186W	19659
8	MCX1	YBR227C	27023
8	RPS6B	YBR181C	27305
8	RPS16B	YDL083C	27480
8	RPL3	YOR063W	27827
8	SUI3	YPL237W	31458
8	LAT1	YNL071W	43665
8	RPL2A	YFR031C-A	46773
8	GPM1	YKL152C	48534
8	RSM7	YJR113C	57946
8	RPL11B	YGR085C	80754
7	RPL17B	YJL177W	14354
7	RPS22A	YJL190C	14532
7	RPL23A	YBL087C	15688
7	RPS17B	YDR447C	20442
7	TIM44	YIL022W	28018
7	RPL8A	YHL033C	39174
7	YPL009C	YPL009C	42685
7	NIP1	YMR309C	82049
7	TFG1	YGR186W	92650
7	ILV5	YLR355C	93036
7	MYO2	YOR326W	119047
7	YGR130C	YGR130C	180525
6	YJL122W	YJL122W	13801
6	DBP2	YNL112W	16913
6	YHR121W	YHR121W	16947
6	ADH1	YOL086C	19169
6	RPL9A	YGL147C	21189
6	RPS18A	YDR450W	21579
6	FUN12	YAL035W	25251
6	RPS0A	YGR214W	27903
6	RPS13	YDR064W	33580
6	RPL35B	YDL136W	36743
6	RPL5	YPL131W	60874
6	CMP2	YML057W	68382
6	RPL10	YLR075W	112249
5	NOP58	YOR310C	11041
5	RPL30	YGL030W	11291
5	RPS15	YOL040C	14662
5	BRE5	YNR051C	15040
5	YOR252W	YOR252W	15245
5	RPS24A	YER074W	15394
5	VMA2	YBR127C	15914
5	RPL36A	YMR194W	16654
5	TPI1	YDR050C	21907
5	TAF61	YDR145W	22181
5	RPL13A	YDL082W	22448
5	RPL27B	YDR471W	23944
5	RPS9B	YBR189W	26661
5	YDJ1	YNL064C	27467
5	TIF34	YMR146C	27807
5	CMK2	YOL016C	28700
5	ECM1	YAL059W	33569
5	RHR2	YIL053W	37547
5	YPL077C	YPL077C	38741
5	YDR101C	YDR101C	39561
5	YCR030C	YCR030C	41707
5	RPP0	YLR340W	44379
5	RPL14B	YHL001W	44382
5	RPC40	YPR110C	50307
5	MRPL6	YHR147C	57552

5	RPL32	YBL092W	57595
5	PSA1	YDL055C	60955
5	NSR1	YGR159C	65076
5	RPS1B	YML063W	96007
5	ACT1	YFL039C	56964
4	RPL12B	YDR418W	8708
4	TIF4631	YGR162W	9976
4	RPL19B	YBL027W	10601
4	ATP7	YKL016C	15835
4	RPL7A	YGL076C	16610
4	ENO1	YGR254W	17646
4	RPL24A	YGL031C	18131
4	RPL15A	YLR029C	19710
4	RPL43B	YJR094W-A	19872
4	SET2	YJL168C	21323
4	YJR083C	YJR083C	21452
4	MRPL13	YKR006C	21605
4	SSC1	YJR045C	21605
4	RPL20A	YMR242C	24331
4	PBP1	YGR178C	27531
4	RPA190	YOR341W	35391
4	PMA1	YGL008C	46679
4	TSA1	YML028W	52636
4	RPS25A	YGR027C	68081
4	RPL38	YLR325C	72928
4	RPL21A	YBR191W	78774
4	ECM16	YMR128W	84420
4	PRT1	YOR361C	88114
4	SWI3	YJL176C	92873
4	RVS167	YDR388W	99584
4	RPL28	YGL103W	106952
4	RGA1	YOR127W	112687
4	DOT6	YER088C	144804
4	RPL6B	YLR448W	186421
3	YGR081C	YGR081C	6614
3	RPL22A	YLR061W	7003
3	SAM2	YDR502C	13560
3	STM1	YLR150W	13870
3	FIP1	YJR093C	22125
3	TSR1	YDL060W	23079
3	FBA1	YKL060C	23952
3	SOD2	YHR008C	24853
3	YER006W	YER006W	26369
3	YER002W	YER002W	26777
3	RPL16A	YIL133C	29300
3	SRV2	YNL138W	29874
3	SRO9	YCL037C	35618
3	CLC1	YGR167W	39477
3	PRP19	YLL036C	41953
3	TUB2	YFL037W	42141
3	ATP15	YPL271W	48454
3	NOP14	YDL148C	49820
3	LYS21	YDL131W	50891
3	RPS30A	YLR287C-A	51791
3	HTA2	YBL003C	56553
3	YDR229W	YDR229W	57381
3	YRA1	YDR381W	57715
3	RPS4B	YHR203C	77746
3	ARC1	YGL105W	88834
3	TAF90	YBR198C	90595
3	CYR1	YJL005W	94169
3	NUP2	YLR335W	227691
2	RPS2	YGL123W	6029
2	PDC6	YGR087C	11035
2	RPL1B	YGL135W	12052
2	RPL16B	YNL069C	12741
2	TIF2	YJL138C	12845
2	YPR143W	YPR143W	13338
2	SUI2	YJR007W	13809
2	RPS10B	YMR230W	15330
2	YKL056C	YKL056C	18252
2	SUB1	YMR039C	18388
2	YJL200C	YJL200C	18594
2	RPS8A	YBL072C	19878
2	DST1	YGL043W	20461
2	ASC1	YMR116C	22174
2	RPP2B	YDR382W	22385
2	NUP60	YAR002W	24399
2	HOR2	YER062C	25323
2	BUD20	YLR074C	27366
2	RPA49	YNL248C	27678
2	FPR4	YLR449W	28076
2	RPS26B	YER131W	34572
2	IDH2	YOR136W	34697
2	BFR2	YDR299W	34852
2	YNL110C	YNL110C	37796
2	HIT1	YJR055W	40660
2	RPL18B	YNL301C	43736
2	RGA2	YDR379W	44551
2	SNF12	YNR023W	46536
2	YEF3	YLR249W	49777
2	RPL40A	YIL148W	56743
2	TUB1	YML085C	57731
2	RPL33B	YOR234C	57753
2	RPL6A	YML073C	58999
2	TAF60	YGL112C	59051
2	BUD3	YCL014W	61161
2	GCD11	YER025W	61185
2	YIL105C	YIL105C	61438
2	SIK1	YLR197W	63723

2	RRP5	YMR229C	77863
2	RPS20	YHL015W	83583
2	LCP5	YER127W	104088
2	RPN2	YIL075C	113161
2	OSH2	YDL019C	115818
2	RPS12	YOR369C	145650
2	SIF2	YBR103W	184692
2	RPL31B	YLR406C	192975
2	PGI1	YBR196C	33096
1	SSA4	YER103W	6538
1	TIM9	YEL020W-A	6553
1	EFB1	YAL003W	6604
1	YPL146C	YPL146C	7598
1	TFG2	YGR005C	8695
1	RPL4B	YDR012W	8870
1	EBS1	YDR206W	9788
1	BMH1	YER177W	10203
1	RPP1B	YDL130W	10519
1	RPL33A	YPL143W	10728
1	TDH1	YJL052W	11283
1	INO2	YDR123C	12067
1	YKR071C	YKR071C	13694
1	YGR002C	YGR002C	13804
1	YMR144W	YMR144W	14120
1	YDL053C	YDL053C	14168
1	LIP5	YOR196C	14918
1	RPL13B	YMR142C	15380
1	SEC1	YDR164C	15718
1	BRX1	YOL077C	16303
1	SSB2	YNL209W	18751
1	MCK1	YNL307C	19768
1	RPS21B	YJL136C	20440
1	RVB2	YPL235W	20526
1	SPT16	YGL207W	22418
1	SAH1	YER043C	22500
1	NPI46	YML074C	24113
1	RPL17A	YKL180W	27513
1	RSA1	YPL193W	27842
1	TDH2	YJR009C	29971
1	MRPL38	YKL170W	30117
1	SAM1	YLR180W	30369
1	MMD1	YIL051C	32159
1	SOD1	YJR104C	32784
1	PHO90	YJL198W	33193
1	MYO4	YAL029C	33332
1	YNR053C	YNR053C	33455
1	ADE5	YGL234W	34219
1	DRS1	YLL008W	34474
1	RLP7	YNL002C	35619
1	YPL070W	YPL070W	35712
1	REX4	YOL080C	35832
1	CST6	YIL036W	36071
1	YKR090W	YKR090W	36438
1	MLC1	YGL106W	36596
1	CDC33	YOL139C	36931
1	YPR169W	YPR169W	37510
1	RVS161	YCR009C	38025
1	RPP2A	YOL039W	38568
1	PFK1	YGR240C	38700
1	NHP2	YDL208W	38814
1	YML093W	YML093W	38992
1	YDR493W	YDR493W	41597
1	PAM1	YDR251W	41671
1	DIM1	YPL266W	41878
1	STE5	YDR103W	42350
1	RPS28B	YLR264W	43010
1	RPA43	YOR340C	43989
1	YMR075W	YMR075W	45212
1	GLN3	YER040W	46363
1	RPT5	YOR117W	46389
1	YNL022C	YNL022C	46473
1	TKL1	YPR074C	48107
1	YMR188C	YMR188C	48690
1	LEU2	YCL018W	48983
1	YPL105C	YPL105C	49671
1	ALD6	YPL061W	49673
1	FAB1	YFR019W	50288
1	IPP1	YBR011C	50433
1	RRP1	YDR087C	50982
1	RPS29A	YLR388W	51464
1	TAL1	YLR354C	52442
1	HHF1	YBR009C	54272
1	RTG2	YGL252C	55096
1	SNF2	YOR290C	55377
1	DBP3	YGL078C	56189
1	KRE35	YGL099W	57357
1	PFK2	YMR205C	58707
1	HTZ1	YOL012C	63643
1	YGR103W	YGR103W	65263
1	PDA1	YER178W	65443
1	IDH1	YNL037C	66479
1	TAF145	YGR274C	69410
1	RPS29B	YDL061C	69526
1	CMK1	YFR014C	72604
1	PET9	YBL030C	73669
1	AAC3	YBR085W	77473
1	ARF2	YDL137W	78851
1	SSE2	YBR169C	79397
1	TIF35	YDR429C	79421
1	URA2	YJL130C	83346

1	BAT1	YHR208W	84385
1	OSH7	YHR001W	84698
1	TEF4	YKL081W	92758
1	THS1	YIL078W	94354
1	PNG1	YPL096W	97684
1	STU2	YLR045C	100013
1	CRP1	YHR146W	100792
1	HCM1	YCR065W	102236
1	ADH2	YMR303C	102696
1	RPL29	YFR032C-A	102844
1	MIS1	YBR084W	104475
1	ADH5	YBR145W	107822
1	BUR6	YER159C	118591
1	ERG10	YPL028W	120561
1	RPS0B	YLR048W	169211
1	RPL26A	YLR344W	194039
1	RPT2	YDL007W	244946
1	RPS31	YLR167W	257328
1	RPL22B	YFL034C-A	38401
1	RPS27B	YHR021C	15912
1	ADA2	YDR448W	86039
1	TUB3	YML124C	69865

Supplemental Table 2. Yeast strains

Strain	Genotype	Reference
SF1	JRY2334, *Mat **a** ade2-1 can1-100 his3-11 leu2-3.112 trp 1 ura3-1 GAL*	J. Rine
SF3	SF1 *sir2Δ::HIS3*	J. Rine
DMY631	SF1 *NET1-HA3-LEU2*	Huang and Moazed 2003
DMY1427	W303a *NET1-GFP-KAN^R*	This work
DMY2733	SF1 *FOB1-MYC13-KAN^R*	Huang and Moazed 2003
DMY2735	DMY631 (*NET1-HA3-LEU2*) with *FOB1-MYC13-KAN^R*	Huang and Moazed 2003
DMY2737	DMY633 (*sir2Δ::HIS3, NET1-HA3::LEU2*) with *FOB1-MYC13-KAN^R*	Huang and Moazed 2003
DMY2889	SF1 *TOF2-HA3-LEU2*	This work
DMY2893	DMY1427 (*NET1-GFP-KAN^R*) with *TOF2-HA3-LEU2*	This work
DMY2909	SF3 (*sir2Δ::HIS3*) with *NET1-GFP-KAN^R* and *TOF2-HA3-LEU2*	Huang and Moazed 2003
DMY2946	DMY2889 (*TOF2-HA3-LEU2*) with *FOB1-MYC13-KAN^R*	This work
DMY2798	W303a *leu2::mURA3*	This work
DMY2804	W303a *RDN1-NTS1::mURA3*	This work
DMY2800	W303a *RDN1-NTS2::mURA3*	This work
DMY2845	DMY2798 (*leu2::mURA3*) with *tof2Δ::KAN^R*	This work
DMY2847	DMY2804 (*RDN1-NTS1::mURA3*) with *tof2Δ::KAN^R*	This work
DMY2849	DMY2800 (*RDN1-NTS2::mURA3*) with *tof2Δ::KAN^R*	This work
DMY2827	DMY2798 (*leu2::mURA3*) with *sir2Δ::KAN^R*	Tanny et al. 2004
DMY2835	DMY2804 (*RDN1-NTS1::mURA3*) with *sir2Δ::KAN^R*	Tanny et al. 2004
DMY2831	DMY2800 (*RDN1-NTS2::mURA3*) with *sir2Δ::KAN^R*	Tanny et al. 2004
DMY2982	DMY2798 (*leu2::mURA3*) with *TOF2-HA3-LEU2*	This work
DMY2983	DMY2804 (*RDN1-NTS1::mURA3*) with *TOF2-HA3-LEU2*	This work
DMY2984	DMY2800 (*RDN1-NTS2::mURA3*) with *TOF2-HA3-LEU2*	This work
DMY2987	DMY2798 (*leu2::mURA3*) with *TOF2-TAP-K.l-TRP1*	This work
DMY2988	DMY2804 (*RDN1-NTS1::mURA3*) with *TOF2-TAP-K.l-TRP1*	This work
DMY2989	DMY2800 (*RDN1-NTS2::mURA3*) with *TOF2-TAP-K.l-TRP1*	This work
DMY3143	DMY2798 (*leu2::mURA3*) with *lrs4Δ::KAN^R*	This work
DMY3145	DMY2804 (*RDN1-NTS1::mURA3*) with *lrs4Δ::KAN^R*	This work
DMY3147	DMY2800 (*RDN1-NTS2::mURA3*) with *lrs4Δ::KAN^R*	This work
DMY3149	DMY2798 (*leu2::mURA3*) with *csm1Δ::KAN^R*	This work

DMY3151	DMY2804 (*RDN1-NTS1::mURA3*) with *csm1Δ::KAN^R*	This work
DMY3153	DMY2800 (*RDN1-NTS2::mURA3*) with *csm1Δ::KAN^R*	This work
DMY2895	W303a *adh4::URA3*	A. Rudner
DMY2896	W303a *TELVIIL::URA3*	A. Rudner
DMY2841	DMY2985 (*adh4::URA3*) with *sir2Δ::KAN^R*	This work
DMY2839	DMY2986 (*TELVIIL::URA3*) with *sir2Δ::KAN^R*	This work
DMY2897	DMY2985 (*adh4::URA3*) with *fob1Δ::KAN^R*	This work
DMY2899	DMY2986 (*TELVIIL::URA3*) with *fob1Δ::KAN^R*	This work
DMY2901	DMY2985 (*adh4::URA3*) with *tof2Δ::KAN^R*	This work
DMY2903	DMY2986 (*TELVIIL::URA3*) with *tof2Δ::KAN^R*	This work
DMY3010	W303a *RAD5$^+$* with *RDN1::ADE2*	L. Guarente
DMY3011	DMY3010 (*RDN1::ADE2*) with *sir2Δ::TRP1*	L. Guarente
DMY3012	DMY3010 (*RDN1::ADE2*) with *fob1Δ::URA3*	L. Guarente
DMY3022	DMY3010 (*RDN1::ADE2*) with *tof2Δ::KAN^R*	This work
DMY3200	DMY3011 (*RDN1::ADE2, sir2Δ::TRP1*) with *lrs4Δ::KAN^R*	This work
DMY3202	DMY3011 (*RDN1::ADE2, sir2Δ::TRP1*) with *csm1Δ::KAN^R*	This work
SF10	BJ5459, *Mat **a** ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*	E. Jones
DMY1690	SF10 *NET1-TAP::K.l-TRP1*	Huang and Moazed 2003
DMY1704	SF10 *SIR2-TAP::K.l-TRP1*	Hoppe et al 2002
DMY3173	DMY1704 (SF10 *SIR2-TAP::K.l-TRP1*) with *tof2Δ::KAN^R*	This work
DMY2334	SF10 *FOB1-TAP::K.l-TRP1*	Huang and Moazed 2003
DMY2883	SF10 *TOF2-TAP::K.l-TRP1*	This work
DMY2924	DMY2883 (*TOF2-TAP::K.l-TRP1*) with *fob1Δ::KAN^R*	This work
DMY3163	DMY2883 (*TOF2-TAP::K.l-TRP1*) with *sir2Δ::KAN^R*	This work
DMY3047	SF10 *LRS4-TAP::K.l-TRP1*	This work
DMY3051	DMY3047 (*LRS4-TAP::K.l-TRP1*) with *fob1Δ::KAN^R*	This work
DMY3053	DMY3047 (*LRS4-TAP::K.l-TRP1*) with *tof2Δ::KAN^R*	This work
DMY3165	DMY3047 (*LRS4-TAP::K.l-TRP1*) with *sir2Δ::KAN^R*	This work
DMY3049	SF10 *CSM1-TAP::K.l-TRP1*	This work
DMY3055	DMY3049 (*CSM1-TAP::K.l-TRP1*) with *fob1Δ::KAN^R*	This work

DMY3057	DMY3049 (*CSM1-TAP::K.l-TRP1*) with *tof2Δ::KAN*^{R}	This work
DMY3167	DMY3049 (*CSM1-TAP::K.l-TRP1*) with *sir2Δ::KAN*^{R}	This work
A13838	W303, MATa *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, LRS4-6HA*	This work
A13839	A13838 with *cdc15-2*	This work
A14158	A13839 with *spo12Δ*	This work
A14204	A13838 with *cdc14-3*	This work
A14566	A13838 with *cdc14-1*	This work
A14568	A13838 with *net1Δ*	This work

Supplemental Table 3. ChIP primer sets

RDN1 (5′→3′)

1)	AAAAGAAACCAACCGGGATT	CCACCCACTTAGAGCTGCAT
2)	GGGAATGCAGCTCTAAGTGG	ATGGATTTATCCTGCCACC
3)	TGCGACGTAAGTCAAGGATG	CTGGCTTCACCCTATTCAGG
4)	TCCCTCAGGATAGCAGAAGC	GTGGTGTCTGATGAGCGTGT
5)	CCGAATGAACTAGCCCTGAA	CGACTAACCCACGTCCAACT
6)	AAAGGTTCCACGTCAACAGC	AGCCATAAGACCCCATCTCCG
7)	ATCCGGAGATGGGGTCTTAT	CTGACCAAGGCCCTCACTAC
8)	TTGTAGACGGCCTTGGTAGG	ATGACGAGGCATTTGGCTAC
9)	CTAGCGAAACCACAGCCAAG	AATGTCTTCAACCCGGATCA
10	ATTGTCAGGTGGGGAGTTTG	TGTCGCTATGAACGCTTGAC
11)	TGGCAGTCAAGCGTTCATAG	CAGCCGCAAAAACCAATTAT
12)	TAATTGGTTTTTGCGGCTGT	ATGATTTATCCCCACGCAAA
13)	TTTGCGTGGGGATAAATCAT	CATGTTTTTACCCGGATCAT
14)	CCGGGGCCTAGTTTAGAGAG	ACCCATCTTTGCAACGAAAA
15)	AGGGCTTTCACAAAGCTTCC	TCCCCACTGTTCACTGTTCA
16)	TGATGATGGCAAGTTCCAGA	CTTATTCCTTCCCGCTTTCC
17)	GGAAAGCGGGAAGGAATAAG	CGATTCAGAAAAATTCGCACT
18)	GTGCGAATTTTTCTGAATCG	CCCTCATATCACCTGCGTTT
19)	GAGGTGTTATGGGTGGAGGA	GCCACCATCCATTTGTCTTT
20)	TGCAAAAGACAAATGGATGG	GCACCTTTTCCTCTGTCCAC
21)	AGAGGAAAAGGTGCGGAAAT	TTTCTGCCTTTTTCGGTGAC
22)	GTTGGTTTTGGTTTCGGTTG	TCGCCGAGAAAAACTTCAAT
23)	GGGAGGTACTTCATGCGAAA	AAGATGCCCACGATGAGACT
24)	AGTCTCATCGTGGGCATCTT	TCCGTCACCATACCATAGCA
25)	GGCAGCAGAGAGACCTGAAA	GAGCCATTCGCAGTTTCACT
26)	TCGACCCTTTGGAAGAGATG	GCCTTCCTTGGATGTGGTAG
27)	AAACGGCTACCACATCCAAG	GGCCCAAAGTTCAACTACGA
28)	CCTTGAGTCCTTGTGGCTCT	TGAAAACGTCCTTGGCAAAT
29)	GGGGATCGAAGATGATCAGA	TTGTGTCTGGACCTGGTGAG
30)	CTCACCAGGTCCAGACACAA	CCAGAACGTCTAAGGGCATC
31)	AGCCAGCGAGTCTAACCTTG	TTGTCCAAATTCTCCGCTCT
32)	TGTTTTGGCAAGAGCATGAG	CTCGAATGCCCAAAGAAAAA
33)	GGCCCAGAGGTAACAAACAC	GGAAATGACGCTCAAACAGG
34)	CTGGCCTTTTCATTGGATGT	ATCCCGGTTGGTTTCTTTTC

CUP1 (5′→3′)

TGAAGGTCATGAGTGCCAAT	TTCGTTTCATTTCCCAGAGCA

Supplemental Figure Legends

Supplemental Figure 1. Tof2 physically associates with Fob1, Net1, and Sir2

(A) Immunoprecipitation of Net1-GFP coprecipitates with Tof2-HA3 and Sir2 (lane 4). Sir2 coprecipitates with Net1-GFP and Tof2-HA3 (lane 8). (-), Untagged; (+), tagged or present; (Δ), *sir2Δ*; (*), cross-reactive band.

(B) Western blots showing that Fob1-Myc13 coprecipitates with Tof2-HA3 and Sir2 from whole-cell extracts. Actin (Act1) serves as loading control for all panels. (-), Untagged; (+), tagged. 1% of whole-cell extract (input) and 25% of bound material (IP) are shown for all panels.

(C) Immunoprecipitation of Fob1-Myc13 coprecipitates similar amounts of Net1-HA3 and Sir2 in the presence (+) or absence (Δ) of *TOF2*. (-), Untagged; (+), tagged or present.

Supplemental Figure 2. Silencing in *tof2Δ* cells is rescued by ectopic expression of *TOF2*

(A) Silencing was assessed as described in Figure 2B. Locations of NTS1 and NTS2 reporters within rDNA are indicated in Figure 2A. Cells were plated on synthetic complete media lacking histidine (-HIS) to maintain *CEN* plasmids, which expressed either no gene (*pCEN-HIS3*) or *TOF2* (*pCEN-TOF2-HIS3*). Silencing was assayed on synthetic complete media lacking histidine and uracil (-HIS-URA).

(B) The level of Sir2 protein does not change in the absence of *TOF2* as shown by Western blotting of whole-cell extracts prepared from cells shown in (A). Act1 is shown as a loading control.

Supplemental Figure 3. Expression of *TOF2* modified with C-terminal HA3 or TAP epitope tags does not affect silencing

Silencing was assessed as described in Figure 2B. Locations of NTS1 and NTS2 reporters within rDNA are indicated in Figure 2A. Cells in which the endogenous copy of *TOF2* was modified to express either *TOF2-HA3* (A) or *TOF2-TAP* (B) maintained wild-type levels of silencing.

Supplemental Figure 4. *TOP1* is required for silencing at both NTS1 and NTS2

Silencing was assessed as described in Figure 2B. Locations of NTS1 and NTS2 reporters within rDNA are indicated in Figure 2A. Cells were plated on synthetic complete media as a plating and growth control and on synthetic complete media lacking uracil (-URA) to assay silencing.

Supplemental Figure 5. Expression of *LRS4* or *CSM1* modified with a C-terminal TAP epitope tag does not affect silencing

Silencing was assessed as described in Figure 2B. Locations of NTS1 and NTS2 reporters within rDNA are indicated in Figure 2A. Cells were plated on synthetic complete media as a plating and growth control and on synthetic complete media lacking uracil (-URA) to assay silencing.

Supplemental Figure 6. Protein sequence homology of LEM domains

Protein sequence homology of the LEM domains of Src1, its *S. cerevisiae* homologue Ydr458c, and the Lap2 proteins of *Xenopus laevis* and *Homo sapiens*. Gray shading indicates identical and similar residues. LEM domains are approximately 40 amino acids in length and primarily found in inner nuclear membrane proteins of metazoans. Although it is presumed that no LEM domain proteins exist in yeast, and thus far, no yeast inner nuclear envelope proteins have been identified (Cohen et al. 2001; Bengtsson and Wilson 2004; Segura-Totten and Wilson 2004), GFP fusions of Src1 and its *S. cerevisiae* homologue Ydr458c localize to the nuclear envelope (Drees et al. 2001; Huh et al. 2003) (data not shown).

Supplemental Figure 7. Lrs4-6HA localization in FEAR network and *cdc14* mutants.

(A) *cdc15-2 spo12Δ* cells (A14158) carrying an *LRS4-6HA* fusion were arrested in G1 in YEPD medium with α factor (5 μg/ml). When arrest was complete, cells were released into YEPD medium lacking pheromone at 37°C. At the indicated times samples were taken to determine the percentages of cells with metaphase and anaphase spindles, as well as the percentage of cells with Lrs4-6HA released from the nucleolus. *SPO12* is a component of the FEAR network that is required for the release of Cdc14 from the nucleolus during early anaphase (Stegmeier et al. 2002).

(B-D) Wild type (A13838), *cdc14-3* (A14204), and *cdc14-1* cells (A14566) carrying an *LRS4-6HA* fusion were arrested in G1 in YEPD medium with α factor (5 μg/ml). When arrest was complete, cells were released into YEPD medium lacking pheromone at 37°C. At the indicated

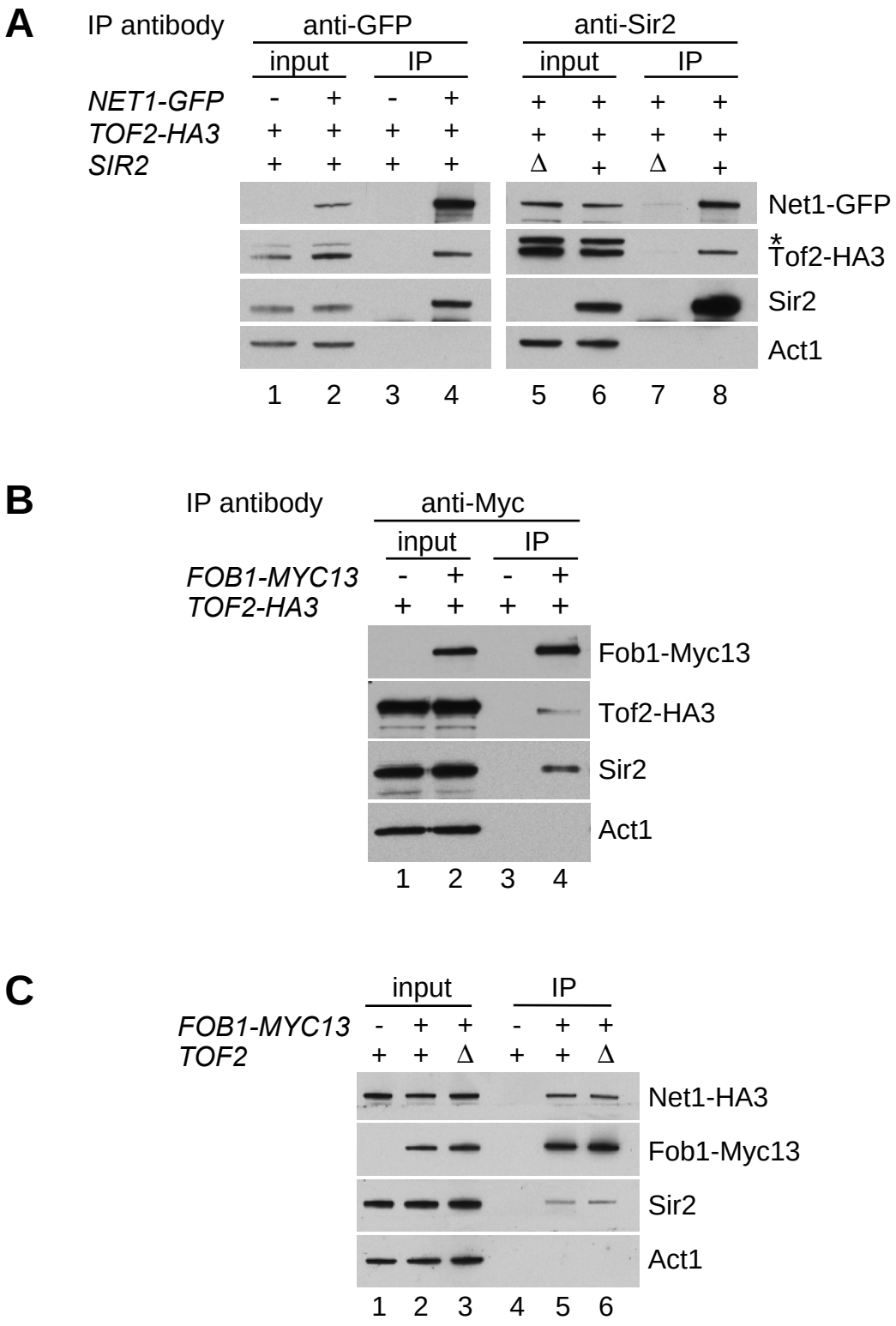
times samples were taken to determine the percentages of cells with metaphase and anaphase spindles, and the percentage of cells with Lrs4-6HA released from the nucleolus.

Supplemental Figure 8. Sir2 localization to rDNA does not require *LRS4/CSM1*

(A) Examples of chromatin immunoprecipitation data showing PCR products amplified from whole-cell extract (WCE) and immunoprecipitated (IP) DNA. Multiplex PCR was performed to amplify *RDN1* and *CUP1* sequences as indicated. PCR products 12-17 and 21-26 are shown.

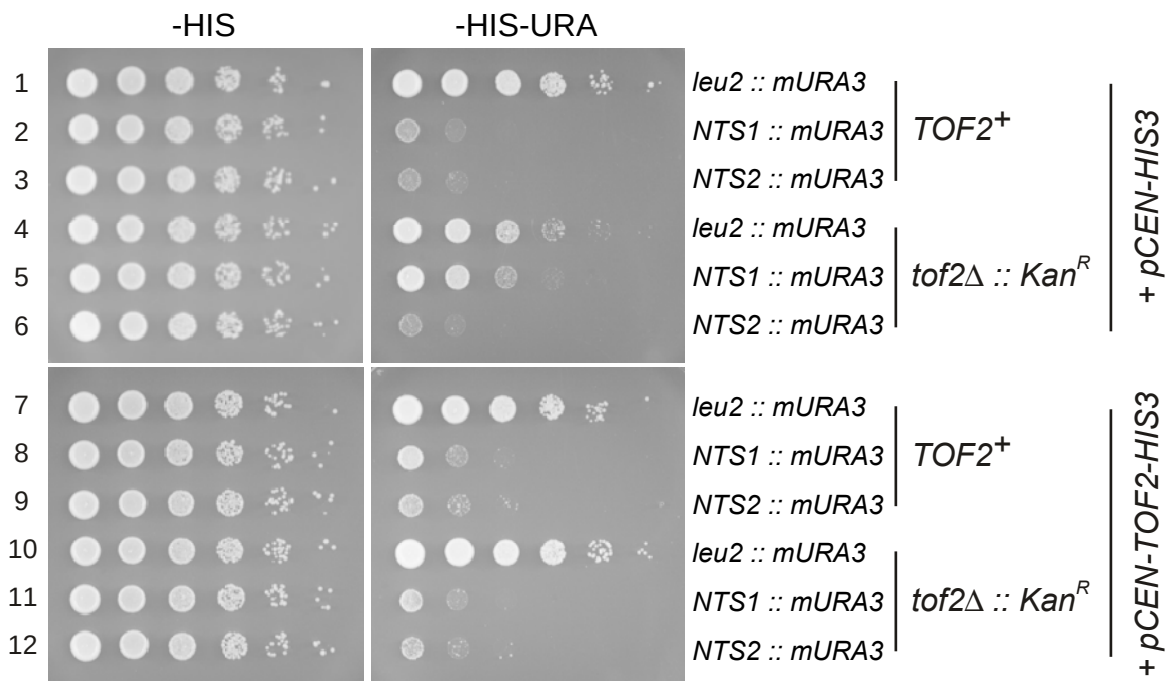
(B) Representative graph showing the association of Sir2-TAP at rDNA in wild-type (solid black line), *lrs4Δ* (solid gray line), or *csm1Δ* (dashed gray line) cells.

Supplemental Figure 1



Supplemental Figure 2

A



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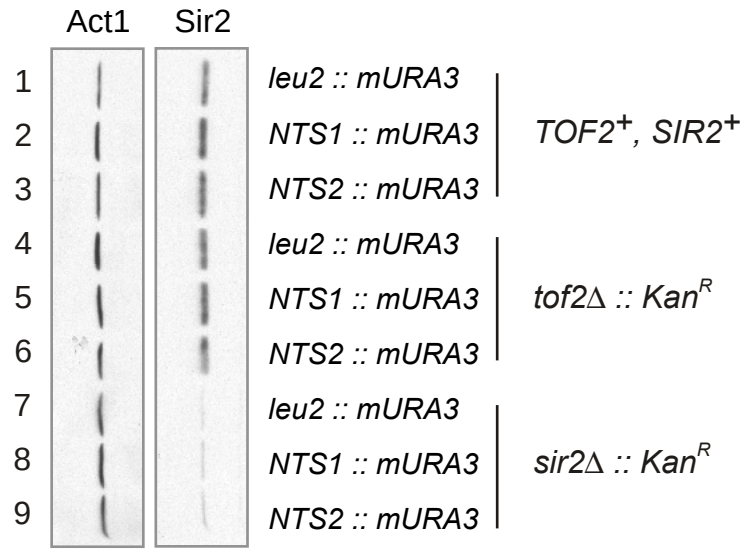
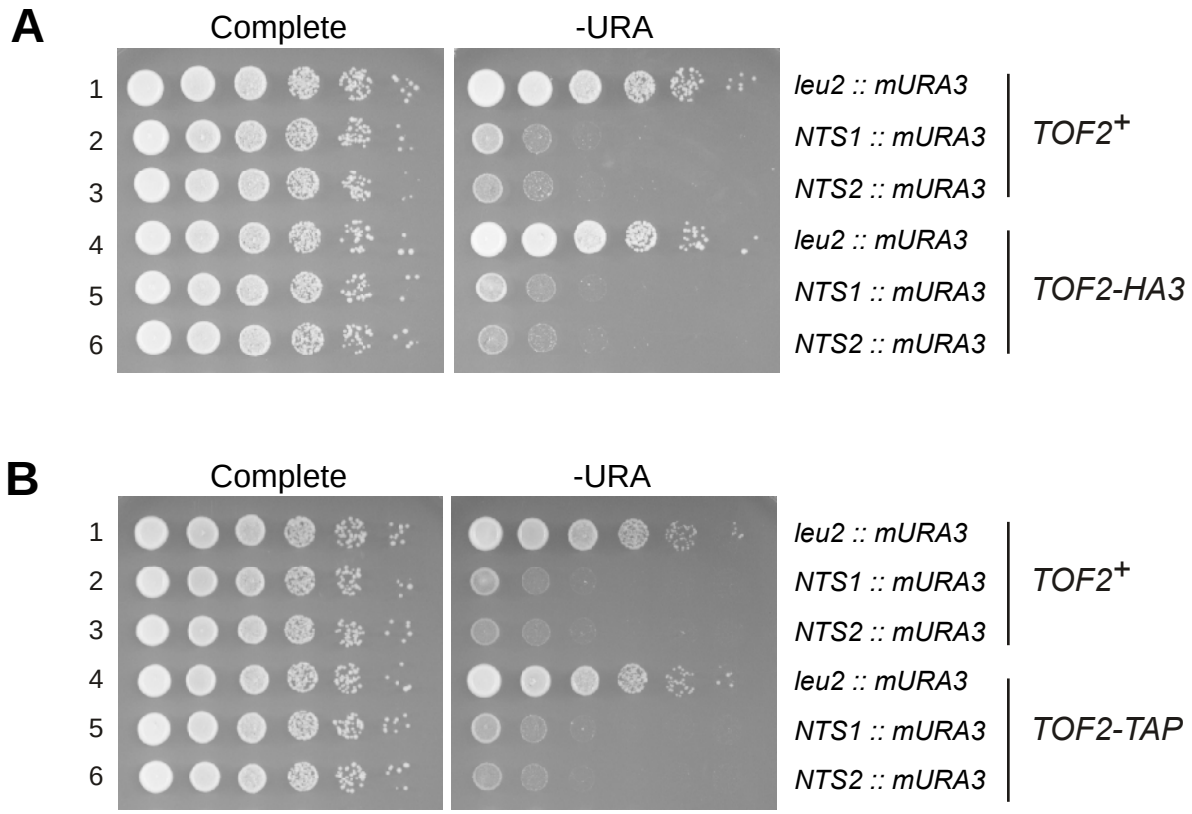
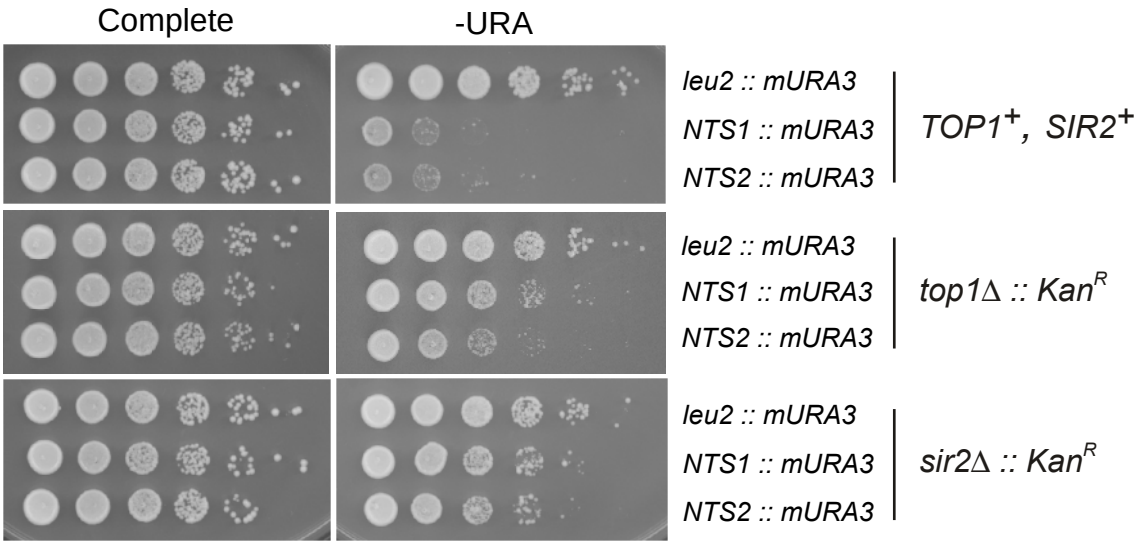


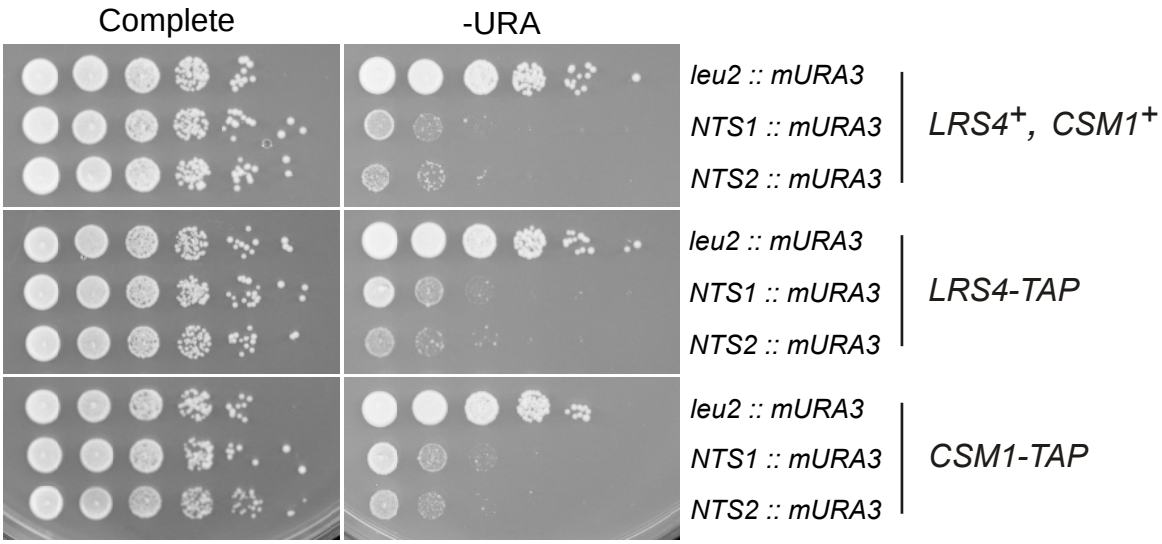
Supplemental Figure 3



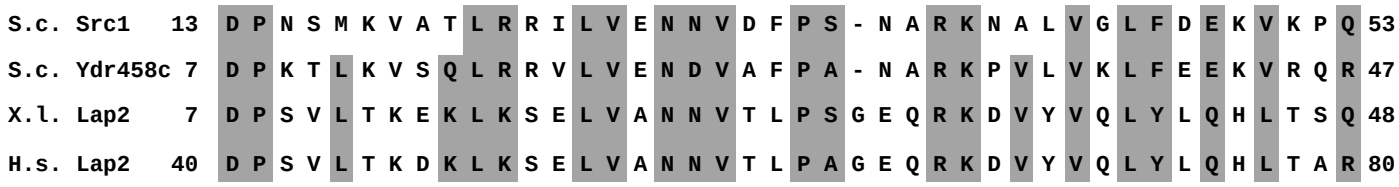
Supplemental Figure 4



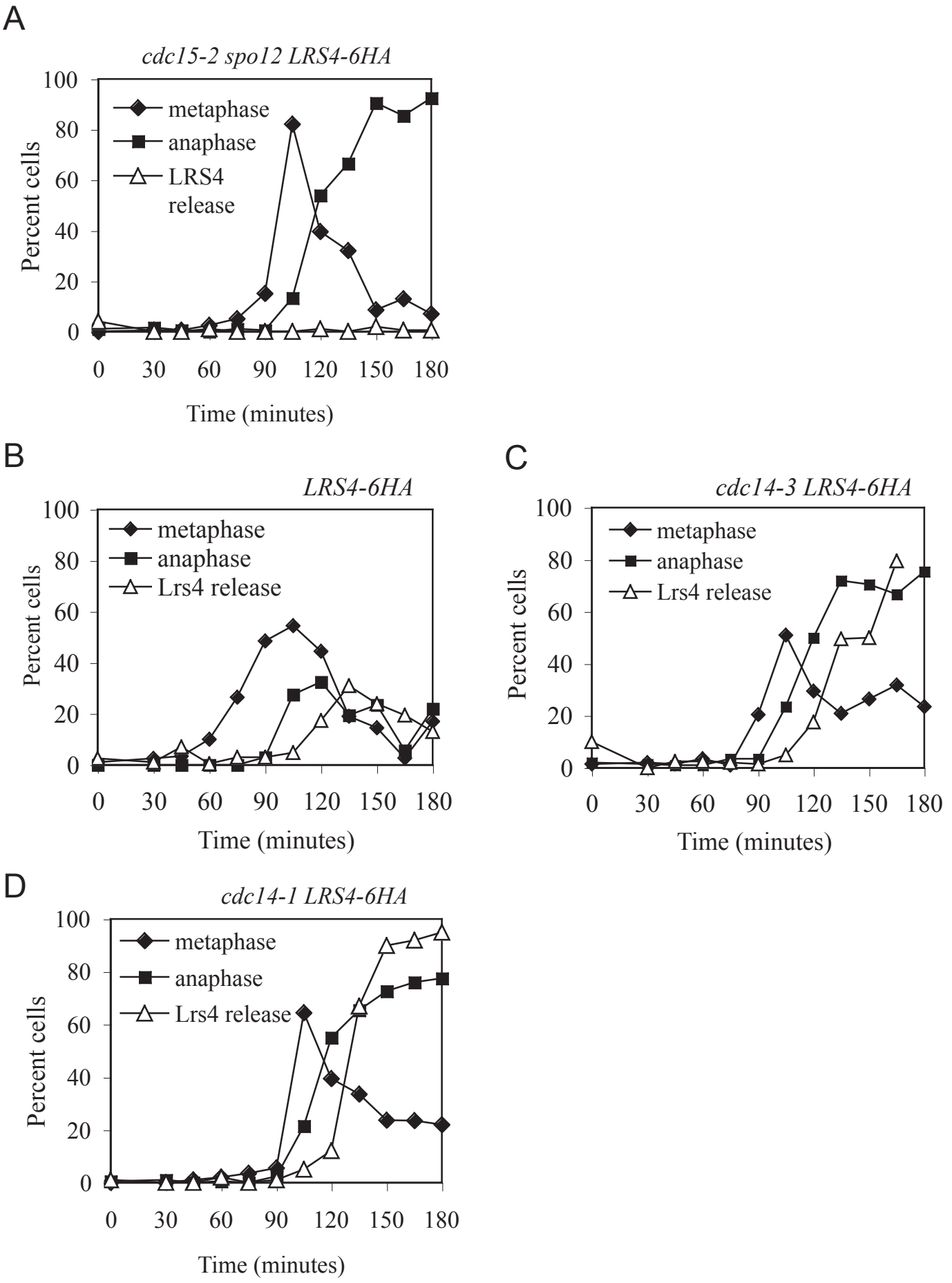
Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7



Supplemental Figure 8