Separation of silencing from perinuclear anchoring functions in yeast Ku80, Sir4 and Esc1 proteins

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In budding yeast, the nuclear periphery forms a subcompartment in which telomeres cluster and SIR proteins concentrate. To identify the proteins that mediate chromatin anchorage to the nuclear envelope, candidates were fused to LexA and targeted to an internal GFP-tagged chromosomal locus. Their ability to shift the locus from a random to a peripheral subnuclear position was monitored in living cells. Using fusions that cannot silence, we identify YKu80 and a 312-aa domain of Sir4 (Sir4^{PAD}) as minimal anchoring elements, each able to relocate an internal chromosomal locus to the nuclear periphery. Sir4^{PAD}-mediated tethering requires either the Ku complex or Esc1, an acidic protein that is localized to the inner face of the nuclear envelope even in the absence of Ku, Sir4 or Nup133. Finally, we demonstrate that Ku- and Esc1-dependent pathways mediate natural telomere anchoring *in vivo*. These data provide the first unambiguous identification of protein interactions that are both necessary and sufficient to localize chromatin to the nuclear envelope.

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Introduction

Chromosomes assume a nonrandom distribution in interphase nuclei (Marshall, 2002). The function of their spatial arrangement is largely unknown, although recent evidence suggests that subnuclear compartments contribute to the establishment and maintenance of epigenetic controls over eukaryotic gene expression (Fisher and Merckenschlager, 2002).

In yeast, flies and man, the protective ends of chromosomes known as telomeres nucleate the formation of an altered chromatin structure that represses the transcription of adjacent RNA pol II genes in a heritable fashion (termed telomere position effect or TPE; reviewed in Huang, 2002;

Perrod and Gasser, 2003). Repressed telomeric chromatin in yeast is found near the nuclear envelope (NE) in discrete foci that are far fewer than the number of chromosomal ends. These clusters of telomeric DNA colocalize with pools of silent information regulatory proteins (Sir2–4; Gotta *et al*, 1996), which are essential for TPE. Intriguingly, a similar spatial juxtaposition of telomeres can be observed in the parasite *Plasmodia*, where the clustering favors subtelomeric gene conversion events between virulence factor loci (Freitas-Junior *et al*, 2000).

In yeast, DNA elements known as silencers bind the sequence-specific factors Abf1, Rap1 and ORC, which nucleate the binding of the SIR complex at the cryptic mating-type loci, *HML* and *HMR* (Rusche *et al*, 2003). At telomeres, on the other hand, Rap1 binds the terminal [TG_{1–3}]_n repeat and cooperates with the end-binding complex YKu70/YKu80 to recruit SIR proteins (Moretti *et al*, 1994; Cockell *et al*, 1995). Once bound, the Sir complex spreads along adjacent nucleosomes preferentially binding underacetylated histone tails (Renaud *et al*, 1993; Hecht *et al*, 1996; Rusche *et al*, 2002).

Several observations support the proposal that subnuclear organization influences silencing. First, the delocalization of SIR factors from telomeres promotes repression at silencer-flanked loci that usually remain active due to their distance from chromosomal ends (Maillet *et al*, 1996, 2001; Marcand *et al*, 1996). This suggests that telomeric foci sequester silencing factors from nontelomeric sites of action. Second, the repression of *HML* and other silencer-flanked reporters is aided by their proximity to telomeres (Thompson *et al*, 1994; Maillet *et al*, 1996; Marcand *et al*, 1996). Third, silencing can be restored to a derepressed allele of *HMR* by targeting proteins of the endoplasmic reticulum to the locus via a Gal4 DNA-binding domain (e.g. Yif1, Yip1; Andrulis *et al*, 1998). These membrane-spanning hybrids accumulate in NE and are thought to promote repression by tethering the reporter gene near telomeric pools of SIR proteins. It has never been demonstrated, however, that the Yif-targeted reporter genes are actually recruited to the NE.

The proteins that mediate the perinuclear anchoring of yeast telomeres and silent loci are unknown, although mutations in *YKU* or *SIR2–4* do affect telomere position. Indeed, the deletion of either subunit of the Ku heterodimer delocalizes roughly half of the yeast telomeres from the NE (Laroche *et al*, 1998), but leaves truncated telomeres tethered (Tham *et al*, 2001; Hediger *et al*, 2002a). The complete delocalization of certain telomeres, such as Tel 14L or a truncated Tel 6R (Tel 6R^Δ), was detected only after deletion of both *sir4* and *yku70*, suggesting that redundant anchoring mechanisms exist (Hediger *et al*, 2002a). Because the restoration of silent chromatin allowed anchoring in the absence of Ku but in a Sir4-dependent manner, it was proposed that chromatin-bound SIR complexes and Ku define two partially redundant tethering pathways.

Among the silencing factors in yeast, Sir4 is most likely to act as a chromatin anchor. First, ectopic expression of its

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coiled-coil carboxy-terminal fragment leads to the release of full-length Sir3 and Sir4 from an insoluble chromatin fraction (Cockell *et al*, 1995) and loss of TPE. Indeed, based on the homology between this domain and human lamins A and C (Diffley and Stillman, 1989), it has been speculated that Sir4 might substitute for the nuclear lamina, which yeast lack. Finally, a penultimate subdomain of Sir4 (aa 950–1262 called Sir4^{PAD} for partitioning and anchoring domain) was found to confer efficient mitotic partitioning on otherwise unstable plasmids and to impair the free rotation of DNA (Ansari and Gartenberg, 1997). One mechanism that could account for both phenotypes would be the anchoring of DNA to a symmetrically segregating nuclear component.

Recent work shows that plasmid partitioning by Sir4^{PAD} requires Esc1 (establishes silent chromatin), a protein that associates with Sir4, imparts silencing when targeted to a crippled silencer-flanked reporter at *HMR*, and accumulates at the nuclear periphery when overexpressed (Andrulis *et al*, 2002). These data gave rise to the proposal that the Sir4–Esc1 interaction might link silent chromatin to a scaffolding at the nuclear periphery. Unfortunately, this hypothesis could not be tested in loss-of-function alleles, because Esc1, like Ku and Sir4, promotes and stabilizes silent chromatin. Indeed, to determine which elements mediate chromatin anchoring, it was necessary to separate the silencing functions of the major telomere-associated proteins from any tethering activity they might have.

We show here that a silencing-incompetent YKu80 monomer and a subdomain of Sir4 can each relocate chromatin to the nuclear periphery without nucleating silent chromatin. Furthermore, we show that Esc1 functions as a perinuclear anchor for this silencing-deficient domain of Sir4, but not as its exclusive binding site. Sir4^{PAD} tethers chromatin through two pathways: one requiring yKu and the second Esc1. Finally, we show that these two pathways cooperate to anchor native telomeres in a cell-cycle-regulated manner. We propose that, unlike the chromatin components Sir4 and yKu, Esc1 plays a structural role determining chromosome domain position *in vivo*.

Results

An assay for chromatin relocation

We have developed a cytological screen for proteins that impart a specific nuclear localization to an otherwise randomly positioned chromosomal segment. Four LexA operators (lexA^{OP}) were linked to an array of 256 lac^{OP}-binding sites and integrated at an internal active locus > 50 kb from either the centromere or the right telomere of chromosome 6, near ARS607 (called Chr6^{int}; Figure 1A). When LacI–GFP is expressed in these cells, it binds the array creating a single focus of GFP fluorescence. The ability of various LexA fusion proteins to influence the subnuclear position of the array can then be assessed.

Three-dimensional (3D) focal stacks were collected to measure the distance between LacI–GFP foci and the nuclear membrane (tagged with GFP–Nup49). Positions measured in several hundred cells show that the Chr6^{int} lac^{OP} array is randomly distributed among three concentric nuclear zones of equal surface, with or without LexA (Figure 1B). Spot position is monitored in one focal plane with zones normalized to the measured nuclear diameter in this plane. To show

Figure 1 An assay for perinuclear chromatin anchoring. (A) Strain GA-1461 bears lac^{OP} repeats and LexA-binding sites at an internal locus on the right arm of Chr 6 (Chr6^{int}) and a GFP–Nup49 fusion. A typical single-plane confocal image superimposed on a phase image is shown. Chr6^{int} can be mapped to one of three concentric zones of equal surface by using the ratio of the spot to pore measurement (black) over the nuclear diameter (red; see Materials and Methods). (B, C) LexA (B) or LexA–Yif1 (C) was expressed in GA-1461, and GFP spot positions were classified by cell-cycle stage. Bar graphs present the percentage of spots (y-axis; n = number of cells analyzed) per zone (x-axis). The dotted bar at 33% indicates a random distribution, and zone 1 distributions that are significantly different from random are indicated by * based on 95% confidence values (p). (D) Characteristic equatorial images of the GFP pore and Chr6^{int} locus in GA-1461 expressing the indicated LexA fusion. In the insets, a 5 min track of Chr6^{int} imaged by confocal microscopy at 1.5 s intervals is projected in red onto a single nuclear section (Heun *et al*, 2001b). Bar = 1 μ m. (E) LexA–Yif1 fusion was overexpressed in GA-1461 (WT) and in isogenic strains bearing complete yku70 or sir4 deletions. Here zone 1 values are compared. See complete statistics in Table 1.

that relocation can occur, we first targeted LexA fused to an integral membrane protein called Yif1 (Andrulis *et al*, 1998) to the Chr6^{int} locus. We observe a significant enrichment of Chr6^{int} in the outermost nuclear zone throughout the cell cycle (Figure 1C). Given that the fluorescent GFP–Nup49 ring occupies a large fraction of zone 1, the presence of a chromosomal signal in this zone indicates close NE–DNA contact (<180 nm). We conclude that the LexA–Yif1 fusion, which promotes repression when targeted to a crippled silencer-flanked reporter gene (Andrulis *et al*, 1998),

can indeed relocalize a chromosomal locus to the nuclear periphery.

Because neither the silencing nor anchorage is 100% efficient, we monitored the residence time of the tagged locus at the periphery using time-lapse microscopy (Hediger *et al*, 2002a; Heun *et al*, 2001b). A total of 300–400 sequential images were collected at 1.5 s intervals from individual cells expressing either LexA or LexA–Yif1. Without LexA–Yif1, the locus moves freely in the nucleoplasm without any discernible perinuclear constraint (Figure 1D). In the presence of LexA–Yif1, the tagged locus oscillates back and forth along the NE, much like the movement of natural telomeres (Hediger *et al*, 2002a). Movements away from the nuclear periphery are occasional. We conclude that an internal non-silenced chromosomal locus becomes significantly but reversibly associated with the NE by binding LexA–Yif1. Importantly, these results demonstrate that our chromatin ‘relocation’ assay can be used to identify protein domains that tether chromosomes to the nuclear periphery.

Identification of silencing-defective *YKU80* and *SIR4* alleles

Sir4 and the Ku complex represented logical anchoring candidates based on loss-of-function analyses. However, YKu80 and full-length Sir4, as well as its N- or C-terminal domains, nucleate silent chromatin when targeted to DNA (Marcand *et al*, 1996; Martin *et al*, 1999). Thus, in order to test a chromatin relocation activity in the absence of silent chromatin, we sought fusions of Sir4 and Ku that would separate silencing and anchoring activities. For Sir4, we fused LexA to the Sir4^{PAD} domain, which unlike other Sir4 domains has no dominant-negative effect on TPE (Ansari and Gartenberg, 1997). For the Ku heterodimer, on the other hand, we created mutant forms of *yku80* by degenerate PCR. Two alleles were identified (*yku80-4* and *yku80-9*; see Figure 2A) that are unable to restore TPE in the *yku80* null background.

To test whether these proteins nucleate silencing when targeted to a crippled HM locus, we inserted a *TRP1* reporter gene at *HMR* and replaced the Rap1 and Abf1 sites of the *E* silencer with four *lexA^{OP}* sites (creating a crippled silencer; *c.f.* Andrulis *et al*, 1998). Growth of serially diluted cultures in the presence or absence of tryptophan allows a qualitative measure of *TRP1* repression. Figure 2B shows that whereas targeted LexA–*yku80-9* confers robust silencing relative to LexA–Yif1 (100 × difference), no silencing was seen with LexA–*yku80-4*, LexA–Sir4^{PAD} or LexA alone. LexA fused to either full-length Sir2 or the C-terminal 540 residues of Esc1 (Esc1^C) also silenced efficiently, in agreement with previous reports (Cockell *et al*, 2000; Andrulis *et al*, 2002).

It was important to show not only that the Sir4^{PAD} domain and *yku80-4* fail to promote repression, but that they also fail to bind other SIR components. By two-hybrid analysis we detect no significant interaction of Sir4^{PAD} with any of the domains of Sir2, Sir3 and Sir4 that are known to be critical for repression (Figure 2C). On the other hand, we detect a strong interaction between Sir4^{PAD} and Esc1^C, as reported, when either protein is used as bait or prey (Andrulis *et al*, 2002; Figure 2C and D). We next tested the two *yku80* mutant proteins for two-hybrid interaction with Sir4C, an interaction previously reported for YKu70 (Tsukamoto *et al*, 1997). The mutant that supports targeted silencing (*yku80-9*) binds Sir4C robustly, while the one deficient for targeted silencing

Figure 2 Silencing-incompetent *yku80-4* and Sir4^{PAD} anchor chromatin at the nuclear periphery. (A) Scheme of the different domains and mutants used in targeted silencing and relocation assays. Dashed boxes correspond to putative coiled-coil domains. (B) Different LexA fusions were expressed from pAT4 for a targeted silencing assay monitored in GA-2050 strain carrying *TRP1* at *HMR* adjacent to a modified *E* silencer: E and B elements are replaced by four *lexA^{OP}* (designated Aeb). Serial 10-fold dilutions of GA-2050 expressing the indicated LexA fusions are grown on SC lacking leucine (–leu) or leucine and tryptophan (–leu –trp) to monitor the level of silencing (no growth on –trp). Sir2 overexpression is known to impair growth (Cockell *et al*, 2000). Efficiencies of *TRP1* expression and of Chr6^{int} relocalization (% zone 1; see Figure 3) are indicated on the right. (C–E) Two-hybrid interactions between the indicated fusion proteins were tested in GA-180 with appropriate bait and prey plasmids, and a lacZ reporter gene. Bait constructs of LexA–Sir4^{PAD} (C)–Esc1^C (D)–YKu70 or –YKu80 (E) fusions are constitutively expressed, while prey constructs (full-length Sir2p, subdomains of Sir3, Sir4 and Esc1) are galactose-inducible (pJG45–pGAL1). β-Galactosidase values are means of quadruplicate assay presented in arbitrary units.

(*yku80-4*) does not. Importantly, the *yku80-9*/Sir4C interaction does not require YKu70 (Figure 2E). Because LexA–*yku80-4* and LexA–Sir4^{PAD} neither promote targeted silencing nor bind the silencing relevant domains of SIR proteins, we are able to test their anchoring activity independently of Sir complex formation.

Chromatin relocation activities of YKu80, Sir4^{PAD}, Esc1 and Sir2

We expressed the LexA fusions discussed above in the tagged Chr6^{int} strain to monitor their abilities to relocalize DNA to the NE (Figure 3). Quantitative analysis shows that both

Figure 3 YKu80, Sir4^{PAD} and Esc1^C relocate chromatin to the nuclear periphery. The position of Chr6^{int} with respect to the three concentric nuclear zones was determined as in Figure 1 in GA-1461 expressing the following fusions: (A) LexA-yku80-4 and LexA-yku80-9; (B) aa 960–1262 of Sir4 (Sir4^{PAD}); (C) aa 1124–1658 of Esc1 (Esc1^C). (D) LexA-Sir2 was expressed either in GA-1461 or its *sir4* derivative (GA-1994). See Table I for statistics. * and the dotted bar are as in Figure 1.

YKu80 fusions produce significant relocation of the GFP focus to the outermost zone of the nucleus. The tethering activity is observed in G1- and S-phase cells (zone 1 values ranging from 51%, $p = 10^{-10}$ to 61%, $p = 10^{-6}$), although not in G2-phase cells, where the locus remains random (24 to 29%, $p = 0.055$). Statistical analyses are summarized in Table I. Importantly, the anchoring efficiencies of the two *yku80* mutants are very similar, despite their opposite behavior with respect to silencing. This demonstrates the separation of function we sought to obtain (Figure 3A).

Sir4^{PAD} and Esc1^C fusions are both extremely efficient at chromatin relocation (Figure 3B and C). The Sir4^{PAD} tethers Chr6^{int} to the NE efficiently throughout the cell cycle (60–65% in zone 1, $p = 10^{-14}$ – 10^{-2}), and Esc1^C does so even better (61–82% in zone 1, $p = 10^{-16}$ – 10^{-5}). Intriguingly, and in contrast to these, a LexA-YKu70 fusion anchors Chr6^{int} at the NE weakly and only during G1 phase (49% in zone 1; Table I).

Sir4^{PAD} is able to promote efficient chromatin association with the NE without interacting with other SIR components. This makes Sir4 a good candidate for the previously reported Ku-independent perinuclear anchor that sequesters silent

chromatin (Hediger *et al*, 2002a). If true, one would expect the tethering by other SIR factors to be *SIR4* dependent. Indeed, LexA-Sir2 can efficiently tether the tagged locus to the nuclear periphery and this is entirely lost in a *sir4* deletion (Figure 3D).

In conclusion, while YKu80, Sir4^{PAD}, Esc1^C and Sir2 all mediate significant chromatin relocation to the NE, we can separate chromatin anchoring from the establishment of silenced chromatin for Sir4^{PAD} and *yku80-4* fusions (Figures 2 and 3). Because they possess silencing-independent anchorage functions, we propose that Sir4 and YKu80 have direct rather than indirect roles in perinuclear tethering of chromatin.

Association of Esc1p with the NE in the absence of SIRs, Ku or silent chromatin

Unlike Sir4^{PAD} and *yku80-4*, Esc1^C promotes both silencing, presumably through its affinity for Sir4, and chromatin anchoring. To distinguish its role as a structural element as opposed to a silent chromatin factor, we determined Esc1 localization in silencing-deficient cells, since integral silent chromatin factors generally become dispersed from perinuclear foci when TPE is lost (Gotta *et al*, 1996; Martin *et al*, 1999).

Previous work has shown that overexpressed Esc1 is perinuclear (Andrulis *et al*, 2002). Fearing artifacts of overexpression, we created 3' fusions of Esc1 with either GFP or Myc epitopes by integration at the endogenous *ESC1* locus. These cells have no defects in TPE nor in NE morphology. Live epifluorescence of Esc1-GFP reveals a patchy perinuclear localization that overlaps only partially with CFP-tagged nuclear pores (Figure 4B), and the double Nop1-Esc1 labelling shows that Esc1-GFP is systematically excluded from the zone adjacent to the nucleolus (Figure 4A). Using an immunofluorescence nanogold labelling technique, we next localized Esc1 by both confocal and electron microscopy (Figure 4C and D). Electron micrographs show that Esc1 localizes mainly to the inner membrane of the NE between nuclear pores. Indeed, scoring gold particles in 50 cells we note that 85% (501/587) are within 100 nm of the NE (see table in Figure 4D). Of these, only 4% are located within 100 nm of a visible nuclear pore.

To determine whether the positioning of Esc1 is influenced by pore distribution, we examined its localization in a *nup133Δ* strain, a mutation that causes nuclear pores to cluster (Doye *et al*, 1994). Esc1-GFP remains evenly distributed around the nucleus despite the concentration of pores in one or two foci (Nup49-CFP; Figure 4B). This shows that Esc1 distribution, like that of telomeres (Hediger *et al*, 2002a), is unlinked to that of nuclear pores. Finally, we show that the localization of Esc1 is unchanged in strains mutant for *yku70*, *sir4* or both, demonstrating that its perinuclear localization does not depend on either the presence of a functional Ku heterodimer or on silencing (Figure 4B, and data not shown). Thus Esc1 is a chromatin anchor whose localization is independent of both telomeric repression and telomere localization.

Esc1 and the Ku complex define two pathways for Sir4^{PAD} anchorage

Because Sir4 interacts with both YKu70/YKu80 and Esc1, it was essential to investigate the interdependence of the

Table 1 Localization of lacop⁻-tagged loci (n) and significance (p value) for zone 1 enrichment

	WT	<i>yku70</i>	<i>esc1</i>	<i>sir4</i>	<i>yku70 esc1</i>
G1 phase					
<i>Chr 6^{int} + anchors</i>					
LexA-Esc1 ^C % zone 1	82	71	42	60	ND
<i>n;p</i>	144;<1 × 10 ⁻¹⁶	78;3 × 10 ⁻¹²	83;0.1	172;2 × 10 ⁻¹³	
LexA-yku70 % zone 1	49	ND	ND	ND	ND
<i>n;p</i>	349;1 × 10 ⁻⁹				
LexA-yku80-4 % zone 1	51	37	50	47	ND
<i>n;p</i>	296;1 × 10 ⁻¹⁰	130;0.4	240;8 × 10 ⁻⁷	274;3 × 10 ⁻⁶	
LexA-yku80-9 % zone 1	53	ND	ND	ND	ND
<i>n;p</i>	296;2 × 10 ⁻⁵				
LexA-Sir4 ^{PAD} % zone 1	65	64	64	77	36
<i>n;p</i>	131;1 × 10 ⁻¹⁴	247;<1 × 10 ⁻¹⁶	182;<1 × 10 ⁻¹⁶	60;<1 × 10 ⁻¹²	140;0.6
LexA-Sir2 % zone 1	56	ND	ND	33	ND
<i>n;p</i>	172;1 × 10 ⁻¹⁰			240;0.8	
LexA-Yif1 % zone 1	54	55	57	51	59
<i>n;p</i>	197;1 × 10 ⁻⁹	92;1 × 10 ⁻⁶	99;9 × 10 ⁻⁷	121;3 × 10 ⁻⁵	141;1 × 10 ⁻¹⁰
<i>Telomeres</i>					
Tel 14L % zone 1	53	42	50	36	40
<i>n;p</i>	257;3 × 10 ⁻¹¹	198;7 × 10 ⁻³	291;2 × 10 ⁻⁹	292;0.34	361;1 × 10 ⁻²
Tel 6R ^t % zone 1	52	47	41	43	38
<i>n;p</i>	431 <1 × 10 ⁻¹⁹	227;2 × 10 ⁻⁵	206;2 × 10 ⁻²	303;4 × 10 ⁻⁴	172;0.2
S phase					
<i>Chr 6^{int} + anchors</i>					
LexA-Esc1 ^C % zone 1	73	62	55	60	ND
<i>n;p</i>	94;1 × 10 ⁻¹⁶	26;2 × 10 ⁻³	38;3 × 10 ⁻³	48;7 × 10 ⁻⁵	
LexA-yku70 % zone 1	39	ND	ND	ND	ND
<i>n;p</i>	155;0.11				
LexA-yku80-4 % zone 1	56	50	30	42	ND
<i>n;p</i>	96;1 × 10 ⁻⁶	66;4 × 10 ⁻³	80;0.7	144;2 × 10 ⁻²	
LexA-yku80-9 % zone 1	60	ND	ND	ND	ND
<i>n;p</i>	38;3 × 10 ⁻⁴				
LexA-Sir4 ^{PAD} % zone 1	61	68	57	70	40
<i>n;p</i>	81;1 × 10 ⁻⁷	98;2 × 10 ⁻¹³	116;7 × 10 ⁻⁸	43;5 × 10 ⁻⁷	63;0.3
LexA-Sir2 % zone 1	50	ND	ND	39	ND
<i>n;p</i>	52;1 × 10 ⁻²			98;0.2	
LexA-Yif1 % zone 1	69	69	51	54	49
<i>n;p</i>	98;1 × 10 ⁻¹⁴	52;4 × 10 ⁻⁸	39;1.3 × 10 ⁻²	46;2 × 10 ⁻³	70;7 × 10 ⁻³
<i>Telomeres</i>					
Tel 14L % zone 1	58	62	61	40	37
<i>n;p</i>	153;2 × 10 ⁻¹¹	111;1 × 10 ⁻¹⁰	135;4 × 10 ⁻¹²	106;0.17	117;0.4
Tel 6R ^t % zone 1	58	59	43	40	36
<i>n;p</i>	195;1 × 10 ⁻¹³	131;7 × 10 ⁻¹⁰	92;4 × 10 ⁻²	127;0.1	97;0.56

Ku-Sir4^{PAD}- and Esc1^C-mediated anchoring functions. To do this, we scored for GFP-Chr6^{int} relocation by all three LexA fusions in strains bearing deletions of *yku70*, *esc1* or *sir4* (Figure 5).

LexA-Sir4^{PAD} chromatin relocation activity was unaffected by loss of either Esc1 or YKu70 individually (Figure 5A), yet we see a complete loss of the Sir4^{PAD} tethering activity in the absence of both Esc1 and Ku, throughout the cell cycle. This result strongly suggests that Esc1 and Ku provide two parallel anchorage pathways for Sir4^{PAD} (Figure 5B). Indeed, the Sir4-Esc1 interaction can account for the yKu-independent anchoring described previously (Tham *et al*, 2001; Hediger *et al*, 2002a). Intriguingly, LexA-Sir4^{PAD} anchors more efficiently in the absence of endogenous Sir4. This latter could reflect competition between the full-length Sir4 and Sir4^{PAD} for a limiting number of perinuclear binding sites.

We next examined whether anchoring via Esc1^C requires Sir4 or Ku. Consistent with Esc1 localization results (Figure 4B), the Esc1^C-mediated relocation of Chr6^{int} shows

little dependence on either *SIR4* or *YKU70* (Figure 5C). It is sensitive, on the other hand, to the deletion of the genomic *ESC1* gene. The simplest explanation for this is that Esc1 homodimerizes through its C-terminal domain, which is present in the LexA fusion, but requires its N-terminus for perinuclear localization. It is not clear why the requirement for full-length Esc1 is more pronounced in G1 phase. To rule out nonspecific effects of these mutations, we show that the anchoring activity of LexA-Yif1 promotes significant anchoring in all deletion strains tested (Figure 1E, Table 1).

The YKu80 anchoring activity requires YKu70 only in G1-phase cells

We next examined whether the chromatin relocation activity associated with yku80-4 requires the Ku heterodimer. Surprisingly, we find that yku80-4-mediated anchoring is lost in the *yku70* mutant in G1 phase, yet is maintained in this strain during S phase (Figure 5D). It appears, therefore, that there is one anchorage site in G1 for the Ku heterodimer,

Figure 4 Esc1 is localized on the nucleoplasmic side of the inner nuclear membrane independently of YKu70 and Sir4. (A) Live visualization of GA-2121 bearing endogenous Esc1-GFP and the nucleolar protein Nop1 fused to CFP. Equatorial sections of nuclei after deconvolution of wide-field Z-stacks of normalized GFP and CFP signals are shown. Bar = 1 μ m. (B) Endogenous Esc1p and Nup49 have been tagged with GFP and CFP, respectively, in wild type (, GA-2122), *sir4* (GA-2125), *yku70* (GA-2126) and *nup133* (GA-2472) strains as indicated. Focal sections after deconvolution are shown. Serial confocal sections (C) or electron micrographs (D) show endogenous Esc1-13Myc immunolocalized using monoclonal 9E10 revealed by a fluoronanogold Alexa⁴⁸⁸ anti-mouse antibody. The arrow indicates a nuclear pore. White bars = 1 μ m. Black bar = 0.2 μ m. Statistics are presented for cells treated identically either with anti-Myc Mab or no primary antibody.

Figure 5 Redundant pathways anchor *yku80-4* and *Sir4^{PAD}* at the nuclear periphery. (A–D) LexA fusions were expressed in GA-1461 bearing complete disruptions of *yku70*, *esc1*, *sir4* and *yku70 esc1*, as indicated. Chr6^{int} position was scored as in Figure 1, and zone 1 values are compared among strains. Statistics are in Table I, and the red * indicates that values are indistinguishable from a random distribution ($p > 0.05$). (B) Scheme of *Sir4^{PAD}* and YKu80 anchoring pathways.

and another in S phase that recognizes YKu80 directly. Coupled with the fact that LexA-YKu70 can relocate chromatin to the periphery only in G1 phase (Table I), we propose that

cell-cycle-regulated changes in the Ku complex influence anchoring. We further observe that the *yku80-4* anchorage function is sensitive to deletion of *ESC1* uniquely in S phase (Figure 5D). This suggests an S-phase-specific interaction between YKu80 and Esc1, or between YKu80 and an Esc1-dependent factor, distinct from the G1-phase Ku anchor (Figure 5D).

In summary, YKu80-mediated anchoring in G1 phase requires YKu70 but is independent of Esc1, while in S-phase cells it requires Esc1 (Figure 5B). Importantly, these cell-cycle variations mirror differences observed for the positioning of GFP-tagged telomeres in *yku70* mutants (Hediger *et al*, 2002a).

Esc1 tethers natural telomeres and promotes repression

Recent analysis of telomere localization in living cells has shown telomere-specific effect of YKU mutations: Tel 6R was randomly localized in a *yku70* deletion strain, while Tel 14L and a truncated Tel 6R^t remained significantly perinuclear. Importantly, however, the residual Ku-independent anchoring was lost upon *SIR4* deletion (Hediger *et al*, 2002a).

To test whether the Sir4-dependent anchorage of telomeres involves the *Sir4^{PAD}*-Esc1 interaction, we deleted *ESC1* and *YKU70* in strains bearing GFP-tagged telomeres and scored telomere position relative to the NE. Whereas the deletion of *ESC1* alone has little effect, the *esc1* and *yku70* effects are additive for Tel 14L delocalization, leading to a random distribution in S phase (Figure 6A). For Tel 6R^t, the Esc1

Figure 6 The Ku heterodimer and Esc1 anchor telomeres *in vivo* and regulate TPE. (A) Tel 14L position was determined in G1- and S-phase cells of GA-1985, *yku70* (GA-1983), *esc1* (GA-2074) and *yku70 esc1* (GA-2082) strains as in Figure 1. Graphs and symbols are as in Figure 5, and for statistics see Table I. (B) Tel 6R¹ localization was determined in WT (GA-1917), *yku70* (GA-1918), *esc1* (GA-2229) and *yku70 esc1* (GA-2230) strains as in (A). (C) Silencing assay on WT (LM11), *yku70* (GA-1055), *esc1* (GA-2411) strains bearing Tel7L::URA3. Serial 10-fold dilutions are grown on plates with or without 5-FOA (growth on 5-FOA reflects silencing). (D) Tel 6R¹::ADE2 silencing assay on WT, *yku70* and *esc1* strains (same strains as in (B)) reveals the degree of TPE (white is depressed). (E) Tel 14L and Tel 6R¹ anchoring depends on YKu70 and Esc1. (F) ChIP was performed $\pm$ CH₂O fixation with either anti-Myc (Esc1-13Myc) or anti-HA (background). Real-time PCR monitors the accumulation rate with primer pairs 800 bp from the Tel 6R TG repeat or within the SMC2 gene. Fold increase reflects the ratio of the Tel 6R signal divided by that of SMC2 after normalization to the ratio obtained in the input fraction. A mean of triplicate real-time PCR from two independent experiments is shown with standard deviation.

dependence even is more pronounced: *esc1* deletion alone delocalizes Tel 6R¹ significantly, and random distributions are detected in both G1- and S-phase *esc1 yku70* cells (Figure 6B). Thus, the *esc1 yku70* double mutant mimics the *sir4 yku70* mutant with respect to both the Tel 6R¹ and Tel 14L localization (Hediger *et al*, 2002a), arguing that the two redundant pathways of telomere anchoring act directly through Ku and Esc1-Sir4, respectively. We observe an inherent cell-cycle variation in their use: the Ku anchor is dominant in G1 phase, while *yku70* deletion has little effect on anchorage in S phase (Figure 6E).

Consistent with this redundancy, we note that the *esc1* deletion induces only a minor reduction in silencing efficiency (10- to 100-fold) for a URA3 reporter at Tel 7L (Andrulis *et al*, 2002; Figure 6C). To correlate the effects on

telomere position and silencing at a single telomere, we monitored repression of an ADE2 reporter inserted in the *lac*^{OP}-tagged Tel 6R¹. Consistent with the partial Tel 6R¹ delocalization observed in the *esc1* null strain, ADE2 becomes partially derepressed, producing pink rather than the red/white sector colonies that characterize wild-type cells (Figure 6D). Loss of *yku70* leads to near total derepression because Sir4 recruitment is impaired. Neither a *yku70* nor *esc1* deletion has any effect on URA3 or ADE2 expression at their normal genomic loci based on genome-wide microarray analysis (data not shown). The fact that repression and telomere positioning are altered at the same telomere (Tel 6R¹::ADE2) in the same strain supports the notion that Esc1-mediated telomere anchoring directly influences TPE.

As further proof that Esc1 is the physiological tether of at least a subset of natural telomeres, we assessed the presence of Esc1 at telomeres by chromatin immunoprecipitation (ChIP). Using an endogenous Myc-tagged Esc1 and quantitative real-time PCR, we find that a DNA fragment located 600 bp away from the TG repeat of the native Tel 6R was enriched 2.8-fold over a non-telomeric fragment (located within SMC2 gene; Figure 6F). Enrichment is calculated from real-time PCR accumulation curves that are normalized to signal ratios in the input DNA. This enrichment is not detected when crosslinking is omitted or a control antibody is used. The presence of Ku and SIR complexes at this telomere was previously shown (Martin *et al*, 1999).

Esc1/Sir4 colocalization increases as TPE improves

If Esc1-Sir4 interaction does anchor a subpopulation of silent yeast telomeres, one might expect that Sir4 foci would colocalize with Esc1 at the NE. To address this point, we performed double immunolocalization of Esc1-13Myc and Sir4 in a GFP-Nup49-tagged strain. We find that 26% of the Sir4 foci colocalize with Esc1 (Figure 7C). This is consistent with the partial colocalization monitored for Sir4 and overexpressed Esc1-GFP (Andrulis *et al*, 2002). In contrast, we find that only 12% of the Sir4 foci colocalize with the pore staining in the same cells (Figure 7B). We then asked if we could improve the amount of Esc1/Sir4 colocalization by increasing the amount of Sir4 associated with telomeres. This can be achieved by eliminating the Rap1 interacting factor Rif1, which competes with Sir4 for binding to the telomere-bound Rap1. Loss of Rif1 is known to improve Sir4 recruitment by Rap1, which in turn improves silencing efficiency (Buck and Shore, 1995). We show in Figure 7D that the colocalization of Esc1 and Sir4 increases to 55% of the Sir4 foci in *rif1* deletion cells. Thus, the number of Sir4 foci that colocalize with Esc1 increases when silencing improves, consistent with the idea that Esc1 anchors telomeric silent chromatin through Sir4.

Discussion

Many results suggest a correlation between perinuclear position and silencing in yeast. Nonetheless, establishing a causal relationship between subnuclear organization and repression has been difficult, because the mutants known to alter the position of silent domains are also directly implicated in the repression machinery. Here we have exploited a chromatin relocation assay that allows us to monitor protein domains that determine subnuclear position independently of

Figure 7 Sir4 foci-Esc1 colocalization increases with increased TPE. (A) Equatorial confocal sections with Sir4 (red) and Esc1-13Myc (blue) revealed by immunofluorescence and Nup49-GFP (green) by direct fluorescence in GA-2110. (B, C) To facilitate comparison between Nup49-GFP/Sir4 and Esc1-13Myc/Sir4 colocalization, the same cells are shown with Sir4 (red) combined with either Nup49-GFP (B) or Esc1-13Myc (C) in green. The inset shows an enlargement ($\times 3$) of overlapping foci. (B', C') Colocalizing foci are pseudocolored in white for each staining, and the percentage of Sir4 foci that overlap with either Nup49 (B') or Esc1 (C') is indicated. n = number of Sir4 foci counted. (D) Immunostaining of Sir4 (red) and Esc1-13Myc (green) in GA-2145 (*rif1*). (D') as in (C'). Bar: 1 μ m. (E) Dual pathways mediated by Ku and Sir4-Esc1 anchor yeast telomeres both in the absence and presence of SIR-mediated repression. Rap1-bound Sir4 can bring a telomere to the NE through interaction with either Ku or Esc1. Sir4^{PAD} promotes the perinuclear anchoring of silent chromatin through either pathway.

their interaction with silencing factors. The minimal protein domains that are sufficient to relocate a tagged locus to the NE include YKu80, Sir4^{PAD} and the C-terminal domain of Esc1. By coupling this chromatin relocation assay with complete gene disruptions of interacting factors, we have identified interactions that can account for the redundant pathways that anchor telomeres in living cells. Indeed, monitoring the position of tagged telomeres in double mutants shows that these same proteins tether yeast telomeres *in vivo*.

Esc1: a nonchromatin element involved in telomere positioning

Esc1 was originally identified as a ligand for the Sir4^{PAD} fragment, and was recovered in a screen for fusion proteins that enhance subtelomeric repression in *rap1 Δ C* cells, in which SIR proteins are dispersed from telomeres (Andrulis *et al*, 2002). Using immunoEM studies, we show that Esc1 is almost exclusively localized along the inner face of the NE, yet it does not colocalize with nuclear pores, nor is its

position affected by mutants that lead to pore clustering (Figure 4). Esc1 has no membrane-spanning domain but appears to bear significant post-translational modification. Its solubilization properties are consistent with membrane association through lipid modification (data not shown).

When fused to a DNA-binding domain, Esc1^C very efficiently relocates nontelomeric chromatin to the nuclear periphery. The chromatin relocation activity of Esc1^C requires full-length Esc1 protein, suggesting that its C-terminus mediates homodimerization. Endogenous Esc1 is also necessary for the perinuclear tethering of chromatin by Sir4^{PAD} in the absence of YKu70 and for the S-phase-specific anchorage mediated by YKu80. Finally, and most importantly, we have shown that Esc1 cooperates with Ku to anchor natural telomeres, being a physiological anchor for both Sir4 and silent chromatin. Consistently, Sir4 is homogeneously distributed throughout the nucleoplasm in a double *yku70 esc1* mutant, whereas residual Sir4 foci can still be detected in the single *yku70* mutant (data not shown). In support of these mutant studies, Esc1 can be recovered crosslinked to

telomeres and we observe a positive correlation between Sir4/Esc1 colocalization and TPE (Figure 7).

The fact that silent telomeres only partially colocalize with Esc1 suggests that there is more than one anchorage site for silent chromatin. Indeed, Sir4 itself can tether chromatin through Ku as well as through Esc1, and we cannot exclude that other weak anchorage sites exist for other telomeres. The dual pathways for tethering Sir4 are mirrored in the dual pathways that tether telomeres (Hediger *et al*, 2002a), and we predict that, as for telomeres, both pathways must be disrupted to release silent chromatin (Figure 7E). In recent studies that allow the separation of a silent chromatin domain from its chromosomal context, we find that the combined deletion of *yku70* and *esc1*, but neither mutation alone, releases an excised silent chromatin ring from perinuclear constraints (Gartenberg *et al*, submitted).

Because endogenous Esc1 localization, as well as chromatin tethering through the targeted LexA-Esc1^C fusion, requires neither Sir4, Ku nor chromatin repression, we propose that Esc1 is a structural component of the yeast nuclear periphery, possibly replacing lamin-associated proteins as a chromatin anchorage site in yeast. Esc1 is highly acidic, with three short coiled-coil domains that may be critical for homodimerization and interaction with other proteins. There are no obvious Esc1 homologs in animal genomes, although recent data suggest that emerin and the lamin B receptor are involved in chromatin anchoring at the mammalian nuclear periphery (Holaska *et al*, 2002). The functional similarities between emerin and Esc1 include evidence that neither is an integral chromatin factor, yet both interact with chromatin components to recruit DNA to the NE. By immunoEM we monitor on average 12 Esc1-specific grains per 90 nm nuclear section. This, together with its weak fluorescence, suggests that Esc1 is a low-abundance protein.

Cell-cycle variations in anchoring pathways

The relative efficiency of the Ku and Sir4-Esc1 anchorage pathways varies with the cell cycle. Strikingly, the cell-cycle variations that were reported for natural telomere anchorage in yeast (Tham *et al*, 2001; Hediger *et al*, 2002a) were accurately reproduced by the efficiency of LexA-yku80-4 tethering. The Ku pathway is prevalent in G1-phase cells, while the Esc1 pathway dominates S-phase anchoring; both are compromised in metaphase cells (Tham *et al*, 2001; Hediger *et al*, 2002a).

Cell-cycle modification of Ku, Sir4 or their NE partners could be responsible for these cell-cycle variations in chromatin anchoring pathways. Indeed, Ku interaction with the RNA component of telomerase may be under cell-cycle control (Stellwagen *et al*, 2003), and telomeric chromatin has been shown to change its composition through the cell cycle (Smith *et al*, 2003). We feel it is unlikely that the S-phase changes in anchoring reflect a destabilization of chromatin structure due to passage of the replication fork, because telomeres are replicated late in S phase, while the change in telomere/chromatin anchoring is detected as soon as buds emerge. On the other hand, these cell-cycle fluctuations in anchorage mechanisms correlate well with the fact that yeast chromatin must traverse S phase, but not necessarily replicate, to establish SIR-mediated repression (Kirchmaier and Rine, 2001; Li *et al*, 2001).

Ku and Sir4 function as perinuclear anchors independently of silencing function

The separation of tethering and silencing activities of the Ku and SIR complexes is manifest in the *yku80-4* allele and the Sir4^{PAD} domain, which efficiently relocate a targeted locus to the NE, but which are unable to promote targeted silencing or bind other SIR proteins. Recent reports have described four mutant forms of *yku80* bearing substitutions in a conserved region of the gene. Of these, two coincide fortuitously with our *yku80-4* mutation (residue 437; Bertuch and Lundblad, 2003; Roy *et al*, 2004). Bertuch and Lundblad show that the *yku80-4* mutant loses TPE and telomere protection activities, but not the nonhomologous end-joining function of Ku. In a related *yku80* allele that bears P₄₃₇A and F₄₃₈A substitutions, interaction with the N-terminus of Sir4 is impaired (Roy *et al*, 2004). These results support our argument that this domain contributes to silencing by mediating YKu80-Sir4 interactions.

Anchoring and silencing: the self-perpetuation of subnuclear compartmentation

An important conclusion of this study is that perinuclear chromatin anchoring can occur prior to or independently of transcriptional repression (see Figure 7E). Nonetheless, our results suggest a functional relationship between anchoring and silencing, because the two are promoted by the same proteins (Ku and Sir4). Moreover, silencing and anchoring, while separable, reinforce each other.

We propose that pools of SIR factors result from the initial anchoring of telomeres through Ku-NE interactions. Indeed, by grouping nonsilent telomeres at the NE, the TG repeat-bound Rap1 can directly recruit Sir4 to this zone. The resulting high density of Sir4-binding chromatin would in turn attract Sir2 and Sir3 through protein-protein interactions. The concentrated Sir factors should then reinforce repression of loci located within this compartment. As silent chromatin spreads, the number of Sir4 molecules bound will increase, thus reinforcing interaction with the NE. Consistent with this model, ChIP studies suggest that Sir4 is the first component bound, and it may be limiting for nucleating the assembly of the Sir2/3/4 complex (Bourns *et al*, 1998; Hoppe *et al*, 2002; Luo *et al*, 2002; Rusche *et al*, 2002).

The dual mechanism for localization described here (i.e. the ability of Ku to anchor in the absence of silencing and the binding of silent chromatin through Sir4) suggests a self-perpetuating mechanism for the formation telomere-associated SIR foci. We suggest that the clustering of repetitive sequence elements and spatial concentration of their ligands may be a general mechanism for the formation of nuclear subcompartments with biochemically distinct characters. In higher eucaryotes, similar mechanisms may facilitate the clustering of centromeric satellite and the propagation of heterochromatin, although in some cases the association of repetitive sequences *in trans* might eliminate the need for NE anchoring domains.

Materials and methods

Plasmid, strains and yeast methods

All strains used are indicated in Table II. PCR-based gene deletions (Longtine *et al*, 1998) created complete null alleles of *yku70*, *esc1* and *sir4* that were checked by PCR and phenotypic assays.

The endogenous *ESC1* gene was tagged C-terminally with either GFP or 13Myc epitope as described (Goldstein and McCusker, 1999).

Standard culture conditions at 30°C were used unless otherwise indicated (Rose *et al*, 1990). Silencing assays were performed as described (Gotta *et al*, 1998). All *lac*^{op}-tagged strains are based on GA-1320 (Heun *et al*, 2001a). Integration of <10 kb of multimerized *lac* operators and four LexA operators 272 bp upstream ARS607 (Chr6^{int}) into GA-1320 (Heun *et al*, 2001a) gave rise to GA-1461.

All LexA fusions were cloned into pAT4, a 2 µm *LEU2* vector expressing LexA from the ADH promoter, which was obtained by fragment fusion from pBMT116 and pRS425. Fusion proteins were created by cloning appropriate gene fragments of Sir4^{PAD} (aa 960–1262), Esc1^C (aa 1124–1658), Yif1 and full-length Sir2 from existing plasmids: pLS4, pEDA114 and pGBC12–YIF1 (Ansari and Gartenberg, 1997; Andrulis *et al*, 1998, 2002; Cockell *et al*, 2000). The mutants *yku80-4* and *yku80-9* were produced by degenerate PCR and chosen for study because they do not complement TPE. Bidirectional sequencing identified three substitutions (L149V, N349D and E518A) in *yku80-9*, and one in *yku80-4* (P437L), which by chance is identical to and named for one recently published (Bertuch and Lundblad, 2003).

Liquid culture two-hybrid analysis was performed as described (Aushubel *et al*, 1994). The *lacZ*-reporter pSH18-34, pEG-202-derived bait, and pJG4-6-derived prey plasmids were transformed into GA-180. Quantitative β-galactosidase assays were performed on permeabilized cells, using four independent transformants per sample (Adams *et al*, 1997) after 6 h on 2% galactose.

Immunological techniques

For IF assay, cells were fixed prior to spheroplasting, and stained with affinity-purified rabbit anti-Sir4 whose specificity has been previously characterized (Gotta *et al*, 1996), the anti-pore MAb414 monoclonal (CRP Inc.) or the anti-Myc MAb 9E10.

For ImmunoEM, cells were permeabilized for 1 min with 0.1% Triton X-100 in phosphate buffer after fixation and spheroplasting (Gotta *et al*, 1996), and incubated sequentially with MAb 9E10 and IgG Fab fluoronanogold antibody coupled to Alexa 488 (Nanoprobe, Inc., Yaphank, NY). After washing, part of the cells was analyzed by confocal microscopy (Leica SP2 AOBS; Leica Microsystems, Mannheim, Germany), and part fixed with 2% glutar-

aldehyde for 20 min. Gold enhancement was performed with Goldenhance-EM (Nanoprobe, Inc., Yaphank, NY) followed by fixation with OsO₄ and uranyl acetate. Epon embedding, ultrathin sectioning and EM images capture (Philips TEM 410, Eindhoven, Netherlands) were as described (Bauer *et al*, 2001).

Live fluorescence microscopy and quantitation

Cultures were grown in SD-His, to <1 × 10⁷ cells/ml. Live microscopy was performed between 22 and 25°C as described (Heun *et al*, 2001a). A Tillvision driven Olympus IX70 microscope was used to capture 19-image stacks of 170 nm step size. Cell-cycle stage classification was as follows: unbudded cells = G1 phase; budded cells with round nucleus not at the bud neck = S phase; large bud with bud-neck nucleus = G2 phase. In the focal plane in which the GFP spot is brightest, its position is mapped to one of three concentric zones of equal surface by dividing the spot-to-pore distance by the nuclear diameter (the width of the three equal surface zones is also normalized to the diameter; Hediger *et al*, 2002a). We eliminate cells in which the GFP spot falls into the top or bottom three focal planes because of the error arising from the small zone width and background signal from the pore. Statistical analysis used a proportion method to compare zone I percentages among different samples or with the predicted random distribution. Significance was determined with a 95% confidence interval. Time-lapse imaging was performed using a Zeiss LSM510 confocal microscope as described (Heun *et al*, 2001b). Deconvolution was performed using Metamorph Software with the following parameters: fast algorithm with five iterations, sigma 0.7 and a frequency of 4.

Chromatin immunoprecipitation

ChIP was performed with or without a 15 min fixation with formaldehyde on GA-2141 as described by Cobb *et al* (2003) using Mab against Myc (9E10) or HA epitopes (12C5A), and real-time PCR (Perkin-Elmer ABI Prism 7700 Sequence Detector System and software). Real-time PCR monitors the 'threshold cycle (C_T)' at which the exponential curve of the accumulated product passes a threshold. Bar heights represent the ratio of the specific signal divided by that of an internal locus (*SMC2*), after normalization to the ratio obtained in the input fraction, which monitors relative probe efficiency. Fold increase = 2^[(C_T^{IP_{SMC2}} - C_T^{IP_{telo}}) - (C_T^{input_{SMC2}} - C_T^{input_{telo}})].

Table II Yeast strains used in this study

Name	Genotype	Reference
GA-1320	MATa ade2-1 can1-100 his3-11,-15::GFP-LacI-HIS3 trp1-1, ura3-1, leu2-3,-112 nup49::NUP49-GFP-URA3	Heun <i>et al</i> (2001a)
GA-1461	GA-1320 Chr6 ^{int} ::lac ^{op} -lexA ^{op} -TRP1 (Chr6 ^{int} is 272 bp upstream ARS607).	This study
GA-1949	GA-1461 yku70::URA3c (= URA3 from <i>C. albicans</i>)	This study
GA-1950	GA-1461 <i>esc1::kanMX6</i>	This study
GA-1994	GA-1461 <i>sir4::kanMX6</i>	This study
GA-1996	GA-1950 <i>yku70::URA3c</i>	This study
GA-2111	GA-1461 <i>sir2::kanMX6</i>	This study
GA-1985	GA-1320 TEL 14L::lacO-lexA ^{op} -TRP1	Hediger <i>et al</i> (2002a)
GA-1983	GA-1985 <i>yku70::URA3c</i>	Hediger <i>et al</i> (2002a)
GA-2074	GA-1985 <i>esc1::kanMX6</i>	This study
GA-2082	GA-2074 <i>yku70::URA3c</i>	This study
GA-2110	GA-1461 <i>ESC1-13myc::kanMX6</i>	This study
GA-2050	MATα hmr Aeb 4lexA ^{op} hmr::TRP1	This study
GA-2122	GA-2050 Nup49::Nup49-CFP URA3 ESC1-GFP::kanMX6	This study
GA-2125	GA-2122 <i>sir4::HIS5</i>	This study
GA-2126	GA-2122 <i>yku70::HIS5</i>	This study
GA-180	MATa ade2-1 can1-100 his3-11,-15, trp1-1, ura3-1, leu2-3,-112	R Rothstein (W3031a)
GA-1917	GA-1320 TEL 6R::lac ^{op} -lexA ^{op} -TRP1-/-ADE2-TG = Tel 6R ^t	Hediger <i>et al</i> (2002a)
GA-1918	GA-1917 <i>yku70::URA3</i>	Hediger <i>et al</i> (2002a)
	GA-1917 <i>esc1::kanMX6</i>	This study
	GA-1918 <i>esc1::kanMX6</i>	This study
LM11	MATα ade2-1 can1-100 his3-11,-15, trp1-1, ura3-1, leu2-3,-112 Tel7L::URA3 lys2::HMLEI::ADE2	Maillet <i>et al</i> (1996)
GA-1055	LM11 <i>yku70::kanMX6</i>	Maillet <i>et al</i> (2001)
GA-2411	LM11 <i>esc1::kanMX6</i>	This study
GA-2141	LM11 <i>ESC1-13myc::kanMX6</i>	This study
GA-2145	GA-2141 rif1::TRP1	This study

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