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Ku-deficient yeast strains exhibit alternative states of silencing competence

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In *Saccharomyces cerevisiae*, efficient silencer function requires telomere proximity, i.e. compartments of the nucleoplasm enriched in silencing factors. Accordingly, silencers located far from telomeres function inefficiently. We show here that cells lacking yKu balance between two mitotically stable states of silencing competence. In one, a partial delocalization of telomeres and silencing factors throughout the nucleoplasm correlates with enhanced silencing at a non-telomeric locus, while in the other, telomeres retain their focal pattern of distribution and there is no repression at the non-telomeric locus, as observed in wild-type cells. The two states also differ in their level of residual telomeric silencing. These findings indicate the existence of a yKu-independent pathway of telomere clustering and Sir localization. Interestingly, this pathway appears to be under epigenetic control.

INTRODUCTION

The gene repression at specific loci plays a critical role in various cell programs. In the yeast *Saccharomyces cerevisiae*, silencing at telomeres and at the *HML* and *HMR* silent mating-type loci depends upon a proper intranuclear distribution of silencing factors (reviewed in Gotta and Gasser, 1996). At the silent mating-type loci, silencing is directed by *cis*-acting elements, termed the E and I silencers, while for telomeric position effect (TPE), multiple Rap1p molecules bind the telomeric TG_{1-3} repeats to nucleate silencing. Genetic studies have identified a number of *trans*-acting factors that are essential to silencing, including three silent information regulator (Sir) proteins (Sir2, 3 and 4) and the histones H3 and H4. Silencers and telomeres promote the formation of nucleoprotein complexes that are believed to nucleate the spreading of silent chromatin outward. The distance covered is limited by the dosage of certain *trans*-acting silencing factors and by *cis*-acting insulators (Strahl-Bolsinger *et al.*, 1997; Donze *et al.*, 1999; Fourel *et al.*, 1999; Pryde and Louis, 1999).

Rap1p, Sir3p and Sir4p are enriched in a limited number of foci located near the nuclear envelope that colocalize with clustered telomeres (Gotta *et al.*, 1996). The unequal distribution of these factors within the nucleoplasm delimits subnuclear compartments which are competent for silencing. Indeed, the focal pattern of Rap1 and Sir proteins is perturbed in a range of mutants that impair silencing (Palladino *et al.*, 1993; Cockell *et al.*, 1995; Hecht *et al.*, 1995). Furthermore, the capacity of a silencer to repress transcription is dependent upon its proximity to telomeres and to the nuclear periphery (Maillet *et al.*, 1996; Marcand *et al.*, 1996; Andrulis *et al.*, 1998). The weakness of silencers in a non-telomeric, ectopic context is best explained by a sequestration of most of the available Sir3 and Sir4 proteins at telomeric clusters. Interestingly, aging (Kennedy et al., 1997) and the response to double-strand breaks (Martin *et al.*, 1999; McAinsh *et al.*, 1999; Mills *et al.*, 1999) induce a redistribution of telomeric factors within the nucleus. The formation of a silencing compartment at telomeres depends upon both the capacity of silencing factors to bind telomeric components and an appropriate subnuclear localization of telomeres. Interestingly, the yKu complex appears to be involved in both these processes.

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The yKu heterodimer binds to chromosome ends, and deletions of either *YKU70* or *YKU80*, which encode the two yKu subunits, affect the configuration of telomeric DNA by decreasing its size and increasing its single-strand character (Porter *et al.*, 1996; Boulton and Jackson, 1998; Gravel *et al.*, 1998). Furthermore, in cells lacking yKu, subtelomeric silencing is severely compromised (Gravel *et al.*, 1998; Laroche *et al.*, 1998; Nugent *et al.*, 1998; Fourel *et al.*, 1999; Mishra and Shore, 1999; Pryde and Louis, 1999; Galy *et al.*, 2000). Interestingly, in yKu-deficient cells, the perinuclear localization of telomeric DNA, Sir proteins and Rap1p is lost, such that all have a more dispersed pattern throughout the nucleoplasm (Laroche *et al.*, 1998). The delocalization of telomeres cannot be interpreted solely as a silencing defect, since the deletion of *SIR3* or *SIR4* does not greatly modify the focal pattern of telomeric DNA (Gotta *et al.*, 1996). Rather, it appears that yKu helps tether telomeric DNA to the nuclear envelope (Laroche *et al.*, 1998; Galy *et al.*, 2000).

Since the lack of ectopic silencing in wild-type cells is likely to reflect competition between telomeres and internal silencers for the pool of Sir proteins, we reasoned that the release of Rap1p and Sir from telomeres in yKu-deficient mutants should lead to efficient ectopic silencing. Our results indeed confirm the existence of a subpopulation of yKu$^-$ cells that is competent for ectopic silencing. Surprisingly, these cells can switch to another state which is incompetent for ectopic silencing and has a more wild-type subnuclear distribution of telomeric components.

RESULTS

An *ADE2* expression color assay for ectopic silencing

In order to monitor silencing at non-telomeric or ectopic sites, we took advantage of the fact that the *ADE2* gene expression can be visualized at the clonal level by a colony color assay (Gottschling *et al.*, 1990). When an *ADE2* gene bracketed by two silencers is integrated at the non-telomeric *LYS2* locus (Figure 1A), the resulting colonies are white, indicative of full *ADE2* expression (Figure 1B, lines a–c). Overexpression of Sir3p, or Sir3p and Sir4p, induces ectopic silencing, as visualized by red or pink colony color (Figure 1B, lines d and e). The overexpression of Sir4p alone also results in an increase in ectopic silencing but to a lesser extent than Sir3p (Figure 1B, line f). These results confirm that Sir-dependent silencing is inoperative when silencers are integrated far from a telomere, but can be revealed by increasing the dosage of Sir proteins (Maillet *et al.*, 1996). Using the same *ADE2*/silencer construct, it was shown that the release of telomeric Sir proteins upon DNA damage also partially restores ectopic silencing (Martin *et al.*, 1999).

Inactivation of yKu results in a variegated ectopic silencing

Seven out of 17 *yku70*::*kanMX4* (hereafter *yku70*) primary transformants of a W303-1B derivative strain carrying the *ADE2*/silencer construct (LM11) formed pink colonies, while 10 other transformants produced white colonies, indistinguishable from the parental strain (data not shown). These were obtained in three independent experiments and were confirmed as genomic null alleles by Southern blot. The ectopic silencing of *ADE2*, as revealed by the pink color of some *yku70* transformants of LM11, was lost upon overexpression of the C-terminal domain of Sir4p (data not shown), consistent with a negative *trans*-dominant effect on Sir-dependent silencing (Cockell *et al.*, 1995), or upon deletion of *SIR4* (F. Hediger, personal communication). These data confirm that *ADE2* repression in pink *yku70* cells reflects a genuine Sir-mediated silencing. Importantly, the ability to produce two color phenotypes in a *YKU70* disrupt is not restricted to a W303-1B genetic background: when the *ADE2*/silencers construct is inserted at *LYS2* in a S150-2B derivative strain (EG204), 7 out of 9 primary *yku70* transformants analyzed are pink (data not shown).

The ectopic silencing detected in pink *yku70* cells is not due to a general improvement of silencer-mediated repression. Indeed, a *HML*-I silencer deleted for the Abf1-binding site, known to confer an attenuated silencing in wild-type cells (Boscheron *et al.*, 1996), displays a further decreased silencing capacity in *yku70* cells (data not shown). Therefore, the effects of yKu deficiency on silencer-mediated repression appear to be the opposite at *HML* and at *LYS2*: a leaky silencer becomes weaker at *HML*, while wild-type silencer function is stronger at *LYS2*. Since *yku70* disruption leads to the partial delocalization of telomeric DNA and silencing factors throughout the nucleoplasm (Laroche *et al.*, 1998), and since *HML* is sequestered at the nuclear periphery (Laroche *et al.*, 2000), this difference might be explained by a decrease in the concentration of silencing factors at *HML* concomitant with an elevated dosage of these factors at non-telomeric locations.

Several arguments indicate that the pink/white phenotypes arise from a loss-of-function of yKu. (i) The repression at *LYS2* is dependent upon the absence of *YKU70*, since the introduction of a complementing *YKU70* gene reverts the pink phenotype to white (Figure 1B, lines g and h). (ii) The pink or white phenotypes can be observed when *YKU80* instead of *YKU70* is disrupted (data not shown). (iii) The *yku70yku80* double disruption also gives rise to both pink and white colonies (data not shown). (iv) Back-crosses with either white or pink clones produced white diploid colonies, and their subsequent sporulation leads to both pink and white *yku70* colonies with a majority of white (Figure 2A). The fact that yKu$^-$ spores exhibit both phenotypes shows that the pink/white phenotype is unstable through meiosis, and suggests that there is no secondary mutation, unlinked to the *YKU70* locus, that is responsible for promoting ectopic silencing.

Upon serial replica plating steps, the patches corresponding to pink and white primary transformants tend to remain pink or white. We consider these cells as the first generation after *yku70* disruption (G1). The colonies isolated after re-streaking the G1 patches define the second generation (G2). Importantly, white or sectored G2 colonies can be recovered from pink G1 cells and pink or sectored G2 colonies can be recovered from white G1 cells (Figure 2B). Starting from G2 cells, G3 colonies with a switched color can also be recovered (Figure 2B). Therefore, although the color phenotype of *yku70* cells seems stable in populations of cells cultivated in patches, switching events from white to pink or from pink to white can be observed upon re-streaking the original transformants. The switch rate in LM79 was determined by using the half-sector assay (Hieter *et al.*,

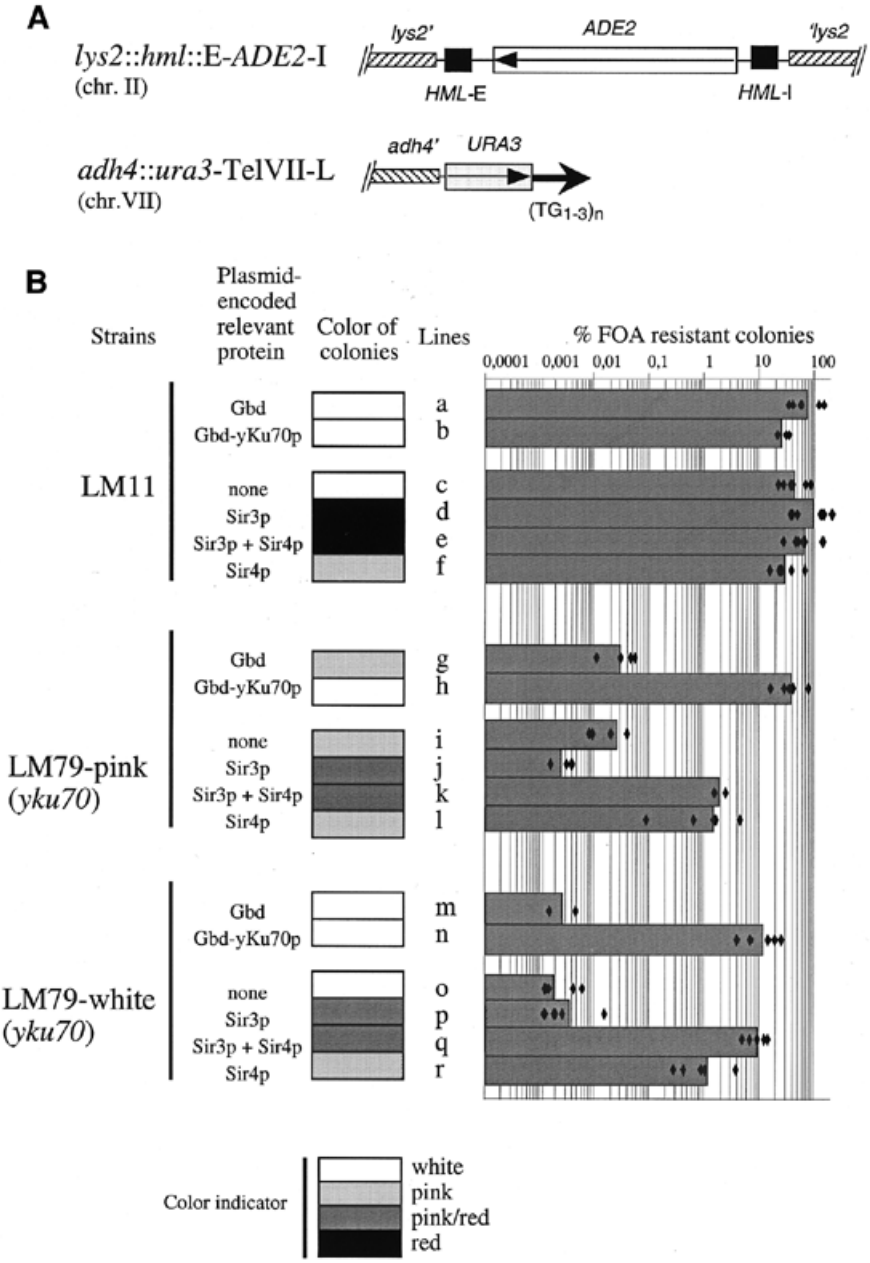

Fig. 1. *ADE2* and telomeric silencing (TPE) in LM11 and in *yku70* derivatives (LM79). (**A**) Structure of the two silencer reporter constructs used in this study. (**B**) The rectangles indicate the color phenotype of colonies of the corresponding strain. The histogram bar represents the average of the % FOA-resistant colonies obtained for a given strain. The relevant proteins (see text) encoded by the plasmids introduced into either LM11 or LM79 are indicated. These plasmids are as follows: pAS2 (lanes a, g and m); pGBD-YKU70 (lanes b, h and n); pAAH5 and pRS424 (lanes c, i and o); p2μ-ASir3 and pRS424 (lanes d, j and p); pFP320 and pAAH5 (lanes f, l and r); p2μ-ASir3 and pFP320 (lanes e, k and q).

1985), leading to values of ~0.6% per cell division from pink to white and ~0.4% from white to pink.

Alternative states of telomeric organization in *yku70* cells

The above data suggest that a *yku70* strain can comprise two populations of cells, as indicated by two alternative states of the ectopic silencing reporter gene. Therefore, we asked whether either the pink or the white phenotype correlates with any of the other well-characterized phenotypes of *yku70* mutants.

Pink and white colonies do not exhibit any difference in their thermosensitivity (our unpublished data). When the mean length of Y′-associated telomeres or the *URA3*-associated VII-L telomere was measured, no change could be detected between pink and white *yku70* cells obtained from either LM11 or EG204. In addition, telomeric DNA of both pink and white *yku70* asynchronous populations of cells exhibits about the same increase in single-

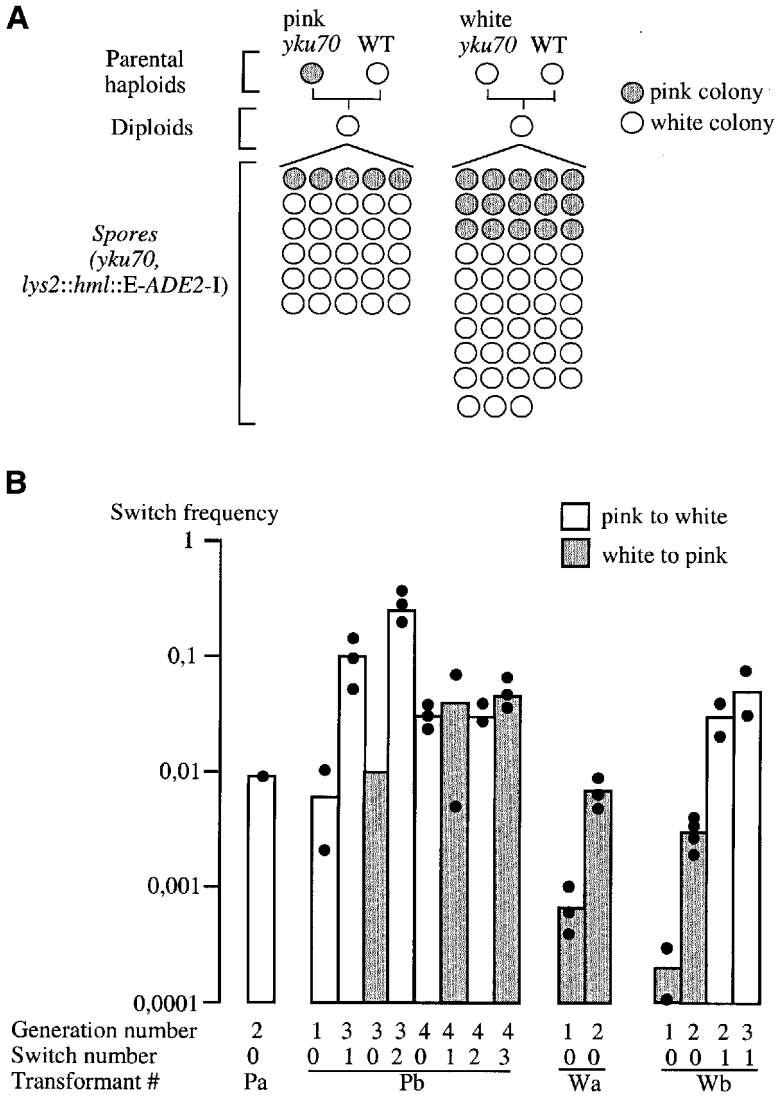

Fig. 2. The pink and white phenotypes are reversible. (**A**) When a pink or a white *yku70* derivative of LM11 was crossed to W303*a* cells, the resulting diploid cells form white colonies. Spores containing the *ADE2* silencing construct at *LYS2* and the *yku70* null allele can exhibit either phenotype, but with a majority of white colonies. (**B**) The frequency of switching in successive generations was estimated roughly by spreading 10^3–5×10^3 cells on YPD-ADE 24 × 24 cm plates and incubating at 30°C for 3 days. The inspection of the colonies was performed after three weeks at 4°C. Between 10^3 and 10^4 colonies were analyzed for each experiment. Starting from pink cells, white colonies and pink colonies with white sectors were counted as white; starting from white cells, pink colonies and white colonies with pink sectors were counted as pink. Two pink primary transformants (named Pa and Pb) and two white primary transformants (named Wa and Wb) were used. 'Generation number' refers to the number of times the patches were re-streaked to yield isolated colonies. 'Switch number' indicates the number of times a switch event (from pink to white or from white to pink) occurred in the lineage of generations. The dots represent independent experiments.

stranded character when compared with wild-type cells (data not shown). Finally, immunoblotting confirms that there is no difference in the steady-state levels of Sir or Rap1 proteins in white and pink *yku70* cells (our unpublished data).

Strikingly, the pink *yku70* or *yku80* cells exhibit a higher level of residual telomeric silencing when compared with white colonies [Figure 1B, compare the % of fluoro-orotic acid (FOA)-resistant colonies of lines g or i with m or o, and Figure 3], although TPE is still largely compromised in both. This result suggests that telomeres in pink and white colonies behave slightly differently, at least with respect to their residual silencing capacity.

A further function proposed for yeast Ku is to anchor telomeres at the nuclear periphery, which helps maintain pools of Rap1p and Sir proteins at these sites (Laroche et al, 1998). Immunofluorescence and fluorescence *in situ* hybridization (FISH) with a subtelomeric Y′ repeat indicate that this function is also differentially affected in white and pink *yku70* cells (Figure 4). As expected, immunostaining for Sir4p and Rap1p clearly shows a delocalized pattern of silencing factors in the pink *yku70* populations (Figure 4, compare panels a and b, d and e). Surprisingly, in the *yku70* white population, the dispersed signal for Sir4p and Rap1p is weaker and is often dominated by one very large focus of silencing proteins (visible as a bright green spot in Figure 4,

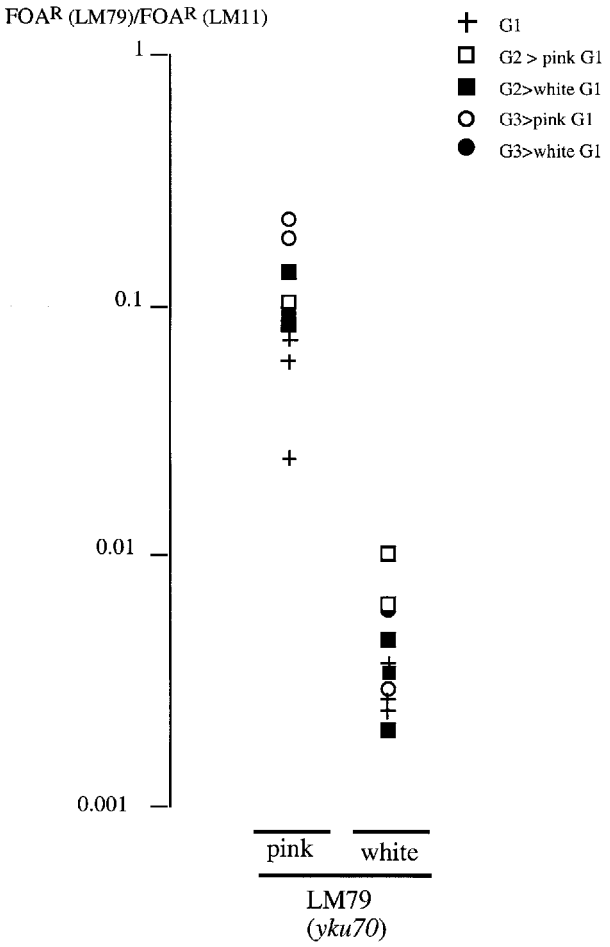

Fig. 3. Residual TPE differs in pink and white *yku70* cells. TPE was assayed in *yku70* derivatives of LM11 as the fraction of cells growing on FOA plates (FOA^R). The values are normalized to the the parental *YKU70* strain, LM11 [FOA^R (LM79)/FOA^R (LM11)]. The primary transformants of the first generation are named G1, and G2/G3 refer to the number of generations (see text). The symbol > means that the cells derived from the corresponding G1 cells. The values of the TPE assays were sorted according to the pink/white phenotype of the LM79 cells, as indicated at the bottom of the figure. Each value represents the average of five or more experiments.

panels c and f). This bright spot is often juxtaposed to the nuclear envelope (visualized here by anti-pore staining in red). The different distribution of silencing factors in the pink and white isolates of LM79 correlates well with the reduced availability of Sir proteins for internal silencing in the *yku70* white colonies.

When the position of telomeric DNA is probed in these same strains using a subtelomeric Y′ repeat probe, we see that telomeric foci appear more dispersed in pink as compared with white *yku70* cells (Figure 4, panels g–l). This qualitative difference was confirmed by scoring foci in a large number of nuclei, which reveals a decrease in the percentage of nuclei with only peripheral Y′ foci and a lower number of total peripheral Y′ signals in pink as compared with white cells (Table I). In the white *yku70* cells we also occasionally see one focus that is significantly brighter than the others, consistent with the Rap1p staining (our unpublished data). These findings indicate that yKu-independent pathways for telomere anchoring function in the *yku70* white colonies. It is noteworthy that some telomeric foci are perinuclear in the LM79-pink cells as well (Figure 4, panel h), suggesting that yKu-independent pathways for anchoring are not completely inoperative in these cells.

Sir3p is limiting for ectopic silencing in *yku70* cells

The fact that ectopic silencing is only partially established in *yku70* cells, as revealed by the white/pink phenotypes, may reflect an insufficient or nonfunctional release of silencing factors. Therefore, we investigated whether Sir3p and Sir4p are still limiting for silencing at *LYS2* in *yku70* cells. Overexpression of Sir3p and/or Sir4p in either white or pink *yku70* cells provokes an increase in ectopic silencing (Figure 1B, lines i–l and o–r). The colony color does not change after transformation with the control plasmids lacking *SIR* gene inserts (Figure 1, lanes i and o), indicating that the transformation procedure *per se* does not modified the switch frequency of LM79. We note that overexpression of Sir3p appears to have more effect than Sir4p; this may reflect either differences in the quantities of the overexpressed proteins, negative *trans*-dominant effects on silencer-mediated silencing, or the fact that Sir4p is not limiting for ectopic silencing in *yku70* cells. Interestingly, the reverse is observed for TPE: overexpression of Sir4p, but not Sir3p, rescues TPE in white or in pink *yku70* cells (Figure 1B, lines l and r). This suggests that only Sir4p is limiting for TPE in *yku70* cells, confirming previous observations (Mishra and Shore, 1999). In summary, TPE and ectopic silencing both appear to be stimulated by the increased dosage of Sir3p and/or Sir4p, but differ in their relative dependence upon these two Sir proteins.

DISCUSSION

We report an intriguing characteristic of yKu-deficient cells which was uncovered in strains carrying an *ADE2* reporter for ectopic silencing at *LYS2*. Roughly half of the primary transformants are able to achieve ectopic silencing, as revealed by a pink color, while the others were white and, thus, do not display ectopic silencing like the parental cells. These two phenotypes of otherwise identical *yku70* cells are mitotically stable, although the switches from one to the other can be scored at a low frequency. Since the two phenotypes are observed in primary transformants, and since a cell can switch from one to the other, it is unlikely that additional mutations are responsible for the pink or white phenotype. Therefore, the pink and white phenotypes appear to correspond to two alternative states of genetically identical *yku70* cells.

The two alternative states of *yku70* cells do not reflect a property restricted to the silencing construct inserted at *LYS2*, but reflect bimodality in more general characteristics. First, we observed that pink cells exhibit a slight increase in residual TPE as compared with white cells. Secondly, the nuclear distribution of telomeric DNA and silencing factors is more diffuse in pink than in white cells. We propose that yKu^- cells balance between epigenetic states characterized, at least in part, by the degree to which silencing factors are released from telomeres. Since it is hard to reconcile an increased release of silencing factors from telomeres with the slight improvement of the residual telomeric

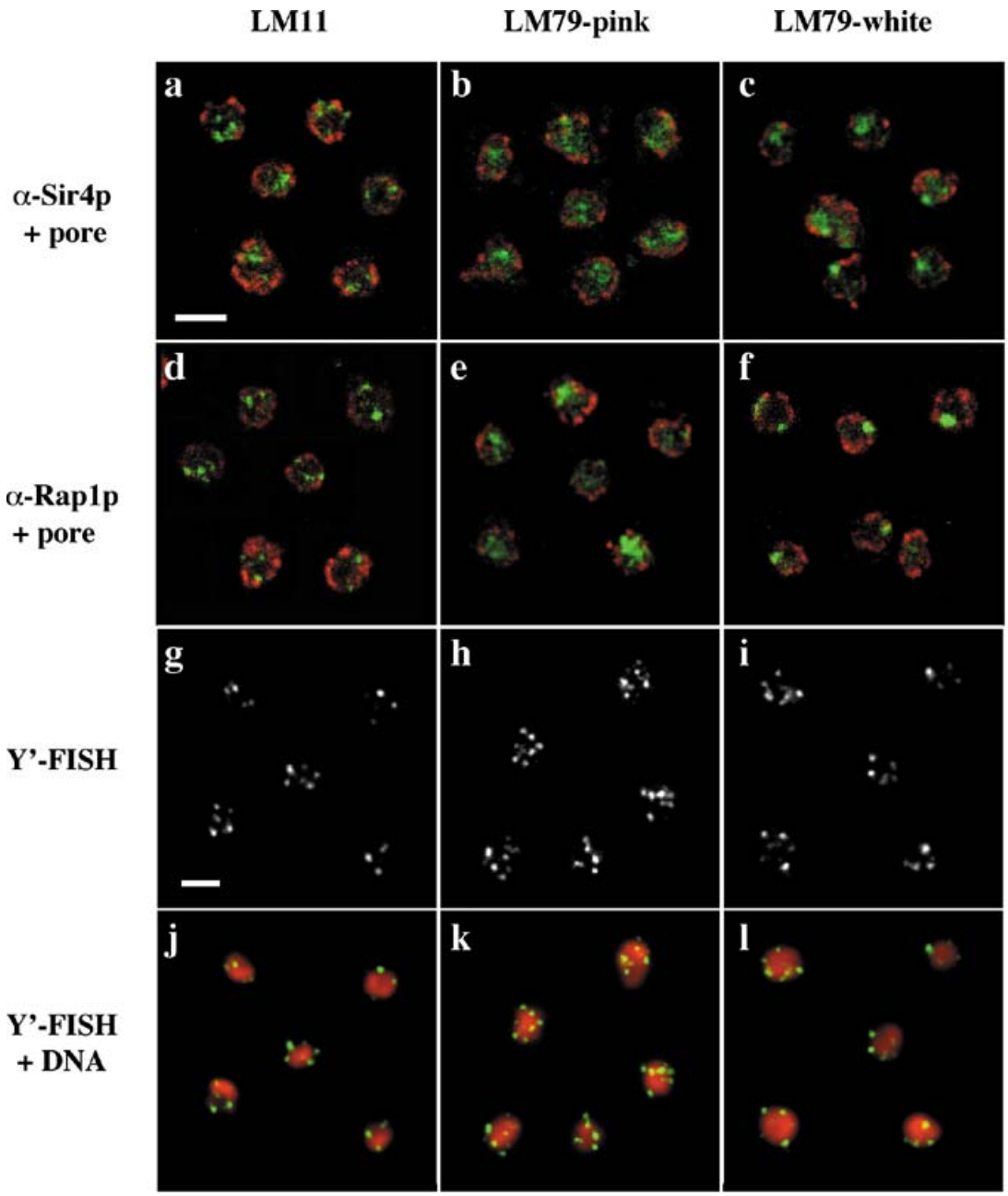

Fig. 4. Two genetically identical *yku70*Δ subpopulations reveal differences in their subnuclear organization. Sir4p and Rap1p immunostaining was performed with affinity-purified rabbit anti-Sir4p or anti-Rap1p as previously described (green staining in panels a–f; Gotta *et al.*, 1996) on LM11 and on genetically identical *hdf::kanMX4* deletion strains (LM79) that are either competent for ectopic silencing (pink) or not (white). The nuclear pore is visualized with a monoclonal that recognizes Nup96p (Mab414, Berkeley Antibody Co; red in panels a–f). Y′ FISH (panels g–l) was performed on the same two strains, as described (Laroche *et al*., 1998). Panels g–i show the FISH probe only, while in the merged images (panels j–l), ethidium bromide is used to label the nuclear DNA. The bar in panel a is valid for panels a–f, and the bar in panel g, for panels g–l. Both indicate 2 μm.

silencing observed in pink *yku70* cells, we suggest further that the two states of *yku70* cells are associated with other, unidentified differences in telomere organization.

That all the telomeres of one cell can establish and maintain a given functional state in a coordinated fashion over long growth periods suggests that long-range mechanisms exist to homogenize the organization of telomeres. The switch between different nuclear states in yKu$^-$ cells is reminiscent of the profound nuclear changes that occur during cellular processes like senescence, differentiation or tumorigenesis in higher eukaryotes (for example see Brown *et al.*, 1999; Bridger *et al.*, 2000). There is no doubt that yeast studies will shed light on the basic principles that underlie these large-scale remodelings of nuclear architecture and their epigenetic control.

Table I. Telomeric foci are less peripheral in pink than in white *yku70::.kanMX4* cells

Strains	No. nuclei (%)		No. foci (%)	
	with only peripheral foci	with both internal and peripheral foci	found at the periphery	found internally
LM11	68 (47.5%)	75 (52.5%)	338 (73%)	125 (27%)
LM79-white	63 (49.5%)	64 (50.5%)	310 (73%)	112 (27%)
LM79-pink	40 (32%)	84 (68%)	273 (64%)	152 (36%)

LM11 and *yku70* derivatives were hybridized with a labeled Y′ probe as in Figure 4.

Table II. Yeast strains used in this study

Strain	Genotype	Source
S150-2B	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3 gal2*	J. Broach
EG204	S150-2B *ade2*::*TRP1 lys2*::*hml*::E-*ADE2*-I	this study
W303-1B	*MAT*α *ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 can1-100*	R. Rothstein
GA210	S150-2B *hml*::e1-*URA3*-I$^{\Delta B}$	Boscheron *et al*. (1996)
YDS631	W303-1B *adh4*::*ura3*-TelVII-L	D. Shore
LM11	YDS631 *lys2*::*hml*::E-*ADE2*-I	this study
LM79	LM11 *yku70*::*kanMX4*	this study

METHODS

Yeast strains and plasmids. Genotypes of strains used in this study are listed in Table II. The plasmid used to complement the *yku70* disruption is pGBD-YKU70, a derivative of pAS2 (Martin *et al.*, 1999). pEADE2I contains an *ADE2* gene flanked by silencers *HML*-E and *HML*-I with *ADE2* promoter proximal to the I silencer (our unpublished construction). To integrate silencer-flanked reporter at the *LYS2* locus, a *Not*I fragment from pEADE2I was integrated into the *Xho*I of the *LYS2* gene DNA (as in Maillet *et al.*, 1996). An *ade2* disruption was created in the S150-2B strain with a linear fragment released from pade2::TRP1 (our unpublished construction).

Null alleles of *YKU70* were created using a PCR product (Wach *et al.*, 1994). In this strategy, the resistance *kanMX4* selectable marker replaces the entire open reading frame. Null mutations of *YKU80* were created with a *yku80*::*HIS3* construct. For overexpression of the C-terminal part of *SIR4*, we used pADH-SIR4C (termed pCSIR4; Cockell *et al.*, 1995). The plasmids used to overexpressed Sir3p and/or Sir4p are the following: p2μ-ASir3, a derivative of pAAH5 (Maillet *et al.*, 1996); pFP320, a derivative of pRS424 (Maillet *et al.*, 1996).

Silencing and microscopic methods. For the ADE color assay, cells were plated on rich medium without supplemented adenine (YPD-ADE) at 30°C for 3 days, and then shifted to 4°C for at least two weeks to allow the red color to develop. Assays to evaluate expression of telomere-proximal *URA3* gene were performed by a spot assay (Gottschling *et al.*, 1990). Immunofluorescence, FISH and confocal microscopy were performed as described (Gotta *et al.*, 1996).

Supplementary data. Data mentioned as not shown in the text are available as supplementary data at *EMBO reports* Online.

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