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Abbreviations used in this paper: 5-FOA, 5-fluoroorotic acid; BP, band pass; CCD, charge-coupled device; ChIP, chromatin immunoprecipitation; mRFP, monomeric RFP; SIR, silent information regulator.

Introduction

A general feature of the nucleus is the organization of repetitive deoxyribonucleic acid sequences in clusters concentrating silencing factors. In budding yeast, we investigated how telomeres cluster in perinuclear foci associated with the silencing complex Sir2–Sir3–Sir4 and found that Sir3 is limiting for telomere clustering. Sir3 overexpression triggers the grouping of telomeric foci into larger foci that relocalize to the nuclear interior and correlate with more stable silencing in subtelomeric regions. Furthermore, we show that Sir3’s ability to mediate telomere clustering can be separated from its role in silencing. Indeed, nonacetylatable Sir3, which is unable to spread into subtelomeric regions, can mediate telomere clustering independently of Sir2–Sir4 as long as it is targeted to telomeres by the Rap1 protein. Thus, arrays of Sir3 binding sites at telomeres appeared as the sole requirement to promote trans-interactions between telomeres. We propose that similar mechanisms involving proteins able to oligomerize account for long-range interactions that impact genomic functions in many organisms.

Clustering heterochromatin: Sir3 promotes telomere clustering independently of silencing in yeast

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the nuclear periphery (Taddei et al., 2004; Bupp et al., 2007; Schober et al., 2008). Sir3 and Sir4 form a stochiometric complex with the NAD+-dependent histone deacetylase Sir2 that deacetylates H3 and H4 histone tails from neighboring nucleosomes, generating histone binding sites for Sir3 and Sir4. This leads to the spreading of the Sir2–3–4 boring nucleosomes, generating histone binding sites for the nuclear periphery.

The SIR complex is also found at the cryptic mating-type loci HML and HMR, where it represses both endo-nucleolytic cleavage and transcription (Haber, 1998; Rusche et al., 2003). In addition, Sir2 is also enriched in the nucleolus (Gotta et al., 1997), where it protects ribosomal DNA from recombination and silences ectopically inserted RNA–polymerase II genes (Gottlieb and Esposito, 1989). Importantly, cellular amounts of Sir proteins, particularly Sir3, are limiting for the spread of silent chromatin from nucleation sites (Renauld et al., 1993; Hecht et al., 1996), and loci associated with Sir3 proteins compete for these limiting pools (Buck and Shore, 1995; Smith et al., 1998; Cockell et al., 2000; Michel et al., 2005).

The mechanism and the proteins that mediate telomere clustering remained elusive. Interactions between subtelomeres have been proposed to be governed only by some physical constraints, including chromosome arm length, centromere attachment to the spindle pole body, and nuclear crowding (Therizols et al., 2010). On the other hand, interaction between HM loci depends on correctly assembled heterochromatin at these loci (Miele et al., 2009). Although mutations in YKU70/YKU80 or SIR3–4 do affect clustering (Gotta et al., 1996; Laroche et al., 1998), these loss-of-function experiments are difficult to interpret because removing any of these proteins from telomeres impacts the recruitment of the others (Hoppe et al., 2002; Luo et al., 2002). However, components of the SIR complex are strong candidates for promoting trans-interactions between telomeres because they all have the ability to interact with each other and among themselves (Rusche et al., 2003; Norris and Boeke, 2010).

To decipher the mechanism underlying the clustering of telomeres, we have investigated the individual contribution of Sir2, Sir3, and Sir4 by monitoring the effect of their overexpression. We show that overexpressing Sir3 leads specifically to the hyperclustering of telomeres and silencing factors in foci mainly localized away from the nuclear periphery. By modulating Sir3 expression, we further show that the cellular amount of Sir3 is a determinant of telomere organization. In addition, we found that nonacetylable Sir3, which is deficient for silencing, is yet efficient for telomere clustering. Moreover, we show that Rap1-mediated recruitment of nonacetylable Sir3 to telomeres can promote telomere clustering in the absence of Sir2 and Sir4. These data lead us to propose a model in which arrays of binding sites for Sir3 are sufficient to promote trans-interactions between telomeres independently of silencing or anchoring to the nuclear periphery.

Results

Sir3 overexpression leads to the grouping of Rap1 foci

To test the individual contribution of the Sir2, Sir3, and Sir4 protein to telomere clustering, we overexpressed each individually by replacing the endogenous promoters of the SIR genes. We constructed a set of strains in which the strong inducible promoter of GAL1 (GALIp) replaces the endogenous promoters of the SIR genes. Importantly, although overexpression of Sir3 from a multicopy plasmid was reported to be toxic (Holmes et al., 1997), the overexpression from the unique genomic copy of SIR2, SIR3, or SIR4 is not toxic (Fig. 1 A). Upon inducing conditions, these strains are competent for silencing at the cryptic mating-type loci (Fig. S1 A) and at telomere VII except for the strain overexpressing Sir4 (Fig. 1 A) as previously reported (Marshall et al., 1987; Cockell et al., 1995).

We studied telomere foci organization in those strains by following the telomere-bound protein Rap1 fused to GFP in living cells (Hayashi et al., 1998). As previously reported for cells grown in glucose-containing medium (Gotta et al., 1996), wild-type cells grown in galactose medium show a diffuse distribution of Rap1-GFP throughout the nucleoplasm with a limited number of bright spots or foci (Fig. 1 B). Strikingly, whereas Sir2 overexpression had almost no effect, overexpressing either Sir3 or Sir4 profoundly affects Rap1-GFP foci but in opposite ways (Fig. 1, B and C). Indeed, these foci decreased in intensity upon Sir4 overexpression, coinciding with the absence of telomeric silencing (Fig. 1 A). In contrast, in a strain overexpressing Sir3, Rap1 foci are brighter and fewer in number. Importantly, the brightness of the Rap1-GFP clusters observed in strains with high Sir3 levels is not caused by increased levels of Rap1-GFP (Fig. 1 D). Furthermore, Sir3 overexpression does not have a major effect on the overall nuclear organization, as the nuclear diameter, the nucleolus, and centromere localization appeared normal under those conditions (Fig. S1, B–D). Thus, Sir3 overexpression appears to affect specifically the distribution of telomeres.

To quantify these observations, we developed a numerical method (see Materials and methods) that allowed the automatic detection of Rap1-GFP foci in interphase cells. Fig. 1 C illustrates the distribution of cells sorted according to the number of foci in different genetic contexts, with gray levels representing foci intensities. The wild-type population shows an expected distribution centered around 3.5 foci per cell (mean = 3.47 ± 0.03 foci). Importantly, foci intensities show a narrow distribution (Fig. 1 E), indicating that the variation of the number of telomeres per wild-type focus is limited. Furthermore, foci intensities are independent of the number of foci per cell from cells with one to eight foci, suggesting that most of the telomeres are not detected as Rap1-GFP foci in cells with few visible foci. Indeed, our simulation suggests that single telomeres or pairs of telomeres are hidden by the diffuse part of Rap1-GFP fluorescence and, thus, are not detectable as Rap1-GFP foci in this assay (Fig. S1 E).

In contrast to the wild-type situation, cells overexpressing Sir3 show fewer foci (mean = 2.48 ± 0.03 foci per cell) with a...
the number of detected telomeres and decreases the number of total clusters.

Finally, Sir3 overexpression induces this hyperclustering even in the presence of high levels of Sir2 and/or Sir4 as shown by co-overexpressing Sir3 with Sir2 and/or Sir4 (Fig. S3 A). Thus, high levels of the other Sir proteins cannot counteract the hyperclustering caused by high Sir3 levels. In conclusion, we propose that Sir3 overexpression specifically induces the hyperclustering of Rap1 foci.

Sir3 overexpression induces telomere hyperclustering in foci containing Sir2, Sir3, and Sir4

To test whether the grouping of Rap1 foci observed upon Sir3 overexpression coincides with the hyperclustering of silent chromatin, we studied the localization of Sir2, Sir3, Sir4, and telomeres in strains with either endogenous or high levels of Sir3. As expected, in wild-type cells grown in galactose,
distance between telomeres VIL and XIVL tagged with the tetracycline operator/tetracycline repressor and lactose operator/lactose repressor systems (Belmont, 2001), respectively. We found that these two telomeres were closer in cells overexpressing Sir3 than in wild-type cells, with a median distance decreasing from 1 µm in wild-type cells (as previously reported by Bystricky et al., 2005) to 550 nm in cells overexpressing Sir3 (Fig. 2 B). The distribution of these distances in a cell population indicated that telomeres remain dynamic and are probabilistically associated only transiently, which is consistent with the dynamics of Rap1-GFP foci (Videos 1 and 2). Importantly, Rap1-GFP foci coincided with Y′ clusters and Sir3 foci in strains overexpressing Sir3, as shown by immuno-FISH and in vivo imaging, respectively (Fig. 2 C). Thus, Sir3 overexpression leads to telomere hyperclustering in foci containing Rap1 and the Sir2–3–4 complex.

GFP-tagged Sir2, Sir3, and Sir4 are found in several foci, whereas upon Sir3 overexpression, most of the cells show one bright nuclear dot (Fig. 2 A). Thus, the overexpression of Sir3 leads to the grouping of Rap1-, Sir2-, Sir3-, and Sir4-containing foci. To rule out the possibility that the hyperclustering of these proteins was independent of the telomeres themselves, we evaluated the status of the telomeric chromatin by FISH experiments. In situ hybridizations were performed with a Y′-repeat telomeric probe on wild-type (yAT126) and GAL1p-SIR3 strains grown in YPGal. [B] 3D position of telomeres VII and XIVL relative to each other in living cells expressing endogenous levels of Sir3 (wild type, yAT56) and in strains overexpressing Sir3 (GAL1p-SIR3, yAT690). YFP-tetracycline repressor and CFP-lactose repressor fusions allowed the visualization of tetOP and lacOP arrays inserted at telomeres VII and XIVL, respectively, as previously described (Bystricky et al., 2005). Cells were grown in galactose before imaging. Shown on the bottom are box plots for distances between telomeres VII and XIVL. The line in the middle of the box represents the median of the values; the bottom and the top of the box are the 25th and 75th percentiles. The whiskers indicate the minimum and maximum data values. [C] Colocalization of telomeres with Rap1-GFP foci (top): GAL1p-SIR3 RAP1-GFP (yAT208) cells were grown in YPGal for immuno-FISH experiments. Colocalization of Sir3-mCherry foci with Rap1-GFP foci (bottom): GAL1p-SIR3-mCherry RAP1-GFP (yAT330) cells were grown in galactose synthetic medium for live-cell imaging. Bars, 2 µm.
The cellular amount of Sir3 is a determinant of telomere organization. Next, we investigated the effect of inducing SIR3 at different levels by the strong GAL1 promoter or its weaker derivative the GALS promoter (Mumberg et al., 1994). Quantification by Western blot analysis showed that GAL1p and GALSp lead, respectively, to Sir3 amounts 15-fold and 6-fold higher than endogenous levels (Fig. 3 A). The sixfold increase of Sir3 amount obtained when GALSp drove SIR3 expression leads to some grouping of Rap1 foci, which are more intense and lower in number than those observed in wild-type cells (Fig. 3, B–D). However, the hyperclustering was more pronounced when Sir3 was overexpressed using the GAL1p promoter, indicating that the degree of telomere clustering is a reflection of the cellular amount of Sir3.

The behavior of the bright Rap1-GFP foci was tracked after Sir3 shutoff and revealed that only 31% of the cells still
showed a bright focus after 6 h of repression and that the disappearance of the Rap1-GFP foci correlated with the dilution of Sir3 upon cell divisions (Fig. S3 B and Videos 2 and 3). Therefore, Sir3 levels correlate with the degree of telomere clustering, and high levels of Sir3 are necessary to maintain telomere hyperclustering.

**Telomere hyperclusters are internal and correlate with more stable silencing**

Because Sir3 overexpression modified the grouping of telomeres, we considered the possibility that the subnuclear position of telomeres was altered under these conditions. We thus monitored the position of the brightest telomere cluster relative to the nuclear envelope as previously described (Hediger et al., 2004) in cells overexpressing Sir3 or in wild-type cells (Fig. 4 A). Strikingly, although the brightest Rap1-GFP focus was mainly found adjacent to the nuclear envelope in wild-type cells (75% in zone 1), the telomere hypercluster was found in the innermost zone in most of the cells overexpressing Sir3 (>90% in zone 3; Fig. 4 A). Thus, Sir3 overexpression leads to the relocalization of telomeres from the nuclear envelope to the nuclear interior.

We then addressed the functional consequence of overexpressing Sir3 on the telomeric position effect. Using either the GAL1p or GALSp promoter to induce Sir3, we monitored silencing at the ADE2 reporter gene inserted at telomere VR by performing a colony color assay (Gottschling et al., 1990). Under inducing conditions, both inducible strains showed stronger ADE2 silencing than the wild-type strain (Fig. 4 B). We noticed that colonies from a wild-type strain showed pink and white sectors reflecting the variegated expression of the reporter gene (Gottschling et al., 1990), whereas strains overexpressing Sir3 through either GAL1p or GALSp showed uniformly pink colonies indicative of a more stable silencing (Fig. 4 B, bottom). This is consistent with a previous study showing that Sir3 overexpression increases the silencing of native telomeres up to 9 kb from the TG repeats at telomere XVR (Przyd and Louis, 1999). Thus, despite the internal localization of the telomere hypercluster, the transcriptional repression that characterizes usually peripheral subtelomeric sequences (Gottschling et al., 1990; Aparicio et al., 1991; Ottaviani et al., 2008) is not impaired upon Sir3 overexpression. This demonstrates that, as previously shown for silencing at HM loci (Gartenberg et al., 2004), efficient and stable silencing of telomeres can also be achieved internally when Sir3 is not limiting.

**Separation-of-function mutants uncouple Sir3 silencing function from clustering**

Because Sir3 overexpression increases both the clustering and the stability of telomeric silencing, we wondered whether the formation of a more stable heterochromatin structure could be the cause of the hyperclustering. To test this hypothesis, we assessed the ability of silencing-defective mutants to promote telomere clustering, and interestingly, we identified several alleles of SIR3 that are efficient for telomere clustering but not for telomere silencing. All of them were modified in their N terminus (unpublished data). As Sir3 is acetylated on Ala2 by the

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**Figure 4.** The telomere hypercluster is internal and correlates with more stable silencing. (A) Rap1-GFP hypercluster localization relative to the nuclear pore. Two-color z-stack images were acquired on strains expressing Rap1-GFP, Nup49-mCherry, and either endogenous levels of Sir3 or high levels of Sir3 (yAT222 and yAT223 transformed with the NUP49-mCherry plasmid). The localization of the brightest Rap1-GFP spot in one of the three equal concentric zones was scored on the corresponding focal plane. This experiment was repeated twice: for experiment 1, nAT222 = 69 and nAT223 = 98, and for experiment 2, nAT222 = 77 and nAT223 = 173 (n is the number of nuclei analyzed). Error bars represent means ± SEM. Bar, 2 µm. (B) Telomeric silencing at the telVR::ADE2 (YPH499 background) in wild-type (WT; yAT7), GALSp-SIR3 (yAT369), and GAL1p-SIR3 (yAT370) strains. Cells were grown in YPGal medium and plated onto YPGal plates. The color of the colonies is indicative of the state of silencing of the ADE2 reporter gene at telVR: the ADE2 gene is expressed in white colonies and repressed in pink colonies. Fivefold dilution assay (top). Single-colony plating (bottom).
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Nat1–Ard1 complex and this modification is essential for its function in silencing at telomeres (Geissenhöner et al., 2004; Wang et al., 2004), we tested whether the A2Q substitution could recapitulate this phenotype. As shown in Fig. 5 A, a strain overexpressing Sir3-A2Q had a severe defect for silencing at HML and was completely deficient for silencing at telomeres. However, this mutant protein was very efficient to promote telomere hyperclustering, as shown by following Rap1-GFP foci in vivo (Fig. 5 B) and telomeres by immuno-FISH (Figs. 5 C and S4 A). Importantly, when expressed on a centromeric plasmid under the control of the SIR3 promoter, Sir3-A2Q was also able to promote telomere clustering in the absence of wild-type Sir3 (Fig. S4 B). These results demonstrate that Sir3 has two distinct functions that can be separated: a function in telomere silencing and a function in telomere clustering.

The second residue of Sir3 has been shown to be important for binding to nucleosomes (Sampath et al., 2009). We thus tested the ability of Sir3-A2Q to spread in subtelomeric regions when overexpressed by performing chromatin immunoprecipitation (ChIP) against Sir3. As was previously described (Hecht et al., 1996; Strahl-Bolsinger et al., 1997; Katan-Khaykovich and Struhl, 2005), overexpressed Sir3 spreads over 15 kb away from telomere VIR. In contrast, overexpressed Sir3-A2Q showed a twofold decrease in recruitment 200 bp away from the TG repeats and background levels of recruitment in subtelomeric regions (Fig. 5 D), which was consistent with its telomeric silencing defect (Fig. 5 A). Thus, Sir3-A2Q is found only at the very end of telomeres, where it promotes telomere clustering without detectable spreading in the subtelomeric regions, demonstrating that stable binding of the SIR complex in subtelomeric regions is not necessary for telomere clustering. In conclusion, Sir3’s ability to mediate telomere clustering can be separated from its role in silencing and spreading into subtelomeric regions.

The nonacetylable Sir3 promotes telomere clustering independently of Sir2 and Sir4 but requires Rap1 C terminus

Having shown that nonacetylable Sir3 can promote telomere clustering independently of stable spreading in subtelomeric regions, we tested whether this activity requires an intact SIR
complex. Intriguingly, overexpression of Sir3-A2Q, but not wild-type Sir3, leads to the grouping of Rap1-GFP foci in the absence of Sir2 (Fig. 6 A). Western blot analysis showed that this difference was not caused by a difference of the cellular amount of Sir3 versus Sir3-A2Q in a sir2 mutant (Fig. S4 C). An alternative explanation could be that the unacetylable Sir3 is recruited better to telomeres than wild-type Sir3 in the absence of Sir2. To test this model, we monitored the recruitment of Sir3 or Sir3-A2Q at telomere VIR by ChIP in a sir2Δ strain. As expected, Sir3 recruitment was severely decreased 200 bp away from the TG repeats (5.5-fold) and did not spread in subtelomeric regions in the absence of Sir2 (Fig. 6 B). In contrast, Sir3-A2Q recruitment was unaffected at the very end of telomeres in the absence of Sir2. Importantly, neither Sir3-A2Q nor Sir3 was complex.
recruited at telomeres in strains bearing the rap1–17 mutation (Liu et al., 1994), which results in the truncation of the Rap1 C-terminal part thought to contain sites for Sir3p and Sir4p association (Moretti et al., 1994). Thus, Sir3-A2Q is recruited to telomeres through interaction with the C terminus of Rap1, independently of Sir2.

To determine the nuclear distribution of Sir3-A2Q, we introduced a GFP tag in the C terminus of this mutant and found that, similar to Sir3-GFP, Sir3-A2Q-GFP formed bright foci when overexpressed (Fig. 6 C). However, contrary to Sir3-GFP, Sir3-A2Q-GFP formed bright foci when overexpressed in the absence of Sir2 (Fig. 6 C), which is consistent with the formation of Rap1-GFP hyperclusters independent of Sir2 upon Sir3-A2Q overexpression (Fig. 6 A). Importantly, Sir3-A2Q-GFP, as Sir3-GFP, did not form any detectable foci in rap1–17 strains (Fig. 6 C). Thus, Sir3-A2Q formed foci only when recruited to telomeres via its interaction with the C terminus of Rap1, ruling out the possibility that this protein forms aggregates when overexpressed. Furthermore, both overexpressed Sir3-A2Q-GFP and Rap1-GFP upon Sir3-A2Q overexpression formed bright foci in a sir2Δ sir4Δ strain, showing that Sir2 and Sir4 are not required for nonacetylatable Sir3-promoted telomere clustering (Fig. 6, D and E). Together, these data strongly suggest that recruiting Sir3 to telomeres is the only requirement to promote trans-interactions between telomeres.

Discussion

Sir3 is a determinant of telomere clustering

Sir3 was previously shown to be limiting for silencing adjacent to telomeres (Renauld et al., 1993). When overexpressed, Sir3 extends silenced regions by spreading over 15 kb in subtelomeric regions (Hecht et al., 1996; Strahl-Bolsinger et al., 1997; Katan-Khaykovich and Struhl, 2005). Here, we show, in two different genetic backgrounds (W303 and YPH499), that Sir3 is also limiting for telomere clustering. Indeed, overexpressing Sir3 with the strong GAL1 promoter leads to a 15-fold increase in Sir3 levels and to the hyperclustering of telomeres. This hyperclustering corresponds to the grouping of wild-type telomere foci into one or two hyperclusters per cell as shown by DNA FISH and localization of telomere-associated proteins. Mild overexpression of Sir3 (sixfold above the endogenous level) through an attenuated version of the GAL1 promoter leads to an intermediate effect. Thus, the cellular amount of Sir3 is a determinant of the extent of telomere clustering.

Silencing occurs away from the nuclear periphery when Sir3 is overexpressed

In wild-type cells, telomeric foci are mainly found at the nuclear periphery (Gotta et al., 1996), where telomeres and silent chromatin are tethered through redundant pathways (Andrulis et al., 2002; Taddei et al., 2004; Bupp et al., 2007; Schober et al., 2009). Unexpectedly, telomere hyperclusters observed upon Sir3 overexpression are internally located, suggesting that an excess of Sir3 counteracts telomere-anchoring pathways. One of these pathways involves Sir4 through its binding with both the inner nuclear envelope–associated protein Esc1 (Andrulis et al., 2002; Taddei et al., 2004) and the transmembrane protein Mps3 (Bupp et al., 2007). It is possible that Sir3 competes with Mps3 and Esc1 for the binding of Sir4 because the Sir4 domains reported to interact with Esc1, Mps3, and Sir3 are all located in the C-terminal half of Sir4 (Moazed and Johnson, 1996; Andrulis et al., 2002; Bupp et al., 2007). Although the mechanism leading to the internal localization of these hyperclusters remains to be elucidated, this observation shows that telomere clustering can occur away from the nuclear periphery. Consistent with this, none of the proteins involved in telomere anchoring (Yku70, Sir4, Esc1, or Mps3) are essential for telomere clustering (Figs. 6 and S4, D–F).

Telomere clustering has been shown to promote and restrict silencing to specific regions by concentrating silencing factors (Maillet et al., 1996; Marcand et al., 1996; Andrulis et al., 1998; Taddei et al., 2009). Consistent with this notion, we show that the hyperclustering of telomeres in the nuclear interior leads to a strong enrichment of silencing factors in this subnuclear region and correlates with a more stable silencing at telomeres. Thus, stable silencing at telomeres occurs away from the nuclear envelope, possibly thanks to the hyperclustering of telomeres allowing the internal concentration of silencing factors.

Silencing and clustering functions of Sir3 can be separated

Sir3 has been the focus of numerous studies addressing its possible mode of action in transcriptional silencing (Norris and Boeke, 2010). However, the possible role of Sir3 in promoting trans-interaction between telomeres has not been specifically addressed in vivo. In this study, we demonstrate that Sir3 promotes telomere clustering and that this function is independent of its activity in silencing, as illustrated by the Sir3-A2Q substitution. This substitution impairs the N-terminal acetylation of Sir3 on Ala2 by the Nat1–Ard1 complex, which is essential for Sir3 function in telomeric and HML silencing (Geissenhöner et al., 2004; Wang et al., 2004). Here, we show that, although Sir3-A2Q is unable to spread in subtelomeric regions, it is efficient for telomere clustering when expressed at endogenous levels and leads to hyperclustering when overexpressed. Sir3 thus appears to have a dual function in silencing and clustering, which could be mediated by distinct domains.

Interestingly, the N and C termini of Sir3p have been shown to perform different and independent functions within the silencing complex, and expression of the two halves of Sir3 in trans partially complements a Sir3 deletion for silencing at HML (Gotta et al., 1998). On the one hand, the N-terminal part of Sir3 contains the conserved bromo-adjacent homology domain that is also found in Orc1 (Zhang et al., 2002) and shows histone tail– and nucleosome-binding activity (Onishi et al., 2007; Sampath et al., 2009). The bromo-adjacent homology domain has been proposed to play an essential role in Sir spreading and can silence HML and HMR in the absence of full-length Sir3 (Connelly et al., 2006; Buchberger et al., 2008). On the other hand, the 144–amino acid C-terminal domain of Sir3 represents the minimum domain for Sir3 homodimerization, a function that is conserved in related yeasts (Liaw and Lustig, 2006).
Mechanism of telomere clustering

Together, these results led us to propose a model (Fig. 7) in which Sir3–Sir3 interactions (Liou et al., 2005; King et al., 2006; Liaw and Lustig, 2006; McBryant et al., 2008) promote telomere clustering in a dose-dependent manner (Fig. 7, A and B). However, nonacetylable Sir3 is well recruited at the TG repeats independently of Sir2 and mediates telomere hyperclustering when overexpressed through Sir3–Sir3 interactions occurring only at the very end of telomeres. CEN, centromere. TPE, telomeric position effect.

In addition, the C-terminal domain can interact with a more internal part of Sir3 (King et al., 2006). These Sir3–Sir3 interactions could promote trans-interactions between Sir3-bound regions as suggested by in vitro studies (Georgel et al., 2001; McBryant et al., 2008; Adkins et al., 2009). Consistent with this hypothesis, overexpressing the N terminal part of Sir3 in the presence of endogenous Sir3 improves telomere silencing with almost the same efficiency as full-length Sir3 (Gotta et al., 1998). However, we observed no improvement of telomere clustering in this case (Fig. 5B), demonstrating that the C-terminal part of Sir3 is necessary for telomere clustering.

Together, these data show that Sir3 function in clustering can be separated from silencing. Therefore, telomere clustering is not a consequence of silencing but can rather favor silencing by concentrating silencing factors.
interactions and/or its binding to Rap1 by eliminating competitive interactions. Furthermore, unacetylable Sir3 can mediate telomere clustering in the absence of Sir4 and Sir2 but requires the C terminus of Rap1, which is necessary to recruit Sir3 to telomeres (Moretti et al., 1994). Thus, recruiting Sir3 to telomeres appears to be the only requirement to promote trans-interactions between telomeres. In the future, it will be interesting to explore how the cell regulates telomere clustering in response to various stresses, knowing that the degree of Sir sequestration in telomeric foci varies in response to nutrient- and damage-induced stress, responding in part to a phosphorylation cascade that targets Sir3 (Stone and Pillus, 1996; Martin et al., 1999; Mills et al., 1999; Ai et al., 2002).

In conclusion, we propose that arrays of chromatin-bound proteins with the ability to oligomerize are sufficient to promote trans-interactions between chromatin regions, which in turn favors the concentration of factors associated with these regions, such as silencing factors. Such a mechanism could account for the clustering of heterochromatin in other species given that many heterochromatin proteins involved in long-range chromatin interactions, including HP1 and Polycomb group proteins, have the ability to self-interact.

Materials and methods

Media and growth conditions
Yeast cells were grown either in rich medium (YPD [yeast extract–peptone-dextrose]) or in synthetic medium (yeast nitrogen base; MP Biomedicals) supplemented with 2% glucose, raffinose, or galactose [wt/vol] and the appropriate supplement mixture (complete or lacking a nutrient; Bio 101). Liquid synthetic media were enriched for complete synthetic medium (2x complete synthetic medium as final concentration; Gomes et al., 2007).

For galactose induction in rich medium, cells were precultured in YPD and switched to YPGal medium (yeast extract–peptone–2% galactose [wt/vol]) for induction of the GAL1 promoter. For time course experiments, cells were precultured in synthetic medium containing 2% raffinose (wt/vol) and galactose was added to a final concentration of 2% [wt/vol] to start the induction. For telomeric silencing assays, 5-fluoroorotic acid (5-FOA; Zymo Research) plates were prepared by adding 5-FOA to a final concentration of 50 µg/ml of lysis buffer (20 mM Hepes KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% N-deoxycholate, and 2.5 µl protease inhibitors [P-1860; Sigma-Aldrich]) and lysed with 0.5 mm zirconium/silica beads (Biospec Products) for three times at 30 s in the FastPrep instrument (MP Biomedicals). The chromatin was isolated by means of sonication in the Bioruptor sample processor (Diagenode) for 14 min at high power with 30 s on/30 s off. For Sir3 ChIP, cleared lysate was added to 50 µl of magnetic beads (Dynabeads Protein A; Invitrogen) preincubated for 4 h at 4°C with 4 µg polyclonal antibody anti-Sir3 (raised against the full-length untagged protein expressed in baculovirus; a gift from F. Martino, Medical Research Council, Cambridge, England, UK). Precipitates were washed, and reversal cross-linking was performed by heating overnight at 65°C. Proteins were digested with proteinase K in the presence of glycerol, and the remaining DNA was purified on columns (QIAquick PCR Purification kit; Qiagen). Finally, samples were treated with RNase.

ChIP and quantitative PCR analyses
ChIP was adapted from Borde et al. (2008). Cells were grown on a YPGal plate for 24 h, seeded in liquid YPGal at OD600 = 0.005, and grown overnight to OD600 = 1. Cells were cross-linked with 1% paraformaldehyde [30 min at 30°C (Sigma-Aldrich)], quenched in 2× SSC, 25 mM glycine for 5 min (Invitrogen), and washed twice in TBS. Pellets were resuspended in 500 µl of lysis buffer (20 mM Hepes KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% N-deoxycholate, and 2.5 µl protease inhibitors [P-1860; Sigma-Aldrich]) and lysed with 0.5 mm zirconium/silica beads (Biospec Products) for three times at 30 s in the FastPrep instrument (MP Biomedicals). The chromatin was isolated by means of sonication in the Bioruptor sample processor (Diagenode) for 14 min at high power with 30 s on/30 s off. For Sir3 ChIP, cleared lysate was added to 50 µl of magnetic beads (Dynabeads Protein A; Invitrogen) preincubated for 4 h at 4°C with 4 µg polyclonal antibody anti-Sir3 (raised against the full-length untagged protein expressed in baculovirus; a gift from F. Martino, Medical Research Council, Cambridge, England, UK). Precipitates were washed, and reversal cross-linking was performed by heating overnight at 65°C. Proteins were digested with proteinase K in the presence of glycerol, and the remaining DNA was purified on columns (QIAquick PCR Purification kit; Qiagen). Finally, samples were treated with RNase.

ChIP quantification by quantitative PCR was performed on 1/20 of the immunoprecipitated DNA or 1/1,800 of the DNA from the whole-cell extract. Primers were designed with the Primer Express software (Applied Biosystems); primer sequences used in this study are the following: for OLI1, am648-GAGAGGAGTTATGGTTATGCTAGG/am649-TTGGTGG- TCTTGGTACACCA; for OGG1, am643-CTAAGGGATGGCCCGCAAG/am644-ACGATGGCGCTCCTGTGGA; for OGG2, am615-TGAGGCGCACATCCGTTG/am616-CAGGAACTGTCATCCATCA; for 14β-tel, am617-TGAGTGGATCAGAGGACACAG/am618-CTCATACA- CAAAGTGAGGAAGC; for 4.2 kb tel, am619-TTCCTGGTCTGAGTCAAGTAGACCCT; for 10 kb tel, am645-ATCCGTAGTTGAGCTGTGCTTATCAA; for 100 kb tel, am646-GGATGATGCTGCGCCGCGCCGCGCGC; for 0.2-kb tel, am620-GTCTCGCTGTGCGAAAACAACAAAGAGAAGTAGTGAAGATC; for 1.2-kb tel, am621-GTCTCGCTGTGCGAAAACAACAAAGAGAAGTAGTGAAGATC.

Real-Time PCR; Applied Biosystems). Sequences of interest were amplified using the SYBR Green PCR Master Mix (Applied Biosystems). Each real-time PCR reaction was performed in triplicate. Triplicates generating cycle threshold (Ct) values from two other regions were eliminated. Each experiment was conducted at least three times.

A dilution series of genomic DNA from 1 to 10−4 ng was used to generate a standard curve. The log (concentration of template) was plotted against the Ct for each dilution. The curve was then used to calculate the threshold (Ct) values differing >0.2 from two other triplicates were eliminated.

Protein immunoblotting
Ct values of the diluted experimental samples were normalized against the Ct for each dilution. The curve was then used to calculate the threshold (Ct) values differing >0.2 from two other triplicates were eliminated. Each experiment was conducted at least three times.

A dilution series of genomic DNA from 1 to 10−4 ng was used to generate a standard curve. The log (concentration of template) was plotted against the Ct for each dilution. The curve was then used to calculate the efficiency for each primer pair (10−1/slope). The Ct values of the diluted genomic DNA were then used to normalize the experimental samples. The signal from a given region was normalized to the one from the OLI1 (Q0130) control locus in immunoprecipitated and input DNA samples. Plots represent the mean value obtained for at least three independent experiments; error bars correspond to SEM.

Immuno-FISH
Immuno-FISH was performed according to Gotta et al. (1999) with a few variations. The probe was obtained by PCR on a plasmid containing 4.8 kb of Y′ element and TG repeats [pEL42110; Louis and Borts, 1995] with
primer pair am151-GAAGAATTGGCCTGCTCTTG/am152-CCGTAAG-
CTGTGCAATTAT. The PCR purification was followed by a nick translation labeling reaction using the Nick Translation kit from Vysis (Abbott Molecular, Inc.). The fluorophore used in the reaction was SpectrumRed (Vysis). The probe was denatured for 5 min at 98°C, purified by ethanol precipitation, and resuspended in the hybridization mix (50% formamide, 10% dextran sulfate, and 2x SSC). 30 OD 1 OD corresponding to 10^6 cells) of cells was grown overnight to mid-logarithmic phase (~1×10^11 cells/ml) in 30 ml YPD or YPGal and harvested at 1,200 g for 5 min at RT. Cells were resuspended in 25 ml of 4% paraformaldehyde for 20 min at RT, washed twice with 20 ml H_2O, and resuspended in 2 ml of 0.1-M EDTA-KOH, pH 8.0, and 10 mM DTT for 10 min at 30°C with gentle agitation. Cells were then collected on 800 g at RT, and the pellet was carefully resus-
pended in 2 ml YPD and 1.2-M sorbitol. Next, cells were spheroplasticized by adding 60 ml YPD and 1.2-M sorbitol. Cells were washed twice in YPD and 1.2-M sorbitol, and the pellet was resuspended in 1 ml YPD. Cells were dropped on diagnostic microscope slides and superficially air dried for 2 min. The slides were put in methanol at −20°C for 6 min, transferred to acetone at −20°C for 30 s, and air dried for 3 min. For immunofluorescence, the slides were incubated in PBS, 1% BSA, and 0.1% Triton X-100 for 20 min and overlayed with anti-GFP at 1:500 (rabbit, fraction A11122; Invitro-
gen) overnight at 4°C. The slides were covered with a coverslip to avoid drying of the antibody solution. After the primary antibody incubation, the slides were washed three times in PBS and 0.1% Triton X-100 for 5 min and an anti-rabbit FITC was added at 1:100 for 1 h at 37°C. The second-
ary antibody was then washed three times in PBS and 0.1% Triton X-100 for 5 min before proceeding to the FISH. The cells were fixed afterward in 4x SSC and 4% paraformaldehyde during 20 min at RT and rinsed three times for 3 min in 4x SSC. After an overnight incubation at RT in 4x SSC, 0.1% Tween, and 20 µg/ml RNase A, the slides were washed in H_2O and dehydrated in ethanol 70, 80, 90, and 100% consecutively at −20°C for 1 min in each bath. Slides were air dried, and a solution of 2x SSC and 70% formamide was added for 5 min at 72°C. After a second step of de-
hydation, the denatured probe was added to the slides for 10 min at 72°C followed by a 37°C incubation for 24–60 h at 37°C in a humid chamber. The slides were then washed twice in 0.05x SSC at 40°C for 5 min and an overnight twice in BT buffer (0.15-M NaHCO_3 for 30 min, 0.1% Tween, and 0.05% BSA) at 37°C. 15 µl of spot of antifading compound in glycerol, pH 7.5 (DABCO), was added before imaging.

Microscopy
Sets of images from any given figure panel were acquired the same day using identical acquisition parameters on cells grown in the same culture conditions. The live-cell images were acquired using a wide-field microscope based on an inverted microscope (TE2000; Nikon) equipped with a 100x/1.4 NA oil immersion objective, a charge-coupled device (CCD) camera (Coolsnap HQ2; Photometrics), and a xenon arc lamp for fluorescence (Lambda LS; Sutter Instrument Co.), a collimated white light-emitting diode for the transmission, and a UV filter on the two illumination paths (EP 400 and GG440; Nikon). A Dual-View microimager (Optical Insights) was positioned in the optical path. When used, this device spatially split emitted light and allowed the simultaneous measurement of two-color information on the same sample. Single-color images were acquired using either a GFP filter block (excitation: band pass (BP), 465–500 nm and di-
achroic, 506 nm; emission: BP, 516–556 nm; Semrock) for green fluores-
cence or a G2-A filter block for red fluorescence (excitation: BP, 510–560 nm and dichroic 565 nm; emission: long pass, 590 nm; Chroma Technol-
ogy Corp.).

GFP-mCherry two-color images were acquired simultaneously on two parallel channels using a GFP-mCherry filter block (excitation: double band, BP 460–490/550–590 nm and dichroic double band BP 500–550/ 600–665 nm) and the Dual View. The Dual View was equipped with adapted filter sets to observe green fluorescence (GFP, dichroic 565 nm and em-
ission BP 499–529 nm; Semrock) on the left channel and red fluorescence (mCherry, dichroic 565 nm and emission BP 604–656 nm; Semrock) on the right channel. A custom macro (National Institutes of Health) was used to align and recombine channels. The position shift was estimated using the correlation function peak in transmitted light data (which is the same in the two channels) and used for fluorescent image alignment.

Immuno-FISH images were acquired with a wide-field microscope (Deltavision RT; Applied Precision) using a 100x/1.4 NA oil immersion objective (Olympus) and a CCD camera (CameraLink) (Applied Precision). The filters comprised the standard filter set suitable for FITC and RD-TRE (rhodamine, Texas red, and phycoerythrin). Immuno-FISH images were deconvolved with softWoRx (additive method; eight iterations).

CFP–YFP two-color images were acquired on a spinning-disk con-
focal microscope (Revolution XD Confocal System; ANDOR) equipped with a spinning-disk unit (CSU-X1; Yokogawa), a microscope (Ti 2000; Nikon) with a 100x/1.4 NA oil immersion objective, and an EM CCD camera (IXON DU-88S; ANDOR). CFP and YFP signals were acquired sequentially for each z-section using solid-state 445- and 514-nm diodes and appropriate filters (confocal scanner unit triple dichroic mirror for 445, 514, and 640 nm and a double BP 464/547 emission filter from Semrock).

For fluorescent images, the axial (z) step is 200 nm, except for 4D movies, which have an axial (z) step of 300 nm. All fluorescent images are a z projection of z-stack images.

Microscopy data processing
Deconvolution was made using the Meinel algorithm in Metamorph (eight iterations; σ = 0.8; frequency 3; MDS Analytical Technologies). Videos were denoised using the Salfit-D algorithm (Institut National de Recherche en Informatique et en Automatique Vila).

Telomere cluster quantification
Analyses have been performed using a home-made Matlab (MathWorks) application (Q-foci). A smoothing of data using a double Gaussian model, whose parameters were determined according to Zhang et al. (2007), was applied on deconvolved images. For segmentation and labeling of individual nuclei in 3D images, the diffuse Rap1-GFP fluorescence signal was considered as a nucleoplasm staining. Otus thresholding was used for nuclear segmentation (Hoebeke et al., 1997). Additional filters were used to discard nonvalid objects. First, a morphological opening (disk kernel, radius of 4 pixels) was used to suppress segmentation artifacts. Incomplete objects touching the border of the 3D data stack and adjacent nuclei were also discarded. Local intensity maxima detected in segmented nuclei were considered as telomere cluster candidates. They were then attributed a score according to local curvature and mean intensity (Thomann et al., 2002). Because Rap1-GFP foci brightness is highly variable (depending on the number of telomeres in the cluster), results did not show a clear cut-off in scores between small clusters and false positives, as in other studies (Thomann et al., 2002; Berger et al., 2008). Consequently, the threshold for classification of a candidate as a telomere cluster was set manually based on the control (wild-type) of the experiment and then applied on data corresponding to other conditions. The resulting data file lists all nuc-
leoli present in a series of 3D data stacks, each representing tenths of cells, along with the number of telomere clusters each cell contains and the in-
tensity corresponding to these clusters, which were measured as the inten-
sity component in the scoring method. 3D distances between telomeres VL and XLV were quantified using the SpotDistance ImageJ plugin with a visual inspection.

Simulations for the detection of telomere clusters in synthetic parameters
Parameters required for these simulations were fitted experimentally on 150 microscopy images presented in Fig. 1, including noise, nucleus size, intensity, and microscope characteristics. Here, noise was considered as following a normal distribution; nuclei were considered as a sphere of radius 800 nm; single telomeres and clusters as subresolution particles; total intensity of nuclei presented in Fig. 1 is equivalent to 4,390 Rap1 molecules per cell as previously described (Ghaemmaghami et al., 2003). The number of Rap1 molecules bound to each telomere was set to 40, assuming that 15–20 Rap1 molecules bind TG repeats (Shore and Nasmyth, 1987) and 10–15 are spreading on neighboring nucleosomes (Peters et al., 1996). The remaining pool of Rap1 was considered as dif-
fusing freely in the nucleus. These simulations were then convolved using the measured point spread function of the microscope to reproduce as accurately as possible the experimental conditions.

Online supplemental material
Fig. S1 shows characterization of Sir3 overexpression and simulations of Rap1-GFP clusters. Fig. S2 shows the dynamics of telomere foci forma-
tion after Sir3 induction. Immuno-FISH images and immunofluorescences using Q-foci. Fig. S3 shows the effect of Sir3 overexpression and Sir3 cellular amount on telomere clustering. Fig. S4 shows the requirements for Sir3 acetylation, Sir1, Esc1, yku70, and mps3 for telomere cluster-
ing. Fig. S5 shows that the overexpression of the N-terminal domain of Sir3 strengthens telomeric silencing without improving telomere cluster-
ing. Video 1 shows the appearance of Rap1-GFP hyperclusters upon Sir3 induction. Video 2 shows the disappearance of Rap1-GFP hyperclusters

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