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Nuclear organization and transcriptional silencing in yeast

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Abstract. Transcriptional repression at the yeast silent mating type loci requires the formation of a nucleoprotein complex at specific cis-acting elements called silencers, which in turn promotes the binding of a histone-associated Sir-protein complex to adjacent chromatin. A similar mechanism of long-range transcriptional repression appears to function near telomeric repeat sequences, where it has been demonstrated that Sir3p is a limiting factor for the propagation of silencing. A combined immunofluorescence/in situ hybridization method for budding yeast was developed that maintains the three-dimensional structure of the nucleus. In wild-type cells the immunostaining of Sir3p, Sir4p and Rap1 colocalizes with Y′ subtelomeric sequences detected by in situ hybridization. All three antigens and the subtelomeric in situ hybridization signals are clustered in foci, which are often adjacent to, but not coincident with, nuclear pores. This colocalization of Rap1, Sir3p and Sir4p with telomeres is lost in *sir* mutants, and also when Sir4p is overexpressed. To test whether the natural positioning of the two *HM* loci, located roughly 10 and 25 kb from the ends of chromosome III, is important for silencer function, a reporter gene flanked by wild-type silencer elements was integrated at various internal sites on other yeast chromosomes. We find that integration at internal loci situated far from telomeres abrogates the ability of silencers to repress the reporter gene. Silencing can be restored by creation of a telomere at 13 kb from the reporter construct, or by insertion of 340 bp of yeast telomeric repeat sequence at this site without chromosomal truncation. Elevation of the internal nuclear pools of Sir1p, Sir3p and Sir4p can relieve the lack of repression at the *LYS2* locus in an additive manner, suggesting that in wild-type cells silencer function is facilitated by its juxtaposition to a pool of highly concentrated Sir proteins, such as those created by telomere clustering.

Key words. Silencing; Sir; yeast mating type; telomere position effect; subnuclear organization; chromatin.

Introduction

Compartmentation of cellular functions is the biological feature that most clearly differentiates eukaryotic from prokaryotic cells. In contrast to the single cytoplasmic compartment of a prokaryote, a eukaryotic cell is composed of numerous membrane-bound organelles which are distinct both functionally and structurally. Among these organelles is the nucleus, which itself reveals a remarkable degree of suborganellar organization. Using antibodies and in situ hybridization techniques it is possible to define subdomains within the yeast nucleus, as shown in figure 1. We observe at least two functional subnuclear compartments, one containing the majority of the chromatin (approximately two-thirds by volume, red fluorescence), and one containing the nucleolus, where rDNA is transcribed and ribosomes are assembled (approximately one-third by volume, blue fluorescence from anti-Nop1 [1]). Recent studies have demonstrated that telomeres also create subdomains of organization within the nucleus, as demonstrated in figure 1 by the immunodetection of Repressor activator protein 1 (Rap1, green fluorescence), an abundant nuclear protein that binds to the repetitive sequence found at all yeast telomeres [2–4].

In this paper we will review results indicating that the clustered signal of Rap1 immunofluorescence reflects the positioning of telomeres in wild-type yeast nuclei [5] (see below). We propose that the binding of Rap1 and two silent information regulatory proteins, Sir3p and Sir4p, at telomeres creates a high local concentration of the factors required for chromatin-mediated silencing in yeast. We suggest that this clustering is functionally important for establishing domains of silent chromatin near telomeric repeats.

Genome and chromatin organization in yeast

The yeast haploid genome contains 1.4×10^7 bp divided among 16 chromosomes, ranging in size from 0.23 to over 1 Mbp. The yeast genome encodes roughly 6000 proteins but contains little simple or middle repetitive DNA. One of the rare examples is the 300 ± 75 bp of an irregular TG_{1-3} repeat found at the very end of all yeast chromosomes (reviewed in ref. 6). Immediately adjacent to the terminal telomeric repeat are highly conserved subtelomeric sequences called Y′ elements, which can be either 5.4 or 6.7 kb in length, and the X element, which

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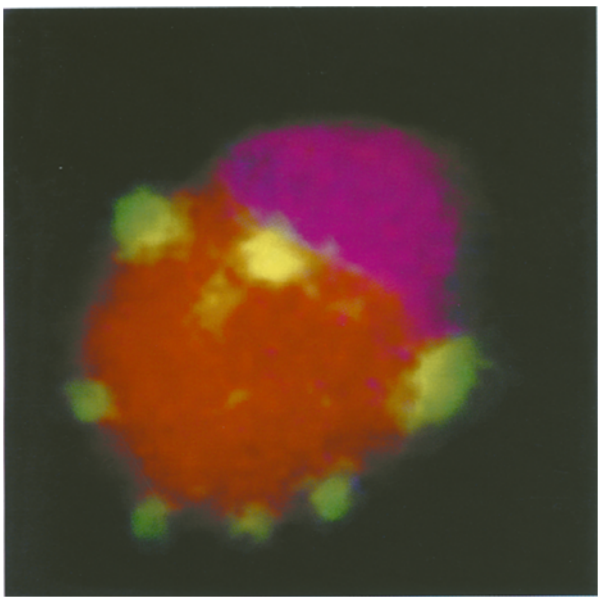

Figure 1. Organization of subcompartments of the yeast nucleus visualized by indirect immunofluorescence. A wild-type diploid yeast strain was prepared for immunofluorescence by a brief incubation with β-glucanase, followed by fixation with 3.7% formaldehyde in growth media. Immunodetection of Rap1, and Nop1 [1] using affinity purified rabbit anti-Rap1 antibodies and a mouse monoclonal specific for Nop1, was performed as described in Gotta et al. [5]. Anti-Rap1 is shown in green (FITC) and anti-Nop1 is shown in blue (CY5). Ethidium bromide (visualized in red) is used as a general DNA stain. Shown is a merger of the three images captured on a Zeiss Axiovert 100 LSM confocal microscope using the 100× Plan-Apochromat objective. The Rap1 signals are not randomly distributed throughout the wild-type yeast cell nucleus, but localize in six to eight foci, many of which are found near the nuclear periphery in G_1-phase nuclei [49, 62, 63].

carries a conserved core element of 500 bp. While the primary sequences of Y′ and core the X elements are highly conserved, their copy number and presence vary among chromosomes and strains [6].

Like all eukaryotic genomes, yeast DNA is organized at the most basic level into the nucleosome, the core of which is defined by 146 bp wrapped in two turns around a complex composed of two copies of each of the four core histones, H2A, H2B, H3 and H4 [7]. Histones H3 and H4 are among the most highly conserved proteins known, and even the N-terminal tails, which are not necessary for forming the core nucleosome, reveal highly conserved patterns of sequence and residue specific modifications (reviewed in ref. 8). Nucleosomal core particles are separated by a stretch of linker DNA, the mean size of which can differ from species to species and among different tissues [9]. In isolated nuclei, this linker DNA is more sensitive to attack by micrococcal nuclease, allowing definition of the nucleosomal repeat. As in other lower eukaryotes, this value is about 165 bp in *Saccharomyces cerevisiae* [10], while in mammalian cells it ranges between 180 and 200 bp [9]. Despite extensive study, the physiological significance of the variation between mean nucleosomal repeat sizes is not understood. It has been demonstrated that the spacing of individual nucleosomes in *S. cerevisiae* is influenced by three parameters; these are the nature of the DNA sequence, chromatin folding and so-called boundary elements, which can be promoter regions, terminators or origins of replication (reviewed in ref. 11). Early attempts to determine whether nucleosomes were specifically positioned with respect to primary sequence suggested that the majority were not. Nonetheless, the translational and rotational settings of certain, individual nucleosomes with respect to strategically located regulatory sequences can play an important role in transcriptional control [12].

It has been proposed that the shorter unit of the nucleosome in yeast may reflect the absence of histone H1, a slightly larger histone, which binds the nucleosome in a ratio of 1 to 1, at the point where the DNA enters and leaves the core particle. Although early attempts to identify a budding yeast equivalent to H1 failed, the genome project has recently identified an open reading frame with 36% identity to the human histone H1 globular domain [13]. The yeast H1 has two such globular domains separated by a highly charged spacer, resembling the lysine-rich tail of histone H1 [13]. It remains to be seen if this molecule is functional in the yeast nucleus, or whether it modifies the nucleosomal repeat length. Heterologous expression of sea urchin H1 at high levels in yeast results in growth inhibition and transcriptional repression, yet it does not alter the nucleosome repeat unit [14], suggesting that histone H1 is not sufficient to determine nucleosomal positioning. Other typical chromatin proteins like the HMG (high mobility group) family are found in yeast, and at least four genes encoding HMG1-like proteins have been cloned [15–17]. These highly charged DNA-binding proteins are essential for viability, and are thought to modulate gene expression by altering local nucleosome positioning [18] or stability [17]. On the other hand, no homologues for HMGI/Y, proteins which bind preferentially to the adenine-thymidine-rich, late-replicating Giemsa-dark bands of facultative heterochromatin in human and mouse mitotic chromosomes [19], have been reported for budding yeast.

Although no yeast protein is known to stabilize yeast nucleosomes in a fibrelike 30-nm solenoid structure [20, 21], yeast nucleosomes do fold spontaneously into a solenoidal structure in a salt-dependent manner in vitro [22, 23]. Consistently, a simple calculation based on the volume of the nucleus and the length of DNA found

within, suggests that yeast may have a level of chromatin compaction roughly equivalent to that in higher eukaryotes. For instance, the linear compaction ratio required to fit the human genome in a nucleus of 10 μm in diameter, is only two- to threefold higher than that of a haploid yeast genome in a nucleus of slightly under 2 μm in diameter. Supporting this, in situ hybridization on flattened yeast nuclei using probes from chromosome XVI [24] reveals a compaction ratio around 80, which is only two- to threefold less than the ratios calculated for regions of the mammalian genome based on in situ hybridization in interphase nuclei [25, 26]. Even if some domains of the two genomes appear equally compact, it is nonetheless clear that certain regions of mammalian genomes remain in a fully condensed, heterochromatic state throughout interphase, reflecting a level of compaction unlikely to be found in yeast.

Chromatin organization

Despite the parallels described above between yeast and mammalian chromatin, the majority of yeast genome shows little differential sensitivity to DNase I digestion, with both transcribed and nontranscribed sequences allowing similar accessibility to exogenously added nucleases [27]. This has led to the proposal that the majority of the yeast genome is in a transcriptionally poised state, whether the genes are actively transcribed or not [28]. Specific nuclease hypersensitive sites, on the other hand, do show increased sensitivity upon the induction of transcription of specific genes. These nuclease hypersensitive sites in yeast often correlate with sites of transcription initiation, and with binding sites for sequence-specific transactivators [28–31].

Occasionally, changes in nucleosomal organization are observed coincident with high levels of transcription. Importantly, the boundaries of the altered nucleosomal organization, such as the phased nucleosomes at the *HSP82* locus [32], appear to correlate precisely with the transcription unit itself, starting at the upstream activating sequences and extending to the site of transcriptional termination. In this way each yeast gene, whether its nucleosomes are positioned or not, appears to act as a chromatin microenvironment, with the boundaries of a transcriptional unit providing the boundaries of the nucleosomal alterations. This is in contrast to the chromatin domains found in mammalian cells, which can include up to 100 kb of flanking DNA that reflects fluctuations in nuclease sensitivity, indicative of the gene's potential for transcription.

In yeast, once a highly transcribed gene has been switched off by the removal of its activator, a delay of 10–50 min can ensue prior to restoration of the typical inactive chromatin structure, which in the case of the *GAL10* promoter involves a positioned nucleosome over the TATA box [33]. This delay may indicate a requirement for the passage of a replication fork to remodel the nucleosomal pattern from an active to a repressed pattern. A similar requirement for DNA replication (again, presumably to allow nucleosomal assembly following DNA synthesis) was observed for the restoration of a repressed chromatin state at the silent mating type loci, after a shift from the restrictive to the permissive temperature for a mutation in *SIR3* [33]. It is important to note, however, that passage through S-phase is not necessary to derepress silent genes after inactivation of a silencer factor.

Repressed chromatin in yeast

Most of the yeast genome appears to be in an open or poised transcriptional state, although two classes of sites show position-dependent transcriptional repression and a less accessible chromatin structure. The first class of sites is the transcriptionally repressed mating type loci on chromosome III (*HML* and *HMR*, collectively termed the *HM* loci), which show a reduced accessibility for the yeast endonuclease HO and for an ectopically expressed *Escherichia coli dam* methylase in vivo. In lysed cells, as well, exogenous restriction enzymes cut less readily in the repressed domain than in flanking regions (reviewed in ref. 35). In a similar fashion, Pol II genes positioned adjacent to the poly(TG_{1-3}) tracts at yeast telomeres were found to succumb to a heritable but reversible transcriptional inactivation that also correlates with reduced accessibility to the ectopically expressed *dam* methylase [36, 37]. This phenomenon is part of a *t*elomeric *p*osition *e*ffect (TPE) which, like *p*osition *e*ffect *v*ariegation (PEV) in *Drosophila*, appears to spread into adjacent euchromatin from a transcriptionally inactive, repetitive element, in this case the TG_{1-3} repeat at the chromosomal end [38]. The regulation of gene expression by alterations in chromatin structure is a universal phenomenon in eukaryotic cells, and is responsible for the proper activation and inactivation of genes in the developmental program of multicellular organisms [39], for position effect variegation in flies [40] and the variable expression of foreign genes integrated into chromosomes [41].

The nucleation of the repressed chromatin state at telomeres requires the cis-acting telomeric repeat, to which bind multiple copies of Rap1 [3, 4, 36, 42–44]. At *HML* and *HMR*, short cis-acting silencer elements serve the same purpose, and bind trans-acting factors needed for the establishment of silencing, namely Rap1 [45], Abf1 (ARS binding factor 1 [46]), and the origin recognition complex (ORC) [47], a six-polypeptide complex that recognizes the ARS consensus [48]. In addition to these cis-acting sequences and their ligands, both TPE and mating type silencing require the silent information regulator genes, *SIR2*, *SIR3* and *SIR4*, the *NAT1*/*ARD1* N-terminal acetylase, and intact N-termini of

histones H3 and H4. *SIR1* is necessary for repression at *HM* loci, but not for genes inserted near TG_{1-3} tracts at telomeres (reviewed in ref. 35).

None of the Sir proteins appear to bind DNA directly, but their exquisite sensitivity to gene dosage suggests that their roles are likely to be structural, at least for Sir1p, Sir3p and Sir4p. For instance, Renauld et al. [38] observed that strains overexpressing *SIR3* extend telomere-proximal silencing up to 25 kb from the terminus of the marked chromosome, while in wild-type strains silencing decreases rapidly beyond distances of 3.5 kb. Overexpression of either full-length Sir4p, or the carboxy-terminal fragment of it that contains a coiled-coil domain, abrogates transcriptional repression at both telomeres [49] and at HM loci, similar to what is observed in a *sir4Δ* strain [50]. Moreover, the anti-SIR activity of the Sir4p C-terminus can be overcome by increased *SIR3* dosage (ref. 50; M. Gotta, unpublished). Such dosage-dependent phenotypes are consistent with a model in which the Sir4p and Sir3p proteins associate with other components in a precisely balanced fashion. Consistently, Sir3p and Sir4p were found to interact in two-hybrid assays with themselves, with each other and with Rap1 [51]. Moreover, Sir4p and Rap1 coprecipitate in a DNase-insensitive complex from yeast nuclear extracts [49]. Finally, both Sir3p and Sir4p were shown to selectively bind the N-termini of histones H3 and H4 in vitro, and mutations in the N-termini that disrupt the interaction and derepress silencing in vivo lead to a redistribution of Sir3p throughout the nucleus [52].

Although less is known about the *SIR2* gene product, recent evidence suggests that it also affects chromatin structure. Braunstein et al. [53] have shown that lysines in the N-termini of histones H3 and H4 bound to both the silent mating type loci and subtelomeric regions are hypoacetylated relative to active chromatin, in a manner dependent on both *SIR2* and *SIR3*. The overexpression of *SIR2* resulted in a more extensive deacetylation of the N-terminal tail of histone H4, suggesting that Sir2p may either be a histone deacetylase or a regulator of a histone acetylase. A homologue of *SIR2* has been identified in a related budding yeast, *Kluyveromyces lactis*, although the two only partially cross-complement [54]. Finally, it has been noted that one of the *Drosophila* suppressor of variegation genes [*su-var(2)1*] also modifies the acetylation state of the ε-amino group of lysines in the N-terminus of histone H4 [55]. It remains to be seen how closely related *su-var(2)1* and *SIR2* are.

Based on the observations summarized above, one can propose a working model for silent chromatin (see fig. 2). According to the model, association of Sir proteins with chromosomes is achieved in part through binding to Rap1 and in part through an interaction with the N-termini of histones H3 and H4. The Sir3p/Sir4p

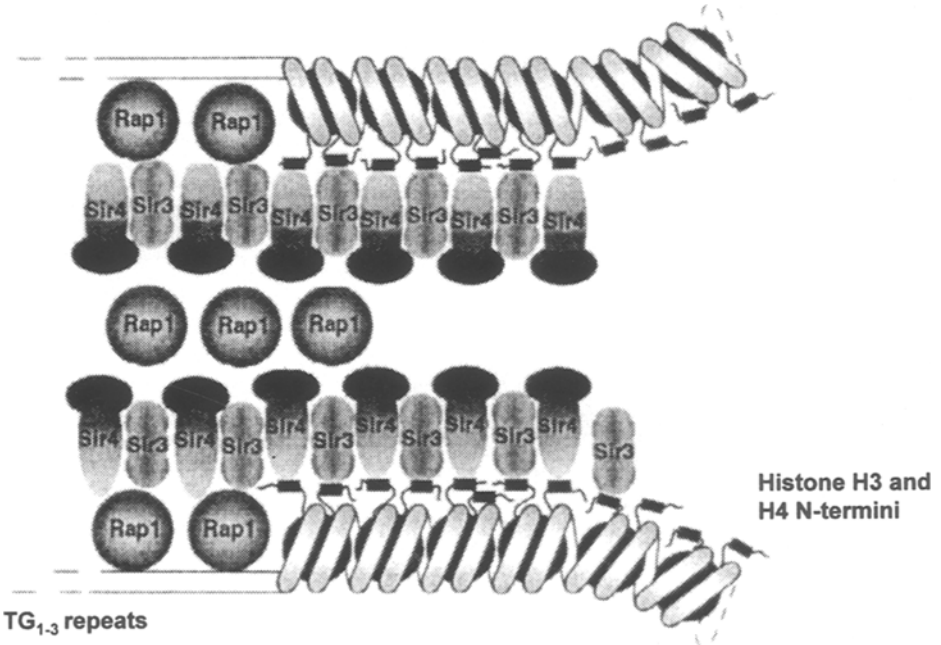

Figure 2. A model of how protein-protein interactions may lead to the nucleation and propagation of chromatin-mediated gene repression near telomeres in yeast. Repressor-activator protein 1 (Rap1) binds multiple sites in the telomeric repeat, where it is implicated in establishing the repressed state through interaction with the products of the *SIR* genes. Sir3p and Sir4p appear to form a complex that interacts both with Rap1 and directly with the N-terminal tails of histones H3 and H4. Since the overexpression of *SIR3* can extend telomeric silencing, it is limiting in this context. Its association with nucleosomes may not require, but be stabilized by, Sir4p. At telomeres it appears that a population of Rap1 can be displaced from the telomeric foci without disrupting the Rap1 that stabilizes the chromosomal end by binding to the TG_{1-3} repeat. This is shown by the second population of Rap1 that is held at telomere clusters by protein-protein interactions involving Sir3p and Sir4p. Adapted from Hecht et al. [52].

complex propagates along the underacetylated nucleosomes to repress transcription. Rap1-Sir protein interaction is presumably not affected by the histone mutations, since Rap1 does not bind to the histone N-termini and histones appear not to be part of the telosome [56].

Results and discussion

Telomeres and functional nuclear compartments

In addition to their role in chromatin-mediated repression, Rap1, Sir3p and Sir4p also appear to play a role in the structural integrity of telomeres. Although Rap1 [45] was first characterized as a transcriptional enhancer [57, 58], and as a repressor that binds at *HM* loci, Rap1 also plays an important role in telomere maintenance and chromosome stability [59, 60]. The association of Rap1 with yeast telomeres was first suggested by its copurification with the telosome, a non-nucleosomal chromatin structure present at the telomeric TG_{1-3} tract [56] and by the fact that mutations in *RAP1* confer substantial effect on telomere tract size [59–61]. We have shown by immunofluorescence using Rap1 antibodies on a spread pachytene nucleus, that a large fraction of this protein is found at the ends of all chromosomes, confirming a role for Rap1 at telomeres [62] (as shown in fig. 3). Both immunofluorescence and electron microscopy on vegetatively growing diploid cells reveal intense anti-Rap1 staining in a limited number of foci [49, 62, 63] (six to eight per nucleus, see fig. 1). Consistent with the notion that the Rap1 staining reflects a clustering of telomeres, the Rap1 immunoreactive foci were lost concomitantly with the loss of telomeric repeats in the yeast mutant *est1* (*ever shorter telomeres 1* [64]). Survivors of the *est1* deficiency recover chromosome stability by *RAD52*-dependent recombination of Y′ elements [65]. In these survivors, the focal staining of Rap1 is restored as the cells regain telomere stability (T. Laroche and S. M. Gasser, unpublished data).

The localization of Rap1 in foci is intriguing, because telomeres are the site of a Rap1-dependent chromatin mediated gene repression. Immunofluorescence using

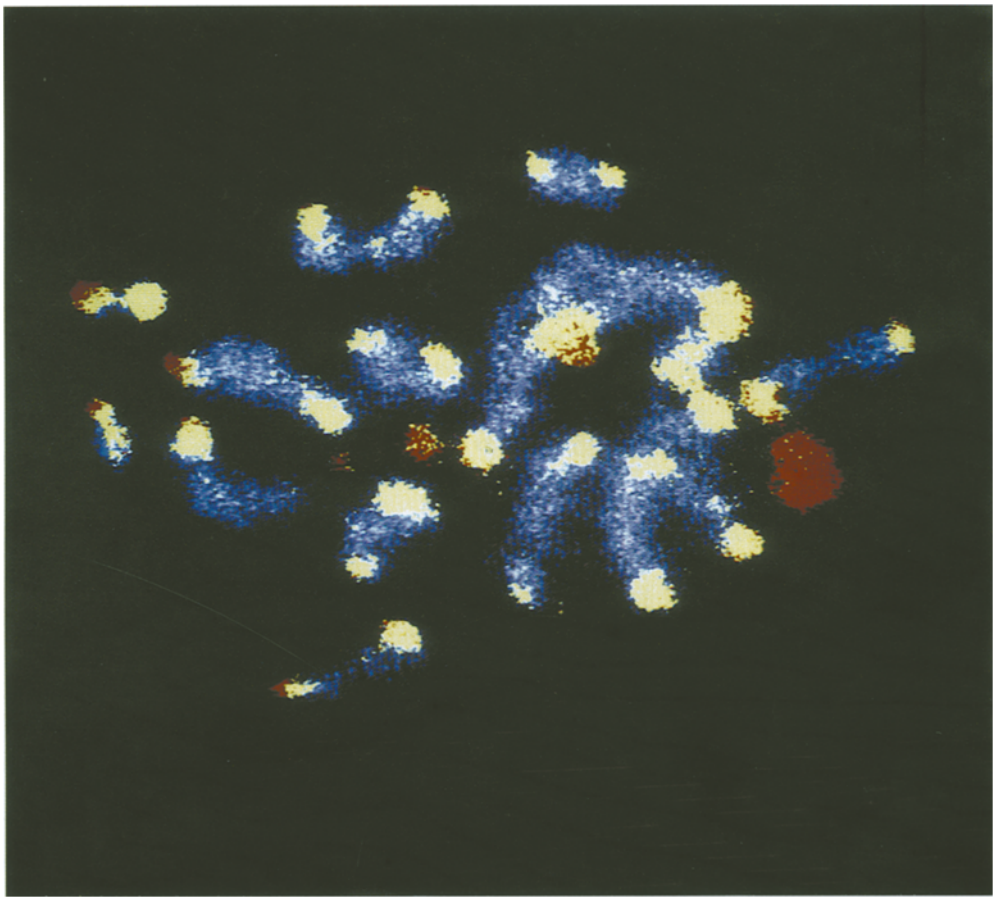

Figure 3. Rap1 localization on a spread pachytene yeast nucleus. Indirect immunofluorescence on a chromosomal spread from a pachytene-arrested SK1 yeast strain using anti-Rap1 antibodies (visualized with a fluorescein-conjugated secondary antibody). Blue areas indicate DNA staining (DAPI); yellow areas represent Rap1 staining. The meiotic nucleus showing its 16 bivalent synoptonemal complexes was also stained with propidium iodide, which gives a red staining indicative of RNA and/or single-stranded DNA. This was reproduced with permission from the J. of Cell Biology (Klein et al. [62]).

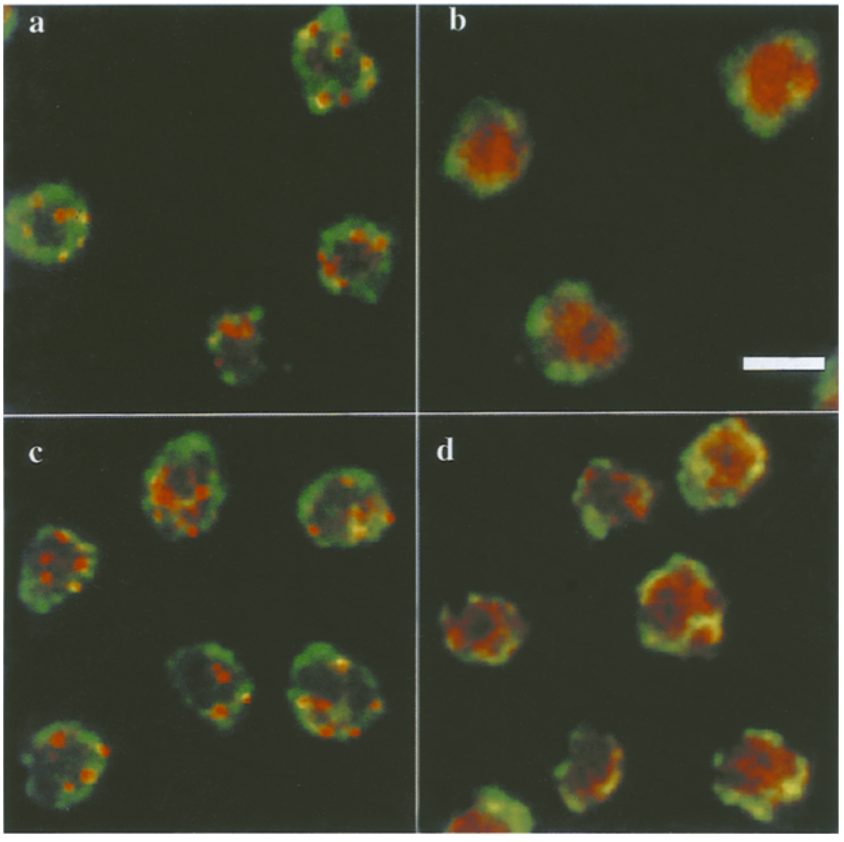

Figure 4. Rap1 and Sir4p immunofluorescence shows discrete foci in wild-type cells and a diffuse staining in *sir3* disruption mutant. A wild-type diploid strain (RS543, panels *a*, *c*) and a diploid *sir3* disruption strain (GA192, panels *b*, *d*) were treated with β-glucanase and Zymolyase before fixation, and incubated with affinity-purified anti-Rap1 antibodies (panels *a*, *b*; in red), affinity purified anti-Sir4p (panels *c*, *d*; in red) and with a mouse antibody raised against p62, a protein of the nuclear pore complex, detected by a Cy5-conjugated secondary antibody (in green). Anti-p62 gives the characteristic ringlike staining in both wild-type and mutant strains. The anti-Rap1 (panels *a*, *b*), and anti-Sir4p (panels *c*, *d*) were visualized through a Texas Red conjugated secondary antibody. Both anti-Rap1 and -Sir4p stainings are diffuse in *sir3* cells (panels *b*, *d*). Shown are images taken on a Zeiss Axiovert 100 LSM confocal microscope using 100× Plan-Apochromat objective. Bar = 2 μm. Adapted from Gotta et al. [5] and reproduced with permission from the J. of Cell Biology.

antibodies against Sir3p and Sir4p also shows a limited number of bright foci in vegetatively growing cells [63] (see fig. 4 for Sir4p staining). In strains deficient for either *SIR3* or *SIR4*, telomeres shorten, chromosome stability decreases slightly and Rap1 staining becomes diffuse throughout the nucleus (see fig. 4b for Rap1 in a *sir3Δ* strain). When unfixed yeast spheroplasts are treated with nonionic detergents, such as digitonin or Triton X-100, prior to and during the reaction with Rap1 antibodies, the integrity of the nuclear membrane is lost, and as nuclei become flattened, the number of detectable Rap1 foci increases to ~16. These are still fewer foci than the 64 telomeres in these diploid cells, but more than the 8 found in intact nuclei. One explanation for this may be that some protein-protein interactions required for telomere clustering are disrupted by solubilization of the nuclear envelope [66], while others are not.

Is there a relationship between the localization of Rap1, Sir3p and Sir4p and telomeric silencing? We have examined the immunolocalization of Rap1, Sir3p and Sir4p in various mutants of *S. cerevisiae*, in which telomeric silencing is altered due to impairment of Rap1, Sir or histone function [49, 52, 63]. In brief, our results show that in every case that silencing is disrupted – by mutation in *RAP1*, overexpression of the Sir4p C-terminus, or deletions or point mutation in the histone H3 and H4 N-termini – we observe a pronounced delocalization of Sir3p staining from perinuclear foci to a general nuclear staining pattern (summarized in table 1; D indicates delocalization from foci). In *sir1*, *rif1* and *rap1*^{s} mutants, situations in which telomeric silencing is not disrupted, Rap1 immunofluorescence is not altered. The *rap1-17* mutation, which removes 165 amino acids of the Rap1 C-terminus, results in high rates of chromosome loss, full derepression of telomeric silencing and dramatic lengthening of the telomeric repeat length [61]. In this mutant, Rap1 staining produces extremely bright immunofluorescence, with two distinct staining patterns:

either an enlarged, barlike staining pattern at the periphery of the nucleus or else numerous small foci distributed randomly throughout the nucleoplasm. This correlates with two classes of telomere behaviour, those that recombine and lose extra telomere length, and those that do not [49]. In *rap1-17* mutants anti-Sir3p and anti-Sir4p immunofluorescence patterns both reveal loss of the discrete punctate staining observed in wild-type cells [49]. In the *rap1-21* mutant, which is a truncation of the C-terminal 28 amino acids, subtelomeric regions are again fully derepressed and Sir3p staining is diffuse, while Rap1 staining localizes in foci. These results confirm two-hybrid data showing interaction between the C-terminus of Rap1 and Sir3p and suggest that Sir3p localization in foci correlates with silencing better than Rap1 localization. Sir3p staining is always delocalized in mutants which abolish silencing, while Rap1 can still be localized in foci in some mutants (see table 1).

It has been shown that Sir3p and Sir4p bind nucleosomes, in particular histones H3 and H4 [52]. N-terminal deletions of histones H3 and H4 abolish TPE and delocalize Rap1 and Sir3p staining (see table 1). In a strain carrying the H4K16Q mutation in the H4 N-terminus, silencing is abolished, Rap1 staining is somewhat altered but still largely punctate, whereas Sir3p staining is delocalized and diffuse in the nucleus. Mutations in histones H2A and H2B do not affect TPE and do not alter Rap1 and Sir3p localization. From these and the above-mentioned results, we propose that Sir3p is targeted to telomeres by Rap1, with additional binding to nucleosomes adjacent to the TG_{1-3} repeat. This second interaction between Sir3p and histone N-termini contributes significantly to the signal seen in immunofluorescence with anti-Sir3p antibodies (see model, fig. 2).

Table 1. Diffuse staining patterns of Sir3p correlates with loss of telomeric repression.

IF:	Rap1	Sir3p	Sir4p
sir3Δ	**D**	-	**D**
sir4Δ	**D**	~**D**	-
rap1ᵗ	**aberrant**	**D**	**D**
rap1-21	+/−	**D**	**n.d.**
Sir4C overexp.	**D**	**D**	**n.d.**
Sir4N overexp.	+/−	**D**	**n.d.**
HHTΔ4-30	+/−	**D**	**n.d.**
HHFΔ4-28	+/−	**D**	**n.d.**
HHFK16Q	+/−	**D**	**n.d.**
HTAΔ4-20	+	+	n.d.
HTBΔ3-32	+	+	n.d.
sir1Δ	+	+	n.d.
rif1Δ	+	+	n.d.
nup133	+	n.d.	n.d.
prp20	+	+	n.d.

This is a summary of Rap1, Sir3p and Sir4p immunofluorescence (IF) results derived from studies published in Palladino et al. [63, 66]; Cockell et al. [49]; Hecht et al. [52]; Gotta et al. [5]. The strains used are described in the indicated references: *sir3Δ* and *sir4Δ* [63, 66]; *rap1t* = *rap1-17* [49, 83]; *rap1-21* [43, 49]; Sir4C overexpression [49]; Sir4N over-expression (M. Cockell, personal communication); *HHTΔ4-30, HHFΔ4-28, HHFK16Q, HTAΔ4-20, HTBΔ3-32* [52]; *sir1Δ* [49]; *rif1Δ* [49, 84]; *nup133* (ref. 67 and T. Laroche, personal communication); *prp20* (T. Laroche, personal communication). Immunolocalization was done with a standard protocol and affinity purified antibodies are routinely used. D = delocalized or a diffuse, uniform nuclear staining; n.d. = not done; +/− indicates a pattern that is slightly altered with less distinct foci or foci displaced from the peripheral zone of the nucleus. The mutations indicated in bold face are conditions that derepress telomeric silencing. *Δ* indicates a deletion in the indicated gene.

Rap1, Sir3p and Sir4p colocalize with Y′ sequences

In mutants where the focal staining patterns of Sir3p, Sir4p and Rap1 are disrupted, are these proteins displaced from telomeres or are telomeres themselves delocalized? Double-labelling experiments using immunofluorescence with Rap1, Sir3p or Sir4p antibodies combined with fluorescence in situ hybridization (FISH), using Y′ subtelomeric sequences as a probe, have shown that these foci in fact reflect telomere position in wild-type cells [5]. Seventy-five percent of the Rap1 and Sir4p foci and 54% of the Sir3p foci colocalize with telomeric sequences (see fig. 5 and table 2). Although there is not a 100% coincidence, this probably reflects the less than 100% efficiency of both in situ and immunofluorescence staining techniques, and the fact that not all telomeres contain Y′ sequences. The possibility also exists that not all the Rap1, Sir3p and Sir4p foci correspond to telomeric foci. Nonetheless, these frequencies are highly significant when compared with the frequencies obtained by computer simulation of randomly distributed foci and with the frequency of colocalization of an internal sequence (*LYS2*) with Rap1 foci (see table 2). In these double-labelling experiments, staining the spheroplast with an antibody against a protein of the nuclear pore complex has allowed us to monitor the maintenance of nuclear integrity and the relationship of the immunofluorescent foci to the nuclear periphery as defined by the nuclear pore staining [5] (see, for example, the green pore staining in fig. 4).

In *sir3* and *sir4* mutants, although telomeres are still clustered in foci, Rap1, Sir3p and Sir4p are diffuse in the nucleus, indicating that at least one population of these proteins is displaced from telomeric sequences [5] (see, for example, Rap1 and Y′ stainings in fig. 5). The fact that Rap1 can be displaced from telomeres in *sir* mutants was an unexpected result, since *rap1* mutants and overexpression of the Rap1 C-terminus have higher chromosome loss rates and a more pronounced telomere size deregulation phenotype than do *sir* mutants. This suggests that in *sir* mutants not all the Rap1 is displaced from telomeres, but a population of this protein, which is normally held at telomeres by

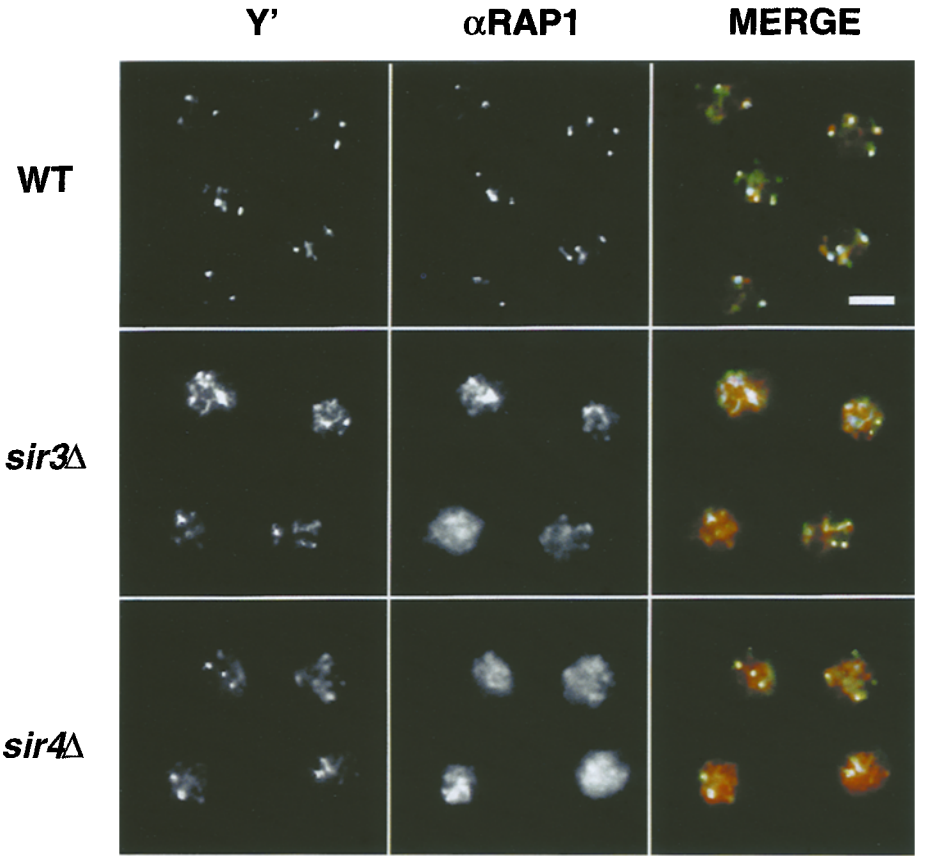

Figure 5. Y′ in situ hybridization coincides with Rap1 in wild-type cells but reveals a qualitatively altered pattern in *sir3* and *sir4* mutants. The wild-type diploid strain RS453 (WT), a *sir3*$^-$ diploid (GA192, *sir3Δ*) and a *sir4*$^-$ diploid (GA202, *sir4Δ*) were first stained with affinity-purified anti-Rap1 antibodies, which are detected by a Texas Red-conjugated secondary antibody (vertical column αRAP1), and subsequently hybridized with a DIG-dUTP labeled Y′ probe, detected by fluorescein-coupled anti-DIG F(ab) fragments (vertical column Y′). The column labelled Merge is the merger of the Y′ FISH and the antibody staining patterns, and the anti-Rap1 is now in red and the Y′ in green. Where signals coincide they are white. The total FISH signal integrated over 20 nuclei from each labelling varied less than 10% among strains. Shown are images taken on a Zeiss Axiovert 100 LSM confocal microscope using 100× Plan-Apochromat objective. Bar = 2 μm. Adapted from Gotta et al. [5], and reproduced with permission from the J. of Cell Biology.

protein-protein interaction (possibly through Sir3p and Sir4p), can be delocalized (see model, fig. 2). It is worth noting that Y′ FISH is nonetheless altered in *sir3* and *sir4* mutants, giving a more diffuse signal, although clustering per se is not lost (fig. 5).

Are Rap1 and telomeric foci at the nuclear envelope?

Quantitation of the immunoelectron microscopy shows that over 70% of these clusters of Rap1 staining are found within a peripheral zone measuring one-fifth of the nuclear radius, which comprises 50% of the spherical nuclear volume [62]. This suggests a nonrandom distribution of telomeres, at least at certain periods of the cell cycle. However, since most studies to date on telomere positioning depend on fluorescence microscopy, which has a resolution of 200 nm, it is impossible to judge whether the foci of telomeres, Rap1 or Sir proteins directly abut the nuclear envelope. Rap1 foci do not appear to be directly associated with nuclear pore complexes, since double-immunostaining with anti-Rap1 and antipore antibodies primarily label nonoverlapping foci at the nuclear periphery, with only 14% direct coincidence. In addition, Rap1 immunofluorescence on a strain carrying a mutation in *nup133*, which provokes the clustering of nuclear pores on one side of the nucleus [67], has a wild-type focal pattern, suggesting that Rap1/telomere complexes are positioned independently of the pore complexes (cited in ref. 5). Despite the fact that Sir4p has a coiled-coil domain in its essential C-terminal domain, which has been likened to the α-helical domain of a nuclear lamin [68], Sir4p staining does not form the ringlike pattern typical for lamin staining, suggesting that it does not form a stable link to a peripheral nuclear substructure. Whether there exist proteins associated with the nuclear envelope responsible for the clustering of yeast telomeres remains to be seen.

What is the significance of telomere clustering?

A question which arises from these results is whether the organization of telomeres within the nucleus is

important for either the establishment or maintenance of repressed chromatin. When long stretches of the TG_{1-3} telomeric repeat are integrated within the genome, they also confer a Sir-dependent repression of adjacent promoters [69], although a minimum of 828 bp (containing over 40 potential Rap1 binding sites) is required. A reporter gene flanked by two functional E and I silencers, or a natural *HM* cassette, is not repressed when integrated more than 200 kb from a telomere, suggesting that silencer function depends on the chromosomal context [70, 71]. Repression could be restored when a new telomere is created at 13 kb from the integrated reporter [70]. It was also observed that by enlarging the pool of Sir3p or Sir4p that is not bound to telomeric sites, either by overexpression of the Sir proteins or by mutations in *rap1*, Sir-dependent repression becomes more efficient at nontelomeric sites in the genome [70, 72, 73]. Thus it appears likely that the subnuclear organization of telomeres and the resulting unequal distribution of chromatin binding factors are capable of modulating transcription patterns in yeast. Ongoing FISH experiments will help to answer whether the re-established repression is caused by the 'looping' of this region into the repressed telomeric domains where Sir proteins are concentrated or whether this region itself forms a subdomain in the nucleus.

Table 2. Colocalization of FISH and IF foci.

FISH	Y′		*LYS2*	
IF	Obs	Sim	Obs	Sim
Rap1	73%[a] (3.4; 3.2; 76)	3.9% (3; 3; 10^4)	9% (2.4; 2.0; 41)	9.3%[b] (2; 2; 10^4)
Sir3p	54%[c] (1.6; 1.6; 160)	2.6% (2; 2; 10^4)	n.d.	n.d.
Sir4p	74% (3.6; 2.1; 96)	5.8% (4; 2; 10^4)	n.d.	n.d.

Shown are the observed (Obs) percentages of colocalization between the foci identified by FISH (Y′ or *LYS2* probes) and the immunofluorescence (IF) signals of Rap1, Sir3p or Sir4p. The observed colocalization corresponds to the fraction of FISH signals that have a surface overlap of more than 50% with a given IF signal. In parentheses are indicated n, m and T, where n = the number of IF foci per nuclear plane; m = the number of FISH foci per nuclear plane, and T = the total number of nuclei scored. Also shown is the percentage of colocalization calculated from a computer simulation of randomly distributed foci (Sim). For the computer simulation, Poisson-distributed numbers of circles ranging from 2 to 4, as indicated in the brackets, were used to calculate the frequency of overlap that would occur by chance in 10^4 nuclei containing two classes of circles in randomly generated positions. For the simulation, circles of 0.2 μm diameter were used and the nuclear midsection circle was 2 μm in diameter.
[a]The converse value for the % of Rap1 foci on Y′ spots is 76%.
[b]For the simulation of the *LYS2* probe, circles of 0.25 μm diameter were used, only to demonstrate the effect of slightly larger signals on the chance of colocalization (compare 5.8% with 9.3%). *LYS2* signals were not on average larger than the Y′ signals, however.
[c]The converse value for the % Sir3p foci on Y′ foci is 65%. This table is from Gotta et al. [5]. Reproduced with permission from the J. of Cell Biology.

Even if internal sequences do not need to move to the telomeric foci to be repressed, the unequal distribution of silencing proteins is likely to influence the repression event. The focal staining pattern of Sir3p and Sir4p observed in immunofluorescence suggests the presence of a high concentration of these proteins in the region of clustered telomeres. Telomeric clusters may be able to create the critical Sir concentration for repression by providing multiple binding sites through Rap1 and through various interactions with themselves. By creating this reservoir of Sir proteins at telomeres, telomeric bound Rap1 will more frequently encounter a Sir complex than a cotransactivator, improving chances that a nearby promoter will be repressed rather than transcribed. Moreover, high concentrations of Sir proteins may be necessary for cooperative binding to nucleosomes.

Nuclear organization in other systems

Specific organization of chromosomal domains is not unique to budding yeast. Indeed, the idea that chromatin is not randomly distributed in the interphase nucleus was first expressed in the classical paper by Rabl [74]. Rabl proposed that each chromosome, in interphase, occupies a distinct domain that reflect its mitotic orientation. These studies were later confirmed by Boveri, who showed that chromosomes maintained relatively fixed positions in nuclei of *Ascaris* embryos (for reviews see ref. 75). These studies also suggested that telomeres could be associated with the nuclear envelope at one side of the nucleus, with the centromeres at the opposite side, but no components have been isolated so far which are essential for this association.

In *Schizosaccharomyces pombe* it has been shown using FISH that centromeres are clustered and are located near the SPB (spindle pole body) throughout the cell cycle except in mitosis. Upon entry into mitosis, the clustered centromere structure is disrupted, centromeres dissociate from the SPB and then distribute along the spindle [76]. Telomeres are also clustered in G2; their position relative to the SPB is apparently not fixed, but they invariably localize at the periphery of the nuclear chromatin. In mitotic cells, however, the telomeric FISH signals are found throughout the nucleus [76]. This is intriguing, because fission yeast telomeres and centromeres are also sites of chromatin-mediated gene repression [77, 78].

In *Drosophila melanogaster* the Polycomb-group (Pc-G) genes play a major role in homeotic gene regulation. In the bithorax domain where particular homeotic genes are kept silent by an early initiation process, the Pc-G is then responsible for maintaining the repressed state through the rest of development [39]. Polycomb (Pc), a protein of this group, localizes mainly in bright spots in

Drosophila SL2 cells [79]. These structures are most probably large Pc-chromatin complexes which may reflect the formation of a subnuclear compartment. The nuclear distribution of Pc is disrupted by mutation in the chromodomain, a domain of unknown function found in many chromatin binding proteins. Mutated Pc is homogeneously distributed in the nucleus or is found in globular structures varying in number and size [79]. This aberrant localization could suggest that the chromodomain is involved in a 'packaging' mechanism and in the formation of a subnuclear compartment similar to that proposed for Sir3p and Sir4p in yeast.

Recently, Dernburg et al. [80] have combined FISH and three-dimensional microscopy techniques to test a structural model for position effect variegation (PEV) in *Drosophila*. PEV is a metastable but heritable repression of a euchromatic gene resulting from its relocation near heterochromatin by a chromosome rearrangement. The model for this phenomenon invokes the spreading of the silent state of heterochromatin along the length of the chromosome into euchromatin [40]. However, there are particular examples of PEV that do not fit this simple model. The $brown^D$ allele is a null mutation caused by an insertion of a large block of heterochromatin into the coding sequence of the gene. This insertion causes variegated inactivation of the normal copy of the gene in the homologous chromosome [81]. To explain the trans-inactivation caused by $brown^D$, it has been proposed that the insertion of heterochromatin into one copy of *bw* causes it to move to a heterochromatic compartment of the nucleus [82]. Due to pairing of the mutant allele with the wild-type copy, the intact gene is also repressed. Using FISH, Dernburg et al. have confirmed that in interphase nuclei the organization of chromosome II (where the *brown* gene lies) is dramatically altered by the insertion of this block of heterochromatin. In both bw^D/bw^D and $bw^D/+$ flies the *bw* locus associates with centromeric heterochromatin, indicating that bw^+ is silenced by contact with heterochromatin. Furthermore, this association is never detected in early embryonic development, suggesting that nuclear architecture varies at different stages of development.

Conclusions

A number of studies published in the past few years have suggested that the position of a gene within the nucleus, not just within the euchromatic or heterochromatic regions of chromosomes, is important for normal patterns of gene expression. However, how the nucleus is organized, which factors drive this organization and how this organization controls the different nuclear functions are still unclear. The study of mating type silencing and telomeric position effect in yeast should help us understand these phenomena. By combining genetic and structural analyses we hope be able to shed light on the mechanics of nuclear architecture and on how this organization controls different nuclear functions.

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