Two paralogs involved in transcriptional silencing that antagonistically control yeast life span

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In the yeast *Saccharomyces cerevisiae*, one determinant of aging or life span is the accumulation of extrachromosomal copies of rDNA circles in old mother cells [1]. The production of rDNA circles depends upon intrachromosomal recombination within the rDNA tandem array, a process regulated by the protein Sir2 (Sir2p). Together with Sir1p, Sir3p, Sir4p and Orc1p, Sir2p is also involved in transcriptional silencing of genes at the silent mating-type cassettes, in the rDNA array, and at telomeres. Using a 'triple silencer' strain that can monitor an increase or decrease in gene expression at these three loci, we found that deletion of the *ZDS1* gene caused an increase in silencing in the rDNA and at a silent mating-type cassette at the expense of telomere silencing. The *zds1* deletion also resulted in an increase in life span and a decrease in Sir3p phosphorylation. In contrast, deletion of its paralog *ZDS2* caused a decrease in rDNA silencing, a decrease in life span and an increase in Sir3p phosphorylation. As Zds2p, but not Zds1p, had strong two-hybrid interactions with Orc1p and the four Sir proteins, Zds1p might indirectly control Sir3p through a Sir3p kinase.

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Results and discussion

We cloned *ZDS1* and *ZDS2* while searching for genes that increased the stability of 15 kb linear centromeric plasmids in *S. cerevisiae* [2]. Zds1p and Zds2p are two proteins of unknown function with 34% sequence identity to each other [3,4]. We previously reported that Zds2p interacts with Sir2p, Sir3p and Sir4p in the yeast two-hybrid assay, whereas Zds1p shows little or no interaction with Sir proteins [2]. Neither Zds1p or Zds2p show strong interactions with the telomere proteins Rif1p or Rif2p [2]. Further analysis revealed that Zds2p also interacts strongly with two other silencing proteins, Orc1p and Sir1p [5,6], whereas Zds1p shows no interaction (see Supplementary material). These data suggested that these paralogs might play a role in gene silencing.

ZDS1 and *ZDS2* have antagonistic effects on rDNA silencing

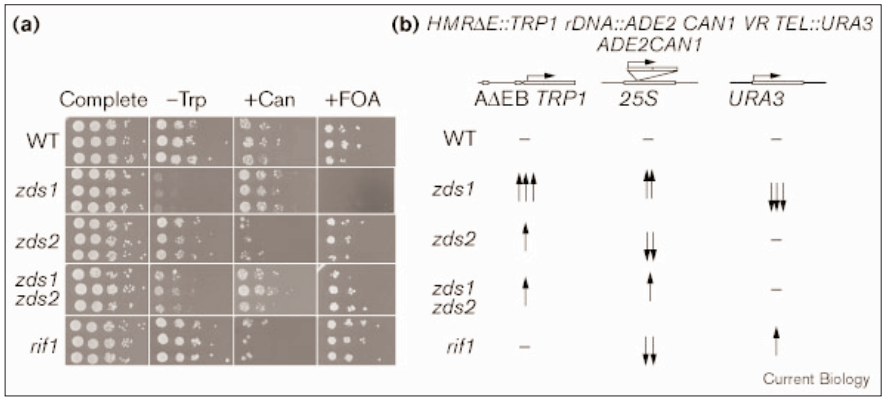
We constructed a yeast strain containing reporter genes at three loci known to be silenced by Sir proteins: an *HM* locus (*hmrΔE*), the 25S rRNA gene in the rDNA array — a tandem array of 100–200 genes encoding ribosomal RNAs — and a telomere. Silencing at *HM* loci requires Orc1p and all four Sir proteins [5], silencing in the rDNA requires Sir2p but not Sir1p, Sir3p or Sir4p [7], and silencing at the telomeres requires Sir2p–Sir4p but not Sir1p [8]. Only incomplete silencing of these reporters was observed in the wild-type strain, so these constructs can detect both an increase and a decrease in silencing (Figure 1). To test the effect of *ZDS* gene dosage on silencing, the *ZDS1* and *ZDS2* genes were either deleted from this strain or introduced on multicopy plasmids.

The *zds1* deletion had a dramatic effect on silencing at all three loci. Silencing at the telomere was reduced by at least 100-fold while silencing at *hmrΔE* was increased by almost

due to changes in transcription, the combined RNA levels of each reporter and its mutant chromosomal locus (for example, *TRP1* and *trp1-289*) were determined. The RNA levels of the *TRP1*, *CAN1* and *URA3* genes in *zds1* cells were, respectively, 0.40, 0.48 and 1.4 times those in wild-type cells (see Supplementary material), which is consistent with the silencing phenotypes observed (Figure 1). In contrast to the *zds1* deletion, multiple copies of the *ZDS1* gene caused a ninefold decrease in rDNA silencing, a fourfold decrease in *hmr* silencing and a fivefold increase in telomeric silencing (see Supplementary material). Thus, *ZDS1* has the genetic properties of an antagonizer of rDNA silencing because *ZDS1* function shifts silencing towards the telomere at the expense of *hmr* and the rDNA.

The *zds2* deletion caused a decrease in silencing in the rDNA array and a threefold increase in silencing at the *hmr* locus (see Supplementary material). The RNA levels of the *TRP1*, *CAN1* and *URA3* genes in *zds2* cells were, respectively, 0.78, 1.4 and 0.91 times those of wild-type cells (see Supplementary material), consistent with the silencing phenotypes. Increased *ZDS2* gene dosage caused an approximately fourfold decrease in *hmr* silencing, whereas silencing in the rDNA and at the telomere was similar to that of the wild-type control (see Supplementary material). Thus, the *zds2* deletion shows that *ZDS2* has

Figure 1



Deletion of the *ZDS1* or *ZDS2* gene causes changes in silencing at *hmrΔE*, the rDNA array and a telomere. The wild-type triple silencer strain CCFY100 (W303–1A *MATa ade2–1 ura3–1 trp1–289 leu2–3,112 his3–11,15 can1–100*) contains the *TRP1* gene in the *hmrΔE* silencer (which lacks the Rap1p-binding site but retains the ARS consensus (A) and Abf1p-binding site (B) [26], the *ADE2–CAN1* double gene cassette in the 25S rRNA gene within the rDNA tandem array [13] and the *URA3* gene followed by TG_{1-3} repeats replacing the right telomere of chromosome V (the VR telomere [21]). The effects on silencing of deleting either *ZDS1* or *ZDS2* [2], or expressing multiple copies of each *ZDS* gene (see Supplementary material) in CCFY100 cells were then determined. CCFY100 cells bearing a *rif1* deletion [20] were generated for comparison because the *rif1* mutation is known to cause decreased life span in this strain background [12]. **(a)** Serial dilutions of single colonies spotted onto the indicated media. For each row, a single colony was resuspended in 1 ml of water and then 5 µl aliquots of undiluted cells and tenfold serial dilutions were spotted. Complete medium shows the total number of cells spotted on each plate; −Trp, medium selecting for *TRP1* expression in *hmrΔE::TRP1* (where more growth means less silencing at this locus); +Can, medium selecting against *CAN1* expression (that is, for *CAN1* silencing in the rDNA where more growth means more silencing); and +FOA, medium selecting against *URA3* expression (that is, for *URA3* silencing at the VR telomere where more growth means more silencing). The deletion mutation in each strain is indicated on the left. WT, wild type. **(b)** Diagrams showing the three silencer constructs. The arrows indicate a reproducible increase (up arrows), decrease (down arrows) or no change (hyphen) in silencing compared with wild-type cells, as determined from at least six colonies (for quantitation see Supplementary material). One arrow indicates a change of threefold to fivefold, two arrows indicate a change of greater than fivefold to ninefold and three arrows indicate a change of more than 80-fold (no single colonies were detected).

the genetic properties of a promoter of rDNA silencing with effects opposite to those of *ZDS1*.

The *zds1 zds2* double mutant had a silencing phenotype distinct from that of either single mutant (see Supplementary material). In the absence of both Zds1p and Zds2p, telomere silencing was indistinguishable from that of wild-type cells, whereas *hmrΔE* silencing and rDNA silencing both increased by about fourfold. If Zds1p and Zds2p both acted in the same pathway, the double-deletion mutant should have the same phenotype as one of the single mutants. Thus, Zds1p and Zds2p alter silencing at telomeres, rDNA and *hmrΔE* by affecting more than one genetic pathway. The *zds1 zds2* double mutant has budding, cell-cycle, and other growth defects that are not present in the single mutants [3,4,9]. Therefore, the remaining analyses focused on the single mutants.

The *zds1* and *zds2* mutations have opposite effects on rDNA recombination and life span

Silencing proteins at the rDNA play a role in the control of rDNA recombination [10,11] and life span [1,12]. Sir2p modulates both rDNA silencing and rDNA recombination [7,10,11]. Because each yeast rDNA repeat contains an origin of DNA replication, intrachromatid crossing over can generate a circular rDNA repeat that can autonomously replicate and be maintained as an extrachromosomal plasmid [1,13,14]. Cell division in budding yeast is asymmetric and the larger mother cell tends to retain a higher fraction of such autonomously replicating plasmids at each cell division, resulting in increased plasmid copy number [15]. The accumulation of extrachromosomal rDNA circles (ERCs) has been shown to cause rapid aging in yeast cells [1]. Sir3p, which is not required for rDNA silencing [7], is required for normal life span — a *sir3* deletion reduces mean life span by ~20% [12]. Sir3p localizes to the nucleolus in old cells [16], and this localization is thought to reduce the production or accumulation of ERCs. A correlation between the extent of rDNA silencing and life span has not been shown, however (reviewed in [17]). Because *ZDS1* and *ZDS2* had antagonistic effects on silencing in the rDNA, we determined whether rDNA recombination and cell life span were also altered in *zds1* or *zds2* cells.

Recombination within the rDNA array was determined by measuring the frequency at which cells lost the *ADE2–CAN1* cassette. Intrachromatid crossing over followed by the generation of plasmid-free daughter cells can generate Ade^- colonies that turn red on rich medium [13]. Because the total number of Ade^- cells generated depends on when the recombination event occurs as a single cell grows into a colony, the median recombination frequency for five independent cultures was determined (Figure 2a). Wild-type cells lost the *ADE2–CAN1* cassette at a frequency of 76 events per 10^4 cells. The *zds1* cells, which showed an increase in rDNA silencing, showed a decrease in rDNA recombination and lost the *ADE2-CAN1* cassette

at a frequency of 14 events per 10^4 cells. In contrast, cells bearing the *zds2* gene deletion, which showed a decrease in rDNA silencing, showed an increase in rDNA recombination and lost *ADE2* at a frequency of 216 events per 10^4 cells. Thus, the *zds1* and *zds2* mutations had opposite effects on rDNA recombination.

The *zds1* and *zds2* mutations also had opposing effects on cell life span. In budding yeast, cell life span is determined by measuring the number of times a newly born cell can give rise to daughter cells before it ceases to divide. To allow for variation between individuals, life span is determined for a population of cells [18]. The mean life span of *zds1* cells was 37% longer than that of wild-type cells, whereas the mean life span of *zds2* cells was 20% shorter than that of wild-type cells (Figure 2b). Shorter life span is also associated with decreased resistance to stress such as heat shock [19]. The short-lived *zds2* cells showed decreased stress resistance, whereas the longer-lived *zds1* cells showed the same resistance as wild-type cells (see Supplementary material). Thus, the decreased stress resistance, decreased rDNA silencing and increased rDNA recombination seen in *zds2* cells correlated with a decreased life span.

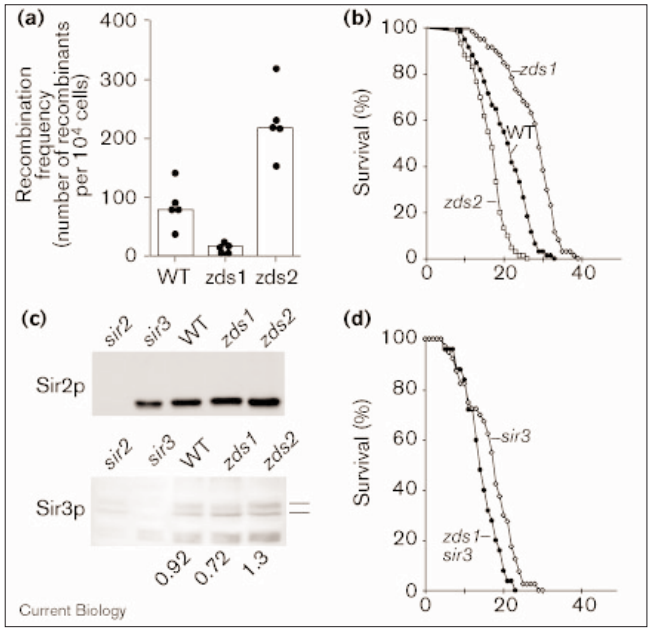
These results suggested that the change in functional rDNA silencing observed in the triple-silencer strain was a predictor of cell life span. Alternatively, the correlation between rDNA silencing levels and life-span length observed in *zds1* and *zds2* cells might be coincidental. To distinguish between these two possibilities, the *RIF1* gene was deleted from the triple-silencer strain. The *RIF1* gene encodes a protein that associates with the major telomere-binding protein Rap1p, and deletion of *RIF1* reduces mean cell life span by ~25% [12] and causes an increase in telomere length [20]. In contrast, the *zds1* or *zds2* deletions do not alter telomere length [2], so the direct effects of these deletion mutations are distinct. If reduced rDNA silencing is correlated with reduced life span, then a short-lived *rif1* strain should have a silencing phenotype similar to the *zds2* strain.

The *rif1* deletion caused a sixfold decrease in rDNA silencing, a fivefold increase in telomere silencing and had no effect on *hmr* silencing (see Supplementary material). Thus, the decrease in functional rDNA silencing was correlated with short life span in the *rif1* and *zds2* strains.

The level of phosphorylated Sir3p is reduced in *zds1* cells

Because Sir2p is limiting for rDNA silencing [17] and Sir3p is limiting for telomeric silencing [21], the levels of Sir2p and Sir3p in *zds1* and *zds2* cells were analyzed by western blotting. Sir2p levels were not changed by deletion of either the *ZDS1* gene or the *ZDS2* gene (Figure 2c). Sir3p has been previously shown to occur in two forms: a faster migrating form and a slower migrating phosphorylated form [22]. Constitutive activation of the Fus3p and Kss1p mitogen-activated protein (MAP) kinase cascades by the *STE11–4* mutation increases the amount of the slower migrating phosphorylated form of Sir3p and the level of telomeric silencing [22]. When compared to Sir3p from wild-type cells, a reproducible decrease in the slower migrating Sir3p band was seen in *zds1* cells, whereas a reproducible increase in this band was seen in *zds2* cells (Figure 2c). These data suggest that Zds1p is

Figure 2



Opposing effects of *zds1* and *zds2* deletions on rDNA recombination, life span and Sir3p phosphorylation. **(a)** The frequency of recombination in the rDNA for individual colonies of W303 wild-type (WT), *zds1* or *zds2* cells, as judged by loss of the *ADE2* gene. Recombination frequency is expressed as the number of recombinants per 10^4 cells. Each dot represents the recombination frequency determined from an individual colony while each bar represents the median recombination frequency for the five colonies analyzed (described in the Supplementary material). **(b)** Survival curves for W303 wild-type, *zds1* and *zds2* cells showing the percentage of cells surviving after a given number of divisions. The mean (and maximum) life spans were 20.6 (33) divisions for wild-type cells, 27.6 (39) for *zds1* cells and 16.5 (26) for *zds2* cells. Each curve represents a sample of 60 cells analyzed as described [19,25]. **(c)** Western analysis of Sir2p and Sir3p. Whole cell extracts from cells bearing a *sir2* or *sir3* mutation [2] or W303 wild-type, *zds1* or *zds2* cells were examined for Sir2p and Sir3p levels, using antibodies against Sir2p (Santa Cruz) or Sir3p (kindly provided by L. Pillus [22]). The two lines indicate the positions of the two forms of Sir3p while the lower bands are unrelated to Sir3p [22]. The ratio of intensities for the slower migrating Sir3p form over the faster migrating Sir3p form are given below (determined using Kodak 1D software on two independent experiments). The ratios obtained from two different western blots differed by less than 5%. **(d)** Survival curves for W303 *sir3* and *zds1 sir3* cells analyzed as in (b). The mean (and maximum) life spans were 16.9 (28) divisions for *sir3* cells and 14.5 (22) divisions for *zds1 sir3* cells, a reduction from the 20.6 (33) divisions for wild-type cells (b). The curves represent samples of 40 cells for *sir3* and 25 cells for *zds1 sir3*.

required in wild-type cells for high levels of Sir3p phosphorylation. The mean life spans of *sir3* cells and *zds1 sir3* cells were similar (Figure 2d) and both were reduced compared to the wild-type control cells (Figure 2b). Thus, the life span extension caused by the *zds1* mutation required Sir3p, consistent with Zds1p acting through Sir3p. In contrast, the normal function of Zds2p results in decreased phosphorylation of Sir3p.

Two models can account for how *ZDS1* and *ZDS2* antagonistically affect cell life span. Zds1p and Zds2p might independently control rDNA silencing and life span. For example, Zds1p might regulate a signaling pathway that alters Sir3p phosphorylation while Zds2p promotes the deposition of *SIR* proteins onto newly synthesized rDNA chromatin. Alternatively, Zds1p and Zds2p might both interact with an unknown protein that regulates rDNA silencing, with Zds1p and Zds2p causing opposite effects.

The result suggesting that Zds1p affects the activity of a Sir3p kinase can help explain the diverse array of *ZDS* phenotypes. The *ZDS* genes were named because their overexpression suppressed a number of phenotypes that allowed their cloning in a zillion different screens ([3,4], reviewed in [2]). Zds1p is concentrated at the cell surface [4] and could affect signaling pathways. Mutations in signaling proteins, such as Ras1p or Ras2p, can have pleiotropic effects on cell growth and life span [23–25]. An alteration in cell signaling by *ZDS1* overexpression could also lead to diverse phenotypes.

Supplementary material

Supplementary material including a figure showing the effects on silencing of expressing multiple copies of either *ZDS1* or *ZDS2* in CCFY100 cells and additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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