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Enforcement of a lifespan-sustaining distribution of Sir2 between telomeres, mating-type loci, and rDNA repeats by Rif1

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Abstract

Telomere dysfunction is linked with genome instability and premature aging. Roles for sirtuin proteins at telomeres are thought to promote lifespan in yeast and mammals. However, replicative lifespan of the budding yeast *Saccharomyces cerevisiae* shortens upon deletion of Rif1, a protein that limits the recruitment of the sirtuin histone deacetylase Sir2 to telomeres. Here we show that Rif1 maintains replicative lifespan by ultimately stabilizing another age-related chromosomal domain harboring the ribosomal DNA (rDNA) repeats. Deletion of Rif1 increases Sir2 localization to telomeres and the silent mating-type loci, while releasing a pool of the histone deacetylase from the intergenic spacer 1 (IGS1) of rDNA. This is accompanied by a disruption of IGS1 silent chromatin assembly and increases in aberrant recombination within rDNA repeats. Lifespan defects linked with Rif1 deletion are abolished if rDNA repeats are forcibly stabilized via deletion of the replication fork-blocking protein Fob1. In addition, Sir2 overexpression prevents Rif1 deletion from disrupting Sir2 at IGS1 and shortening lifespan. Moreover, subjecting cells lacking Rif1 to caloric restriction increases IGS1 histone deacetylation and lifespan, while uncovering novel genetic interactions between *RIF1* and *SIR2*. Our data indicate that Rif1 maintains lifespan-sustaining levels of Sir2 at rDNA by preventing excessive recruitment of the histone deacetylase to telomeric and silent mating-type loci. As sirtuin histone deacetylases, such as Sir2 or mammalian SIRT6, each operate at multiple age-related loci, we propose that factors limiting the localization of sirtuins to certain age-related loci can promote lifespan-sustaining roles of these sirtuins elsewhere in the genome.

Key words: calorie restriction; DNA recombination; replicative lifespan; ribosomal DNA (rDNA); Sirtuin 2 (Sir2); telomeres.

Introduction

Gene expression patterns and cellular fate are thought to be intimately connected to the establishment and maintenance of silent chromatin or heterochromatin domains (Kurdistani & Grunstein, 2003). Silencing within these regions is mediated by several enzymes including histone deacetylases (Kurdistani & Grunstein, 2003). However, the level of a given histone deacetylase, which may act simultaneously at multiple silent chromatin loci, is often limiting (Moazed, 2001). In addition, certain factors can directly limit the recruitment of histone deacetylases to specific silent chromatin domains (Moazed, 2001). How such factors affect the distribution of histone deacetylases between all the major silent chromatin domains at which the enzyme typically operates and how this affects the cell overall is unclear (Buck & Shore, 1995; Smith *et al.*, 1998; Michel *et al.*, 2005). The budding yeast *Saccharomyces cerevisiae* has three major silent chromatin domains, namely the rDNA repeats, telomeres, and mating-type loci (Moazed, 2001). All three domains, which are nonrandomly arranged within the nucleus, are controlled by the conserved histone deacetylase Sir2 (Rine & Herskowitz, 1987; Aparicio *et al.*, 1991; Imai *et al.*, 2000; Moazed, 2001; Mekhail & Moazed, 2010). Sir2 is required for the maintenance of replicative lifespan, a measure of cellular longevity that reflects the number of times that a yeast mother cell replicates its DNA and yields progeny before reaching senescence (Kaeberlein, 2010).

Replicative lifespan is highly dependent on the stability of the ribosomal DNA (rDNA) repeats, which consist of ~190 rRNA genes that are tandemly arranged on chromosome XII and reside within the nucleolus, a major nuclear compartment that is also the site of ribosomal biogenesis (Mekhail & Moazed, 2010). RNA polymerase (Pol) I and III transcribe rRNA genes, while transcription from promoters within intergenic spacers (IGS1 and IGS2) flanking rRNA genes is mediated by RNA Pol II (Mekhail & Moazed, 2010). Expansion or contraction of the rDNA repeats via recombination can be beneficial under stress (Kobayashi *et al.*, 1998). However, hyperactive or aberrant recombination can lead to chromosomal instability and a shorter replicative lifespan (Gottlieb & Esposito, 1989; Kaeberlein *et al.*, 1999). Ribosomal DNA instability can also lead to the formation of extrachromosomal rDNA circles, which can promote but are not required for lifespan shortening (Sinclair & Guarente, 1997; Ganley *et al.*, 2009). Thus, recombination within the rDNA repeats is tightly regulated. Recombination within the repeats is promoted by an open chromatin structure and is dependent on binding of the fork block 1 (Fob1) protein to IGS1 sequences (Kobayashi & Horiuchi, 1996; Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith & Boeke, 1997; Huang & Moazed, 2003). Sir2 operates at rDNA as part of a complex called RENT, which also contains Net1/Cfi1 and Cdc14 (Shou *et al.*, 1999; Straight *et al.*, 1999; Visintin *et al.*, 1999). Through a process termed rDNA silencing, compaction of IGS1 chromatin by Sir2 limits access to RNA Pol II and to the recombination machinery preserving chromosome stability (Gottlieb & Esposito, 1989; Bryk *et al.*, 1997; Smith & Boeke, 1997). Other nucleolar factors implicated in rDNA silencing include the Cohibin complex, which is composed of Lrs4 and Csm1 proteins and operates at least partly independently of Sir2 (Huang *et al.*, 2006; Mekhail *et al.*, 2008; Chan *et al.*, 2011; Poon & Mekhail, 2011). Thus, disruption of nucleolar Sir2/Cohibin or Fob1, respectively, decreases and increases replicative lifespan (Defossez *et al.*, 1999; Kaeberlein *et al.*, 1999; Chan *et al.*, 2011).

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The function of telomeric Sir2 is also required for the maintenance of replicative lifespan (Gottschling *et al.*, 1990; Dang *et al.*, 2009; Chan *et al.*, 2011). Telomeric Sir2 is the enzymatic subunit of the SIR complex (*s*ilent *i*nformation *r*egulator), which also contains Sir3 and Sir4 (Moazed, 2001). Through a process termed telomere position effect or telomeric silencing, SIR is recruited to telomeres and spreads into subtelomeric regions while promoting silent chromatin assembly and silencing RNA Pol II-transcribed subtelomeric genes (Gottschling *et al.*, 1990; Moazed, 2001). In addition, perinuclear telomere clustering, which relies in part on telomeric Cohibin complexes, increases the local concentration of SIR proteins ensuring efficient telomeric silencing (Mekhail & Moazed, 2010; Chan *et al.*, 2011; Poon & Mekhail, 2012). Disruption of this Sir2-dependent telomeric silencing is linked with subtelomeric DNA instability and a shorter lifespan (Kaeberlein *et al.*, 1999; Dang *et al.*, 2009; Chan *et al.*, 2011). Sir2 also localizes to and represses gene expression at the silent mating-type loci, *HMR* and *HML*, which are commonly referred to as HM loci (Rine & Herskowitz, 1987). How this impacts lifespan is unclear (Kaeberlein *et al.*, 1999). Thus, Sir2 promotes lifespan at least via rDNA- and telomere-related processes.

Regulation of telomeric silencing is also partly mediated by the protein Rap1 (*r*epressor *a*ctivator *p*rotein 1), which binds to sites within the terminal $C_{1-3}A$ telomeric repeats (Longtine *et al.*, 1989). Rap1 can recruit Sir4, which then recruits the rest of the SIR complex promoting its spreading into subtelomeric regions (Moretti *et al.*, 1994; Buck & Shore, 1995; Hoppe *et al.*, 2002). In addition, different domains of Rap1 can recruit the telomeric Rif1 and Rif2 proteins forming a complex that is functionally analogous to shelterin with regard to its ability to limit access to telomerase and prevent excessive telomere lengthening (Hardy *et al.*, 1992; Wotton & Shore, 1997; Martinez & Blasco, 2010; Gallardo *et al.*, 2011). However, Sir4 and Rif1, but not Rif2, compete to bind the same domain of Rap1 and thus Rif1 can directly limit the Sir4-dependent recruitment of Sir2 to telomeres (Buck & Shore, 1995; Wotton & Shore, 1997). While the physiological relevance of this antagonistic role of Rif1 with respect to telomeric silencing is unclear, deletion of Rif1, but not Rif2, has been linked with a shortened lifespan suggesting that hyper-recruitment of Sir2 to chromosome ends underlies the shortened lifespan of *rif1*Δ cells (Austriaco & Guarente, 1997; Managbanag *et al.*, 2008). However, as the recruitment of Sir2 to telomeres is positively linked with lifespan, we tested whether the hyper-recruitment of Sir2 to chromosome ends in *rif1*Δ cells occurs at the expense of other Sir2-regulated chromosomal domains that are also critical to lifespan maintenance.

By examining how Rif1 affects the localization and function of Sir2 at major silent chromatin domains, our work reveals that cells utilize factors such as Rif1 to enforce lifespan-sustaining distributions of key chromatin-modulatory enzymes between their principal sites of action. We also identify various genetic and environmental approaches that can prevent or counteract chromosomal and cellular defects typically observed following the mislocalization of Sir2 across the genome in cells lacking Rif1. Overall, our work reveals lifespan-sustaining relationships between histone deacetylases, their regulatory factors, nonrandom spatial genome organization, and the cellular environment.

Results

Rif1 affects Sir2 localization and histone acetylation at all major silent chromatin domains

To gain insight into the role of Rif1 in lifespan maintenance, we first examined the genetic relationship between *RIF1* and *SIR2*. The loss of Rif1 significantly shortened replicative lifespan, albeit not to the same extent as deleting Sir2, as expected (Fig. 1A and Table S1) (Austriaco & Guarente, 1997; Kaeberlein *et al.*, 1999). More importantly, *rif1*Δ *sir2*Δ cells exhibited a lifespan similar to that of *sir2*Δ cells (Fig. 1A and Table S1). Controls for lifespan analyses were *lrs4*Δ and *hxk2*Δ cells, which exhibited a respectively shortened and extended lifespan relative to wild-type cells, as expected (Fig. 1A; Table S1) (Lin *et al.*, 2002; Chan *et al.*, 2011). These findings suggest that Rif1 may operate via Sir2 to maintain replicative lifespan.

The levels of chromatin silencing proteins such as Sir2 can be limiting (Maillet *et al.*, 1996; Smith *et al.*, 1998). Sir2 is implicated in silent chromatin assembly at all of the three major silent chromatin domains of *S. cerevisiae*, namely the rDNA repeats, subtelomeres, and silent mating-type loci (Rine & Herskowitz, 1987; Gottschling *et al.*, 1990; Aparicio *et al.*, 1991; Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith & Boeke, 1997). Thus, we examined if the loss of Rif1 impacts the localization and function of the Sir2 histone deacetylase at all three major silent chromatin domains. We conducted chromatin cross-linking and immunoprecipitation (ChIP) analyses employing antibodies specific to Sir2 or, as a Sir2 functional readout, to histone H3 acetylated on lysines 9 and 14 (diAcH3K9/K14) (Braunstein *et al.*, 1993; Imai *et al.*, 2000; Suka *et al.*, 2001; Armstrong *et al.*, 2002; Huang & Moazed, 2003; Mekhail *et al.*, 2008; Chan *et al.*, 2011). Loss of Rif1 increased the level of Sir2 proteins associated with subtelomeres while decreasing histone H3 acetylation near chromosome ends (Fig. 1B,E). Surprisingly, we also observed a weak increase in Sir2 localization and histone deacetylation at the silent mating-type loci, *HMR* and *HML*, of *rif1*Δ cells (Fig. 1C,E). In contrast, histone acetylation and Sir2 localization were not significantly altered at IGS2 of rDNA (Fig. 1D,E). However, increased Sir2 localization to subtelomeres and HM loci was accompanied by the release of a pool of Sir2 from the rDNA IGS1 region, which exhibited increased histone acetylation (Fig. 1D,E). Differences between the effect of deleting Rif1 on the intergenic spacers of rDNA repeats may be linked with the fact that more Sir2 is normally localized to IGS1 as compared to IGS2 in wild-type cells (Huang & Moazed, 2003). Importantly, pull down of TAP-tagged Rif1 revealed enrichments for DNA sequences at telomeric and mating-type loci but not rDNA repeats, whereas Sir2-TAP localized to telomeres, mating-type loci, as well as the rDNA repeats (Fig. 1H–J). These data suggest that Rif1 proteins at telomeres and mating-type loci may ultimately modulate the distribution of Sir2 between the three major silent chromatin domains.

While an excessive silencing at telomeres is not thought to alter replicative lifespan, loss of silent chromatin assembly at the rDNA repeats leads to an increase in aberrant recombination events such as unequal sister chromatid exchange (USCE) and shortens lifespan (Gottlieb & Esposito, 1989; Kaeberlein *et al.*, 1999; Chan *et al.*, 2011). Thus, we assessed USCE by measuring the rate of loss of an *ADE2* marker gene inserted within the rDNA repeats (Kaeberlein *et al.*, 1999; Mekhail *et al.*, 2008; Chan *et al.*, 2011). Interestingly, Rif1 deletion significantly increased the rate of USCE, although to a lower extent than the deletion of Sir2 (Fig. 1F,G; Table S2). In addition, USCE rates were not statistically different in *sir2*Δ and *sir2*Δ *rif1*Δ cells (Fig. 1G and Table S2). In contrast, USCE rates can be further increased in *sir2*Δ cells upon deletion of other factors such as Lrs4 and Heh1, which are thought to suppress rDNA recombination via processes that are partly independent of Sir2 (Mekhail *et al.*, 2008). Furthermore, consistent with our ChIP and recombination data, Rif1 ensures efficient silencing of a foreign reporter gene inserted within the rDNA repeats but not silent mating-type or subtelomeric regions (Hardy *et al.*, 1992; Smith *et al.*, 1999; Chan *et al.*, 2011; Park *et al.*, 2011). Taken together, our findings suggest that Rif1 suppresses Sir2 recruitment primarily to telomeres and, to a lesser extent, the mating-type loci to maintain Sir2 levels at the rDNA recombination

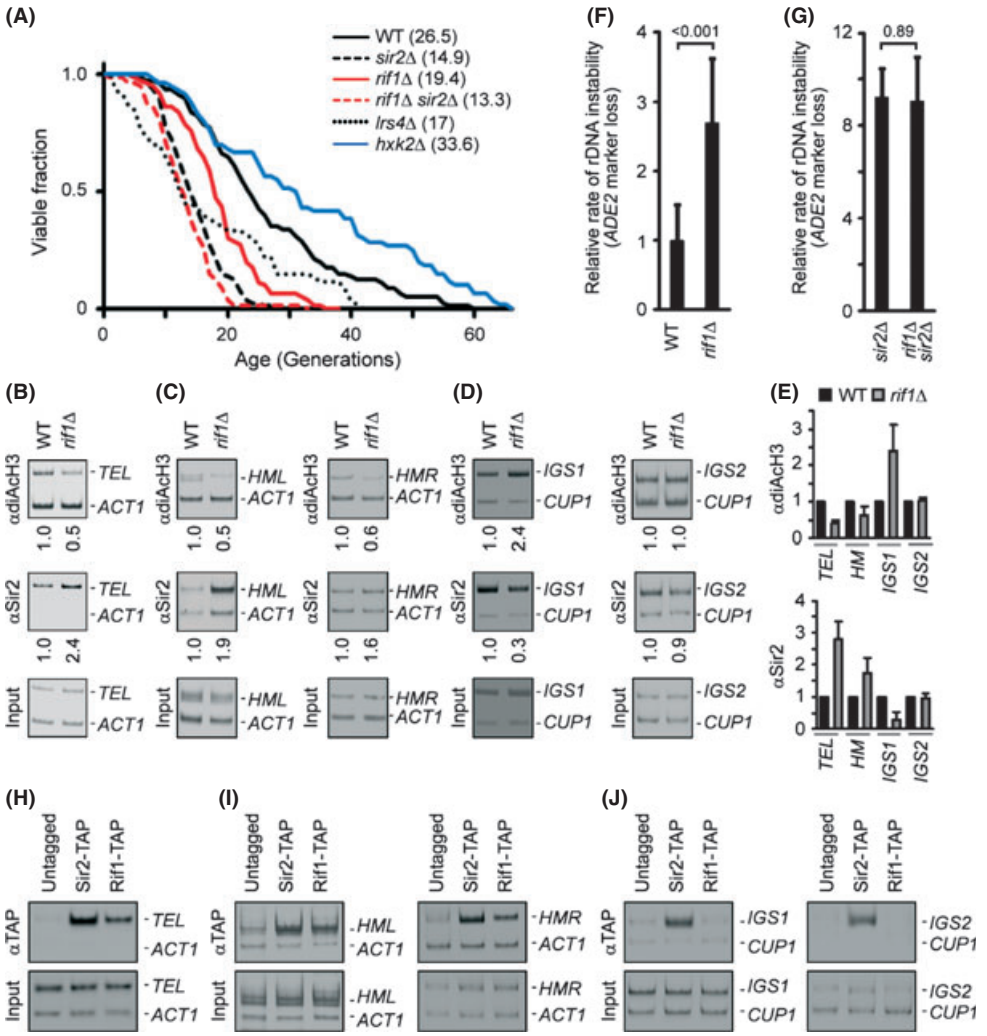

Fig. 1 Rif1 and Sir2 display an epistatic relationship in lifespan regulation and Rif1 deletion is accompanied by changes to Sir2 localization and histone acetylation at all major silent chromatin domains. (A) Disruption of Rif1 in *sir2*Δ cells, unlike in wild-type (WT) cells, does not shorten replicative lifespan. Mean lifespans are shown in parentheses and *P* values are listed in Table S1. BY4741 isogenic strains used: KMY 372, 391, 711, 1052, 1335, and 1055. (B–E) Rif1 regulates Sir2 localization to and histone acetylation at all three major silent chromatin domains. Duplex PCR products for telomeres (B), *HML* or *HMR* (C), and rDNA IGS1 or IGS2 (D) were amplified from input and immunoprecipitated (IP) chromatin. Fold enrichments for telomeric or HM loci (normalized to *ACT1* control) and rDNA sequences (normalized to *CUP1* control) relative to wild-type are shown below gels. (E) Quantifications of ChIP experiments as in (B–D) are shown. Data correspond to means (± SE) from two independent experiments and each point was calculated with at least three PCRs (*t*-test $P \leq 0.05$ for TEL, HM, and IGS1). Strains in B–E: KMY 372 and 711. (F–G) Rif1 deletion alone increases rDNA instability but *sir2*Δ cells and *rif1*Δ *sir2*Δ cells exhibit a similar elevated level of repeat instability. Shown are measurements of rDNA repeat instability (unequal sister chromatid exchange rates normalized to WT) as revealed by the rate of loss (± SD) of an *ADE2* marker gene from the rDNA repeats. *P* values (*t*-test) are on graphs while full counts and statistical analyses are in Table S2. Strains in F–G: KMY 326, 327, 953, and 1333. (H–J) Detection of interactions between Rif1 and telomeric/HM loci but not the rDNA intergenic spacers of rDNA repeats. Input and immunoprecipitated (IP) chromatin were obtained from untagged cells or cells expressing TAP-tagged Sir2 or Rif1 proteins, and duplex PCR products were amplified for telomeres (H), *HML* or *HMR* (I), and rDNA IGS1 or IGS2 (J). Strains in H–J: KMY 255, 282, and 1440.

enhancer sequence-containing IGS1 and thereby ensure efficient histone deacetylation and DNA stability within the rDNA repeats. However, while the loss of rDNA repeat stability may well underlie the lifespan defect observed in *rif1*Δ cells, our data so far do not fully support this notion. In addition, we still cannot completely rule out the possibility that excessive silent chromatin assembly at the telomeres and HM loci of *rif1*Δ cells may at least partly contribute to lifespan shortening.

Rif1 is dispensable for lifespan maintenance in a setting where rDNA repeats are forcibly stabilized via Fob1 deletion

We next set out to uncouple rDNA repeat stability from Rif1 deletion. Binding of the nucleolar protein Fob1 to recombination enhancer elements within IGS1 regions of rDNA repeats is required for recombination within the repeats (Kobayashi & Horiuchi, 1996). However, Fob1 is actually required for the recruitment of chromatin silencing factors including Sir2 and Lrs4 to rDNA repeats (Huang & Moazed, 2003). Thus, although Fob1 deletion abrogates rDNA silencing, it still leads to increased rDNA stability and replicative lifespan (Defossez *et al.*, 1999; Huang *et al.*, 2006). Therefore, we investigated the effects of deleting Rif1 in *fob1*Δ cells. ChIP analyses revealed that the loss of Rif1 in a *fob1*Δ setting still increased Sir2 localization and histone deacetylation at both the subtelomeric and HM loci (Fig. 2A,B, and D). Interestingly, such *rif1*Δ-dependent increases in Sir2 localization and histone deacetylation were higher in the absence of Fob1 than in its presence (compare Figs. 2D to 1E). This is consistent with the release of a significant fraction

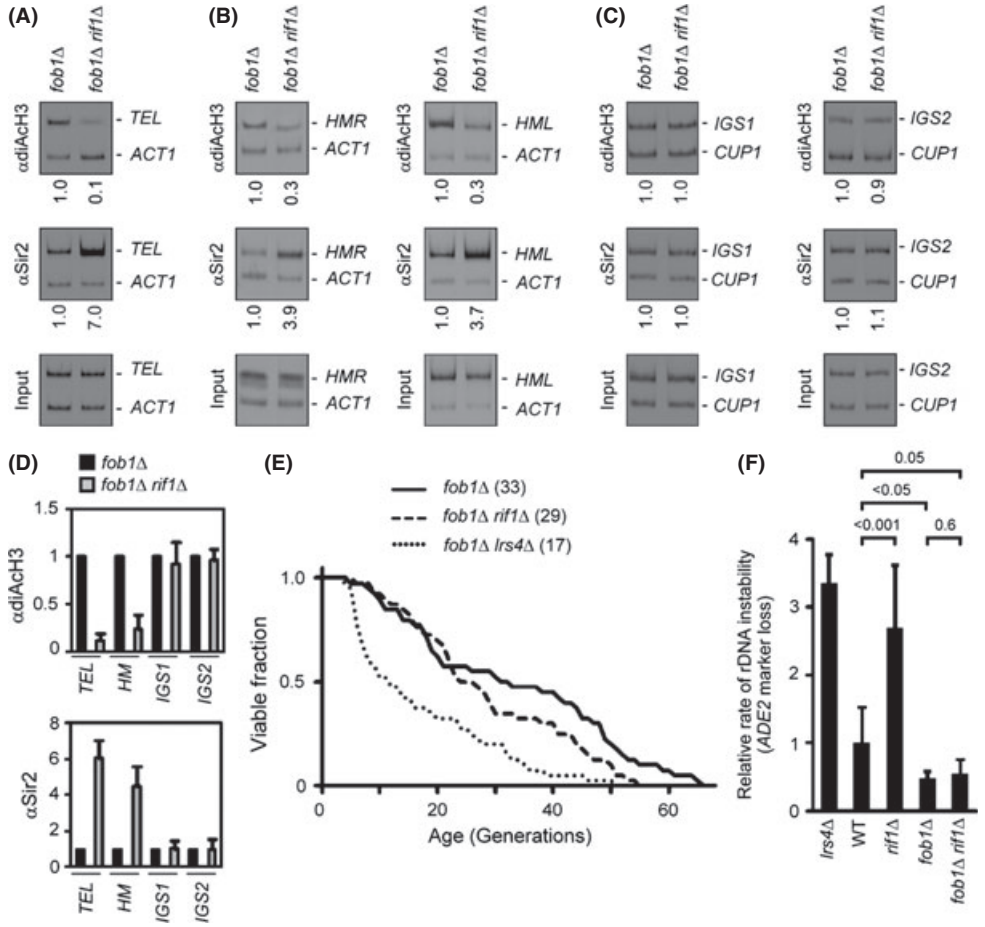

Fig. 2 Fob1 deletion renders *rif1*Δ ineffectual on rDNA repeat stability and replicative lifespan. (A–D) ChIP analysis reveals that deletion of Rif1 in cells already deficient in Fob1 still increases Sir2 levels and histone deacetylation at telomeres (A) and HM loci (B) but fails to alter chromatin at rDNA repeats (C). Representative gels with fold enrichments for telomeric or HM loci (normalized to *ACT1* control) and rDNA sequences (normalized to *CUP1* control) relative to wild-type (A–C) and quantifications (D) are shown. Data correspond to means (± SE) from two independent experiments and each point was calculated with at least three PCRs (*t*-test $P < 0.03$ for TEL and HM). BY4741 isogenic strains in A–D: KMY 1048 and 1534. (E) Disruption of Lrs4, but not Rif1, shortens the lifespan of *fob1*Δ cells. Mean lifespans are in parentheses and *P* values are listed in Table S1. BY4741 isogenic strains analyzed in parallel with cells shown in Fig. 1A are KMY 1048, 1528, and 1534. (F) Rif1 deletion does not destabilize the rDNA repeats of *fob1*Δ cells. Shown are measurements of rDNA repeat instability [unequal sister chromatid exchange rates normalized to wild-type (WT)] as revealed by the rate of loss (± SD) of an *ADE2* marker gene from the rDNA repeats. *P* values (*t*-test) are indicated on the graph while full counts and statistical analyses are in Table S2. Strains: KMY 326, 328, 337, 953, and 955.

of the Sir2 protein population from rDNA repeats in *fob1*Δ cells (Huang & Moazed, 2003). This also suggests that Rif1 deletion in wild-type cells only leads to respectively moderate and weak increases in Sir2 localization to telomeres and HM loci because of a limiting population of Sir2 proteins, a major fraction of which is normally located at rDNA repeats. In addition, the levels of acetylated histones associated with the rDNA repeats, either at IGS1 or IGS2, were similar in *fob1*Δ and *fob1*Δ *rif1*Δ cells (Fig. 2C,D). Together, these data indicate that Rif1 deletion in *fob1*Δ cells is ineffectual on chromatin status at rDNA repeats but still leads to excessive silent chromatin assembly at telomeres and HM loci.

Thus, if Rif1 deletion shortens the lifespan of wild-type cells by primarily promoting excessive silencing at telomeres and HM loci, then Rif1 deletion would be expected to significantly shorten the lifespan of *fob1*Δ cells. However, we found that *fob1*Δ and *fob1*Δ *rif1*Δ cells exhibited a similar extended, replicative lifespan (Fig. 2E and Table S1). In addition, Rif1 deletion in *fob1*Δ cells failed to increase the rate of USCE within rDNA repeats (Fig. 2F and Table S2). In contrast, deletion of Lrs4, which typically maintains rDNA repeat stability but also ensures telomeric silencing, increased the rate of rDNA USCE and decreased the lifespan of Fob1-deficient cells (Fig. 2E,F; Tables S1 and S2) (Chan *et al.*, 2011). ChIP and lifespan experiments conducted in different strain backgrounds yielded similar results (Fig. 2; Figure S1). Taken together, our results suggest that increased rDNA recombination, as a consequence of decreased Sir2-IGS1 interactions, underlies the *rif1*Δ-associated lifespan defect, which can be prevented if the rDNA repeats are forcibly stabilized via Fob1 deletion.

Increasing cellular Sir2 levels prevents *rif1*Δ-associated defects in rDNA silencing/stability and replicative lifespan

We next asked how the effects of deleting Rif1 may be altered in cells already expressing elevated levels of Sir2. We used a strain in which a second copy of *SIR2* is inserted at the *LEU2* locus (designated *SIR2* overexpression or *SIR2*-OE) (Kaeberlein *et al.*, 2004). Reverse transcription PCR (RT-PCR) and western blot analysis confirmed that *SIR2*-OE cells exhibited increased Sir2 mRNA and protein levels (Fig. 3A,B). Importantly, Rif1 deletion was ineffectual on Sir2 mRNA and protein levels both within the wild-type as well as the *SIR2* overexpression settings (Fig. 3A,B).

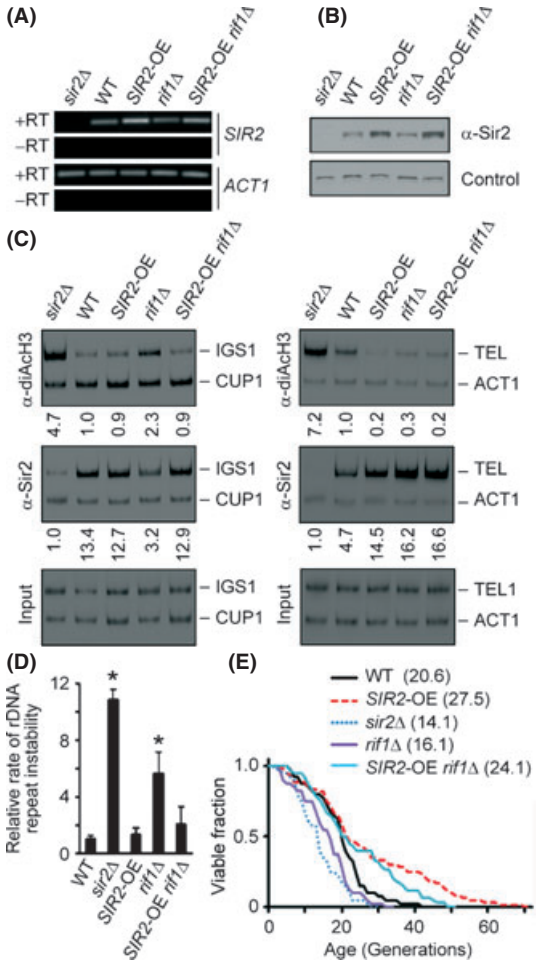

Fig. 3 Sir2 OE abolishes chromatin, rDNA stability, and lifespan defects associated with the loss of Rif1. (A, B) Semi-quantitative RT-PCR (A) and Western blot (B) analyses, respectively, revealing that Sir2 mRNA and protein levels are increased in SIR2-OE strains irrespective of Rif1 presence. (C) ChIP analysis revealing that overexpression of Sir2 prevents *rif1*Δ from decreasing Sir2 levels or increasing histone acetylation at rDNA IGS1 (Left). ChIP also reveals that SIR2-OE increases Sir2 localization/function at telomeres in wild-type (WT) cells while preventing *rif1*Δ from altering Sir2 localization/function near chromosome ends. (D) Analysis of variations to rDNA copy number by qPCR reveals that SIR2-OE prevents Rif1 deletion from destabilizing rDNA repeats. Shown are measurements of rDNA repeat instability (± SD from three independent experiments) as revealed by rDNA copy number variations detected via qPCR analysis with normalization to WT cells. Asterisk, *P* value (*t*-test) <0.05. (E) Replicative lifespan analysis examining the effect of deleting Rif1 in cells overexpressing *SIR2*. Mean lifespans are in parentheses and *P* values are listed in Table S1. Strains in A-E: KMY 1536, 1537, 1542, 1544, and 1546.

ChIP analysis revealed that *SIR2* overexpression does not alter Sir2 levels or histone acetylation at IGS1 within an otherwise wild-type setting (Fig. 3C). However, relative to *rif1*Δ cells, *SIR2-OE rif1*Δ cells exhibited both higher IGS1 Sir2 localization and histone deacetylation levels that were similar to those observed in wild-type cells (Fig. 3C). Thus, within our experimental conditions, IGS1 Sir2-binding sites are likely at or near saturation in the wild-type setting but Rif1 deletion causes the release of a pool of Sir2 from these binding sites only when the overall levels of Sir2 are limiting in the cell. In contrast, *SIR2*-OE increases telomeric Sir2 localization and histone deacetylation in the wild-type setting but not in the *rif1*Δ setting, in which Sir2-telomere interaction levels are already higher than those observed in wild-type cells (Fig. 3C). The mating-type loci exhibited changes similar to those seen at telomeres while IGS2 was unaltered by Sir2 overexpression or Rif1 deletion (Fig. 3C and not shown; also see Figs. 1C–E and 2A–D). More importantly, our ChIP experiments suggest that increasing the levels of Sir2 at IGS1 regions, which contain the rDNA recombination enhancer sequences, could eliminate the rDNA repeat instability and shorter lifespan typically linked with the loss of Rif1. Indeed, we did find that *SIR2* overexpression prevented Rif1 deletion from reducing rDNA repeat stability and excessively shortening cellular lifespan (Fig. 3D,E; Tables S2 and S1). *SIR2* overexpression within the wild-type setting did not alter rDNA repeat stability but increased replicative lifespan likely due to increased telomeric silencing, as expected (Fig. 3C–E). We note that we did observe a statistically insignificant yet qualitative difference between the lifespan of SIR2-OE cells and SIR2-OE *rif1*Δ cells and so this could reflect effects on Sir2 or other processes in the cell within these experimental settings (Fig. 3E). All in all, our data so far suggest that the release of a pool of Sir2 from rDNA IGS1 regions underlies the rDNA repeat instability and, at least in part, the reduced replicative lifespan linked with the loss of Rif1.

Relationships between Rif1, Sir2, and calorie restriction in rDNA and lifespan maintenance

Calorie restriction (CR) is an environmental intervention that restricts the concentration of glucose in the cellular milieu (Kaeberlein, 2010). CR is thought to extend replicative lifespan via processes that are both dependent and independent of Sir2 (Lin *et al.*, 2000; Kaeberlein *et al.*, 2005). In an effort to reveal putative relationships between Rif1, Sir2, and dietary restriction, we set out to test the effect of CR on cells lacking Rif1 and/or Sir2.

Restricting glucose concentrations decreased IGS1 histone acetylation, increased rDNA stability, and extended the lifespan of wild-type cells (Fig. 4A–C; Tables S1 and S2). Interestingly, CR reduced histone acetylation at the rDNA IGS1 of *rif1*Δ cells, which now exhibited a level of rDNA stability and replicative lifespan similar to that of wild-type cells grown under normal glucose conditions (Fig. 4A–C; Tables S1 and S2). We still observed a qualitatively very small, albeit statistically insignificant, difference between the lifespans of calorie restricted *rif1*Δ cells and nonrestricted wild-type cells, which could indicate that CR may be rescuing the lifespan of *rif1*Δ cells via processes that do not, at least in part, typically implicate Rif1 and therefore Sir2 as well (Fig. 4C). Consistent with these findings and with previous reports, we found that CR partly restores rDNA IGS1 histone deacetylation and rDNA repeat stability in *sir2*Δ cells (Fig. 4D–E) (Riesen & Morgan, 2009; Smith *et al.*, 2009). We also found that CR fails to alter the lifespan of *sir2*Δ cells, possibly reflecting the fact that rDNA IGS1 histone acetylation levels as well as rDNA instability in such CR-treated *sir2*Δ cells are still significantly higher than the levels observed in wild-type cells cultured under normal glucose concentrations (Fig. 4D–F; also compare Fig. 4B–E; Table S2). This may also reflect the overall ability of *sir2*Δ to significantly limit or even abolish the lifespan-extending effects of various environmental and genetic interventions (Lin *et al.*, 2000; Delaney *et al.*, 2011). However, the fact that CR partly reduces the levels of acetylated histones at rDNA IGS1 in *sir2*Δ cells, also suggests that CR is able to activate or direct one or several other histone deacetylases to the rDNA repeats, at least in the absence of Sir2. Alternatively, CR may be reducing the function of histone acetyl transferases at the rDNA repeats of *sir2*Δ cells. Interestingly, CR reduces rDNA IGS1 histone acetylation as well as rDNA repeat instability in *rif1*Δ cells and *sir2*Δ cells, but not *rif1*Δ *sir2*Δ cells (Fig. 4D–E; Table S2). This suggests that the loss of Rif1 may

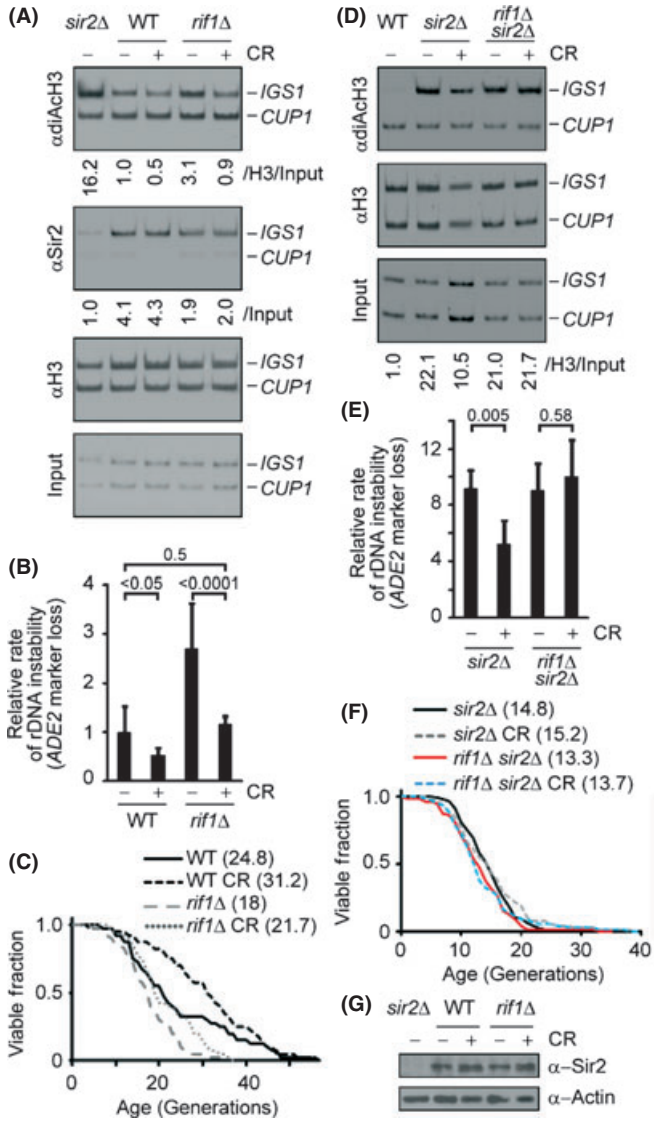

Fig. 4 Calorie restriction suppresses aberrant rDNA recombination events and lifespan defects in *rif1*Δ cells. (A) Representative ChIP gels revealing that calorie restriction strongly decreases histone acetylation within rDNA repeats without altering Sir2 localization to the repeats in *rif1*Δ cells. WT, wild-type. BY4741 isogenic strains: KMY- 372, 711, and 1052. (B) Calorie restriction of *rif1*Δ cells restores rDNA repeat stability. Shown are measurements of rDNA repeat instability (unequal sister chromatid exchange rates normalized to WT) as revealed by the rate of loss (± SD) of an *ADE2* marker gene from the rDNA repeats. Counts and statistical analyses are in Table S2. Strain: KMY 326 and 953. (C) Calorie restriction restores replicative lifespan of *rif1*Δ cells. Mean lifespans are in parentheses and *P* values are listed in Table S1. BY4741 isogenic strains: KMY 372 and 711. (D) ChIP analysis reveals that calorie restricting *sir2*Δ cells, but not *rif1*Δ *sir2*Δ cells, partly decreases histone acetylation at rDNA IGS1. BY4741 isogenic strains: KMY 372, 1052, and 1055. (E) Calorie restriction partly decreases rDNA instability in *sir2*Δ cells, but not *rif1*Δ *sir2*Δ cells. Counts and statistical analyses are in Table S2. Strains: KMY 327 and 1333. (F) Calorie restriction does not alter the lifespan of *sir2*Δ or *rif1*Δ *sir2*Δ cells. BY4741 isogenic strains analyzed in parallel with cells shown in (C) are KMY 1052 and 1055. (G) Western blot analysis indicates that calorie restriction does not affect total Sir2 protein levels. BY4741 isogenic strains: KMY 372, 711, 1052.

somehow inhibit or counteract, at least in part, the CR-triggered processes normally responsible for the reduction in acetylated histone levels at rDNA IGS1 in *sir2*Δ cells. CR also failed to alter the lifespan of *rif1*Δ *sir2*Δ cells (Fig. 4F). This could be due to the fact that CR fails to alter histone acetylation levels or rDNA repeat stability in these cells (Fig. 4D–E; Table S2). Alternatively, this could again be reflective of the inability of cells lacking Sir2 to respond to a myriad of typically lifespan-extending interventions. Overall Sir2 protein levels in either wild-type or *rif1*Δ cells were similar irrespective of glucose concentration (Fig. 4G). We note that we did notice a second lower-migrating and faint band in anti-Sir2 immunoblotting of calorie restricted cells (Fig. 4G). While this may reflect changes to the post-translational modification status of a pool of Sir2 proteins, Sir2-IGS1 interactions were unaltered under CR (Fig. 4A).

These data indicate that calorie restricting *rif1*Δ cells renders their chromatin assembly at rDNA IGS1 as well as their cellular lifespan similar to the phenotypes observed for wild-type cells grown under normal glucose conditions. In addition, our CR experiments reveal possible intersections between Sir2, Rif1, and CR in the maintenance of rDNA repeats.

Discussion

Chromosomal domains are nonrandomly arranged relative to each other and to various nuclear landmarks. In *S. cerevisiae*, this is the case for the three major silent chromatin domains – the nucleolar rDNA repeats, telomeres arranged in 4–8 perinuclear clusters, and HM loci, which tend to be restricted to somewhat peripheral regions of the nucleus (Mekhail & Moazed, 2010). Our results reveal that Rif1 exploits this nonrandom organization to maintain replicative lifespan by enforcing an optimal rDNA-stabilizing and lifespan-sustaining distribution of Sir2 between its major sites of action (Fig. 5A,B). Forced stabilization of rDNA repeats via Fob1 deletion renders the loss of Rif1 ineffectual on lifespan even in the presence of increased Sir2 localization to telomeres and mating-type loci (Fig. 5C). This indicates that the shortened lifespan of *rif1*Δ cells is linked with the release of Sir2 from the recombination modulating IGS1 and not the increased localization of this histone deacetylase to telomeres or mating-type loci. Consistent with these findings, over-expressing Sir2 prevents Rif1 deletion from increasing rDNA IGS1 histone acetylation, compromising rDNA repeat stability, or shortening replicative lifespan relative to wild-type cells (Fig. 5D). Our findings also suggest that the increased lifespan of SIR2-OE cells relative to wild-type cells is more likely to be linked with increased Sir2 localization and histone deacetylation at nonrDNA sites such as telomeres. However, the increased lifespan of SIR2-OE *rif1*Δ cells relative to *rif1*Δ cells is more likely to be linked with the restored localization and function of Sir2 at rDNA IGS1. Moreover, exposing *rif1*Δ cells to environmental CR decreases rDNA IGS1 histone acetylation, possibly through processes that are dependent and independent of Sir2, and restores rDNA repeat stability while extending replicative lifespan (Fig. 5E). Interestingly, unlike *rif1*Δ cells or *sir2*Δ cells, the inability of *rif1*Δ *sir2*Δ cells to decrease rDNA IGS1 histone acetylation under caloric restriction suggests that Rif1 deletion inhibits or counteracts CR-triggered processes that can lower the levels of rDNA IGS1 histone acetylation in the absence Sir2. Such processes could modulate Sir2 homologues such as Hst2, other histone modifying enzymes, or possibly trigger histone modulatory mechanisms linked with the target of rapamycin 1 (Tor1) (Kaeberlein *et al.*, 2005; Lamming *et al.*, 2005). It is important to note that previous studies have revealed that CR relying on different glucose concentrations can yield at least partly different results and so our cells may be similarly affected (Kaeberlein, 2010). In addition, changes to histone acetylation may vary at different locations within the rDNA intergenic spacers. Overall, our findings indicate that Rif1 limits the local recruitment of Sir2 to telomeres and the silent mating-type loci.

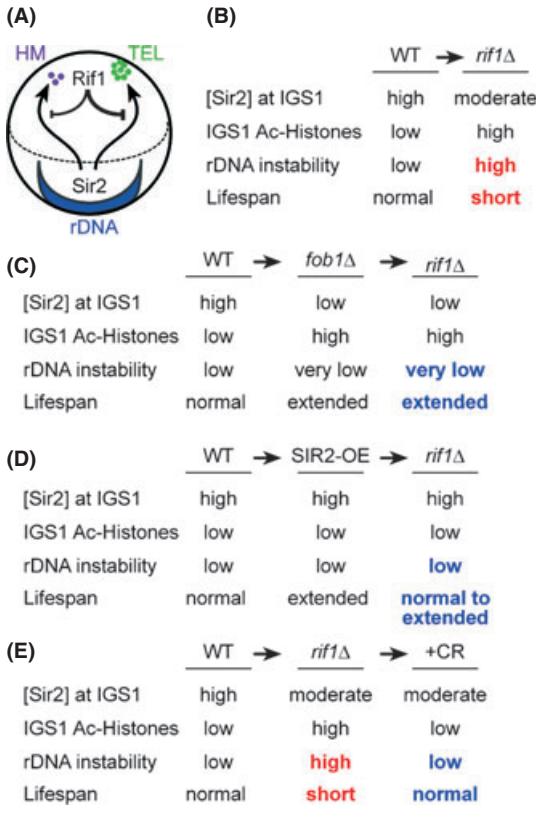

Fig. 5 Model for the role of Rif1 in the modulation of Sir2 and replicative lifespan. (A) Schematic of the nucleus illustrating the subnuclear localization of the three major silent chromatin domains: rDNA repeats (crescent), telomeres (one cluster shown) as well as the silent mating-type HM loci. Excessive relocation (arrows) of the Sir2 histone deacetylase from the rDNA to the other major silent chromatin domains can occur. Rif1 limits Sir2 localization to telomeres (thick stopper) and to a lesser extent to the silent mating-type loci (thin stopper). These actions of Rif1 ensure that a threshold level of Sir2 is retained at rDNA repeats, thereby maintaining repeat stability and overall replicative lifespan. (B) Loss of Rif1 decreases the levels of Sir2 at rDNA IGS1 leading to increases in the levels of acetylated histones at IGS1, repeat instability, and to a shorter lifespan. (C) Forcibly stabilizing the rDNA repeats, via Fob1 deletion, prevents the loss of Rif1 from shortening lifespan. (D) Sir2 overexpression prevents Rif1 deletion from destabilizing the rDNA repeats and excessively shortening lifespan. (E) Calorie restriction decreases rDNA histone acetylation and repeat instability, possibly via Sir2-dependent and independent processes. Calorie restriction also rescues the lifespan of *rif1*Δ cells.

This reinforces the association of this histone deacetylase with rDNA repeats, thereby maintaining chromosome stability and replicative lifespan. Furthermore, *rif1*Δ-related defects in rDNA repeat stability and replicative lifespan can be prevented or counteracted via *fob1*Δ-dependent rDNA repeat stabilization, Sir2 overexpression, as well as Sir2-dependent/independent processes triggered by caloric restriction.

We previously reported that the V-shaped complex Cohibin, which is a structural analogue of the conserved Ndc80 subcomplex Spc24-Spc25, clusters telomeres and links them with the nuclear envelope via interactions with conserved SUN and LEM domain-containing inner nuclear membrane proteins (Chan *et al.*, 2011). This Cohibin-dependent clustering of telomeres increases the local concentration of Sir2 ensuring efficient silent chromatin assembly and DNA stability within subtelomeric regions. This telomeric function of Cohibin is required for the maintenance of replicative lifespan (Chan *et al.*, 2011). Thus, together with the findings that we report here, our work reveals that interplay between the functions of Cohibin and Rif1 at telomeres is critical to lifespan-sustaining processes at both telomeres and rDNA repeats. First, Cohibin ensures telomere clustering to promote silencing at telomeres. However, telomeric Rif1 prevents the excessive recruitment of Sir2 to maintain lifespan-sustaining threshold levels of the histone deacetylase at rDNA repeats. We also note that our data indicate that Rif1 limits the recruitment of Sir2 to telomeres and, to a lesser extent, the silent mating-type loci. As Rap1 is implicated in the recruitment of SIR proteins to telomeres as well as the HM loci (Buck & Shore, 1995), our results point to a model in which Rif1 and SIR proteins compete for Rap1 binding at telomeres and to a lesser extent at HM loci. This model is bolstered by our findings that Rif1 localizes to telomeres and mating-type loci but not rDNA. As to why Rif1 deletion seems to favor Sir2 localization to telomeres more than it does to the HM loci remains unclear. The lengthening of telomeres upon Rif1 deletion may contribute, although it is likely insufficient, to promote the shift of a greater fraction of the Sir2 protein pool released from rDNA to telomeres (Austriaco & Guarente, 1997). Another possibility may also be that telomere clustering significantly increases the local concentration of SIR proteins, making it easier for Rap1/Sir4-dependent Sir2 recruitment upon Rif1 deletion.

Our study highlights the importance of modulating the distribution of sirtuin histone deacetylases across the genome to lifespan regulation and suggests that similar processes may exist in other organisms. Humans have seven sirtuin genes, *SIRT1-SIRT7*, and disruption of the mammalian histone deacetylases SIRT1 or SIRT6 is linked with premature aging phenotypes [Reviewed in (Haigis & Guarente, 2006; Tennen *et al.*, 2011)]. Mammalian SIRT1 regulates rDNA repeats and telomeres while SIRT6 is linked with the regulation of telomeres and the lifespan-modulating NFκB-dependent genes (Murayama *et al.*, 2008; Kawahara *et al.*, 2009; Palacios *et al.*, 2010). Shelterin proteins, which are telomeric factors that are dysfunctional in premature aging patients suffering from Werner or Bloom syndromes, or other telomeric factors, may thus maintain cellular lifespan by controlling the distribution of histone deacetylases such as SIRT1 or SIRT6 between telomeres and other age-related loci (Martinez & Blasco, 2010). In addition, Sir2, SIRT1, SIRT6, and other sirtuins play various roles in regulating metabolic responses to nutrient availability, and it is therefore possible that Rif1 may affect cross talk between chromosomal and metabolic roles of sirtuins (Chalkiadaki & Guarente, 2012).

All in all, our work highlights the importance of fine tuning the distribution of sirtuin histone deacetylases between various age-related loci in yeast and points to possibly similar processes in other organisms. We expect that future work aiming to identify additional factors that directly or indirectly modulate the localization of sirtuins within nuclear space should reveal processes that fine-tune chromatin structure to sustain the lifespan of many organisms.

Experimental procedures

Strains and materials

Endogenous genes were deleted as described (Mekhail *et al.*, 2008). Yeast strains are listed in Table S3. Antibodies used were either commercially available or previously generated. The anti-Sir2 antibody was used previously (Mekhail *et al.*, 2008) and the anti-H3 and anti-diAcH3-K9-K14 antibodies were commercially available (Millipore).

Lifespan

Replicative lifespan of yeast strains were determined by micromanipulation as previously described (Kaeberlein *et al.*, 1999). The number of

times that a new mother cell buds was measured and the Wilcoxon rank-sum test was conducted for statistical analysis. For each genotype, two independent clones (20 cells for each) were analyzed and data was pooled from two to four separate experiments. Only simultaneously counted isogenic strains were compared to each other on graphs and within our statistical analyses.

ChIP

Experiments were performed as described with modifications (Huang & Moazed, 2003; Mekhail *et al.*, 2008; Chan *et al.*, 2011). Relative fold enrichments were obtained according to the following calculations: $[rDNA_{IP}/CUP1_{IP}]/[rDNA_{Input}/CUP1_{Input}]$, $[TEL_{IP}/ACT1_{IP}]/[TEL_{Input}/ACT1_{Input}]$, or $[HM_{IP}/ACT1_{IP}]/[HM_{Input}/ACT1_{Input}]$. Full experimental procedures are in the Supporting Information with primers listed in Table S4.

USCE

Assays were performed essentially as described (Kaeberlein *et al.*, 1999; Mekhail *et al.*, 2008). Cells were grown to OD_{600} = 0.4–0.8, sonicated briefly, and spread (about 400 cells per plate) on thick plates (5 mg L^{-1} adenine). Incubation was at 30°C for 3 days, 4°C for 3 days, then RT for 1 day. Rates were obtained by dividing the number of half-sectored colonies by the total number of colonies excluding completely red colonies. For the calorie restriction USCE experiments, cells were grown overnight under CR conditions before re-diluting, further growing to OD_{600} = 0.4–0.8, and then plating on low adenine media.

Calorie restriction

Calorie restriction consisted of using media containing 0.05% glucose instead of the standard 2% glucose in YEP media. Triggering CR effects was confirmed by the extended lifespan of wild-type and other cells as well as the reduction of histone acetylation marks at silent chromatin domains.

Genomic DNA preparation and RNA extraction

For genomic DNA preparation, cells were subjected to bead-beating in the presence of Triton X-100 and phenol-chloroform-isoamyl alcohol before DNA precipitation. DNA pellets were washed, dried, resuspended in TE and RNAse treated. For RNA extractions, total RNA was prepared via standard hot phenol extraction and EtOH precipitation before re-suspending pellets in 0.1% DEPC and treatment with DNase I. Full experimental procedures for genomic DNA and RNA extractions are included in the Supporting Information section online.

Semi-quantitative RT-PCR and quantitative PCR

Semi-quantitative reverse transcriptase PCR was performed as described with modifications (Xu *et al.*, 2007). RT reactions relied on random nonamers and the M-MLV reverse transcriptase. Quantitative real-time PCR was performed using the CFX Connect Real-Time system (Bio-Rad). Full experimental procedures for RT and quantitative PCR are included in the Supporting Information section online.

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Author contributions

Experimental design by J.S.S., J.N.Y.C., and K.M.; writing by J.S.S. and K.M.; text editing by J.S.S., J.N.Y.C., and K.M; strains by J.S.S. and J.N.Y.C.; ChIP experiments by J.N.Y.C. primarily and also by J.S.S.; USCE by J.S.S. and J.D.W.; RT-PCR, qPCR, and Western Blot analyses by J.N.Y.C.; lifespan analyses by J.S.S., C.P., and T.T.L.

References

Aparicio OM, Billington BL, Gottschling DE (1991) Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell* **66**, 1279–1287.

Armstrong CM, Kaeberlein M, Imai SI, Guarente L (2002) Mutations in *Saccharomyces cerevisiae* gene SIR2 can have differential effects on in vivo silencing phenotypes and in vitro histone deacetylation activity. *Mol. Biol. Cell* **13**, 1427–1438.

Austriaco NR, Jr., Guarente LP (1997) Changes of telomere length cause reciprocal changes in the lifespan of mother cells in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA* **94**, 9768–9772.

Braunstein M, Rose AB, Holmes SG, Allis CD, Broach JR (1993) Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev.* **7**, 592–604.

Bryk M, Banerjee M, Murphy M, Knudsen KE, Garfinkel DJ, Curcio MJ (1997) Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. *Genes Dev.* **11**, 255–269.

Buck SW, Shore D (1995) Action of a RAP1 carboxy-terminal silencing domain reveals an underlying competition between HMR and telomeres in yeast. *Genes Dev.* **9**, 370–384.

Chalkiadaki A, Guarente L (2012) Sirtuins mediate mammalian metabolic responses to nutrient availability. *Nat. Rev. Endocrinol.* **8**, 287–296.

Chan JN, Poon BP, Salvi J, Olsen JB, Emili A, Mekhail K (2011) Perinuclear cohibin complexes maintain replicative life span via roles at distinct silent chromatin domains. *Dev. Cell* **20**, 867–879.

Dang W, Steffen KK, Perry R, Dorsey JA, Johnson FB, Shilatifard A, Kaeberlein M, Kennedy BK, Berger SL (2009) Histone H4 lysine 16 acetylation regulates cellular lifespan. *Nature* **459**, 802–807.

Defossez PA, Prusty R, Kaeberlein M, Lin SJ, Ferrigno P, Silver PA, Keil RL, Guarente L (1999) Elimination of replication block protein Fob1 extends the life span of yeast mother cells. *Mol. Cell* **3**, 447–455.

Delaney JR, Sutphin GL, Dulken B, Sim S, Kim JR, Robison B, Schleit J, Murakami CJ, Carr D, An EH, Choi E, Chou A, Fletcher M, Jelic M, Liu B, Lockshon D, Moller RM, Pak DN, Peng Q, Peng ZJ, Pham KM, Sage M, Solanky A, Steffen KK, Tsuchiya M, Tsuchiyama S, Johnson S, Raabe C, Suh Y, Zhou Z, Liu X, Kennedy BK, Kaeberlein M (2011) Sir2 deletion prevents lifespan extension in 32 long-lived mutants. *Aging Cell* **10**, 1089–1091.

Fritze CE, Verschueren K, Strich R, Easton Esposito R (1997) Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. *EMBO J.* **16**, 6495–6509.

Gallardo F, Laterreur N, Cusanelli E, Ouenzar F, Querido E, Wellinger RJ, Chartrand P (2011) Live cell imaging of telomerase RNA dynamics reveals cell cycle-dependent clustering of telomerase at elongating telomeres. *Mol. Cell* **44**, 819–827.

Ganley AR, Ide S, Saka K, Kobayashi T (2009) The effect of replication initiation on gene amplification in the rDNA and its relationship to aging. *Mol. Cell* **35**, 683–693.

Gottlieb S, Esposito RE (1989) A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. *Cell* **56**, 771–776.

Gottschling DE, Aparicio OM, Billington BL, Zakian VA (1990) Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell* **63**, 751–762.

Haigis MC, Guarente LP (2006) Mammalian sirtuins–emerging roles in physiology, aging, and calorie restriction. *Genes Dev.* **20**, 2913–2921.

Hardy CF, Sussel L, Shore D (1992) A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation. *Genes Dev.* **6**, 801–814.

Hoppe GJ, Tanny JC, Rudner AD, Gerber SA, Danaie S, Gygi SP, Moazed D (2002) Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a

Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. *Mol. Cell. Biol.* **22**, 4167–4180.
Huang J, Moazed D (2003) Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. *Genes Dev.* **17**, 2162–2176.
Huang J, Brito IL, Villen J, Gygi SP, Amon A, Moazed D (2006) Inhibition of homologous recombination by a cohesin-associated clamp complex recruited to the rDNA recombination enhancer. *Genes Dev.* **20**, 2887–2901.
Imai S, Armstrong CM, Kaeberlein M, Guarente L (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**, 795–800.
Kaeberlein M (2010) Lessons on longevity from budding yeast. *Nature* **464**, 513–519.
Kaeberlein M, McVey M, Guarente L (1999) The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev.* **13**, 2570–2580.
Kaeberlein M, Kirkland KT, Fields S, Kennedy BK (2004) Sir2-independent life span extension by calorie restriction in yeast. *PLoS Biol.* **2**, E296.
Kaeberlein M, Powers RW, 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK (2005) Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* **310**, 1193–1196.
Kawahara TL, Michishita E, Adler AS, Damian M, Berber E, Lin M, McCord RA, Ongaigui KC, Boxer LD, Chang HY, Chua KF (2009) SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell* **136**, 62–74.
Kobayashi T, Horiuchi T (1996) A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities. *Genes Cells* **1**, 465–474.
Kobayashi T, Heck DJ, Nomura M, Horiuchi T (1998) Expansion and contraction of ribosomal DNA repeats in *Saccharomyces cerevisiae*: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I. *Genes Dev.* **12**, 3821–3830.
Kurdistani SK, Grunstein M (2003) Histone acetylation and deacetylation in yeast. *Nat. Rev. Mol. Cell Biol.* **4**, 276–284.
Lamming DW, Latorre-Esteves M, Medvedik O, Wong SN, Tsang FA, Wang C, Lin SJ, Sinclair DA (2005) HST2 mediates SIR2-independent life-span extension by calorie restriction. *Science* **309**, 1861–1864.
Lin SJ, Defossez PA, Guarente L (2000) Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science* **289**, 2126–2128.
Lin SJ, Kaeberlein M, Andalis AA, Sturtz LA, Defossez PA, Culotta VC, Fink GR, Guarente L (2002) Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature* **418**, 344–348.
Longtine MS, Wilson NM, Petracek ME, Berman J (1989) A yeast telomere binding activity binds to two related telomere sequence motifs and is indistinguishable from RAP1. *Curr. Genet.* **16**, 225–239.
Maillet L, Boscheron C, Gotta M, Marcand S, Gilson E, Gasser SM (1996) Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression. *Genes Dev.* **10**, 1796–1811.
Managbanag JR, Witten TM, Bonchev D, Fox LA, Tsuchiya M, Kennedy BK, Kaeberlein M (2008) Shortest-path network analysis is a useful approach toward identifying genetic determinants of longevity. *PLoS ONE* **3**, e3802.
Martinez P, Blasco MA (2010) Role of shelterin in cancer and aging. *Aging Cell* **9**, 653–666.
Mekhail K, Moazed D (2010) The nuclear envelope in genome organization, expression and stability. *Nat. Rev. Mol. Cell Biol.* **11**, 317–328.
Mekhail K, Seebacher J, Gygi SP, Moazed D (2008) Role for perinuclear chromosome tethering in maintenance of genome stability. *Nature* **456**, 667–670.
Michel AH, Kornmann B, Dubrana K, Shore D (2005) Spontaneous rDNA copy number variation modulates Sir2 levels and epigenetic gene silencing. *Genes Dev.* **19**, 1199–1210.
Moazed D (2001) Common themes in mechanisms of gene silencing. *Mol. Cell* **8**, 489–498.
Moretti P, Freeman K, Coodly L, Shore D (1994) Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. *Genes Dev.* **8**, 2257–2269.
Murayama A, Ohmori K, Fujimura A, Minami H, Yasuzawa-Tanaka K, Kuroda T, Oie S, Daitoku H, Okuwaki M, Nagata K, Fukamizu A, Kimura K, Shimizu T, Yanagisawa J (2008) Epigenetic control of rDNA loci in response to intracellular energy status. *Cell* **133**, 627–639.
Palacios JA, Herranz D, De Bonis ML, Velasco S, Serrano M, Blasco MA (2010) SIRT1 contributes to telomere maintenance and augments global homologous recombination. *J. Cell Biol.* **191**, 1299–1313.
Park S, Patterson EE, Cobb J, Audhya A, Gartenberg MR, Fox CA (2011) Palmitoylation controls the dynamics of budding-yeast heterochromatin via the telomere-binding protein Rif1. *Proc. Natl Acad. Sci. USA* **108**, 14572–14577.
Poon BP, Mekhail K (2011) Cohesin and related coiled-coil domain-containing complexes physically and functionally connect the dots across the genome. *Cell Cycle* **10**, 2669–2682.
Poon BP, Mekhail K (2012) Effects of perinuclear chromosome tethers in the telomeric URA3/5FOA system reflect changes to gene silencing and not nucleotide metabolism. *Front. Genet.* **3**, 144.
Riesen M, Morgan A (2009) Calorie restriction reduces rDNA recombination independently of rDNA silencing. *Aging Cell* **8**, 624–632.
Rine J, Herskowitz I (1987) Four genes responsible for a position effect on expression from HML and HMR in *Saccharomyces cerevisiae*. *Genetics* **116**, 9–22.
Shou W, Seol JH, Shevchenko A, Baskerville C, Moazed D, Chen ZW, Jang J, Charbonneau H, Deshaies RJ (1999) Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* **97**, 233–244.
Sinclair DA, Guarente L (1997) Extrachromosomal rDNA circles–a cause of aging in yeast. *Cell* **91**, 1033–1042.
Smith JS, Boeke JD (1997) An unusual form of transcriptional silencing in yeast ribosomal DNA. *Genes Dev.* **11**, 241–254.
Smith JS, Brachmann CB, Pillus L, Boeke JD (1998) Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p. *Genetics* **149**, 1205–1219.
Smith JS, Caputo E, Boeke JD (1999) A genetic screen for ribosomal DNA silencing defects identifies multiple DNA replication and chromatin-modulating factors. *Mol. Cell. Biol.* **19**, 3184–3197.
Smith DL, . Jr , Li C, Matecic M, Maqani N, Bryk M, Smith JS (2009) Calorie restriction effects on silencing and recombination at the yeast rDNA. *Aging Cell* **8**, 633–642.
Straight AF, Shou W, Dowd GJ, Turck CW, Deshaies RJ, Johnson AD, Moazed D (1999) Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. *Cell* **97**, 245–256.
Suka N, Suka Y, Carmen AA, Wu J, Grunstein M (2001) Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. *Mol. Cell* **8**, 473–479.
Tennen RI, Bua DJ, Wright WE, Chua KF (2011) SIRT6 is required for maintenance of telomere position effect in human cells. *Nat Commun.* **2**, 433.
Visintin R, Hwang ES, Amon A (1999) Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature* **398**, 818–823.
Wotton D, Shore D (1997) A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in *Saccharomyces cerevisiae*. *Genes Dev.* **11**, 748–760.
Xu F, Zhang Q, Zhang K, Xie W, Grunstein M (2007) Sir2 deacetylates histone H3 lysine 56 to regulate telomeric heterochromatin structure in yeast. *Mol. Cell* **27**, 890–900.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Additional controls related to the demonstration that *fob1*Δ-dependent forced stabilization of rDNA repeats renders *rif1*Δ ineffectual on lifespan.

Figure S2. Effects of calorie restriction on telomeric Sir2 localization as well as histone acetylation in wild-type and *rif1*Δ cells.

Table S1. Mean lifespan and *P* values for Wilcoxon rank-sum test for replicative lifespan analyses.

Table S2. Rates of ADE2 marker loss from rDNA repeats.

Table S3. List of strains used in this study.

Table S4. List of primer pairs used for ChIP.

Supporting Experimental Procedures – ChIP, genomic DNA or total RNA preparation, RT-PCR, and qPCR.