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A cytosolic NAD-dependent deacetylase, Hst2p, can modulate nucleolar and telomeric silencing in yeast

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In budding yeast, the silent information regulator Sir2p is a nuclear NAD-dependent deacetylase that is essential for both telomeric and rDNA silencing. All eukaryotic species examined to date have multiple homologues of Sir two (HSTs), which share a highly conserved globular core domain. Here we report that yeast Hst2p and a mammalian Hst2p homologue, hSirT2p, are cytoplasmic in yeast and human cells, in contrast to yHst1p and ySir2p which are exclusively nuclear. Although yHst2p cannot restore silencing in a *sir2* deletion, overexpression of yHst2p influences nuclear silencing events in a *SIR2* strain, derepressing subtelomeric silencing while increasing repression in the rDNA. In contrast, a form of ySir2p carrying a point mutation in the conserved core domain disrupts both telomeric position effect (TPE) and rDNA repression at low expression levels. This argues that non-nuclear yHst2p can compete for a substrate or ligand specifically required for telomeric, and not rDNA repression.

Keywords: homologues of Sir2p/nucleolus/*SIR2*/telomeric silencing/yeast

Introduction

Chromatin-mediated silencing converts specific domains of the yeast genome into a transcriptionally inactive state that shares several characteristics with heterochromatin. Notably, silent chromatin generally contains underacetylated histones, replicates late in S phase and is refractory to the transcriptional apparatus and DNA-modifying enzymes (reviewed in Lustig, 1998). In several species, this reduced accessibility has been shown to correlate with a characteristic subnuclear localization; for example, in both *Drosophila* and yeast, silent domains on different chromosomes cluster together near the nuclear periphery. Similarly, in mammalian B cells, the association of genes with centromeric heterochromatin correlates with an inactive transcriptional state (reviewed in Cockell and Gasser, 1999; Marshall and Sedat, 1999).

In budding yeast, silencing requires a protein complex that contains balanced proportions of the silent information regulators 2–4 (Sir proteins; Rine and Herskowitz, 1987; Aparicio *et al*., 1991). This complex interacts with underacetylated N-termini of histones H3 and H4, as well as with sequence-specific DNA-binding factors that recruit and nucleate its binding (reviewed in Lustig, 1998). Sir-mediated repression occurs at three different loci: at the two silent mating type cassettes on ChrIII (*HMR* and *HML*), and adjacent to the telomeric $TG_{1–3}$ repeat (called the telomeric position effect or TPE; Gottschling *et al*., 1990). A related type of repression occurs in the repetitive rDNA array on ChrXII (Bryk *et al*., 1997; Fritze *et al*., 1997; Smith and Boeke, 1997), although Sir2p is the only Sir protein required for the repressive chromatin structure in the nucleolus. Cross-linking assays show that Sir2p is associated with silenced reporter genes both at telomeres and in the rDNA, and overexpression studies indicate that Sir2p levels are limiting at both sites (Gotta *et al*., 1997; Strahl-Bolsinger *et al*., 1997; Cockell *et al*., 1998; Smith *et al*., 1998).

Despite its dependence on Sir2p, the mechanism of rDNA repression is clearly distinct from that of TPE. First, the Sir2p-dependent rDNA chromatin structure also suppresses homologous recombination among the tandemly repeated rDNA copies (Gottlieb and Esposito, 1989; Fritze *et al*., 1997; Smith and Boeke, 1997). Secondly, in the nucleolus, Sir2p is complexed with the nucleolar protein Net1p, and a telophase-regulating phosphatase, Cdc14p, which is released in late metaphase (Shou *et al*., 1999; Straight *et al*., 1999). Finally, overexpression of either Sir4p or domains of Sir3p, which derepress silencing at telomeres (Cockell *et al*., 1998; Gotta *et al*., 1998; Smith *et al*., 1998), actually increases repression of a PolII reporter gene in the rDNA. In comparison with TPE, nucleolar repression is relatively unstable, fluctuating in response to chromatin-modifying proteins that act at non-nucleolar sites (Bryk *et al*., 1997; Smith *et al*., 1999).

The protein encoded by the *SIR2* gene is a member of a highly conserved family of proteins called homologues of Sir two in *Saccharomyces cerevisiae* (yHst1–4p; Brachmann *et al*., 1995) or SirTuins in humans (hSirT1-7; Frye, 1999, 2000). The hallmark of the Sir2-like family is a conserved globular core of ~250 amino acids containing a four-Cys Zn^{2+} finger motif. Recently, yeast Sir2p and Hst2p (hereafter ySir2p and yHst2p) as well as mouse Sir2-like proteins have been shown to catalyse an NAD-dependent deacetylation reaction *in vitro* using acetylated histone tails as substrate (Imai *et al*., 2000; Landry *et al*., 2000). A weak ribosyl transferase activity was also detected under some conditions, and may reflect an intermediate state during NAD hydrolysis (Frye, 1999; Tanny *et al*., 1999). Unlike other histone deacetylases,

that of Sir2p activity is stoichiometrically coupled to NAD hydrolysis (Tanner *et al*., 2000) and both enzymatic activities are abrogated by mutations within the core domain that correlate with a loss of silencing (Tanny *et al*., 1999; Imai *et al*., 2000). Although this suggests that a major function of ySir2p *in vivo* is enzymatic, essential non-enzymatic silencing functions have also been assigned to its N- and C-terminal domains (Cockell *et al*., 2000). It remains unclear whether Sir2p prefers acetylated histones over other acetylated substrates, and whether histones are indeed the physiological targets of these enzymes.

Several Sir2p homologues may also modulate chromatin structure, since overexpression of yHst1p, the protein most similar to ySir2p itself, restores silencing at *HMRa* in a *sir2*-deficient strain (Brachmann *et al*., 1995; Derbyshire *et al*., 1996). In addition, yHst1p forms a complex with Sum1p and represses meiosis-specific sporulation genes during mitotic growth (Xie *et al*., 1999). Less is known about the functions of *HST3 and HST4*, although the *hst3hst4* double mutant has increased chromosome instability and strongly reduced TPE (Brachmann *et al*., 1995).

In related yeasts, such as *Kluyveromyces lactis*, the loss of Sir2p renders cells hypersensitive to the DNA-intercalator ethidium bromide and reduces both mating and sporulation efficiency (Chen and Clark-Walker, 1994), while in *Candida albicans* a Sir2-like protein is implicated in the control of phenotypic switching (Perez-Martin *et al*., 1999). Finally, in fission yeast, the mutations of *hst4* correlate with slow growth and fragmented DNA, as well as a decrease in chromatin-mediated repression in subtelomeric and centromeric domains (Freeman-Cook *et al*., 1999). At least partial cross-species complementation for the loss of TPE in *S.cerevisiae* strains lacking Sir2p was shown for genes from these three distantly related fungi, although higher eukaryotic homologues were unable to complement silencing (Chen and Clark-Walker, 1994; Freeman-Cook *et al*., 1999; Perez-Martin *et al*., 1999). Indeed, no cellular function has been assigned to any of the seven mammalian SirTuins.

Here we have analysed the function of yHst2p, the least well characterized yet most universally conserved member of the yeast Sir2 family (Figure 1). yHst2p has a robust deacetylase activity and is more active than ySir2p on histone substrates *in vitro* (Landry *et al*., 2000). Indeed, in yeast cell extracts, yHst2p accounts for the majority of detectable NAD-dependent deacetylase activity (Smith *et al*., 2000). To examine potential roles for both yHst2p and the human homologue hSirT2p in silencing, we have monitored their subcellular localization and their effects on TPE and rDNA silencing in yeast. Surprisingly, we find that both yHst2p and hSirT2p are cytoplasmic enzymes. Nonetheless, elevated levels of yeast Hst2p can modulate TPE and rDNA repression in yeast, albeit in opposite ways. This demonstrates that Sir2-like enzymes can influence silencing without being targeted to the site of repression. Although loss-of-function mutations suggest that ySir2p and yHst2p have distinct physiological roles, we propose that these two homologues share a limiting substrate or ligand, other than NAD, that is necessary for telomeric, but not rDNA repression.

Results

BLAST searches of genomic and expressed sequence tag (EST) databases using either the full-length *SIR2* sequence or the region that encodes the ySir2p core domain (amino acids 255–496, shaded grey in Figure 1A) have identified additional Sir2 family members in vertebrate, fly, worm, yeast and bacterial species, allowing for a more complete alignment of homologous genes. The five related genes in budding yeast (encoding ySir2p and yHst1–4p; Brachmann *et al*., 1995) all share strong sequence identity in a core domain (between 35 and 82% identity). In addition, yHst1p and ySir2p have N- and C-terminal extensions that share 50 and 55% identity, while yHst2p and yHst3p lack N-terminal extensions and have unrelated C-terminal domains. At least some bacterial species have more than one homologue of Sir2p (e.g. *Archeoglobus fulgibus*), although the prokaryotic Sir2p-like proteins generally lack the N- and C-terminal extensions that help distinguish the eukaryotic forms. Intriguingly, in all eukaryotic species examined to date we find members that are much more closely related to the core domain of the yHst2p protein than to the core domain of ySir2p itself (see values in italics for PRSS scores, Figure 1A). These include a Sir2p family member from *Schizosaccharomyces pombe*, *C.albicans*, *Leishmania* (LmSir2rp; Yahiaoui *et al*., 1996) and chicken, as well as the human hSirT2p and hSirT3p, and the closely related mouse proteins, MmSirT2p and MmSirT3p (see Hst2 subfamily in italics, Figure 1B; for accession numbers see Materials and methods). We propose to designate this group as Hst2-like, as it forms an independent branch within the Sir2 family tree.

In addition to the Hst2-like and the Hst1/Sir2 subfamilies, our phylogenetic analysis confirms the designation of a third subfamily that includes yHst3p, yHst4p and the *S.pombe* hst4$^+$, as previously suggested (Sherman *et al*., 1999). Cross-species complementation has been demonstrated among these genes, although their physiological roles are not yet fully understood (Figure 1B; Freeman-Cook *et al*., 1999).

Despite its ubiquitous character, no functional information has been published on any member of the Hst2 subfamily, although a mutated form of a human homologue, hSirT2, has been cloned as a melanoma antigen (T.Woelfel, personal communication). Comparison of the sequence encoding the tumour-specific antigen with the corresponding region of the wild-type gene (identical to hSir2L in Afshar and Murnane, 1999; or hSir2A in Sherman *et al*., 1999) revealed a single point mutation that converts a conserved proline at amino acid 182 to a leucine. This mutation, which will be called *hsirT2^{P182L}*, is responsible for the observed autoantigenicity (T.Woelfel, unpublished data). It is not known whether hsirT2^{P182L}p contributes to the cellular transformation events that led to this melanoma, but previously identified mutations responsible for melanoma antigens have been correlated with oncogenesis (Woelfel *et al*., 1995). Motivated by our identification of a highly conserved Hst2 subfamily, and by the potential medical relevance of the *hsirT2^{P182L}* mutation, we examined whether overexpression of yHst2p, hSirT2p, or mutant forms of these proteins, would modulate silencing functions in yeast.

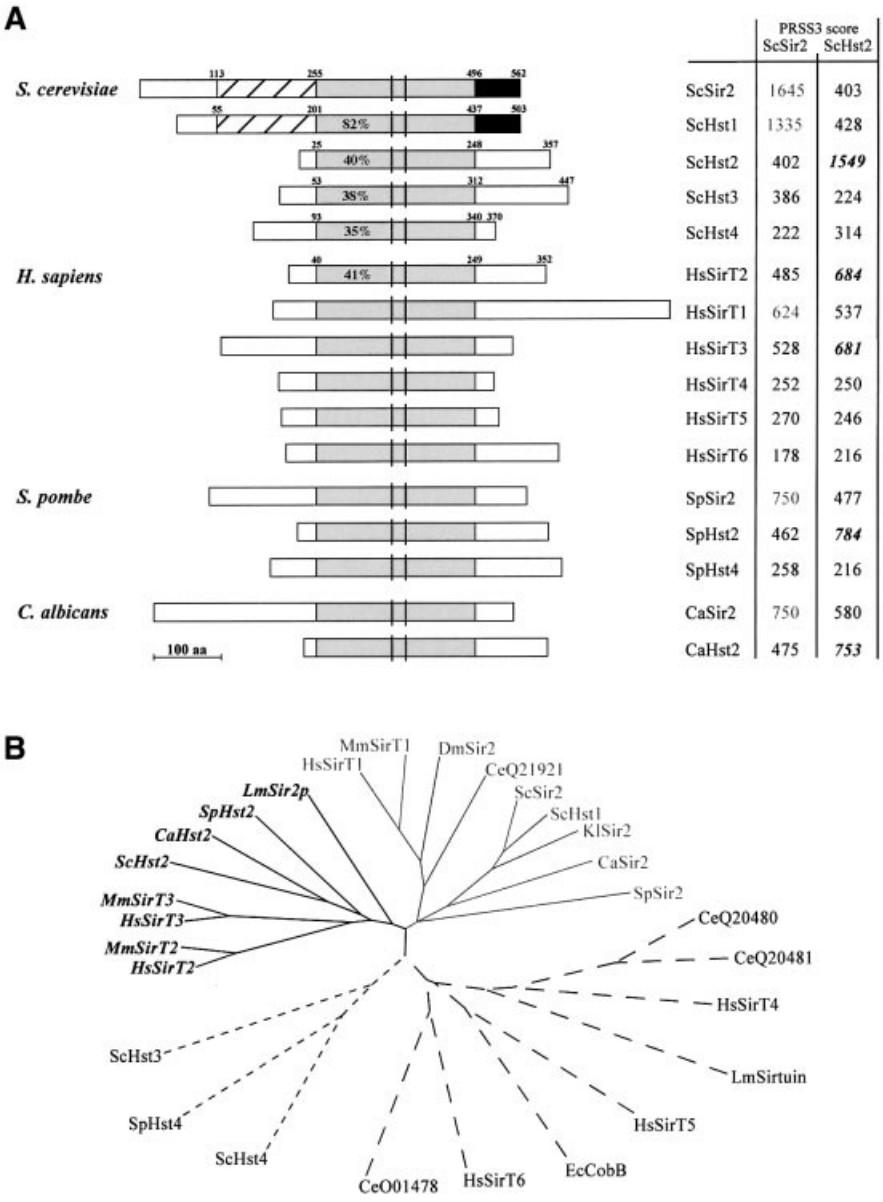

Fig. 1. Comparative alignments and phylogenetic tree of Sir2 family members. (**A**) Diagrammatic representations of Sir2p homologues are aligned with respect to the evolutionarily conserved core domain (light grey boxes). The PRSS3 score is indicated for the pairwise comparison of the indicated core domain with that of the yeast Sir2p or Hst2p (labelled ScSir2 and ScHst2). The PRSS3 program (http://www.ch. embnet.org/software/PRSS) calculates the optimal score of a protein sequence alignment and evaluates the significance of this score. Scores with higher homology to ySir2p are indicated in grey and those closer to yHst2p are in italics. The percentage amino acid identity shared with the ySir2p core domain is calculated by the Gap program of GCG (with ran = 100, gap = 12, gap extension = 2), and is indicated for the yHst family and the *Homo sapiens* SirT2 protein. Unshaded boxes are regions of no significant identity (<20%) with ySir2p. Cross-hatched boxes indicate 50% identity in N-terminal extensions, and black boxes indicate 55% identity to ySir2p. The amino acid boundaries of the domains selected for comparison are indicated. Vertical black bars represent cysteine pairs of a putative zinc finger motif. We have used the nomenclature of Frye (1999) for human SirTuins 1–5, and named a previously unreported human cDNA, HsSirT6. The HsSirT2 is identical to the gene called hSir2L (Afshar and Murnane, 1999) and hSir2A (Sherman *et al*., 1999). (**B**) The phylogenetic unrooted tree of eukaryotic Sir2 homologues was generated using CLUSTALW (Higgins *et al*., 1996) and TREEVIEW (Page, 1996), which compare the core domain sequences of homologues identified in cDNA and unigene libraries from *Escherichia coli*, *S.cerevisiae*, *K.lactis*, *Schizosaccharomyces pombe*, *C.albicans*, *Leishmania major*, *H.sapiens*, *Mus musculus*, *Caenorhabditis elegans* and *Drosophila melanogaster*. The database accession No. for each gene listed is given in Materials and methods. The Sir2 subfamily is indicated in grey and the Hst2 subfamily in italics.

Dominant-negative effects of yHst2p on TPE

Previous studies have shown that normal levels of ySir2p are limiting in the nucleus, since low level overexpression of *SIR2* could improve both TPE and rDNA repression (Cockell *et al*., 1998; Smith *et al*., 1998). In contrast, high levels of ySir2p are dominant-negative at telomeres, presumably due to disruption of the Sir complex. No similar disruption or titration occurs in the rDNA, even at the highest levels of ySir2p overexpression (Cockell *et al*., 2000). Although published studies indicated that yHst2p is not essential for mating type or telomeric silencing (Brachmann *et al*., 1995), it was not tested whether overexpressed yHst2p complements a *sir2::HIS3* strain for TPE, or whether it modulates repression in a wild-type

Table I. Yeast strains used in this study

Strain	Genotype	Reference
GA194	*MAT***a**/*MAT*α *ade2/ADE2 trp1/trp1 his3-11/his3 ura3-1/ura3-52 can1-100/can1-100 leu2-3, 112/leu2, sir2::HIS3/ sir2::HIS3*	(Gotta *et al.*, 1997)
GA225	*MAT***a**/*MAT*α *ade2/ADE2 trp1/trp1 his3-11, 15/his3 ura3-1/ura3-52 can1-100/can1-100*	(Gotta *et al.*, 1997)
GA424	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ1 leu2-Δ1 his3-Δ200 ppr1::HIS3 adh4::URA3-TEL*	(formerly UCC111; D.Gottschling)
GA426	*MAT***a** *ade2::hisG can1::hisG his3-11 leu2 trp1Δ ura3-52 TelVR::ADE2*	(Stone and Pillus, 1996)
GA427	*MAT***a** *ade2::hisG can1::hisG his3-11 leu2 trp1Δ ura3-52 TelVR::ADE2 sir2::HIS3*	(Gotta *et al.*, 1997)
GA503	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 leu2-Δ1 his3-Δ200 ppr1::HIS3 adh4::URA3-TEL TelVR::ADE2*	(formerly UCC3505; Singer and Gottschling, 1994)
GA758	*MAT*α *his3Δ200 leu2Δ1 trp1Δ63 ura3-167 RDN1::mURA3/HIS3*	(Smith *et al.*, 1998)
GA759	*MAT*α *his3Δ200 leu2Δ1 trp1Δ63 ura3-167 RDN1::mURA3/HIS3 sir2::kanMX4*	(J.Smith, unpublished)
GA917	GA758 with *hst1::LEU2*	this study
GA918	GA758 with *hst2::TRP1*	this study
GA921	GA758 with *hst1::LEU2, hst2::TRP1*	this study
GA1154	GA426 with *HST1-13Myc-kanMX6*	this study
GA1155	GA427 with *HST1-13Myc-kanMX6*	this study
GA1229	GA427 with *HST2-13Myc-TRP1*	this study
GA1275	GA426 with *SIR4-13Myc-kanMX6*	this study
GA1276	GA426 with *HST2-13Myc-kanMX6*	this study
GA1281	GA758 with *sir2::TRP1*	this study

Table II. Disruption of *hst1 and hst2* does not significantly affect rDNA repression

Genotype	Repression (FOA^r)		Recombination
	Mean	Normalized	
HST1 HST2 SIR2	0.393 (0.29–0.621)	1.0	0.067
hst1 HST2 SIR2	0.128 (0.054–0.34)	0.33	0.3
HST1 hst2 SIR2	1.0 (0.1–1.04)	2.54	0.0
hst1 hst2 SIR2	0.110 (0.062–0.17)	0.28	0.3
HST1 HST2 sir2	6.4×10^{-7} ($4.7–14.3 \times 10^{-7}$)	1.6×10^{-6}	1.0

SIR2, *HST1* and *HST2* genes were disrupted in the strain GA758. Growth on FOA-containing media monitors the efficiency of repression of the *RDN1::mURA3-HIS3* reporter construct as described in Materials and methods. The left hand column shows the mean and numerical spread of the fraction of a total of 30 colonies in which *URA3* is not expressed. The value for the wild-type strain is used to normalize values to 1. The fraction of the Ura$^-$ cells that have lost *URA3* due to recombination events is indicated in the right hand column (see Materials and methods).

background. Because yHst2p has no readily identifiable nuclear localization signal (NLS), we tested constructs in which full-length yHst2p is fused to a functional NLS (pJG45-yHst2) and one in which it is not (pGAL-yHst2). Both constructs are expressed under the control of the *GAL10* promoter on high copy number (2μ) vectors (pJG45 or pGAL, see Materials and methods). Growth on glucose medium confers a low, but non-negligible level of expression from these vectors, since pJG45-ySir2 is able to complement a *sir2::HIS3* strain for TPE on glucose (Figure 2A; repression of the telomere-proximal reporter *TelVR::ADE2* is monitored by an accumulation of red pigment; see Materials and methods).

In Figure 2A, we show that neither pJG45-yHst2 nor pGAL-yHst2 is able to complement a *sir2::HIS3* strain for TPE, whether expressed at low or high levels (glucose versus galactose, respectively). However, when *HST2* is induced in a Sir2$^+$ strain, which is normally pink or red-sectored, the colonies become completely white, demonstrating a dominant-negative effect of *HST2* induction on telomeric repression (Gal, Figure 2A). This effect is similar to that observed for ySir2p overexpression, is independent of the presence of the NLS (compare pJG45-ySir2 with either pJG45- or pGAL-yHst2; Figure 2A) and does not reflect a non-specific effect of galactose (see vector control). As reported for overexpression of the core domain of ySir2p (Cockell *et al*., 2000), only high levels of yHst2p impair TPE. The simplest interpretation of this result is that yHst2p interferes with the activity of ySir2p by binding to or modifying an essential silencing factor, or by forming a non-productive complex with a common substrate.

yHst2p influences telomeric and rDNA silencing in opposite ways

Although it is well established that ySir2p is essential for rDNA silencing, it has not been reported how the deletion of *HST1* and *HST2* affects the expression of PolII reporters in the rDNA. To this end, we created single and double *hst1* and *hst2* deletions in strains containing the *RDN1::mURA3-HIS3* insert (see Table I). 5-fluoro-orotic acid (FOA) resistance monitors repression of the *mURA3* reporter, while the loss rate for the *mURA3* reporter indicates recombination rates. A complete deletion of *hst1* has a very minor effect on rDNA silencing, producing a 3-fold reduction in FOA resistance, as compared with the >10^6-fold drop that correlates with *sir2* deletion (Table II). Only a third of the FOA-resistant *hst1* colonies reflect excision of the *mURA3* reporter, whereas recombination appears to account for all of the resistance in a *sir2* null (Table I). Previous results also showed no significant increase in recombination rates in the absence of yHst1p (Derbyshire *et al*., 1996). Since disruption of *hst2* alone, or in combination with *hst1*, incurs no significant change in rDNA repression or recombination (Table II), we conclude that neither homologue is essential for nucleolar silencing. The minor changes in the *hst1* mutant are likely to result from indirect effects, as suggested by the immunostaining

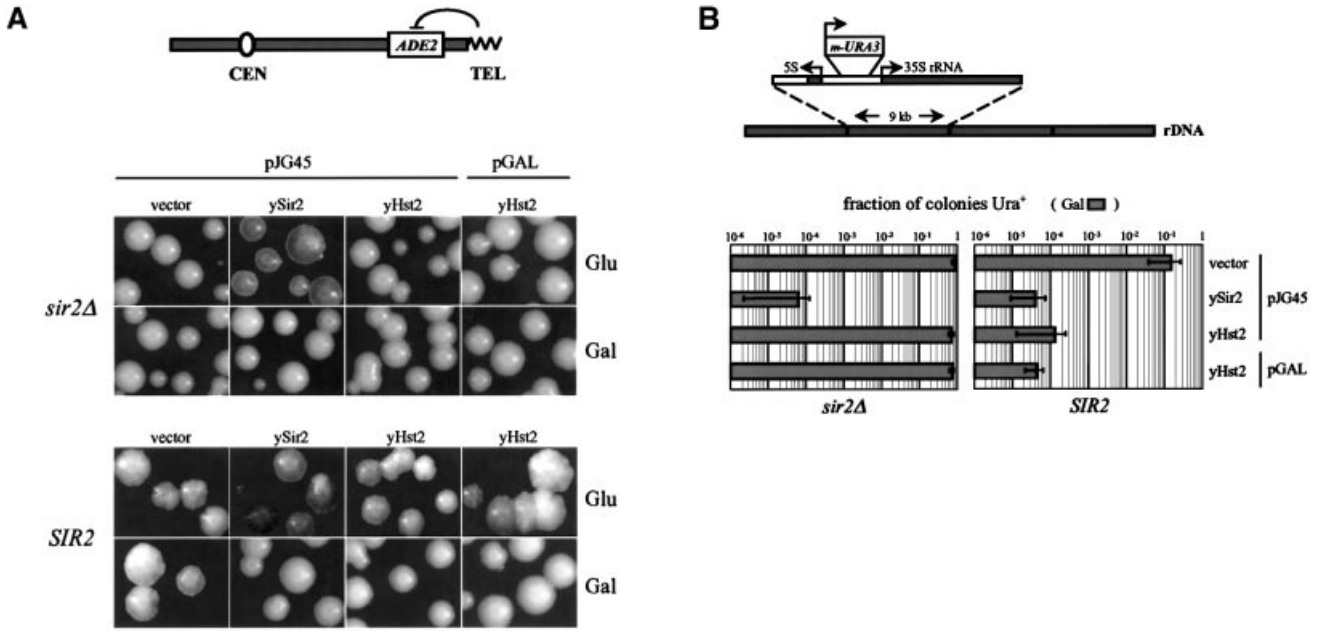

Fig. 2. yHst2p at high levels of overexpression is dominant-negative for TPE. The effects of ectopic expression of yeast Hst2p on silencing of the *TelVR::ADE2* reporter gene (Singer and Gottschling, 1994) were determined after growth on glucose (Glu, low expression levels) and galactose (Gal, high expression levels). Isogenic *sir2::HIS3* (GA427) and *SIR2* (GA426) strains, carrying the *TelVR::ADE2* reporter gene, were used in (A). (**A**) The plasmid indicated above each panel was introduced into the *sir2::HIS3* strain GA427 labelled *sir2Δ* or in the *SIR2* strain GA426, and were grown under limiting adenine conditions (see Materials and methods). pJG45, pJG45-ySir2 and pJG45-yHst2 express the indicated yeast protein fused at their N-termini to the B42-NLS-HA peptide. pGAL-yHst2 encodes an HA-Hst2p fusion. All fusions are expressed under the inducible *GAL10* pomoter, and are expressed at low levels on glucose and induced at least 40-fold on galactose. Both on glucose and galactose media, *sir2Δ* colonies carrying the parental vectors (shown here only for pJG45) are white, indicating *ADE2* expression, whereas $Sir2^+$ colonies carrying the parental vectors alone (shown here only for pJG45) have a red/white sectored appearance like the untransformed strain, indicating metastable *ADE2* silencing. (**B**) The effects of strong ectopic expression of yHst2p, with and without a fused SV40 NLS, on silencing of *RDN1::mURA3/HIS3*, was determined in isogenic *sir2::kanMX4* (GA759) and *SIR2* (GA758) strains. Strains were transformed with the plasmids indicated at the right of the graph and described above in (A). Galactose induces strong expression from the *GAL10* promoter. Five-fold serial dilutions of each transformant were plated on both –TRP and –TRP–URA media with the indicated carbon source. *URA3* expression is calculated as described (see Materials and methods). The *sir2::kanMX4* strain (GA759) carrying either vector alone gives a value of ~1 for this calculation (100% of the colonies express *URA3*), while the *SIR2* strain (GA758) with either vector gives a value of ~0.1 (10% of the cells express *URA3*). The mean and standard deviation were calculated from at least three independent transformants of each plasmid.

data presented below, which localize yHst1p to the non-nucleolar nucleoplasm.

To see if *HST2* overexpression might influence rDNA silencing, isogenic *sir2::kanMX4* (GA759) and *SIR2* (GA758) strains containing the *RDN1::mURA3* insert were used (Figure 2B; see Materials and methods). We confirm that both low and high level expression from pJG45-ySir2 improves rDNA silencing in a $Sir2^+$ background, while neither form of yHst2p (i.e. yHst2p or NLS–Hst2p) can complement the *sir2::kanMX4* strain for rDNA repression (Figure 2B). In striking contrast to the effects at telomeres, however, overexpression of either form of yHst2p significantly improves rDNA silencing (1000-fold; see Figure 2B), almost as efficiently as overexpressed ySir2p in a $Sir2^+$ strain.

yHst2p is cytoplasmically localized, even at high levels of expression

To understand better the differential effects of the Sir2p homologues in the different silencing assays, we investigated the subcellular distributions of ySir2p, yHst1p and yHst2p under conditions of low and high level expression. To this end, the endogenous copies of *HST1* and *HST2* were fused to a sequence encoding a 13 Myc epitope tag, in otherwise isogenic *sir2::HIS3* and *SIR2* strains. Although yHst1p has 61% overall identity to ySir2p and has the N- and C-terminal extensions characteristic of ySir2p, it is not enriched in the nucleolus, but is localized to the non-nucleolar nucleoplasm (Figure 3). This is true in both the presence and absence of Sir2p (Figure 3; see inset), consistent with the minor effects *HST1* deletion has on rDNA repression.

In contrast to yHst1p, however, yHst2p expressed under its endogenous promoter gives a weak but almost entirely cytoplasmic signal (Figure 3, yHst2-myc). Since yHst2p affects both TPE and rDNA repression at high expression levels, we next examined the localization of yHst2p and NLS–Hst2p when they were induced on galactose as pGAL or pJG45 constructs. We first note that in the absence of an NLS, even at very high levels of expression, yHst2p remains cytoplasmic (Figure 3; pGAL-yHst2, low or high). Thus, if its dominant-negative effect on TPE results from competition for a ligand or substrate, that ligand must spend time in both the nucleus and the cytoplasm. Intriguingly, the NLS–Hst2p fusion is also retained in the cytoplasm, except in cells expressing very high amounts (compare pJG45-yHst2 low and high) where it becomes largely nuclear. This suggests that NLS–Hst2p may associate with a cytoplasmic complex that only becomes limiting when the fusion protein is in great excess. Importantly, loss of TPE occurs equally efficiently when either yHst2p or NLS–Hst2p is overexpressed.

The dual effect of yHst2p on silencing might have been explained by an increase in the free pool of ySir2p following the disruption of complexes involved in TPE, analogous to the increase in rDNA repression observed when Sir4p subdomains are overexpressed (Kennedy *et al*., 1997; Smith *et al*., 1998). However, immunolabelling shows that, unlike the situation when ySir2p is overexpressed, ySir4p is not significantly delocalized by high levels of yHst2p (Figure 4). Moreover, there was no detectable increase in non-telomeric ySir2p (data not shown). This is consistent with the fact that yHst2p lacks the N-terminal domain of ySir2p that binds ySir4p (Cockell *et al*., 2000), and argues that the dominant-negative effect of yHst2p overexpression does not simply reflect disruption of the Sir2/3/4 complex.

Human SirT2p is also cytoplasmically localized

The unexpected finding that the most active NAD-dependent Sir2-like deacetylase in yeast is cytoplasmic led us to ask whether other members of the Hst2 subfamily share this characteristic. Wild-type and mutant forms of the *hSIRT2* gene were cloned and sequenced during the analysis of a human melanoma antigen (T.Woelfel, personal communication). Both the wild-type and the mutant gene (carrying the Pro182 to leucine transition) were fused to both His_6 and Myc epitope tags under the control of a cytomegalovirus (CMV) promoter, for transfection into mammalian cells (see Materials and methods). In transfected Phoenix cells, the protein is expressed at high levels. Immunostaining with an anti-Myc epitope reveals a strong cytoplasmic signal (Figure 5C), which does not coincide significantly with nuclear DNA nor with nuclear pores (red, anti-pore; green, hSirT2p; Figure 5C). An identical localization, which is highly reminiscent of that of yHst2p, was obtained for both the wild-type and P182L form of hSirT2p (data not shown).

To see if the endogenous hSirT2p is also cytoplasmic, polyclonal antibodies were raised against hSirT2p fused to the bacterial maltose-binding protein (MBP). The resulting antiserum was purified against recombinant antigen that was either denatured (on nitrocellulose strips) or native (by affinity chromatography). The specificity was confirmed by showing that both strip-purified and column-purified anti-hSirT2p (Figure 5A and B, respectively) react strongly with the epitope-tagged hSirT2p expressed in transfected Phoenix cells. The bands coincide with those labelled by reaction with anti-Myc and anti-His_6 monoclonals (Figure 5A), while in non-transfected cells this band is absent. The endogenous hSirT2p is below the level of detection by western blotting in the Phoenix cell line, although a weak signal at ~68 kDa in HeLa cell extracts resists competition with MBP, and may represent the endogenous hSirT2 protein (Figure 5B, right panel).

Finally, immunofluorescence performed with column-purified anti-hSirT2 antibodies on non-transfected Phoenix cells revealed a general cytoplasmic staining, which is lost when competed by a bacterial extract containing the MBP–hSirT2p fusion (Figure 5C). Similar results were obtained with nitrocellulose-purified antibodies on HeLa cells, human kidney carcinoma cells (RCC7680), in mouse NIH-3T3 and in the human Mel4 fibroblasts, which carry the *sirT2*^{P182L} mutation

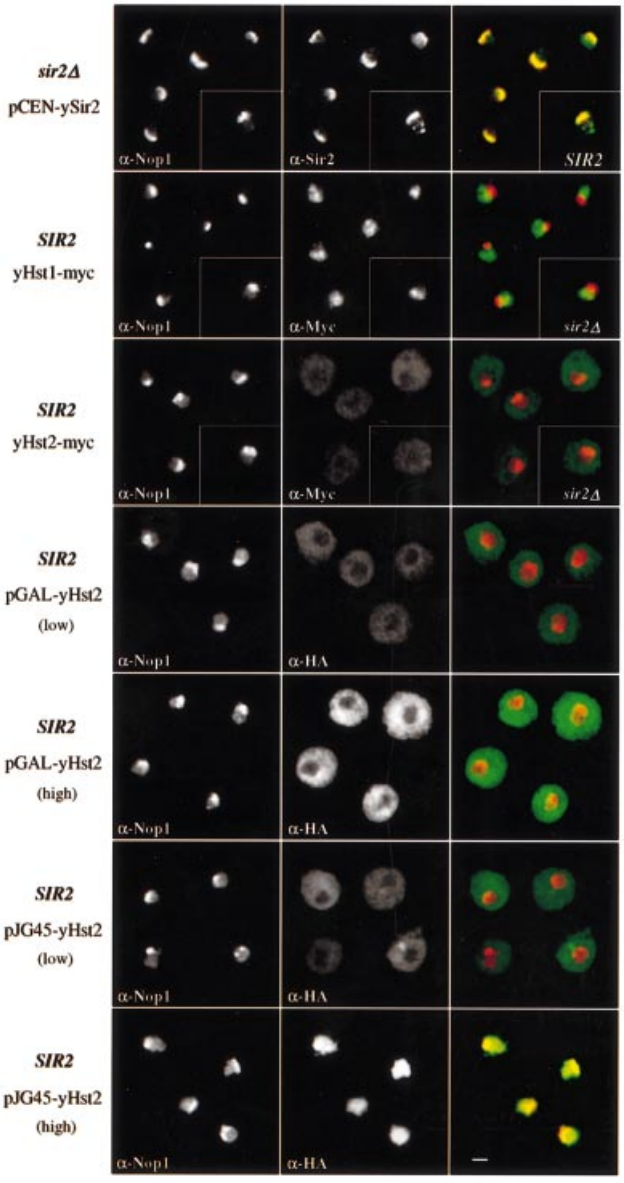

Fig. 3. yHst1p is enriched in the non-nucleolar nucleoplasm and yHst2p is cytoplasmic. The indicated proteins were localized by indirect immunofluorescence on fixed yeast cells as described in Materials and methods. In all cases, the nucleolar marker Nop1p was localized with anti-Nop1 (rabbit antiserum or mouse monoclonal antibody, as appropriate; Gotta *et al*., 1997) and a Cy5-coupled secondary antibody. This is shown in the first panel of each row and is red in the merged images. In the first row, localization of ectopically expressed ySir2p (α-Sir2, green in merged image) was examined in a diploid *sir2::HIS3* strain (GA194) after transformation with pADH-ySir2. The inset shows the localization of ySir2p to the telomeric foci and the nucleolus, when the cells have been washed, after fixation, in 1% Triton–0.02% SDS to improve accessibility (see Gotta *et al*., 1997). yHst1-Myc is detected by the monoclonal 9E10 (α-Myc) in the haploid strain GA1154 (*SIR2*) and the isogenic *sir2::HIS3* strain GA1155 (inset). yHst2-Myc was examined in GA1276 (*SIR2*) and the isogenic *sir2::HIS3* strain GA1229 (inset). Both fusions are genomic and under their endogenous promoters. The NLS-containing HA-tagged yHst2p expressed from pJG45-yHst2 was examined in transformants of the diploid wild-type strain GA225 expressing either low or high levels of the protein after 4 h of galactose induction, as indicated. The localization of the HA-tagged yHst2 fusion protein, which is encoded by pGAL-yHst2 and lacks a detectable NLS, was examined in transformants of GA225 under conditions of low and high expression, as indicated. The merge is shown in colour, with Nop1p in red and ySir2p, c-Myc or HA epitopes in green. Coincidence of the two signals is yellow. Bar = 2 μm.

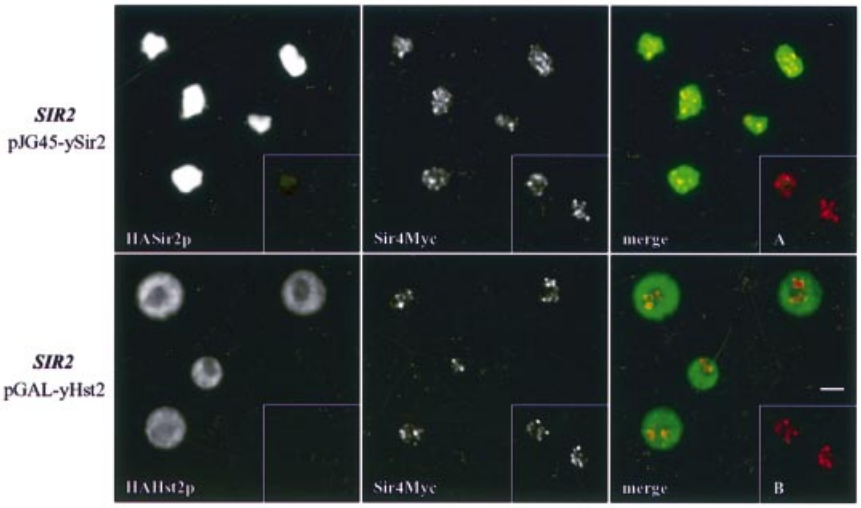

Fig. 4. Telomeric foci remain intact at the nuclear periphery despite yHst2p-mediated disruption of silencing. Upper panels: ySir4p-Myc was localized in *SIR2* cells (GA1275) transformed with pJG45-ySir2 after 4 h induction on galactose, using mouse anti-Myc antibodies (red signal in the merge). Nuclear localization of the highly overexpressed HA-Sir2p is demonstrated by immunostaining with anti-HA in the first panel (visualized in green in the merge). ySir4p-Myc is partially delocalized as compared with the punctate pattern observed in cells that do not overexpress ySir2p (GA1275, inset A). Lower panels: ySir4-Myc was localized in the same cells transformed with pGAL-yHst2 and induced for 4 h on galactose. The cytoplasmically localized HA-yHst2p is visualized in green; anti-Myc (ySir4p-Myc) is in red in the merged image. The inset B shows control cells that do not overexpress yHst2p. Bar = 2 μm.

(T.Laroche and S.M.Gasser, data not shown). Since an excess of MBP–hSirT2 antigen can compete for the cytoplasmic staining pattern, we conclude that the localization of both wild-type and mutant hSirT2p in mammalian cells is cytoplasmic, like that of yHst2p.

A proline to leucine mutation in ySir2p abrogates both rDNA and telomeric silencing in a dominant-negative manner

Does either hSirT2p or its mutant form mimic the effects of yHst2p on silencing in yeast? As shown in Figure 6A, neither low nor high levels of hSirT2p or hsirT2^{P182L}p can complement the *sir2* phenotype in TPE (Figure 6A, *sir2Δ*), nor were dominant-negative effects detected when hSirT2p or its mutant form was overexpressed in a wild-type background (Figure 6A, Sir2$^+$, Gal). Western blots confirm that the galactose-induced human proteins are stable and readily detected in yeast cell extracts (Figure 6D). Since hSirT2p expression had no phenotype, we decided to introduce the equivalent melanoma-associated point mutation into the yeast *SIR2* gene, producing a full-length ysir2^{P394L}p (Pro394 to leucine). The analogous mutation could not be made in yHst2p, since the relevant proline is not conserved; moreover, no silencing phenotypes have been correlated with inactivation of Hst2p to date.

The *sir2^{P394L}* mutation is compared in all assays with a previously characterized triple mutant called *sir2^{LGG}*, which has two arginine to glycine substitutions at amino acids 403 and 404, together with the Pro394 to leucine mutation (Cockell *et al*., 2000). Neither pJG45-sir2^{LGG} nor the single mutant pJG45-sir2^{P394L} can complement TPE in a *sir2::HIS3* strain on glucose (low level expression) or galactose (high level expression; Figure 6A and Cockell *et al*., 2000). In contrast, the same vector expressing wild-type Sir2p on glucose under control of the *GAL10* promoter restores repression in a *sir2Δ* strain (Figure 6A). Thus, both mutant forms inactivate *SIR2* function at telomeres. Unlike wild-type ySir2p, we observed that low level expression of the mutant sir2^{P394L} is strongly dominant-negative for TPE in a Sir2$^+$ strain. This is evidenced by the loss of the red/pink sectors in both GA426 (Sir2$^+$; Figure 6A) and GA503, the latter carrying two independent subtelomeric reporters (*URA3* and *ADE2*; see –TRP, Figure 6B). Since pigment accumulation is qualitative, derepression of the subtelomeric *URA3* reporter was quantified to compare the dominant-negative effects of the two mutant forms. In contrast to pJG45-ySir2 or pJG45-yHst2, which only affect silencing at high levels of expression, the single point mutation confers an ~10-fold loss of TPE on glucose and a 10^4-fold effect at high expression levels, while the triple *sir2^{LGG}* mutant derepresses ~10^3-fold on glucose and up to 10^4-fold on galactose (compare ysir2^{P394L}, ysir2^{LGG} and pJG45 on –TRP–URA /±Gal, Figure 6B). All the yeast proteins are expressed at approximately equal levels, as shown by a western blot in which a cytoplasmic protein (p55$^{RNase\ H}$) serves as a loading control (Figure 6D). In summary, when the mutation correlated with the human melanoma antigen is introduced into ySir2p, we obtain a gain of function that disrupts TPE even at low levels of expression. Intriguingly, both the single and triple mutations tested here render Sir2p entirely deficient for the NAD-dependent deacetylation reaction *in vitro* (J.Landry, R.Sternglanz, M.M.Cockell, S.Perrod and S.M.Gasser, unpublished results).

To examine whether the rDNA silencing behaves similarly in the presence of hSirT2p or the mutant forms of ySir2p, we introduced the expression plasmids into a strain carrying the *RDN1::mURA3* reporter construct. Neither the wild-type nor the mutant forms of hSirT2p (hSirT2-Myc or hsirT^{P182L}-Myc) complement ySir2p for rDNA silencing, nor do they disrupt rDNA repression in a *SIR2* strain (*sir2Δ* and

Sir2$^+$ panels, Figure 6C). Similarly, the mutant ySir2p forms are unable to complement a *sir2* deficiency for rDNA repression (*sir2Δ* panel, Figure 6C). In Sir2$^+$ strains, however, we see that overexpression of ysir2^{LGG}p partially disrupts rDNA repression (a 5- to 10-fold increase in the fraction of Ura$^+$ colonies on galactose) while overexpression of ysir2^{P394L}p has no significant effect. Neither mutant form is excluded from the nucleolus as determined by immunostaining (Cockell *et al*., 2000; S.Perrod, data not shown); thus, we conclude that the strong dominant-negative effect that correlates with the enzymatically inactive ysir2^{P182L}p mutant at telomeres is not manifest in rDNA repression. This, together with the differential effects of yHst2p overexpression, distinguishes the repression mechanism at telomeres from that in the rDNA. Since the enzymatically inactive *sir2* mutants do not improve rDNA repression like yHst2p overexpression, we propose that they modulate silencing by different mechanisms (see Table III).

Discussion

The number of genes characterized as members of the highly conserved Sir2 family continues to expand, and currently includes seven *SIR2*-like enzymes in humans [see Figure 1 for hSirT1-6p; and Frye (2000) for hSirT7]. The initial speculation that Sir2 family members might generally be involved in the modification of chromatin structure (Brachmann *et al*., 1995) has been strengthened by the fact that both mammalian and yeast Sir2p-related homologues have an NAD-dependent deacetylase activity *in vitro* (Imai *et al*., 2000; Landry *et al*., 2000; Smith *et al*., 2000). Because mutations that eliminate this activity correlate with a loss of TPE, it appears likely that the enzymatic activity is involved in the repression mechanism. Moreover, mutations in other enzymes that affect NAD levels abrogate rDNA repression efficiently (Smith *et al*., 2000). Here, we describe in detail the localization and silencing phenotypes of yHst2p, which is a member of a distinct but broadly conserved subfamily of Sir2p homologues, and which constitutes the major NAD-dependent deacetylase in yeast.

ySir2p, yHst1p and yHst2p have distinct patterns of subcellular localization

In yeast, ySir2p, yHst1p and yHst2p each have a unique subcellular distribution. Like ySir2p, yHst1p is nuclear, yet it is not enriched at telomeres or in the nucleolus. In contrast, yHst2p and its closest homo-

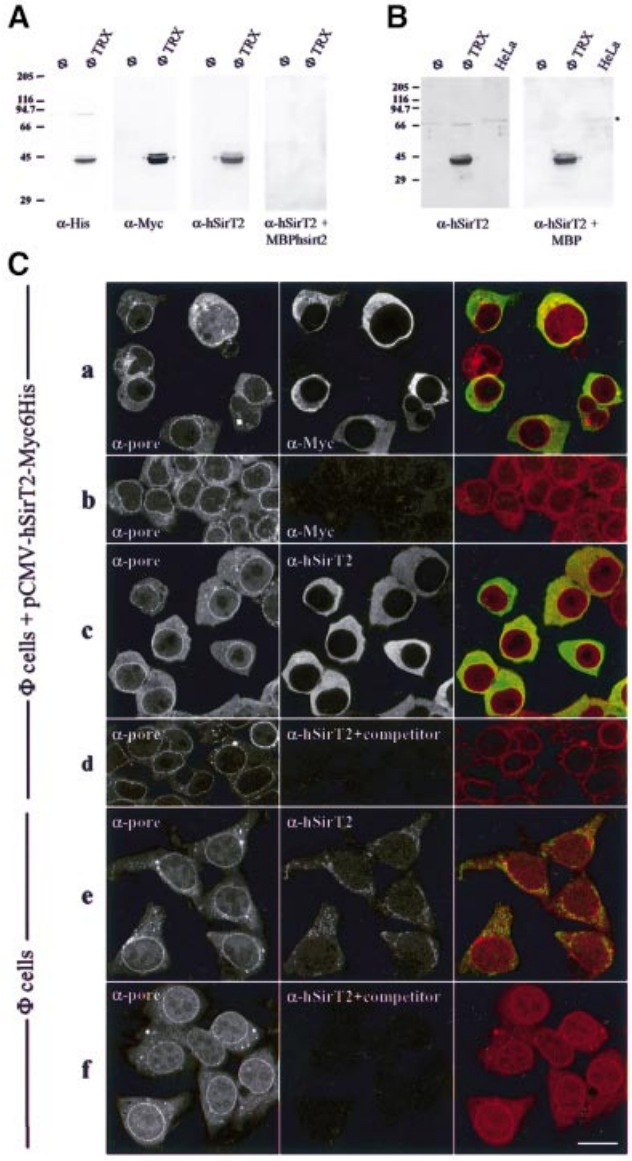

Fig. 5. hSirT2 is cytoplasmic in human cells. (**A**) Whole-cell extracts of a human embryonic kidney cell line (Phoenix cells, labelled Φ, see Materials and methods) and of the same cells transfected with pCMV-hSirT2-Myc6His (labelled ΦTRX) were analysed by western blotting using anti-His, anti-Myc, column-purified anti-hSirT2 (see Materials and methods) and the same anti-hSirT2 mixed with an excess of a bacterial extract expressing MBP–hSirT2, to demonstrate the specificity of the anti-hSirT2 antibody. Each lane was loaded with ~40 μg of total protein. Molecular weight markers (kDa) are indicated on the left of the blot. (**B**) Protein samples are as in (A), with the addition of a total cell extract from HeLa cells. A 40 μg aliquot of protein was analysed in each lane by western blotting using affinity-purified anti-hSirT2 and the same purified antibody mixed with bacterial extract expressing MBP, to identify endogenous hSirT2p. Molecular weight markers (kDa) are indicated on the left of the blot. (**C**) hSirT2-Myc6His and endogenous hSirT2p are cytoplasmic in Phoenix cells. Row a: Phoenix cells transfected with pCMV-hSirT2-Myc6His were stained with anti-pore (detected by Cy5-conjugated secondary antibodies, red in the merge) and anti-Myc (detected by DTAF-conjugated secondary antibodies, green in the merge). Row b: to test the specificity of the anti-Myc, non-transfected Phoenix cells were stained with anti-pore and anti-Myc. Row c: Phoenix cells transfected with pCMV-hSirT2-Myc6His were stained with anti-pore (red in the merge) and column-purified anti-hSirT2 (green in the merge). Row d: as c, except that the column-purified anti-hSirT2 antibodies were pre-incubated with an excess of extract from bacteria overexpressing MBP–hSirT2p. The immune complexes were removed by centrifugation prior to staining the fixed cells. Row e: non-transfected Phoenix cells were stained with anti-pore (red in the merge) and column-purified anti-hSirT2 (green in the merge). To detect the low level signal of endogenous hSirT2, the laser intensity (488 nm) was increased 40-fold over the scanning conditions used in a–d. Row f: the specificity of column-purified anti-hSirT2 is demonstrated by pre-mixing an excess of bacterial extract expressing MBP–hSirT2 with the purified antibody, prior to staining non-transfected Phoenix cells with anti-pore (red in the merge) and the depleted anti-hSirT2 (green in the merge). Bar = 15 μm.

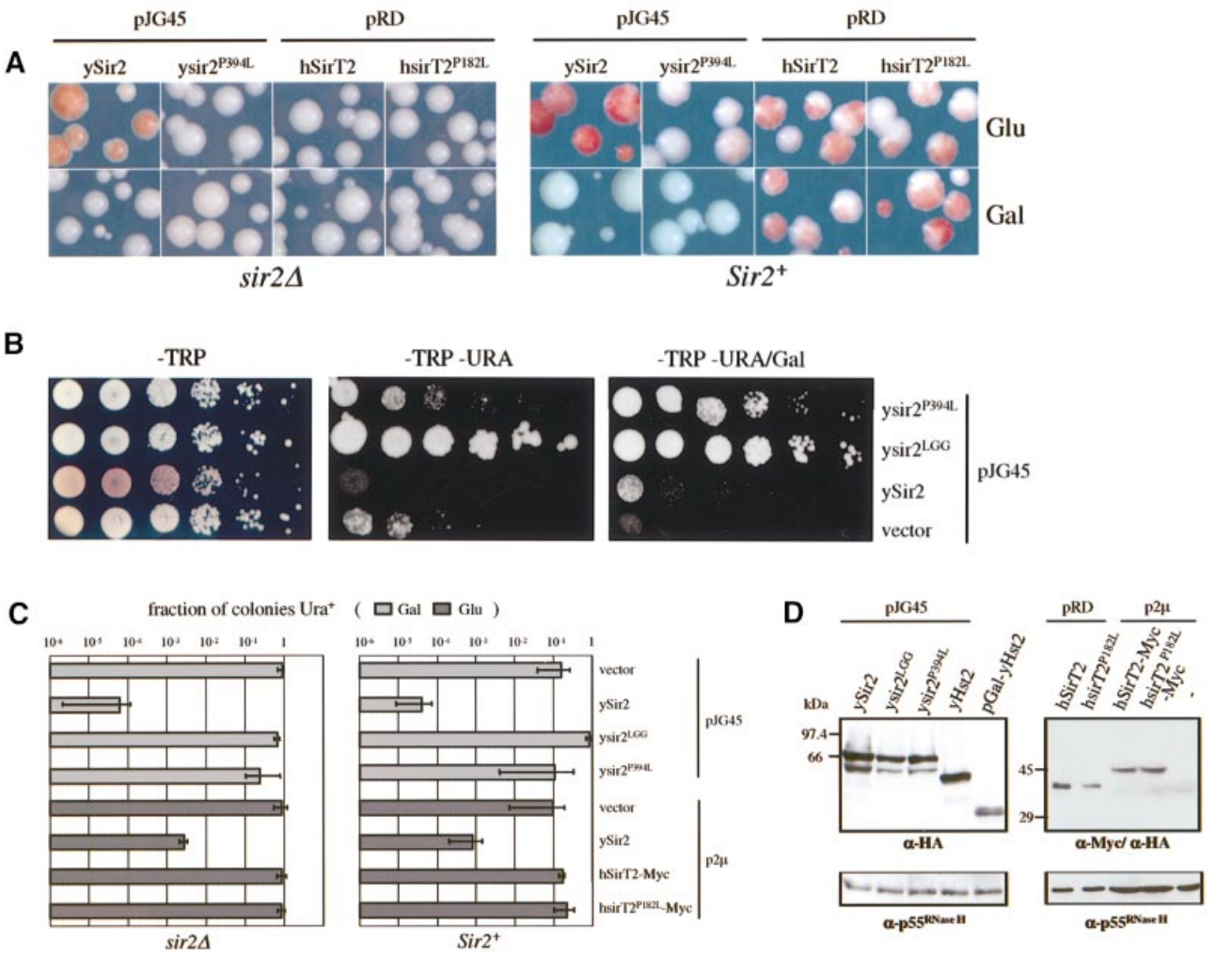

Fig. 6. The ysir2^{P394L} mutant form is non-functional and disrupts TPE in wild-type cells. (**A**) The effects of ectopic expression of yeast Sir2p mutants and human SirT2 on silencing of the *TelVR::ADE2* reporter gene (Singer and Gottschling, 1994) was determined after growth on glucose (Glu, low expression) and galactose (Gal, high expression). Isogenic *sir2::HIS3* (GA427) and Sir2^{+} (GA426) strains were transformed with the plasmids indicated above each panel and were grown under limiting adenine conditions such that colonies accumulate red pigment when *ADE2* is repressed. The plasmids pJG45-ySir2 and pJG45-ysir2^{P394L} express the indicated yeast protein fused at their N-termini to the B42-NLS-HA peptide. pRD-hSirT2 and pRD-hsirT2^{P182L} encode HA epitope-tagged hSirT2p and hsirT2^{P182L}p. All are under *GAL10* control. Although these hSirT2 constructs lack their first 33 amino acids, we obtained identical results by expressing a full-length hSirT2 clone fused to a C-terminal Myc epitope under *ADH* control (data not shown and see below). (**B**) The effects of wild-type and mutated yeast Sir2p (plasmids pJG45-ysir2^{P394L} and pJG45-ysir2^{LGG}) on silencing of the *TelVIIL::URA3* reporter were determined in a Sir2^{+} strain carrying the *TelVR::ADE2* marker (GA503). GA503 was transformed with the plasmids indicated on the right: pJG45-ysir2^{P394L}, pJG45-ysir2^{LGG}, pJG45-ySir2 and the vector alone. Ten-fold serial dilutions starting with 5×10^5 cells of each transformant were plated on glucose plates lacking tryptophan (–TRP), tryptophan and uracil (–TRP–URA), and on galactose plates with the same selectivity (growth on –TRP/Gal was identical to that observed on –TRP, data not shown). Growth on –URA indicates loss of TPE on glucose or galactose in the presence of the mutated forms of ySir2p, while low level expression of wild-type ySir2p improves TPE. On –TRP plates, the reddish colour indicates subtelomeric repression of the *TelVR::ADE2* marker, whereas white indicates full derepression. (**C**) The effect of ectopic expression of wild-type and mutants of ySir2p and hSirT2p on silencing of a *RDN1::mURA3* reporter was determined in isogenic *sir2::kanMX4* (GA759) and Sir2^{+} (GA758) strains, transformed with the plasmids indicated on the right of the graph. Plasmids pJG45, pJG45-ySir2, pJG45-ysir2^{LGG} and pJG45-ysir2^{P394L} are described in (B), and are all galactose inducible. Five-fold serial dilutions of each transformant were plated on both –TRP and –TRP–URA plates on the indicated carbon source. The fraction of colonies in which the *URA3* gene is expressed is described in Materials and methods. For *sir2::kanMX4* strain (GA759), transformation with either vector alone gives a value of ~1 (100% of colonies express *URA3*) while the Sir2^{+} strain (GA758) with either vector alone gives ~0.1 (~10% of cells express *URA3*). Standard deviations and means were calculated from at least three independent transformants of each plasmid. (**D**) Epitope-tagged proteins expressed in GA426 after transformation with the plasmid indicated above each lane were detected by western blots of whole-cell extracts of each transformant. For all constructs, except those labelled p2μ, proteins were extracted after growth on galactose/raffinose. Approximately 30 μg of protein extract was loaded per lane. Equal loading was checked by blotting with a constitutively expressed protein, p55$^{RNase\ H}$ (Karwan *et al*., 1990).

logue in human cells are cytoplasmic. For yHst2p, the cytoplasmic localization is observed for the protein whether it is expressed at low levels or induced from the *GAL10* promoter. Moreover, when a functional NLS is fused artificially to yHst2p, the protein remains cytoplasmic until it is highly overexpressed, suggesting that cytoplasmic retention competes for nuclear translocation. This is consistent with the localization of the epitope-tagged form of hSirT2 in transiently transfected human cells (Figure 5C and Afshar and Murnane,

Table III. Effects on transcriptional silencing in *SIR2* and *sir2Δ* strains

Genotype	*SIR2*						*sir2Δ*				
Locus monitored	*TelVIIL::URA3*		*TelVR::ADE2*		*RDN1::mURA3*		*HMLα*	*TelVR::ADE2*		*RDN1::mURA3*	
Media	Glu	Gal	Glu	Gal	Glu	Gal		Glu	Gal	Glu	Gal
Protein expressed											
none (vector)	wt	wt	sectored	sectored	wt	wt	–	–	–	–	–
ySir2	++	–	red	white/pink	+	++	+	+	–	+	+
ysir2^{LGG}	–	—	white	white	–	–	–	–	–	–	–
ysir2^{P394L}	–	—	white	white	wt	wt	+	–	–	–	–
yHst2	wt	(–/+)[a]	sectored	white	wt	++	–	–	–	–	–
NLS–Hst2	wt	(–/+)[a]	sectored	white	wt	++	–	–	–	–	–
yHst1	–	–	white/pink	white/pink	nd	nd	nd	nd	nd	nd	nd
hSirT2	wt	wt	sectored	sectored	wt	wt	nd	–	–	–	–
hSirT2^{P182L}	wt	wt	sectored	sectored	wt	wt	nd	–	–	–	–

Telomeric silencing was assayed in GA424 (*SIR2*, *TelVIIL::URA3*), GA503 (*SIR2*, *TelVIIL::URA3, TelVR::ADE2*), GA426 (*SIR2*, *TelVR::ADE2*) and GA427 (*sir2::HIS3*, *TelVR::ADE2*), while rDNA repression was measured in GA758 (*SIR2*, *RDN1::mURA3*) and GA759 (*sir2::kanMX4*, *RDN1::mURA3*). Wt, for wild-type levels of silencing based on Sir2$^+$ strains transformed with the empty vector; +, repression better than wt; –, repression less than wt; —, a very pronounced reduction of silencing; (–/+), derepression that is coupled with slow growth and reduced viability.
[a]Cells of GA503 and GA424, which overexpress either yHst2p or NLS–yHst2p, grow ~10 times less efficiently, possibly due to a negative effect of yHst2p on pathways required for growth on galactose. This effect is less pronounced in GA758 in which we test rDNA silencing.

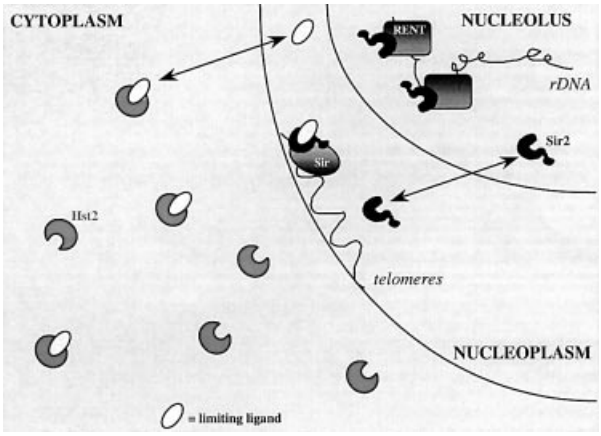

Fig. 7. Model for yHst2p effects on silencing in yeast. A model is shown to account for the dominant-negative effects of cytoplasmic yHst2p on silencing in yeast. yHst2p is cytoplasmic at both low and high levels of expression, and its absence has no effect on TPE or rDNA silencing. At high levels of expression, cytoplasmic yHst2p affects TPE and rDNA silencing much like highly overexpressed ySir2p: rDNA silencing improves while TPE is disrupted. We propose that yHst2p sequesters or modifies a ligand of ySir2p that is essential for TPE, and which shuttles between the nucleoplasm and cytoplasm. By sequestering or modifying this unknown ligand, yHst2p may disrupt TPE, releasing a pool of ySir2p that can relocalize to the nucleolus and improves rDNA silencing. The limiting ySir2p/yHst2p ligand must not be necessary for rDNA repression. In this model, fluctuations in levels of cytoplasmic ySir2-like proteins, as well as changes in the amount of this ligand are predicted to influence Sir protein function at telomeres and at the rDNA.

1999) and with the diffuse cytoplasmic localization detected for endogenous hSirT2p using affinity-purified antibodies on fixed cultured cells. Taken together with a *Leishmania* Sir2-like protein that also appears to be cytoplasmic (Zemzoumi *et al*., 1998), we suggest that the entire Hst2 branch will be cytoplasmic NAD-dependent deacetylases.

Cytoplasmic yHst2p may compete for a telomere-specific silencing ligand

We show that null alleles of *HST1* or *HST2* have only minor effects on rDNA repression, although overexpression of Hst2p in a Sir2$^+$ strain disrupts telomeric silencing while significantly improving repression in the rDNA. We interpret these findings in light of recent studies that show that Sir2-like proteins share one or more enzymatic functions (Frye, 1999; Imai *et al*., 2000; Landry *et al*., 2000; Tanny *et al*., 1999). A robust NAD-dependent deacetylation activity was demonstrated for ySir2p, yHst2p and mouse SirT1p (i.e. mSir2α), and mutations in yeast Sir2p that eliminate deacetylase activity were shown to result in derepression *in vivo* (Imai *et al*., 2000). Since rDNA silencing drops when cellular NAD levels fall (Smith *et al*., 2000), and since hSirT2p itself is able to bind NAD (Frye, 1999), it is unlikely that the loss of TPE elicited by the overexpression of yHst2p reflects titration of NAD. Rather, the dosage-dependent, dominant-negative phenotype suggests that the conserved core domain shared among the Sir2-like enzymes can compete for a ligand or substrate that can be present in both nuclear and cytoplasmic compartments. Importantly, we find that the telomeric localization of ySir4p is not affected by yHst2p overexpression, making it unlikely that yHst2p acts through modulation of the Sir complex. Consistently, yHst2p lacks the ySir2p domain that binds ySir4p and does not interact with ySir4p by two-hybrid analysis (Cockell *et al*., 2000; and data not shown).

Several lines of evidence suggest that histone tails may be a physiological target for deacetylation by ySir2p and yHst2p. First, overexpression of ySir2p resulted in global hypoacetylation of histones (Braunstein *et al*., 1996). Secondly, both yHst2p and ySir2p proteins deacetylate bulk histones efficiently *in vitro* (Landry *et al*., 2000). Finally, Imai *et al*. (2000) have reported a preference of ySir2p for an acetylated K16 of the histone H4 peptide. In this case, however, substrates other than histone N-terminal fragments were not tested and the target

histone was not assembled into nucleosomes. If histones are the critical substrate of ySir2p and overexpressed yHst2p, then it follows from our results that the pattern of deacetylated lysines on the histone H4 tail might have opposite effects on rDNA and telomeric silencing. Consistent with this possibility, mutation of Arg17 to glycine in the histone H4 tail was found to improve rDNA repression while disrupting TPE (H.Renauld, unpublished observations). Clearly additional studies are required to determine which proteins involved in silencing are common targets of the different Sir2 family members.

Although their distinct subcellular distribution suggests that yHst2p might sequester a ySir2p substrate in the cytoplasm, this appears not to be the case. The relocalization of overexpressed yHst2p to the nucleus through its fusion to the SV40 NLS does not abrogate its dominant-negative effect. Alternatively, a silencing-relevant ligand may be modified inappropriately, or bound irreversibly to yHst2p. The proposal that yHst2p modifies a range of substrates is consistent with the observation that cell viability in some yeast backgrounds drops up to 10-fold when *HST2* is overexpressed (data not shown).

From the silencing results and immunolocalization studies, we propose the following model for the effects of yHst2p on gene silencing (Figure 7). yHst2p may have a significant affinity for what is normally a ySir2p-specific ligand required for proper telomeric repression by the Sir complex. Normal levels of cytoplasmic yHst2p are probably not sufficient to compete for this ligand. However, a ligand required for Sir2/3/4-mediated silencing could become modified or bound by overexpressed yHst2p before assembly at its normal site of action in the nucleus. In its absence, nuclear ySir2p would no longer be able to promote Sir complex propagation at telomeres. This might then permit the relocalization of some fraction of the ySir2p pool to the nucleolus to improve rDNA repression. In this way, weak but overlapping substrate specificities between yHst2p and ySir2p could suffice to explain the phenotypes associated with yHst2p overexpression. This demonstrates the importance of the enzymatic activity *per se* in silencing, and dissociates it from the role of ySir2p in Sir complex formation. On the other hand, unlike a Gal4 DNA binding domain fusion to ySir2p, the targeting of an equivalent yHst2p fusion protein to a reporter gene is not sufficient to promote silencing (M.M.Cockell, unpublished data), indicating that even the targeting of a Sir2-like deacetylation activity is not sufficient to promote transcriptional repression.

Inactivating point mutations can render ySir2p dominant-negative for silencing

In contrast to the yeast *HST2*, the introduction of a melanoma-associated point mutation into yeast *SIR2* provokes the loss of both telomeric and rDNA silencing functions, and renders the proteins strongly dominant-negative for telomeric repression, even at low expression levels. These observations indicate that even in heteroallelic human melanoma cells, the mutant form of hSirT2p could influence patterns of gene expression. We have found that the *sir2*^{P394L} and *sir2*^{LGG} mutations in *S.cerevisiae* inactivate the gene product's enzymatic function (J.Landry, R.Sternglanz, M.M.Cockell, S.Perrod and S.M.Gasser, data not shown). These mutations eliminate a function required for both *SIR3/4*-dependent and -independent silencing, but also sequester or inhibit silencing factors in Sir2^{+} strains. This may provide an example of how a single mutant allele of a chromatin factor can have strong repercussions on patterns of gene expression in differentiated cells.

The fact that a non-nuclear NAD-dependent deacetylase can disrupt silencing suggests that there are common substrates for yHst2p and ySir2p, and most probably substrates other than histones, that are likely to influence silencing events. Characterization of these substrates and mutagenesis of the target sites will be needed to decipher the cellular functions of this large and ubiquitous family of enzymes in higher eukaryotes.

Materials and methods

Plasmid constructions

Plasmids used in the study are listed below along with a brief description of their relevant characteristics. The following abbreviations are used: NLS, nuclear localization signal; *GAL10*, the UAS and promoter sequence of the *GAL10* gene; *ADH1*, the UAS and promoter sequence of the *ADH1* gene; B42, the bacterial B42 activation domain; HA, the haemagglutinin epitope; and Myc, the c-Myc epitope. pJG45 (2μARS, *TRP1*, expresses B42-NLS-HA under control of *GAL10*); pJG45-ySir2 (identical to pJG45 but with an in-frame fusion of B42-NLS-HA to full-length *SIR2*); pJG45-ysir2^{P394L} (identical to pJG45-Sir2 but with a Pro394 to leucine substitution); pJG45-ysir2^{LGG} (identical to pJG45-sir2^{P394L} but with two additional mutations of Arg403 and Arg404 to glycines); pJG45-yHst2 (identical to pJG45 but with an in-frame fusion of B42-NLS-HA to full-length *HST2*); pGAL (2μARS, *TRP1*, *GAL10* promoter); pGAL-yHst2 (identical to pGAL with full-length *HST2* fused to HA); pRD (or pRD54C with *CEN3 ARS1*, *URA3* and the HA epitope under *GAL10* control); pRD-hSirT2 (as pRD with *hSIRT2* lacking the N-terminal 33 amino acids fused in-frame to HA); pRD-hsirT2^{P182L} (as pRD-hSirT2 with a Pro182 to leucine substitution); p2μ (equivalent to pRS424, with 2μARS, *TRP1* and *ADH1*); p2μ-hSirT2-Myc (p2μ with the full-length *hSIRT2* fused to a Myc epitope at its C-terminus); p2μ-hsirT2^{P182L}-Myc (identical to p2μ-hSirT2 but with the Pro182 to leucine substitution). These latter were constructed by subcloning *Pme*I fragments from pcDNA3.1/Myc-HisB-hSirT2/hsirT2^{P182L} into *Sma*I sites of p2μ. p2μ-ySir2 was constructed by ligating an *Eco*RI–*Xho*I fragment, encoding *SIR2* subcloned from the pJG45-ySir2 vector into *Eco*RI–*Xho*I sites of p2μ.

The plasmids pJG45-ySir2, pJG45-ysir2^{LGG} (Cockell *et al*., 2000) and pJG45-ysir2^{P394L} (obtained from pJG45-ySir2 by PCR mutagenesis; this study) were constructed by in-frame ligation of the appropriate *SIR2* fragment into the *Eco*RI and *Xho*I sites of the vector pJG45. An *Eco*RI–*Xho*I fragment containing *HST2* was also obtained by PCR and cloned in-frame using the *Eco*RI and *Xho*I sites of pJG45 (Golemis *et al*., 1996). A *Pst*I–*Cla*I fragment encoding HA-yHst2 was obtained from pJG45-yHst2 by PCR, and then cloned into the *Pst*I and *Cla*I sites of pGAL. All constructs were verified by DNA sequence analysis, and western blots on extracts of the yeast transformants verified the correct size of each fusion protein.

pRD-hSirT2 and pRD-hsirT2^{P182L} (*hSIRT2*, 960 bp) were constructed by ligating RT–PCR (from cDNA libraries of a melanoma patient, taking melanoma cells and normal cells of this same patient; T.Woelfel, personal communication) *Eco*RI–*Hin*dIII fragments encoding hSirT2 and hsirT2^{P182L}, respectively, into *Eco*RI–*Hin*dIII sites of pRD (i.e. pRD54C). pCMV-hSirT2-Myc6His (full-length *hSIRT2*, 1059 bp) is also called pcDNA3.1-hSirT2-Myc6His (gift of T.Woelfel and U.Mainz). This was constructed by ligating an *Apa*I–*Xba*I fragment of *hSIRT2* obtained by RT–PCR (as above) into pcDNA3.1/Myc-HisB (Invitrogen).

Accession numbers

EcSir2, SW:P75961; ScSir2, SW:P06700; ScHst1, SW:P53685; ScHst2, SW:P53686; ScHst3, SW:P53687; ScHst4, SW:P53688; KlSir2, SW:P33294; HsSirT1, EM:AF083106; HsSirT2, EM:AF083107; HsSirT3, EM:AF083108; HsSirT4, EM:AF083109; HsSirT5, EM:AF083110; HsSirT6, TREMBL NEW:AAD15478; SpSir2, sp_tr:O94640; SpHst2, emnew:AL121807; SpHst4, emnew:AF117324;

CaSir2, SW:O59923; CaHst2, sp_tr:O94066; LmSir2p, SW:G25337; LmSirTuin, emnew:AL117324; DmSir2, sp_tr:O96505; MmSirT1, EM:AF214646; MmSirT2, munigene:Mm25161; MmSirT3, munigene:Mm41625; CeQ20480, sp_tr:Q20480; CeQ20481, sp_tr:Q20481; CeO01478, sp_tr:O01478; CeQ20480, sp_tr:Q219221; GgSirT2, EM:AI981091.1; GgSirT6, EM:AI981030.1.

Yeast methods and tagging of HST1, HST2 and SIR4

The genotypes of the yeast strains used in this study are indicated in Table I. Media and standard yeast genetic methods were as described (Rose *et al*., 1990). Limiting adenine medium contains 10 μg/ml adenine sulfate, and all yeast incubations were performed at 30°C.

HST1, *HST2* and *SIR4* open reading frames were fused in-frame at their C-termini to a 13 Myc epitope as described (Longtine *et al*., 1998). Clones expressing *kanMX6* were selected by growth on YPAD medium containing 200 μg/ml G418, whereas clones expressing *TRP1* were selected by growth on SC-trp. Correct insertion of the tag was verified by PCR and immunoblotting in each case.

Silencing assays

Expression of the subtelomeric *TelVR::ADE2* reporter gene, the *TelVIIL::URA3* reporter and the *RDN1::mURA3/HIS3* construct (Smith and Boeke, 1997) have been described previously (Cockell *et al*., 1998, 2000). For the assay in Table II, repression of *RDN1::mURA3/HIS3* was monitored by comparing growth on YPD ± 0.1% FOA. A total of 30 colonies were used for each strain in six different experiments. Recombination events that excise *URA3* were monitored by streaking eight FOA^r colonies from each strain on medium lacking uracil.

Protein extraction and western blots

Yeast strains were grown to 1–2 × 10^7 cells/ml and cells were recovered by centrifugation to form a pellet of ~100 μl. Cell extracts were obtained by glass bead breakage in the presence of 50 mM Tris–HCl pH 7.5, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 2 μg/ml antipain, 300 μg/ml benzamidine, 1 μg/ml pepstatin A, 100 μg/ml 1-chloro-3-tosylamido-7-L-2-heptanone (TLCK), 50 μg/ml *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), 1% Trasylol. Protein concentrations were calculated by Bradford assay, and 30–40 μg of protein extract were separated on a 10% SDS–polyacrylamide gel and western blotted by standard enhanced chemiluminescence protocols (ECL, Amersham).

Immunofluorescence microscopy on yeast cells and preparation of antibodies

Yeast immunofluorescence methods and antibodies were described previously (Gotta *et al*., 1996, 1997). Rabbit anti-hSirT2 was raised against recombinant MBP fused to hSirT2p expressed in *E.coli* by standard procedures (New England Biolabs). All rabbit antisera were affinity purified against bacterially expressed fusion proteins transferred to nitrocellulose strips. In the case of anti-MBP-hSirT2, antibodies were affinity purified in two ways: either on nitrocellulose strips or by binding and elution from native MBP–hSirT2 protein that was covalently bound to cyanogen bromide-activated Sepharose beads. For affinity purification, antisera were first depleted for anti-MBP antibodies by incubation with MBP alone. Other antibodies used are: anti-Myc (monoclonal 9E10), anti-Nop1 (A66, gift of John P.Aris, University of Florida, Gainesville, FL; Aris and Blobel, 1988), rabbit anti-Nop1 (RP1-5, gift of Ed Hurt, Heidelberg), anti-HA (HA.11, clone 16B12 monoclonal from BABCO, Berkeley, CA), Cy5-coupled anti-mouse secondary antibody and 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF)-coupled anti-rabbit secondary antibody (both Milan Analytica). Secondary antibodies were pre-absorbed against fixed yeast spheroplasts prior to use. No cross-reactivity among these reagents has been detected, and controls using secondary antibodies alone were carried out. For western blot standardization, a rat antibody that recognizes an abundant RNase H (p55) was used (Karwan *et al*., 1990).

Confocal microscopy was performed on a Zeiss Laser Scanning Microscope 410 and 510 with a 63× Plan-Apochromat objective (1.4 oil), as previously described (Gotta *et al*., 1996). No signal from one fluorochrome could be detected on the other filter set, and image capture and background subtraction were carried out uniformly on all images to allow direct comparisons.

Cell culture and immunofluorescence microscopy on mammalian cells

ΦNX_A cells (Phoenix amphotropic packaging lines, 293T packaging cell line, human embryonic kidney line transformed with adenovirus E1a) were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) with 10% fetal calf serum (FCS) and HEPES. Transfections were done with calcium phosphate precipitates, as described by Jordan *et al*. (1996). Cells were grown on coverslips which were washed twice in phosphate-buffered saline (PBS) and fixed in 4% freshly prepared paraformaldehyde for 15 min at room temperature. All subsequent steps were carried out at this temperature. Cells were washed once with PBS and permeabilized in 0.1% Triton X-100/PBS for 10 min. Cells were washed once in PBS, sites were saturated for 30 min in 2% bovine serum albumin (BSA)/0.1% Tween 20/PBS and subsequently washed twice in PBS. Primary and secondary antibodies were incubated in 1% BSA/0.1% Triton X-100/PBS with washes of 3 × 5 min with PBS (primary antibodies for 2 h, secondary antibodies for 45 min). Images were captured as described above. For samples with competitor, 10 μl of whole bacterial extract dissolved in Laemmli buffer lacking β-mercaptoethanol and bromophenol blue were added to 90 μl of antibody adjusted to 1% Triton X-100. After 2 h, complexes were sedimented by centrifugation, and the supernatant was used to stain the coverslips.

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