

Dimerization of Sir3 via its C-terminal winged helix domain is essential for yeast heterochromatin formation

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Gene silencing in budding yeast relies on the binding of the Silent Information Regulator (Sir) complex to chromatin, which is mediated by extensive interactions between the Sir proteins and nucleosomes. Sir3, a divergent member of the AAA + ATPase-like family, contacts both the histone H4 tail and the nucleosome core. Here, we present the structure and function of the conserved C-terminal domain of Sir3, comprising 138 amino acids. This module adopts a variant winged helix-turn-helix (wH) architecture that exists as a stable homodimer in solution. Mutagenesis shows that the self-association mediated by this domain is essential for holo-Sir3 dimerization. Its loss impairs Sir3 loading onto nucleosomes *in vitro* and eliminates silencing at telomeres and *HM* loci *in vivo*. Replacing the Sir3 wH domain with an unrelated bacterial dimerization motif restores both *HM* and telomeric repression in *sir3Δ* cells. In contrast, related wH domains of archaeal and human members of the Orc1/Sir3 family are monomeric and have DNA binding activity. We speculate that a dimerization function for the wH evolved with Sir3's ability to facilitate heterochromatin formation.

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Introduction

Large regions of eukaryotic chromatin assume an epigenetically heritable structure that is refractory to gene expression, called heterochromatin or, in budding yeast, silent chromatin. From yeast to man, gene silencing is thought to stem from a more compact folding of the chromatin fibre that sterically restricts DNA accessibility (Gottschling, 1992; Singh and Klar, 1992; Loo and Rine, 1994). Covalent modifications of histone proteins play a major role in determining chromatin compaction, and

hypoacetylated histones are characteristic of silent chromatin in budding yeast (Braunstein *et al*, 1993; Suka *et al*, 2001). In addition, the binding of non-histone complexes along the nucleosomal fibre can repress productive transcription. In budding yeast, this is achieved by the binding of a heterotrimeric complex of Silent Information Regulator (Sir) proteins Sir2, Sir3 and Sir4, each of which is essential for gene silencing (Rine and Herskowitz, 1987).

The Sir proteins themselves do not recognize specific DNA sequences, but are recruited to discrete loci through protein–protein interactions with multifunctional factors like Rap1, ORC and Abf1, which recognize specific DNA motifs at telomeres and in silencer elements that flank the silent homothallic mating type (*HM*) loci. At *HM* loci, an intermediary protein called Sir1 bridges between these factors and the Sir complex (reviewed in Rusche *et al*, 2003). From these initial nucleation sites, the Sir complex spreads for 3–20 kb along the chromatin fibre (Hecht *et al*, 1996; Strahl-Bolsinger *et al*, 1997).

The establishment of silent chromatin requires the NAD-dependent deacetylase activity of Sir2 (Tanny *et al*, 1999; Imai *et al*, 2000; Smith *et al*, 2000; Yang and Kirchmaier, 2006), while Sir3 and Sir4 are thought to play structural roles in the silent chromatin formation (reviewed in Gasser and Cockell, 2001; Rusche *et al*, 2003; and Moazed *et al*, 2004). Sir4 forms a tight complex with Sir2, and stimulates Sir2 activity *in vitro* (Ghidelli *et al*, 2001; Hoppe *et al*, 2002; Tanny *et al*, 2004; Cubizolles *et al*, 2006). The C-terminal coiled-coil domain of Sir4 dimerizes and interacts with Sir3; this interaction is essential for silencing, because point mutations in this Sir3–Sir4 interface abolish assembly of the trimeric Sir complex (Chang *et al*, 2003; Rudner *et al*, 2005; Ehrentraut *et al*, 2011).

The spread of Sir-mediated repression is limited both by histone modifications that lower affinity of Sir3 for chromatin and by the limited concentration of Sir proteins in the nucleus (Maillet *et al*, 1996; Marcand *et al*, 1996; van Leeuwen *et al*, 2002; Martino *et al*, 2009; Oppikofer *et al*, 2011). The overexpression of Sir3, or a balanced overexpression of Sir4 and Sir3, leads to the extension of the silent chromatin domains at telomeres or enhanced repression at silencer-flanked reporter genes (Renaud *et al*, 1993; Gotta and Gasser, 1996; Hecht *et al*, 1996; Maillet *et al*, 1996; Strahl-Bolsinger *et al*, 1997), suggesting that Sir3, and its dosage, plays major roles in Sir complex spreading and transcriptional repression.

SIR3 arose from the duplication of the *ORC1* gene (Kellis *et al*, 2004), and both encode a highly conserved N-terminal BAH domain, a C-terminal AAA + ATPase-like domain, plus an extreme C-terminal domain (Gaudier *et al*, 2007). A chimeric protein formed by exchanging the BAH domain of Sir3 with that of Orc1 can restore mating in a *sir3Δ* strain, but no other Orc1 subdomain is able to support silencing when integrated into Sir3 (Bell *et al*, 1995). Overexpression of a N-terminal domain of Sir3 reinforces telomere-proximal silencing in a *SIR*⁺ strain and partially restores mating at

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HM loci in a *sir3Δ* background if Sir1 is overexpressed (Gotta *et al*, 1998; Connelly *et al*, 2006). Consistently, biochemical and structural analyses show that the Sir3 BAH domain interacts with the nucleosome (Onishi *et al*, 2007; Buchberger *et al*, 2008; Sampath *et al*, 2009; Armache *et al*, 2011). Both H3K79 methylation by Dot1 and H4K16 acetylation by Sas2 reduce the association of Sir3 BAH with chromatin (Onishi *et al*, 2007), or of holo-Sir3 with nucleosomes *in vitro* (Martino *et al*, 2009; Oppikofer *et al*, 2011), indicating that the BAH domain helps to restrict Sir3-mediated silencing to unmodified nucleosomes.

The AAA + ATPase-like (AAA) domain of Sir3 (aa 530–845) has lost its ATPase activity, but has gained specific contacts with both Sir4 and unmodified nucleosomes (Ehrentraut *et al*, 2011). Mutagenesis confirmed that both interactions are essential for silencing. A third structural subdomain of Sir3 occupies its extreme C-terminus (aa 843–978). When fused to *lexA* and targeted to a subtelomeric reporter, this fragment can recruit Sir proteins and nucleate transcriptional repression (Liaw and Lustig, 2006). It was also reported to self-associate in a yeast-two-hybrid assay (Liaw and Lustig, 2006). Pull-down experiments argued that a slightly longer Sir3 C-terminal fragment (aa 832–978) might bind a central domain of Sir3 (aa 464–728), which contains the N-terminal portion of the AAA + ATPase module (King *et al*, 2006). While these results were suggestive of homo- or hetero-dimerization activities, the importance of such interactions for silencing or their redundancy with other potential dimerization domains in Sir3 was never rigorously tested. Intriguingly, this C-terminal domain is conserved among Orc1 homologues and corresponds to the domain III of ORC/CDC6, which in *A. pennix* and other archaea binds DNA (Bell *et al*, 1995; Liu *et al*, 2000; De Felice *et al*, 2004; Singleton *et al*, 2004). X-ray structure analysis showed that the archaeal C-terminal domain adopts a winged helix-turn-helix fold (Dueber *et al*, 2007; Gaudier *et al*, 2007). However, the sequence identity between the C-termini of archaeal Orc1 and yeast Sir3 is very low (13%), rendering cross-species structure-function predictions unreliable.

To sort out the role of this extreme C-terminal domain of Sir3, we first solved its X-ray crystal structure to 2.7 Å resolution. These 138 amino acids form a winged helix-turn-helix variant (Sir3 wH) that is reminiscent of domain III of Orc1/Cdc6, yet the Sir3 wH has acquired a strong homodimerization function and fails to bind DNA. We show that deletion of the domain (Sir3ΔwH), or loss of its dimerization potential, ablates silencing, which can be restored by attaching a bacterial dimerization motif to Sir3ΔwH. With a combination of structural, biochemical and genetic analyses we demonstrate that the last 138 aa of Sir3 evolved as the crucial homodimerization motif within Sir3, that is essential for the assembly of Sir-dependent silent chromatin in yeast. Distinct features of the wH structure distinguish human and archaeal Orc1 from the silencing-competent *S. cerevisiae* Sir3 paralogue.

Results

The C-terminal 138 aa of Sir3 form a winged helix-turn-helix variant that homodimerizes

SIR3 arose from the duplication of the *ORC1* gene with which it shares a multidomain organization (Figure 1A; Bell *et al*,

1995; Kellis *et al*, 2004). While the extreme C-terminal region of archaeal *A. pennix* Orc1 folds as a winged helix-turn-helix domain that binds DNA (De Felice *et al*, 2004; Dueber *et al*, 2007; Gaudier *et al*, 2007), the function of the homologous domain in ScSir3 was unclear. The multiple sequence alignment of selected archaea, vertebrate, and yeast Orc1/Sir3 wH domains revealed important evolutionary changes (Figure 1B). Notably, yeast Orc1/Sir3 wH domains possess a ~30 residues insertion (Figure 1B, orange residues) which is missing in archaea and higher eukaryotic Orc1 wH domains. In addition, the specific *ApOrc1* residues implicated in DNA binding (Gaudier *et al*, 2007) are poorly conserved in yeast and higher eukaryotes (Figure 1B, blue residues).

To determine the biochemical and structural features of the predicted Sir3 wH, we expressed the last 138 aa of Sir3 (aa 840–978) in *E. coli* and purified the protein to ≥95% purity (Figure 1C). Surprisingly, the molecular mass of the purified Sir3 840–978 aa (15.9 kDa) fragment, determined by size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) was 33.1 kDa, consistent with recombinant Sir3 840–978 aa being a homodimer in solution (Supplementary Figure S1). The folding of this C-terminal module was determined by solving its X-ray crystal structure.

The extreme C-terminal domain of Sir3 crystallized in space group P3₂12 with one molecule per asymmetric unit and its structure was solved to 2.7 Å resolution by the multi-wavelength anomalous diffraction (MAD) method using a seleno-methionine derivative (Table I). The final crystal structure displays clear electron density for residues 849–975, that fold into a wH variant featuring a total of 7 α-helices and a 3 stranded β-sheet wing (Figure 1D). A search of the Protein Data Bank (PDB) using DALI (Holm and Rosenstrom, 2010) indicated that the wH domains of archaeal Orc1 (*A. pennix*, 2V1U) (Gaudier *et al*, 2007), Orc2 (*A. pennix*, 1W5T) (Singleton *et al*, 2004), CDC6P (*P. aerophilum*, 1FNN) (Liu *et al*, 2000), and CDCP6 (*S. solfataricus*, 2QBY) (Dueber *et al*, 2007) are most similar.

Superposition of Sir3 wH with an archaeal homologue (*ApOrc1* wH; Gaudier *et al*, 2007; Figure 1E) showed that the core helix-turn-helix structures and the β-sheet wing are highly similar (RMSD of 2 Å), but also revealed important structural differences. The most significant differences are due to an additional stretch of 30 aa we identified by sequence alignment (Figure 1B, orange residues), which is absent in the archaeal *ApOrc1*. This insert is found between the wH core α-helices three (α3, aa 883–896) and four (α4, aa 928–938) and forms two additional α-helices (a, b) that are connected by an elongated turn in coiled conformation (Figure 1B and E; highlighted in orange).

Whereas the asymmetric unit of Sir3 in the crystal contains one Sir3 wH module, the crystal packing reveals two symmetry-related Sir3 wH proteins in the crystal lattice (Figure 1F). The ~30 aa α-helical insertion contributes most to the buried solvent-accessible surface area of the protein–protein interface covering 1500 Å², with leucine 861 and valine 909 being located in the hydrophobic core of this contact (Figure 1F; contact surfaces of the two molecules are represented in cyan and dark red). This large contact surface is likely to account for the strong homodimerization observed in solution (Supplementary Figure S1), as predicted by PISA (Protein Interfaces, Surfaces and Assemblies) calculations based on the crystal packing (Krissinel and Henrick, 2007).

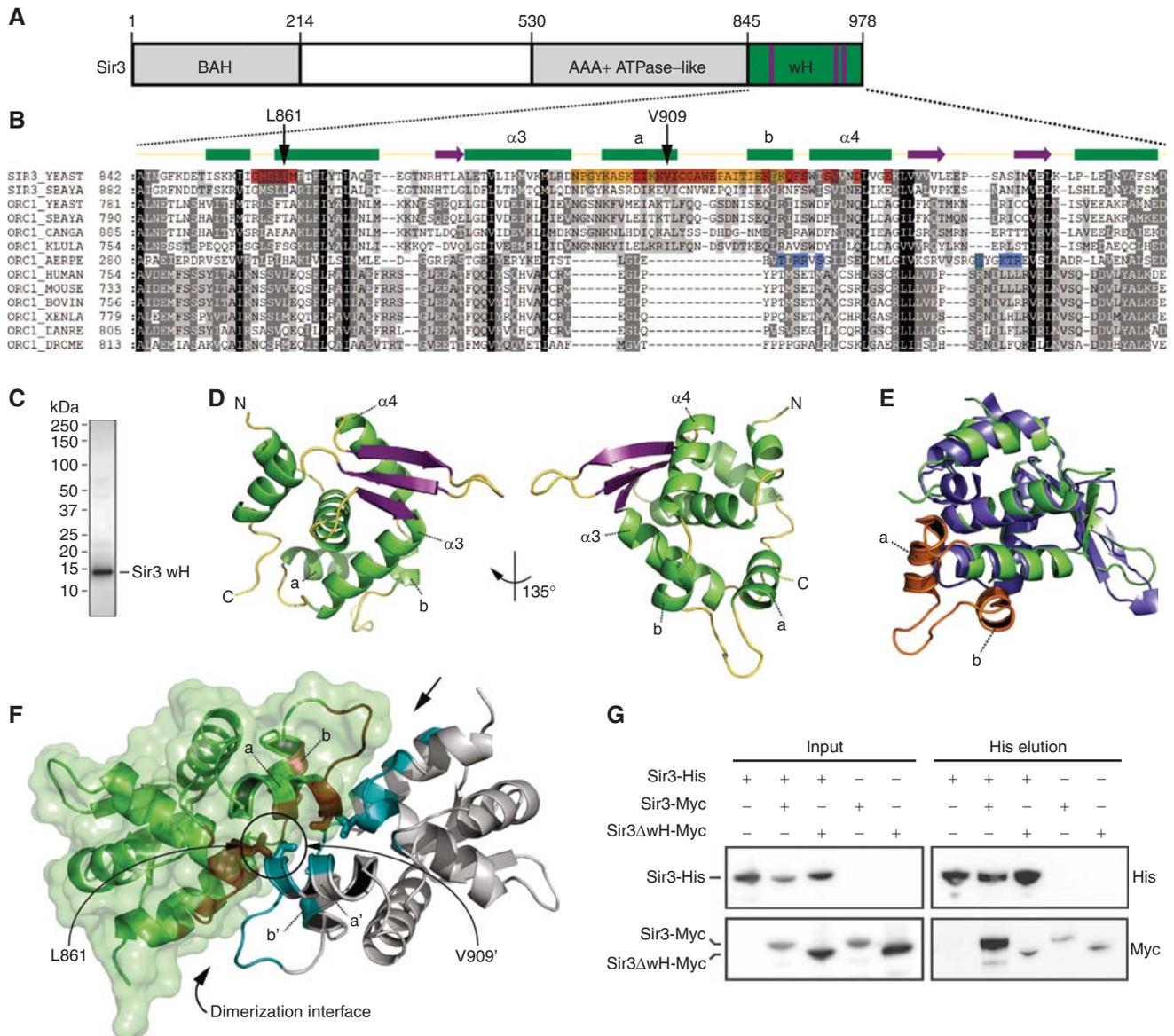


Figure 1 The C-terminal 138 residues of Sir3 fold into a wH domain and homodimerize. (A) Schematic representation of the Sir3 protein. The C-terminal wH domain in green has been crystallized. Purple bars show the position of the 3-stranded β -sheet wing. (B) Multiple sequence alignment of Orc1/Sir3 wH modules from archaea, budding yeasts and higher eukaryotes. Conserved residues are highlighted in black (100%), dark grey (50%) and light grey (20%). ScSir3 wH residues that contribute to the dimerization interface are in red, the 30-aa insertion is highlighted in orange, and ApOrc1 wH residues that contact DNA are in blue (Gaudier *et al*, 2007). Above the alignment is the secondary structure of Sir3 wH colour coded as in (D). The L861 and V909 residues mutated in the Sir3-AA construct are highlighted. (C) 2 μ g of purified Sir3 wH (aa 840–978) was loaded on a SDS-PAGE and stained with Coomassie blue. (D) Representation of the Sir3 wH structure; α -helices are represented in green, β -sheets in purple and coils in yellow. (E) Superposition of Sir3 and ApOrc1 (PDB 2v1u; Gaudier *et al*, 2007). The insertion, absent in ApOrc1, forming most of the Sir3 wH homodimerization interface is in orange (see sequence alignment) and displayed as dumbbell-shaped helices (a, b). (F) Representation of a Sir3 wH dimer, the residues involved in the hydrophobic interface are coloured in red and cyan for the green and grey Sir3 wH monomers, respectively. The surface representation of the green Sir3 wH monomer is displayed in light green. The insertion crucial for Sir3 wH dimerization is displayed as dumbbell-shaped helices. (G) His-tagged full-length Sir3 (Sir3-His) was co-expressed in insect cells with Myc-tagged full-length Sir3 (Sir3-Myc) or Sir3 lacking the wH module (Sir3 Δ wH-Myc; aa 1–850). Sir3-His was affinity purified, input and His elution were analysed by western blotting using the indicated antibody.

In order to test the importance of the wH module for Sir3 dimerization in a competitive assay, we co-expressed in insect cells a His-tagged full-length Sir3 (Sir3-His) with a Myc-tagged full-length Sir3 (Sir3-Myc) or Sir3 lacking the wH module (Sir3 Δ wH-Myc; aa 1–850). Affinity purified Sir3-His was tested for interaction with Sir3-Myc and Sir3 Δ wH-Myc using an anti-Myc antibody on proteins recovered on Ni beads, which bind the His tag. Strikingly, we observed that the deletion of the wH domain reduced Sir3 self-association

to background levels, whereas full-length Sir3-Myc was efficiently recovered with full-length Sir3-His (Figure 1G). Sedimentation analysis by glycerol density gradients showed in addition that Sir3 Δ wH-His sedimented more slowly than full-length Sir3-His, consistent with a loss of dimerization (Supplementary Figure S2B). Moreover, we were unable to detect any interaction of Sir3 wH with the Sir3 AAA domain by pull-down assays or isothermal titration calorimetry (Supplementary Figure S3B and C). This suggests that Sir3

Table 1 Data collection and refinement statistics

	Sir3 wH native	Sir3 wH Se-Met peak ^a	Sir3 wH Se-Met inflection ^a	Sir3 wH Se-Met remote ^a
<i>Data collection</i>				
Space group	P3 ₂ 12	P3 ₂ 12	P3 ₂ 12	P3 ₂ 12
Cell const. <i>a</i> , <i>b</i> , <i>c</i> (Å)	75.3, 75.3, 55.6	75.1, 75.1, 55.3	75.2, 75.2, 55.3	75.2, 75.2, 55.4
Wavelength (Å)	1.000	0.9793	0.9798	0.9716
Resolution range (Å) ^b	30.0–2.7 (2.77–2.70)	30.0–3.1 (3.21–3.10)	30.0–3.1 (3.21–3.10)	30.0–3.1 (3.21–3.10)
Unique reflections	5076	6078	5990	5854
Completeness (%) ^b	99.9 (100.0)	96.3 (73.1)	94.4 (64.8)	91.9 (50.6)
Multiplicity	8.2	5.8	5.8	5.7
R _{sym} (%) ^b	4.6 (53.6)	6.9 (60.3)	6.8 (63.5)	7.0 (78.8)
I/σ(I) ^b	25.7 (4.7)	34.8 (1.4)	33.6 (1.2)	31.6 (0.84)
Phasing power ano	—	1.46	0.82	1.19
<i>Refinement</i>				
Resolution range (Å)	30.0–2.7			
Reflections (all)	5076			
Reflections (test set)	728 (14.3%)			
R _{crys} (%)	21.1			
R _{free} (%)	26.3			
RMSDs				
Bond lengths (Å)	0.01			
Bond angles (deg)	1.34			
Ramachandran plot (%)				
Allowed	96.0			
Outliers	4.0			

^aData collection statistics is reported for unmerged Friedel pairs.

^bValues in parentheses refer to the highest resolution shell.

wH-mediated homodimerization accounts for most, if not all, Sir3 self-interaction, at least in its soluble form.

The wH fails to interact with DNA or chromatin but promotes Sir3 loading onto nucleosomes

Past work has implicated both the BAH and the AAA domains in dimerization, and in the spreading of Sir3 along the nucleosomal fibre (Connelly *et al*, 2006; Armache *et al*, 2011; Ehrentraut *et al*, 2011). In addition, the Sir3 wH has been proposed to heterodimerize by binding a central Sir3 domain (King *et al*, 2006; Liaw and Lustig, 2006), while the archaeal ORC/CDC6 wH modules were shown to bind to DNA in concert with the AAA + ATPase-like domain (De Felice *et al*, 2004; Singleton *et al*, 2004; Dueber *et al*, 2007; Gaudier *et al*, 2007). Importantly, however, the residues implicated in DNA binding within *ApOrc1* (labelled in blue), are not conserved in Sir3 wH (Figures 1B and 2A; Gaudier *et al*, 2007).

To test various models of DNA binding, domain interaction and cooperation in chromatin association, we purified two additional Sir3 C-terminal fragments: the AAA + ATPase-like (AAA) domain alone (aa 530–845; Ehrentraut *et al*, 2011) and the entire Sir3 C-terminal half, which includes both AAA and wH domains (Sir3C; aa 527–978; Figure 2B; Supplementary Figure S3A). We first incubated increasing amounts of the Sir3 wH and the AAA module with a constant amount of a Cy3-labelled 147 bp DNA (Huynh *et al*, 2005; Martino *et al*, 2009), and monitored interaction by gel shift assay. Scoring the loss of unbound DNA template, we found that Sir3 AAA readily binds DNA, while Sir3 wH does not, even at 2500-fold molar excess (Figure 2C). To see if the Sir3 wH instead binds a chromatin template, we repeated the titration into a constant amount of hexameric (6-mer) nucleosomal arrays, reconstituted from bacterially expressed histones and the Widom 601 repeat (Martino *et al*, 2009). The AAA domain

efficiently interacted with this array (see also Ehrentraut *et al*, 2011), while Sir3 wH was unable to bind chromatin, even at 2500-fold molar excess (Figure 2D).

In order to test whether the dimerization capacity of the wH domain contributes to the DNA and chromatin binding properties of Sir3 AAA, we compared the binding of the AAA domain with the larger Sir3C fragment, which includes the wH domain (Figure 2B). Strikingly, the presence of the wH, which does not interact with DNA *per se*, increased Sir3C-DNA binding affinity by approximately 3- to 4-fold over that of the AAA module alone (based on apparent IC₅₀ from gel shift quantitation, Figure 2E). Similarly, when we compared the interaction of the AAA and the larger Sir3C domains (containing the wH domain) with the 6-mer array, the loading of Sir3C onto chromatin was strikingly more efficient (Figure 2F). We found no evidence for heterodimerization between the Sir3 wH and AAA domains by pull-down and isothermal titration calorimetry experiments (Figure 1G; Supplementary Figures S2 and S3). Thus, while we cannot exclude that wH-AAA interactions might occur after the Sir complex is loaded onto chromatin, our data argue that homodimerization of the wH module within the full Sir3C domain stabilizes the association of Sir3C molecules with chromatin, indirectly increasing affinity for the 6-mer array. Contacts with nucleosomes and DNA, on the other hand, are mediated exclusively by the AAA domain (Figure 2, see also Ehrentraut *et al*, 2011).

Sir3 wH is essential for silencing *in vivo*, and can be substituted by a bacterial dimerization motif

To test whether Sir3 dimerization through its wH module is important to form silent chromatin *in vivo*, we examined the restoration of silencing in a *sir3Δ* strain complemented with various Sir3 constructs expressed from a plasmid (Figure 3A). The constructs were cloned together with 1 kb of Sir3 5'- and

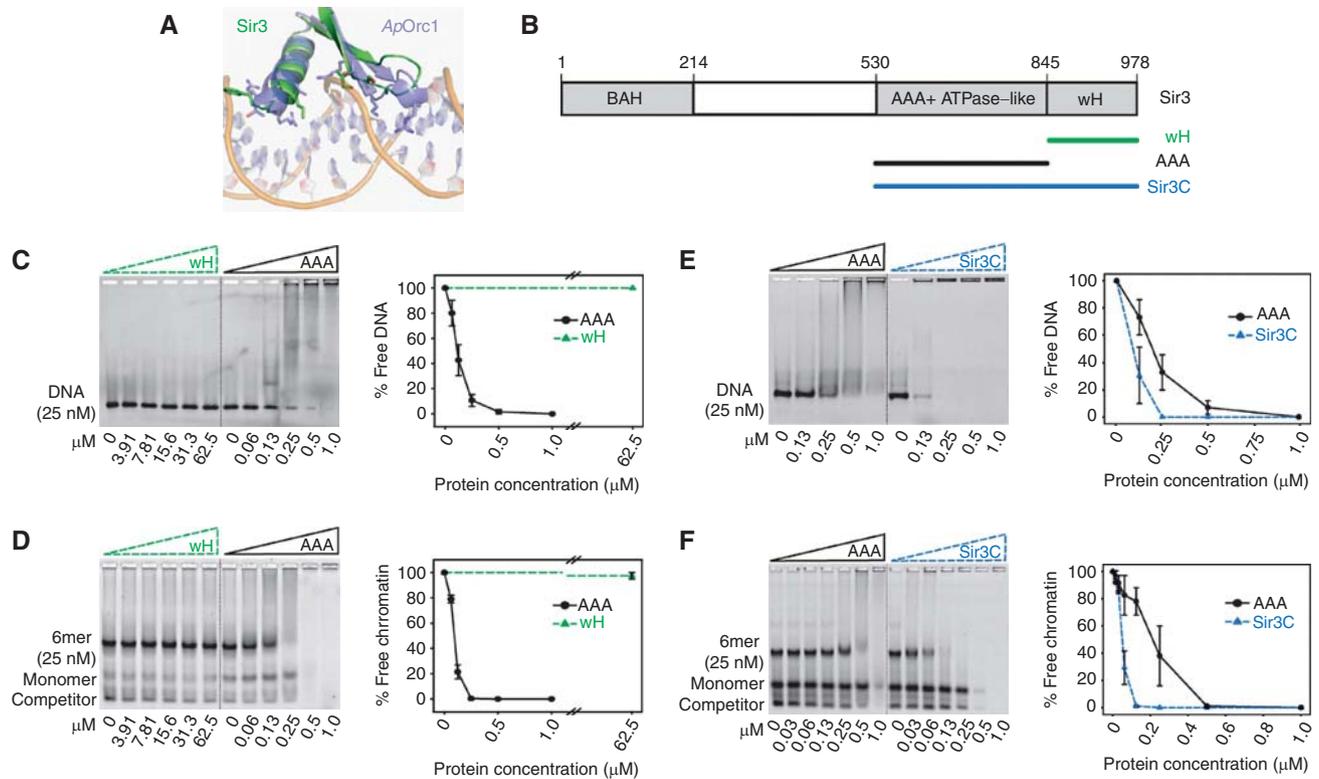


Figure 2 The association of the Sir3 C-terminus to DNA and chromatin is promoted by wH homodimerization. (A) Details on the residues of *ApOrc1* wH (blue) interacting with DNA and superposition with the corresponding region of Sir3 (green). (B) Scheme of the modular organization of the Sir3 protein. Constructs used are highlighted. The indicated Sir3 domains were titrated onto a constant amount of Cy3-labelled 147 bp DNA (C, E) or a 6-mer of nucleosomes (D, F). Samples were separated on a native agarose gel, the nucleosomal template was stained with SYBR[®] safe and the amount of unbound probe was quantified by the intensity of the fluorescent signal. The images are representatives for three or more experiments; quantification represents mean values and s.e.m. of the % of free probe normalized to the input.

3'-UTR to ensure endogenous expression levels, which were checked routinely by western blot (Figure 3B). As expected, expression of full-length *SIR3* fully restored silencing of reporter genes inserted at telomere 7L and to a large extent at the *HMR* locus (Figure 3E). We then tested the complementation efficiency of a construct lacking the wH module (Sir3ΔwH; aa 1–850) which fails to dimerize *in vitro* (Supplementary Figure S2B). Importantly, we found that Sir3ΔwH, which was expressed at wild-type Sir3 levels, failed to restore silencing at telomere 7L and *HMR* (Figure 3B and E).

We next examined whether Sir3 wH homodimerization *per se* was necessary for silencing. Because PISA analysis suggested that leucine 861 (L861) and valine 909 (V909) contribute strongly to the wH-wH hydrophobic interface, we mutated these two key residues to alanines (Sir3-AA; L861A-V909A). V909 is located at the C-terminal end of the α -helix 'a', which is part of the yeast-specific insert described above. It contacts L861 of the other Sir3 wH unit within the crystallized homodimer (Figures 1B, F and 3C). Like Sir3ΔwH, the Sir3-AA mutant had impaired dimerization capacity and sedimented as a monomer in a glycerol gradient (Supplementary Figure S2A and B). Importantly, the Sir3-AA double mutant, which was again expressed at levels comparable to wild-type Sir3 (Figure 3B), led to a complete loss of silencing of the telomere 7L reporter gene, mimicking a full deletion of *SIR3* (Figure 3E; Supplementary Figure S4A and B). Sir3-AA also weakly decreased silencing at *HMR*

compared to wild-type Sir3 (Figure 3E). Several other mutations, such as the triple alanine substitution mutant, L861A-V909A-F926A, or mutation of L861 and V909, singly or in combination, into tryptophan (W), arginine (R) or aspartic acid (D) residues, as well as swapping the residues 900–914 with a short glycine-serine-glycine (GSG) linker, instead reduced Sir3 protein levels, although they were expressed from the same promoters. These mutations, which rendered Sir3 unstable, of course, also failed to repress at telomere 7L (hereafter, TPE for Telomere position effect; Gottschling *et al*, 1990; Supplementary Figure S4A and B).

We reasoned that if Sir3 wH homodimerization was the only essential function of the wH in gene repression, a chimeric protein consisting of the Sir3ΔwH construct fused to an ectopic dimerization domain might be able to restore silencing. To test this, we made use of the well-characterized transcription factor HlyU from *Vibrio vulnificus*, which dimerizes readily and is similar in overall size to Sir3 wH, although it shares no sequence identity (Nishi *et al*, 2010; Liu *et al*, 2011; Figure 3D). This chimeric construct (*sir3-HlyU*), like the Sir3-AA mutant, was expressed at wild-type Sir3 levels (Figure 3B) in a *sir3Δ* strain. Using reporters at both telomere 7L and at the *HMR* locus, we found that the hybrid protein Sir3-HlyU was able to restore silencing completely, with an efficiency indistinguishable from that of full-length Sir3 protein (Figure 3E). The fact that an ectopic bacterial dimerization module can functionally replace the wH domain of Sir3, conferring silencing function and protein stability,

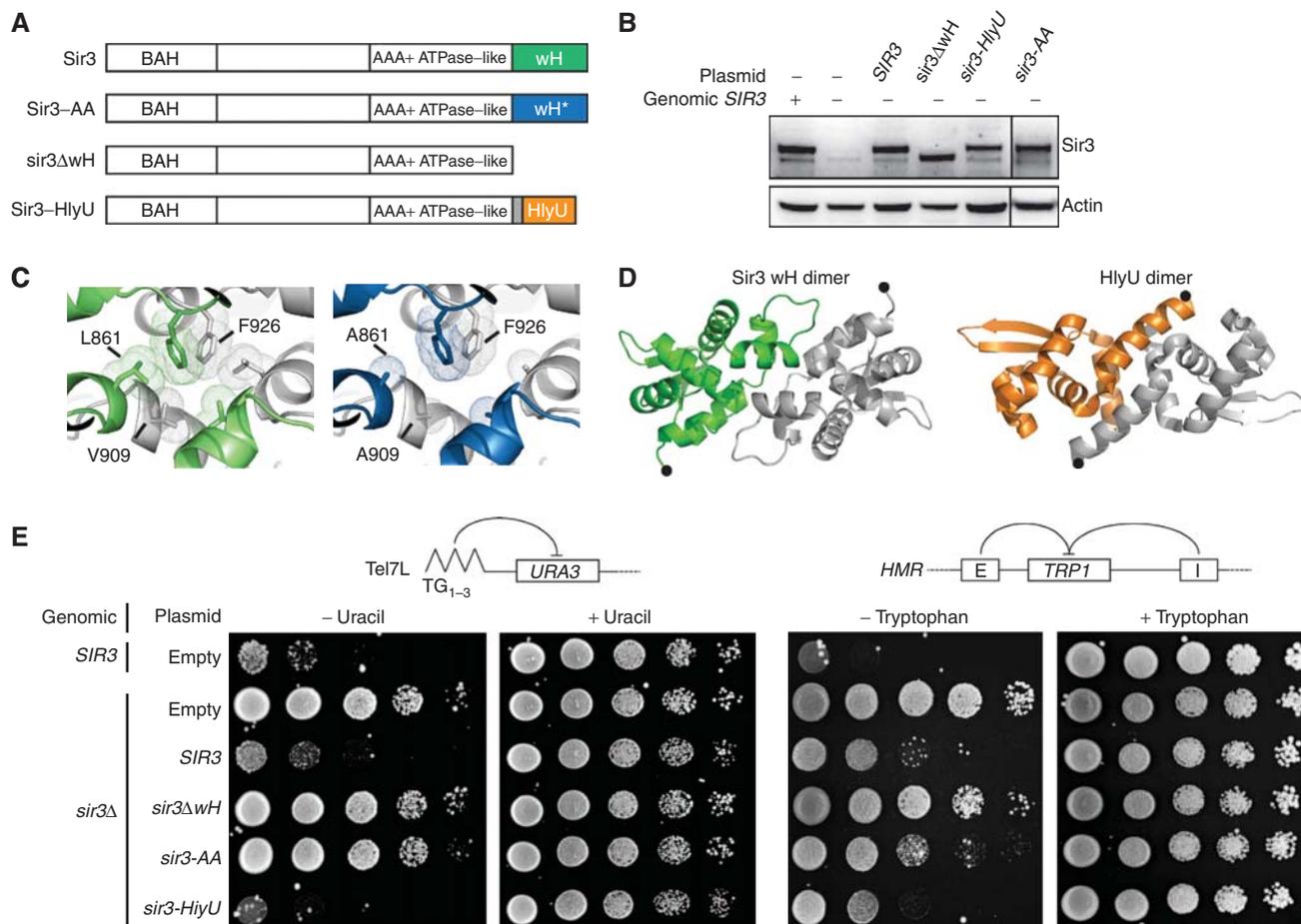


Figure 3 Sir3 wH homodimerization is essential for telomeric and HMR silencing. (A) Schematic representation of the Sir3 constructs used. Sir3 wH wild-type (green), the L861A-V909A mutant (blue) and the HlyU module from *Vibrio vulnificus* (orange) are highlighted. (B) Western blot analysis of alkaline yeast extracts (Ruault *et al*, 2011) of the strains used in (E) show wild-type levels of Sir3 expression, using a polyclonal antibody raised against full-length Sir3 (a kind gift from L Pillus). (C) Modelled representation of the impact L861A and V909A mutations on the extent of the hydrophobic Sir3 wH dimerization interface. (D) Representation of the Sir3 wH or the HlyU dimer. The most C-terminal residues are highlighted by a black dot. (E) The indicated strain was grown overnight in selective media and 10-fold dilutions were dropped on the indicated media. Ability to repress a *URA3* gene inserted at the telomere 7L was tested by growth on synthetic media lacking uracil while ability to repress a *TRP1* gene inserted at the *HMR* locus was tested by growth on synthetic media lacking tryptophan. Synthetic complete media (only selecting for plasmids) was used to control for equal deposition.

strongly suggests that dimerization is the only essential function of the Sir3 wH domain for Sir-mediated repression *in vivo*.

Overexpression of Sir3 wH competes with endogenous Sir3 dimerization to relieve TPE

Given the above findings, and past results showing that *SIR3* gene dosage affects repression (Renault *et al*, 1993; Hecht *et al*, 1996; Maillet *et al*, 1996; Strahl-Bolsinger *et al*, 1997), we reasoned that an excess of Sir3 wH might compete for endogenous full-length Sir3 dimerization and impair silencing. To test this hypothesis, we cloned the wH module under the control of the *GAL1* promoter, which allows overexpression in galactose-containing media (Mumberg *et al*, 1994). We introduced this construct in a *SIR3*⁺ strain and monitored TPE at telomere 7L and 5R, as well as silencing at the *HMR* locus on plates containing galactose to induce Sir3 wH expression. Indeed, overexpression of Sir3 wH abrogated TPE at the two telomeres scored (Figure 4A; Supplementary Figure S5). On the other hand, Sir3 wH overexpression did not affect repression at *HMR*, where redundant

silencers ensure more stable repression (Figure 4B; Maillet *et al*, 1996; Marcand *et al*, 1996).

To confirm that the Sir3 wH-provoked derepression stems from its ability to dimerize and thereby disrupt essential Sir3–Sir3 interactions, we introduced structure-based point mutations in the dimerization interface of Sir3 wH. As detailed in Figure 3, by introducing the same L861A and V909A mutations as we did in full-length Sir3 (Sir3-AA) in the overexpressed wH module alone (wH-AA) we reduced the extent of the wH–wH hydrophobic dimerization interface (Figure 3C). We also replaced V909 by an aspartic acid (wH-D), destabilizing Sir3 wH dimerization by inserting a negative charge at the hydrophobic interface. Both mutated Sir3 wH domains, wH-AA and wH-D, were expressed at similar levels as the wild-type Sir3 wH domain (Figure 4C). Overexpression of the wH-D mutant failed to impair TPE at telomeres 7L and 5R (Figure 4A; Supplementary Figure S5), and the wH-AA mutant derepressed both sites, albeit less efficiently than the wild-type domain (Figure 4A; Supplementary Figure S5). This is consistent with the notion that overexpression of the Sir3 wH domain has a

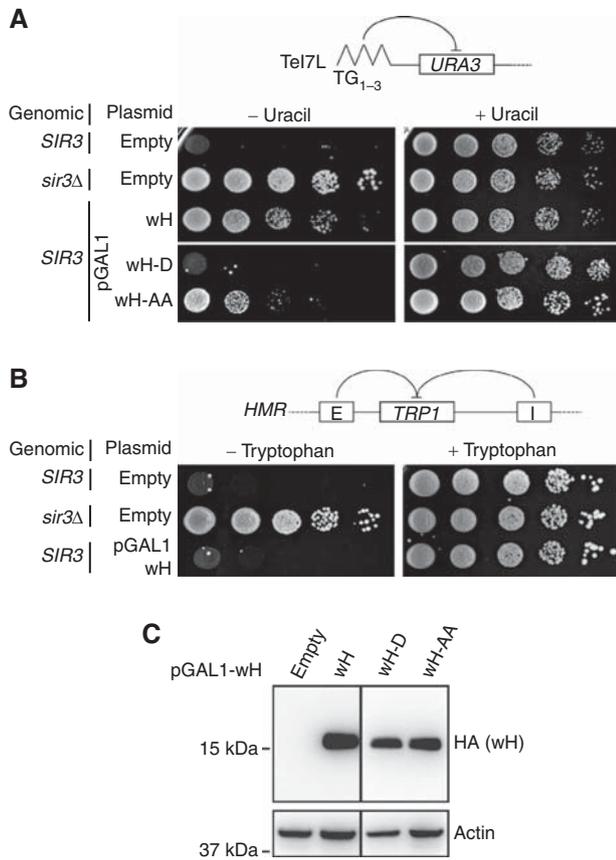


Figure 4 Overexpression of Sir3 wH, but not of dimerization mutants, derepresses silencing. (A) The indicated strain was grown overnight in selective media and 10-fold dilutions were dropped on the indicated media containing galactose as carbon source, to induce pGAL1^{promoter}-*SIR3*wH construct overexpression. Ability to repress a *URA3* gene at telomere 7L was tested as in Figure 3E. (B) The ability to repress *TRP1* inserted at the *HMR* locus was tested as in Figure 3E. (C) Western blot analysis from alkaline yeast extracts (Ruault *et al*, 2011) of strains showed in Figure 4A, grown on galactose, to confirm domain expression. wH-D and wH-AA refer to the overexpression of the Sir3 wH domain carrying the single V909D or the double L861A-V909A mutations, respectively.

dominant-negative effect on TPE by interfering with endogenous Sir3 dimerization, and again confirms the importance of the large dimerization interface within Sir3 wH domains for Sir-mediated repression.

Sir3 dimerization is dispensable for Sir complex formation, but is essential for Sir complex assembly on chromatin *in vivo*

Our *in vitro* assays revealed that the Sir3 wH domain promotes the loading of the Sir3 C-terminal region onto a reconstituted chromatin template (Figure 2). Given the multiplicity of contacts between the Sir complex and chromatin, we next examined whether Sir3 wH homodimerization also promotes the binding of the holo-Sir complex to chromatin *in vitro*. We first expressed Sir3-AA, Sir3ΔwH or wild-type Sir3 in insect cells, together with Sir2 and Sir4, and found that all generate stable complexes with Sir2-4 that can be purified from cell extracts (Figure 5A), indicating that the Sir3-Sir4 interaction does not depend on Sir3 dimerization capacity. We then incubated purified Sir2-4 complexes and the corresponding equimolar amount of Sir3, Sir3-AA or Sir3ΔwH

(Supplementary Figure S2A) with the 6-mer of reconstituted nucleosomes. Strikingly, disruption of Sir3 dimerization by deletion (Sir3ΔwH) or mutation (Sir3-AA) of the Sir3 wH domain consistently reduced the binding affinity of the holo-Sir complex to chromatin *in vitro* (Figure 5B). This is consistent with the loss of TPE in cells expressing only the Sir3ΔwH or Sir3-AA forms of Sir3.

Given the fact that loss of Sir3 wH homodimerization weakened Sir complex loading *in vitro* (Figure 5B) and compromised TPE *in vivo* (Figure 3), we asked whether Sir3 wH-mediated dimerization was also necessary for the stable binding and spread of Sir complexes along subtelomeric chromatin *in vivo*. Alternatively, the mutant Sir complexes might bind chromatin, but simply fail to repress. To address this, we performed chromatin immunoprecipitation (ChIP) analysis using an antibody against Sir4 in strains that express the Sir3 constructs described above (Figure 3), driven by the *SIR3* promoter and flanked by *SIR3* terminator sequences. All constructs are expressed at levels comparable to wild-type Sir3 (Figure 3B). Using qPCR on chromatin sheared to obtain a resolution of roughly 500 bp, we monitored Sir4 binding to the subtelomeric *URA3* reporter gene that monitors silencing at telomere 7L, along with other genomic locations that support Sir-mediated repression.

We found that Sir3ΔwH failed to support Sir4 loading at native telomeres 6R and 9R, and at the *HML-α1* gene, while there was reduced Sir4 binding at telomere 7L and at the *HML-E* silencer (Figure 5C). In addition, the dimerization mutant *sir3-AA* reduced Sir4 loading at all telomeres tested (Tel6R, 7L, 9R) as well as at *HML-α1*, while it did not affect binding of Sir4 to the *HML-E* silencer. Consistent with its ability to restore repression, expression of Sir3-HlyU restored binding of the Sir4 protein at all loci tested (Figure 5C). This confirms that Sir3 dimerization through the wH domain is essential for Sir complex spreading *in vivo*, although Sir4-silencer binding is largely independent of Sir3 dimerization activity. Our ChIP results correlate perfectly with the silencing phenotypes described in Figure 3, and reveal that Sir3 wH-mediated dimerization is critical for holo-Sir complex loading or stable association on chromatin *in vivo*. Since the bacterial dimerization domain (HlyU) restores holo-Sir binding, our data argue that dimerization is the essential silencing-specific function of Sir3 wH.

The *S. cerevisiae* Orc1/Sir3 wH domain evolved to self-associate prior to gene duplication

As discussed above, the Orc1/Sir3 family is conserved throughout evolution. The archaeal *A. pernix* Orc1 wH was shown to bind DNA (Gaudier *et al*, 2007), and based on this observation, a similar function was predicted for other Orc1 homologues, including the Sir3 wH domain (Norris and Boeke, 2010; Hickman *et al*, 2011). Having shown here that Sir3 wH does not bind DNA, and evolved instead into a dimerization module that is crucial for Sir-mediated silencing, we were curious whether the *S. cerevisiae* and *H. sapiens* Orc1 wH domains would be more similar to Sir3 or to the archaeal protein.

To shed light on the functional conservation of these wH modules, we purified the equivalent domains of ScOrc1 and HsOrc1 after expression in *E. coli* (Figure 6A). To test whether the ScOrc1 or HsOrc1 wH domains interact with DNA, we titrated an increasing amount of each purified protein into a

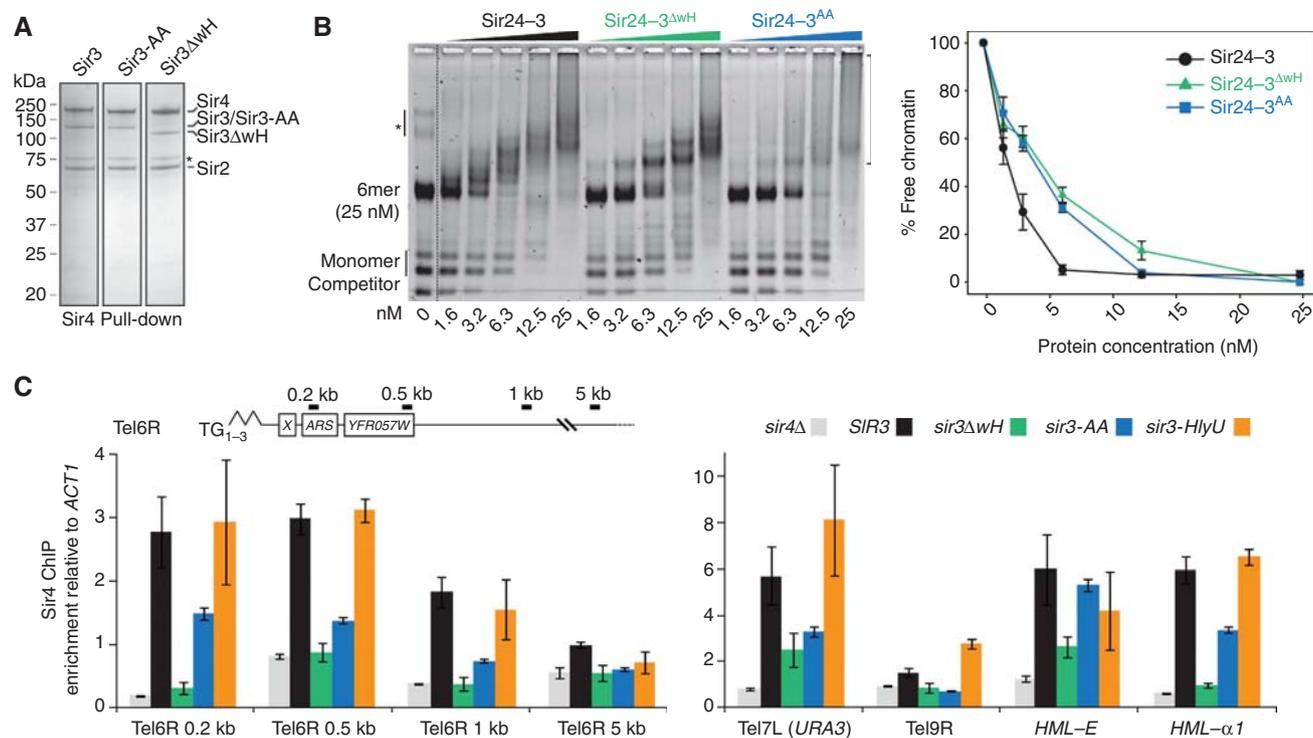


Figure 5 Sir3 wH homodimerization is critical for loading or stable binding of Sir complex onto chromatin *in vivo*. (A) Hi5 insect cells were co-infected with Sir2, Sir4 and the indicated Sir3 expressing virus. Sir4 was affinity purified as described previously (Martino *et al*, 2009) and a sample elution was loaded on an SDS-PAGE and stained with Coomassie blue. The asterisk indicates a common Sir4 degradation product. (B) The holo-Sir complex or mutant complexes containing either Sir3 Δ wH (aa 1–850) or Sir3-AA (L861A and V909A) were titrated into a constant amount of 6-mer of nucleosomes. The images are representative of at least three independent experiments; quantifications show the mean value \pm s.e.m. of the % of unbound chromatin compared to the input. The asterisk indicates a contaminant DNA, and the bracket on the right indicates Sir/chromatin complexes. (C) Sir4 ChIP analysis of the same strains used for silencing assay in Figure 3E, the amount of precipitated DNA was measured by qPCR. Enrichment over the *ACT1* locus was normalized to the input.

constant amount of Cy3-labelled 147-bp DNA, and monitored interaction by gel shift assay. We found that the human Orc1 wH domain bound DNA readily, while the yeast Orc1 wH did not (Figure 6B). Next, we investigated the oligomeric states of ScOrc1 and HsOrc1 by SEC-MALS. We found that ScOrc1 wH oligomerizes in solution and, in the conditions used here, forms a range of oligomers, primarily consistent with 5–7 molecules, but ranging in one experiment up to 27 (Figure 6C). This may reflect a need for other subunits of ORC for proper oligomerization. In contrast, the HsOrc1 wH, purified under the same conditions and with homologous boundaries as ScOrc1 and ScSir3, showed no self-association and retained a stable monomeric state in solution (Figure 6D). We conclude that there is a fundamental difference between ScOrc1/Sir3 and both the archaeal and human Orc1 proteins, in that the ScOrc1/Sir3 wH evolved to promote protein–protein interactions, losing DNA binding ability. This most likely occurred before an ancient yeast underwent the genome duplication that generated the two related *SIR3* and *ORC1* genes.

Discussion

The formation of yeast silent chromatin depends on the recruitment of the Sir proteins by factors that bind specific DNA elements, followed by the establishment of Sir–chromatin and Sir–Sir contacts. These layers of interaction contribute to a stable chromatin structure that impairs transcription and protects the linker DNA from nuclease attack.

Whereas Sir protein recruitment by Rap1, ORC, Abf1 and Yku has been studied extensively, as have interactions between Sir domains and chromatin, only a few studies have examined the importance of Sir protein dimerization for repression.

The Sir3 C-terminal wH domain is necessary and sufficient for Sir3 dimerization

Sir4 homodimerizes through a C-terminal coiled-coil domain, which also interacts with Sir3, Yku70 and Rap1 (Moretti *et al*, 1994; Tsukamoto *et al*, 1997; Chang *et al*, 2003; Murphy *et al*, 2003; Rudner *et al*, 2005). Sir4 dimerization has been proposed to be necessary for silencing by providing a binding site for Sir3 (Chang *et al*, 2003; Murphy *et al*, 2003). The importance of Sir3 dimerization has never been tested directly, even though Sir3 has the ability to spread along chromatin with substoichiometric amounts of Sir2 and Sir4 (Hecht *et al*, 1996; Strahl-Bolsinger *et al*, 1997). This implied that Sir3–Sir3 interaction should be important for its function *in vivo*.

Over the years, various Sir3 domains have been proposed to mediate homodimerization or heterodimerization. Both the AAA domain and the BAH domain made suggestive contacts in the crystal packing when crystallized alone (Connelly *et al*, 2006; Ehrentraut *et al*, 2011) or together with a nucleosome (Armache *et al*, 2011). However, the relevance of this finding outside of the crystal context is unclear. Dimerization of the Sir3 BAH could not be detected by co-IP (Buchberger *et al*, 2008) and only a very weak

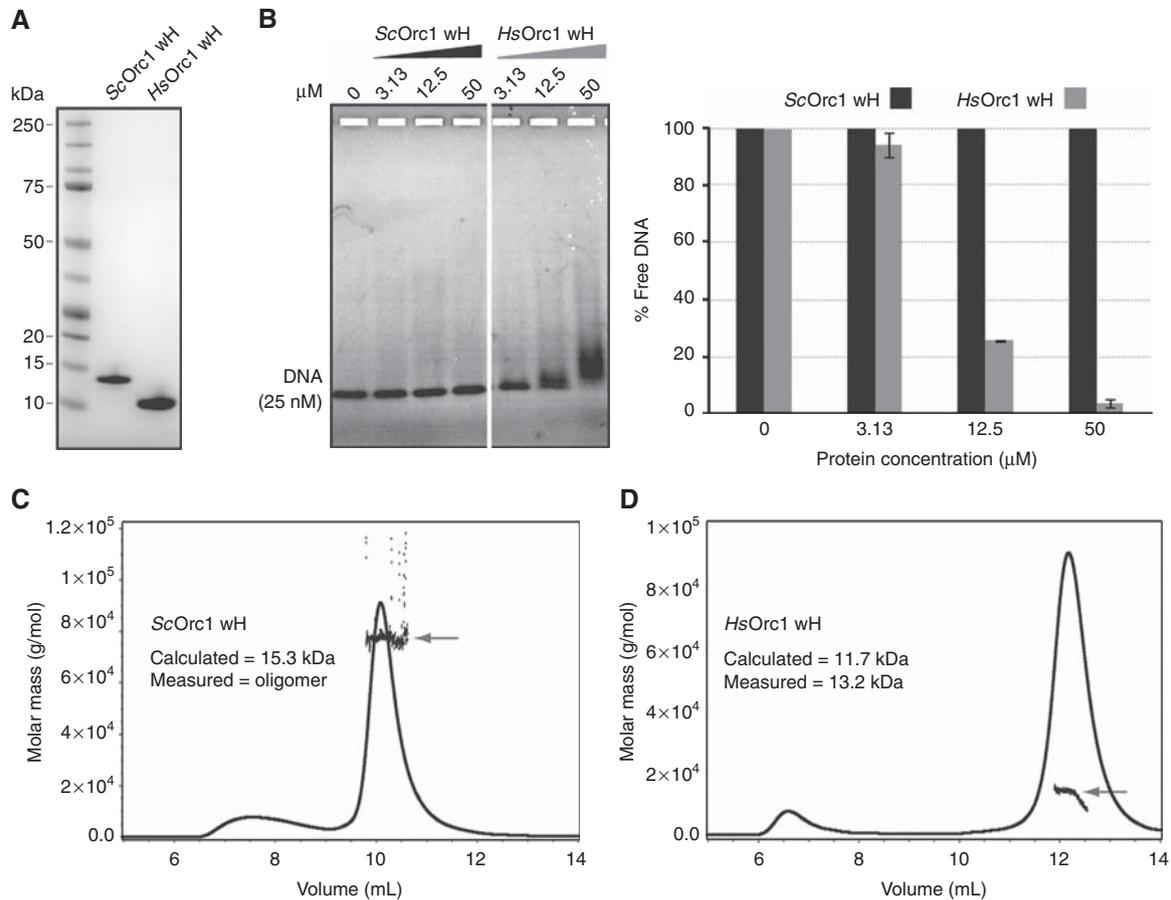


Figure 6 Evidence for a functional conservation of Sir3 wH in *Saccharomycetales* species. (A) 2 μg of the indicated domains was run on an SDS-PAGE and stained with Coomassie blue. (B) The indicated domain was titrated onto a constant amount of a 147-bp Cy3-DNA probe; binding was scored as in Figure 2. Purified ScOrc1 wH, 3 mg/ml (C) or HsOrc1 wH, 7.8 mg/ml (D) was analysed by SEC-MALS as described in Materials and methods. The measured molar mass is represented by black dots (grey arrow) and superposed to the UV (280 nm) trace. The ScOrc1 wH domain was injected five times yielding the following results: 78, 81, 108, 123 and 417 g/mol.

interaction was observed by analytical ultracentrifugation (Armache *et al*, 2011). A Sir3 C-terminal fragment that contained both the AAA and the wH domains appeared to dimerize in solution (Chang *et al*, 2003), while the Sir3 wH and a fragment containing a large N-terminal portion of the AAA domain showed some interaction upon bacterial co-overexpression (King *et al*, 2006). Indeed, no study to date had carefully mapped the dimerization interface between Sir3 molecules, nor mutated important residues to monitor both a loss of dimerization and of silencing function *in vivo*. Here, we demonstrate unequivocally that the Sir3 C-terminal 138 residues form a wH module that is necessary and sufficient for dimerization, and that Sir3 homodimerization is crucial for Sir complex loading along nucleosomes *in vivo*. Genetic, biochemical and structural data support this conclusion rigorously.

The crystal structure of the wH domain presented here reveals a large hydrophobic dimerization interface (1500 Å²), which we show is biologically relevant in contrast to the contact surfaces observed for the BAH and AAA domains (Connelly *et al*, 2006; Ehrentraut *et al*, 2011). We confirmed Sir3 wH dimerization in solution by SEC-MALS. Importantly, Sir3 dimerization in insect cell extracts was reduced to background levels when full-length Sir3 was co-expressed

with Sir3 lacking the wH domain (Figure 1G). Finally, while holo-Sir3 was shown to migrate as a dimer in a glycerol gradient, Sir3ΔwH or Sir3-AA shifted the Sir3 protein towards a monomeric state. Together, these studies indicate that dimerization of Sir3 is mostly, if not entirely, mediated by the wH domain. In contrast, we found that the AAA domain does not stably dimerizes (Ehrentraut *et al*, 2011), nor does it interact with the wH domain by either pull-down or isothermal titration calorimetry (this study). While we cannot rule out that novel dimerization interfaces are revealed in Sir3 upon assembly into the holo-Sir complex or upon binding to chromatin, it is made less likely by the fact that either deletion or mutation of the Sir3 wH domain reduces the binding affinity of the holo-Sir complex to chromatin *in vitro*.

The Sir3 wH does not bind DNA or chromatin, but stabilizes the Sir complex on chromatin

Due to its homology with the crystallized archaeal ApOrc1 C-terminus (Gaudier *et al*, 2007), the Sir3 wH domain was predicted to bind DNA. However, Sir3 wH neither binds DNA nor chromatin *in vitro*. Instead it forms a homodimer that reinforces the loading of the Sir3 C-terminal domain onto both templates in binding assays (Figure 2). Previous work

using full-length Sir3 showed that holo-Sir3 can bind DNA and nucleosomal templates, forming oligomeric structures *in vitro* (Georgel *et al*, 2001; McBryant *et al*, 2006, 2008). We attribute these chromatin interactions to contacts demonstrated for the BAH and AAA domains (Connelly *et al*, 2006; Armache *et al*, 2011; Ehrentraut *et al*, 2011), while Sir3 wH specifically mediates dimerization.

Our biochemical data suggest that the dimerization of Sir3 wH promotes Sir3 and holo-Sir complex loading onto chromatin *in vitro*, while ChIP data show that it also promotes the propagation and/or stable binding of the holo-Sir complex along the chromatin fibre *in vivo*. Given that the Sir4 C-terminal domain has been reported to dimerize as well (Chang *et al*, 2003; Murphy *et al*, 2003), it is tempting to speculate that Sir3 and Sir4 dimerization capacities jointly promote the spread of the Sir complex along chromatin, possibly by creating Sir complex dimers (i.e., hexamers) and then interactions between them along the chromatin fibre. While the precise role of Sir4 dimerization in establishing Sir-repressed chromatin remains to be investigated, reducing the Sir3 wH dimerization interface by targeted mutagenesis (Sir3-AA) or deletion of the wH domain, significantly decreased the loading of Sir4 along subtelomeric nucleosomes at telomeres 6R, 7L, 9R and across the *HML* locus *in vivo*. Consistently, the Sir3-AA or Sir3 Δ wH mutants failed to silence subtelomeric reporter genes. Remarkably, repression could be completely restored to the wH-deficient Sir3 mutant by attaching a bacterial dimerization motif that has no homology with the native Sir3 domain. Reinforcing our conclusion that dimerization of Sir3 is crucial for repression, it was reported that expression of the BAH domain could support measurable *HM* silencing only when fused to the dimerization-competent *lexA* module (Connelly *et al*, 2006). Taken together, we conclude that Sir3 dimerization capacity is a general requirement for gene repression, even when it is mediated by an alternative mechanism that involves only a subdomain of Sir3. In summary, our data show unequivocally that Sir3 wH-mediated homodimerization is essential for loading of Sir proteins onto chromatin and for the silencing of underlying genes.

Consistent with the loss- and gain-of function constructs described above, we also demonstrate that overexpression of the Sir3 wH domain ablates TPE, while a dimerization deficient mutant does not. This refines previous data showing that overexpression of a large Sir3 C-terminal construct (aa 437–978) derepresses TPE, while if it includes the Sir3 wH domain (Enomoto *et al*, 2000). Although TPE has been shown to be more easily disrupted than *HMR* silencing in overexpression studies, we note that the deletion of the wH domain fully derepressed both *HMR* and telomeric reporters, demonstrating the general need of Sir3 wH dimerization for silencing.

A previous study showed that a few amino-acid substitutions that do not affect Sir3 wH dimerization in yeast-2-hybrid assays, interfered with a tethered repression assay (Y900A, Y964A and K973A; Liaw and Lustig, 2006). Our structural studies reveal that these residues are not involved in dimerization, and thus their effects may stem instead from altered intramolecular secondary structure within Sir3, or Sir3 interfaces with other proteins. However, the fact that the *HlyU* dimerization motif can replace wH to

restore TPE, argues against other silencing-specific functions in this C-terminal domain.

Evolutionarily distinct wH domains among *Orc1/Sir3* family members

The basic wH fold that we present here for the Sir3 C-terminal domain is conserved from archaea to man, and generally mediates macromolecular interactions (Aravind *et al*, 2005). The yeast Sir3 wH domain, however, binds neither chromatin nor DNA, in striking contrast to the wH domain of archaeal (De Felice *et al*, 2004; Dueber *et al*, 2007; Gaudier *et al*, 2007) or human *Orc1* (this study). Indeed, we show here that a recombinant wH domain of *HsOrc1*, like the archaeal *Orc1* wH, forms a stable monomer in solution and readily binds DNA under the conditions tested here.

Sequence analysis predicts that only budding yeast lineage (*Saccharomycetales*) homologues, that is, those bearing an insertion in the wH domain, will have a sufficiently large hydrophobic surface to mediate dimerization (Figure 1B). Consistently, the *ScOrc1* wH, unlike *HsOrc1* wH, self-associates in solution, and fails to bind DNA. On the basis of these functional and sequence similarities between *ScOrc1* and *ScSir3*, and their dissimilarity with *Orc1* homologues in other species, we propose that yeast *Orc1* wH is closer to Sir3 than to the human *Orc1* wH. In fact, the human *Orc1* wH more closely resembles that of archaea, at least with respect to macromolecular interactions. However, given that the critical residues for *ApOrc1*–DNA interaction are not conserved in man (Figure 1B), the two may bind DNA in different ways.

The fact that the wH domains of both yeast Sir3 and *Orc1* acquired a ~30aa insert that generates a large protein–protein interface, and the ability to self-interact, rather than bind DNA, makes it highly likely that these changes in the wH domain sequence arose prior to the genome duplication event that generated *S. cerevisiae* and the *SIR3/ORC1* gene pair (Kellis *et al*, 2004). Consistently, the characteristic insertion in the wH domain is also found in the budding yeast *Kluyveromyces lactis*, even though its genome did not undergo endoduplication (Hickman and Rusche, 2010). Intriguingly, *Orc1* from *K. lactis* acts in conjunction with the deacetylase Sir2 and the histone-binding protein Sir4 to generate telomeric and mating type heterochromatin (Hickman and Rusche, 2010). The fact that *ScOrc1* cannot substitute for Sir3 in the budding yeast SIR complex, despite having a similar wH domain, most likely reflects the well-characterized specialization of the Sir3 AAA domain for Sir4 interaction (Ehrentraut *et al*, 2011).

We still do not know how important *ScOrc1* self-association is for its function in either replication or silencing, although we show here that dimerization of the same domain in Sir3, is crucial for silencing in yeast. We note that the changes in the wH domain that promote dimerization, correlate well with the appearance of a divergent ‘silencing’ branch of the *Orc1/Sir3* family. While other mechanisms of chromatin-mediated repression, such as those involving Heterochromatin Protein 1 or Polycomb repressive complex 1, also depend on components that have homodimerization potential, it has never been shown that the dimerization domain can be swapped for a dimerization motif from bacteria (Fujisaki *et al*, 2003; Czypionka *et al*, 2007; Canzio *et al*, 2011). This demonstration for yeast Sir3 confirms that its dimerization capacity is essential for efficient Sir complex loading onto chromatin,

that is, for both telomeric and mating type silencing. By ensuring the ability of haploid spores to switch mating type and conjugate, mating type silencing is, of course, essential for the survival of the species.

Materials and methods

Multiple sequence alignment

Orc1 wH sequences from higher eukaryotes were aligned with ClustalW (www.ebi.ac.uk) and submitted to HHPRED (Soding *et al*, 2006) to yield an alignment with the *A. pernix* Orc1 wH sequence of which the structure is known (Gaudier *et al*, 2007). A ClustalW alignment of yeast Sir3 and Orc1 wH sequences was then added according to a structural alignment computed with DALI (Holm and Rosenstrom, 2010) using crystal structures of the *A. pernix* Orc1 and Sir3 wH domains.

Purification of protein domains

The Sir3 wH (aa 840–978), Sir3C (aa 527–978), ScOrc1 wH (aa 783–914) and HsOrc1 wH (aa 760–861) constructs were cloned into a pOPINF vector using the In-Fusion system (Clontech) (Berrow *et al*, 2007), expressed in the *E. coli* strain BL21 Rosetta pLysS and affinity purified *via* a His tag using ProBond Ni-NTA resin (Invitrogen) according to manufacturer's instructions. Tags were removed by HRV 3C protease digestion (Novagen) and Sir3 domains were further purified by gel filtration on a HiLoad 16/60 Superdex 75 column in 50 mM Tris pH 7.5, 200 mM NaCl, 0.02% Na₂S₂O₈ and 1 mM TCEP. The Sir3 AAA (aa 530–845) was purified as described previously (Ehrentraut *et al*, 2011). Point mutations were introduced by site directed mutagenesis using *Pfu* DNA polymerase (Promega). For the chimera Sir3-HlyU, the coding region for aa 851–978 was deleted and replaced with *AscI* and *XbaI* sites using site directed mutagenesis. The cDNA of HlyU-Vv was amplified by PCR (without the ATG start codon) inserting *AscI* and *XbaI* sites and a SGSG linker. The cDNA of HsOrc1 (IRAK013J04) was obtained through RIKEN BRC, Japan (Ota *et al*, 2004; Otsuki *et al*, 2005; Itoh *et al*, 2006; Kimura *et al*, 2006). Purity was confirmed by SDS-PAGE and Coomassie blue staining; protein concentration was measured by UV absorbance (280 nm).

Structure determination

Native and selenomethionine substituted Sir3 wH crystals were grown at 20°C by sitting-drop vapour diffusion in 96-well crystallization plates from mixtures containing equal volumes of protein (22 mg/ml in 20 mM Tris pH 8.5, 200 mM NaCl, 0.02% Na₂S₂O₈ and 1 mM TCEP) and reservoir solutions (0.1 M sodium citrate pH 5.6, 0.2 M ammonium acetate and 30% (w/v) PEG-4000) and were flash-frozen in mother liquor made up to 25% (v/v) ethylene glycol. Diffraction data were collected at beam lines X06DA and X10SA at the Swiss Light Source synchrotron in Villigen, Switzerland. Detailed Materials and methods for structure determination and refinement can be found in Supplementary data. Coordinates have been deposited in the PDB database under accession code 3ZCO.

SEC-MALS experiments

Purified domains were concentrated to 2–8 mg/ml and filtered through a 0.1 µm Amicon filter before injection. In all, 100 µl of each protein was separated on a Wyatt SEC 300A 7.8 × 300 mm column equilibrated in 20 mM Tris pH 7.4, 200 mM NaCl, 0.02% Na₂S₂O₈ at a flow rate of 0.5 ml/min at 4°C. Light scattering was recorded on an in-line miniDAWN TREOS 3-angle light scattering detector (Wyatt Technology) and protein concentration detected with an in-line Optilab Trex refractive index detector. Data analysis was done using the Zimm fitting method in Astra V software (Wyatt Technology).

Co-immunoprecipitation from insect cells

Full-length or truncated Sir3-ΔwH was co-infected in Hi5 cells as indicated in Figure 1G. Cells were lysed by gentle sonication in 50 mM HEPES pH 7.5, 150 mM NaCl, 0.1% NP-40, and Sir3-HIS-HA purified in batch using ProBond Ni-NTA resin (Invitrogen). Beads were washed with 50 mM Na₂HPO₄ pH 8.0, 500 mM NaCl, 50 mM imidazole, 0.05% NP-40 and eluted in 2 × the bead volume with 50 mM Na₂HPO₄ buffer pH 8.0, 300 mM NaCl, 250 mM imidazole, 0.05% NP-40. Co-purification of Myc-tagged Sir3 constructs was

analysed by immunoblotting using HIS-hrp (Abcam, ab1187) or Myc (9E10) antibodies.

Chromatin and DNA interaction assays

In vitro reconstitution of nucleosomal arrays and binding assays were performed as described previously (Oppikofer *et al*, 2011). See also description in Supplementary data.

Overexpression and complementation assays in yeast

For overexpression assays, the genomic sequence coding for Sir3 wH (aa 840–978) was HA tagged, amplified by PCR and cloned into the pRS425-GAL1 vector (Mumberg *et al*, 1994) using *PstI* and *SalI* sites. For complementation assays, the *SIR3* gene together with 1 kb 5'- and 3'-UTR was amplified by PCR and cloned into pRS415 using *BamHI* and *NotI* sites. Deletion and point mutations were introduced by site directed mutagenesis using *Pfu* DNA polymerase (Promega). Telomeric silencing used GA503 (*SIR3*⁺) and GA7055 (*sir3Δ*) strains, while *HMR* silencing used GA484 (*SIR3*⁺) and GA7292 (*sir3Δ*) strains. *SIR3* deletion was achieved by replacing the endogenous gene together with 1 kb 5'- and 3'-UTR with a kanamycin resistance cassette (Supplementary Table S1). Silencing of the indicated reporter genes (*URA3* or *TRP1*) was monitored as described (Gotta *et al*, 1998), by growth on synthetic media lacking either uracil or tryptophan.

ChIP and qPCR experiments

ChIP experiments were carried out as previously described (Braunstein *et al*, 1993), with an antibody raised against GST-Sir4 C-terminus used at 750 ng/20 µl MagSi-proteinA 1.0 beads (MagnaMedics). Precipitated DNA was purified using the AccuPrep PCR purification kit (Bioneer) after overnight incubation at 65°C to reverse the formaldehyde cross-linking. The GA5822 *sir4Δ* strain (Kueng *et al*, 2012) was used as control. Quantification of the precipitated DNA was performed by qPCR on a StepOnePlus instrument (Applied Biosystems); enrichments were normalized to input and the *ACT1* locus. Primers have been used previously (see Supplementary Table S2). Experiments were performed in biological and technical duplicates.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: MO, SK and SMG designed most experiments and interpreted results. MO performed most experiments with the following exceptions. HG collected X-ray diffraction data, solved the Sir3 wH structure, produced the wH alignment and supervised the structural work. SK performed pull-down experiments from insect cells, and helped with *in vivo* assays and ChIP. JJK performed SEC-MALS analysis and helped with cloning and purification of proteins from *E. coli*. MH purified the Sir3 AAA domain and performed ITC analysis under the supervision of AGL. MO, SK, HG, AGL and SMG wrote the manuscript. SMG supervised the work.

Conflict of interest

The authors declare that they have no conflict of interest.

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