Functional Complementation of sir2Δ Yeast Mutation by the Human Orthologous Gene SIRT1

Davide Gaglio1,2, Anna D’Alfonso1, Giorgio Camilloni1,2,3*

1 Department of Biology and Biotechnology Charles Darwin, Sapienza-Università di Roma, Rome, Italy, 2 Istituto Pasteur - Fondazione Cenci Bolognetti, Sapienza-Università di Roma, Rome, Italy, 3 Institute of Molecular Biology and Pathology, The National Research Council (CNR), Rome, Italy

Abstract

Sirtuins, class III histone deacetylases, are proteins homologous to the yeast protein Sir2p. Mammalian Sirt1 has been shown to be involved in energy metabolism, brain functions, inflammation and aging through its deacetylase activity, acting on both histone and non-histone substrates. In order to verify whether Sirt1 can replace Sir2p in the yeast cells, we expressed the full-length human Sirt1 protein in S.cerevisiae sir2Δ mutant strain. The structure of chromatin is basically maintained from yeast to human. Thus, yeast chromatin is a favourable environment to evaluate, inhibit or activate an ectopic histone deacetylase activity in an in vivo substrate. Mutant sir2Δ shows a series of different phenotypes, all dependent on the deacetylase activity of Sir2p. We analyzed the three silent loci where normally Sir2p acts: ribosomal DNA, telomeres and the mating type loci. Moreover, we verified extrachromosomal ribosomal DNA circles production and histone hyperacetylation levels, typical marks of sir2Δ strains. By strong SIRT1 overexpression in sir2Δ cells, we found that specific molecular phenotypes of the mutant revert almost to a wild-type condition: in particular, transcriptional silencing at rDNA was restored, extrachromosomal rDNA circles formation was repressed and histone acetylation at H3K9 and H4K16 decreased. The complementation at the other studied loci: HM loci, telomere and sub-telomere does not occur. Overall, our observations indicate that: i) SIRT1 gene is able to complement different molecular phenotypes of the sir2Δ mutant at rDNA ii) the in vivo screening of Sirt1 activity is possible in yeast.

Citation: Gaglio D, D’Alfonso A, Camilloni G (2013) Functional Complementation of sir2Δ Yeast Mutation by the Human Orthologous Gene SIRT1. PLoS ONE 8(12): e83114. doi:10.1371/journal.pone.0083114

Editor: Keqiang Wu, National Taiwan University, Taiwan
Received: June 18, 2013; Accepted: October 30, 2013; Published: December 11, 2013
Copyright: © 2013 Gaglio et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was partially supported by: the Istituto Pasteur-Fondazione Cenci Bolognetti, Università di Roma La Sapienza (http://www.istitutopasteur.it/) and by the Epigenomics Flagship Project EpiGen, the Italian Ministry of Education and Research, National Research Council (http://www.epigen.it/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

* E-mail: giorgio.camilloni@uniroma1.it

Introduction

Sirtuins, class III histone deacetylases (HDAC III), are proteins homologous to the yeast protein Sir2p. Enzymes belonging to this family show strong NAD-dependent activity and are involved in the control of a series of basic functional and metabolic pathways. These activities have been reported for almost all organisms, from bacterial to human, in which they belong to this family show strong NAD-dependent activity and rely on, directly connects the cellular energetic status with the chromatin structure and with the transcriptional repression [3]. For this reason sirtuins are considered a putative enzymatic system that may adapt genetic programs to the metabolic status of the cell [2,3].

Sirt1 is the best-characterized member of the family, it acts on a wide variety of protein substrates, including histones and shows a high percent identity with the yeast protein Sir2p [2]. Moreover, human Sirt1 and yeast Sir2p have a partially overlapping specificity for histone residues, specifically for H4K16Ac and H3K9Ac [2].

However, Sirt1 also deacetylates transcription factors and cofactors, triggering their activation or inactivation, with relevant consequences on gene expression [8]. In mammals,
Sirt1 activity has been associated with a series of disease-related processes, e.g., chromatin/epigenetic modifications in neural functions (Parkinson’s and Alzheimer’s diseases), metabolism (Diabetes syndrome), cancer (prostate cancer), cardiovascular function and inflammation or stress responses [1,2,3].

The relevance of the sirtuin family is supported by the ever-growing number of studies in the literature. In addition, the discovery of a series of molecules that inhibit or activate sirtuins has had a strong impact on biological and biomedical research [8,9].

Excellent reviews describing both the biology and the chemistry of sirtuins are available [1,11,12].

When considering the wide implication of sirtuins in biomedical research, the possibility to obtain specific and potent regulators becomes an important pursuit that will certainly open up new therapeutic perspectives. In spite of the fact that inhibitor and activator molecules have been found very early in the history of sirtuins, in vivo assays on chromatin substrates are still missing. At present, Sir1 activity assays are based on in vitro deacetylation reaction with peptide substrates [13]. We decided to express the SIRT1 gene in S. cerevisiae since the yeast model presents many advantages [14,15], i.e. the availability of specific mutants, the highly characterized genetic environment and the easy genetic manipulation. In view of these observations we deemed this simple biological model capable of furnishing detailed information on the basic mechanisms of Sir1 enzymatic reaction in vivo.

A previous study by Sherman J.M. et al. [16] described the molecular cloning of a human sirtuin in S. cerevisiae sir2Δ mutant. Specifically they did not express Sir1 but hSir2Ap, also known today as Sir2 [16,17]. They discovered that only a chimera with N/C-terminal of yeast Sir2p and the core portion of hSir2A (human Sir2) was able to replace Sir2p activity on a subset of genetic loci. Actually, Sir1 shows a higher percent identity with yeast Sir2p than Sir2 and is considered the phylogenetic ortholog of yeast Sir2p [2]. Although Sir2 still shows a high similarity with the yeast protein Sir2p, it is considered more similar to another yeast sirtuin, Hst2p [2].

In this study we expressed the full-length form of SIRT1 in yeast sir2Δ mutant cells under the inducible GAL1 promoter, and studied the complementation of sir2Δ molecular phenotypes.

The yeast sir2Δ mutant shows a series of different phenotypes, all depending on the deacetylase activity of Sir2p. The phenotypes we considered were: transcriptional silencing at HYMLT1 gene, the telomeric transcript YFR057W, the subtelomeric IRC7 lying on TEL VI, and the ribosomal non coding transcripts of both NTS1 and NTS2 regions. We also studied other phenotypic marks of the sir2Δ strain such as extrachromosomal rDNA circles (ERCs) production and histone hyperacetylation levels.

Here we showed that ectopic SIRT1 expression is able to rescue some of sir2Δ mutation phenotypes with different efficiency in different chromosomal regions.

Our study demonstrated that human Sir1 in S. cerevisiae acts on some chromatin substrates, and therefore, the yeast system may be exploited for screening.

**Results**

**SIRT1 cloning, expression and toxicity in S. cerevisiae**

In order to verify whether the full length human SIRT1 gene complements all or part of sir2Δ mutant phenotypes in S. cerevisiae, we inserted the SIRT1 coding sequence from p1791 plasmid [18] into the pYES2 plasmid to yield pDGSIRT1 (Figure 1A; details in M&M). WT and sir2Δ cells were transformed with pDGSIRT1 (+), or with the empty vector (-) and a dilution spot assay was performed (Figure 1B). Yeast cells grown to logarithmic phase were initially diluted to 4×10^3 cells/µl. Six serial five-fold dilutions were made and 5 µl of each were spotted onto minimal medium plates containing glucose or galactose as carbon source, and then incubated at 30°C. Both strains transformed with empty or SIRT1 gene-containing vectors did not show significant differences in growth efficiency when plated on glucose-supplemented medium. However, when galactose plates were analyzed, a reduction in colony forming efficiency was evident in WT+ cells when compared to WT− (Figure 1B).

We also cloned the non-catalytic version of Sir1, (Sirt1-H363Y), into the pYES2 plasmid to check which complemented phenotypes are due to the catalytic activity of the human protein (Figure S1, D). The SIRT1-H363Y containing plasmid, pDGSIRT1-H363Y, has been used to transform sir2Δ cells in order to yield the control strain sir2Δ+. We further tested the toxicity of this non-catalytic Sir1 version by spot assay (Figure S1, E). The sir2Δ− (empty vector), sir2Δ+ (catalytic Sir1) and sir2Δ++ (non-catalytic Sir1 version) strains did not exhibit growth defects when plated on glucose medium or in galactose medium (Figure S1, E).

Taken together, these data suggest that Sir1 and the non-catalytic mutant Sir1-H363Y are not toxic in sir2Δ mutant cells. In WT+ cells, however, a slight decrease in cell growth was observed when compared to WT−, possibly attributable to the physical or genetic interaction between the endogenous Sir2p and the ectopically expressed Sir1 (Figure 1B).

In order to verify the correct mRNA expression of SIRT1 and SIRT1-H363Y, we analyzed their transcripts by reverse transcription PCR (Figure S1A). Moreover the presence of Sir1 protein in strains transformed with empty plasmid (-), SIRT1 gene-containing vectors (+) or SIRT1-H363Y gene (++) was evaluated by Western blot. In figure S1, panels B-C show a time-course analysis of Sir1 expression after switching the carbon source from glucose (GLU) to galactose (GAL) in the growth medium. Sir1 expression was evident between 6 and 9 hours of GAL induction both in sir2Δ+ and sir2Δ++ cells. However, after overnight induction the protein was still abundantly present (Figure 2A). In order to analyze complementation phenotypes we also checked the protein expression level of the human Sir1 and the non-catalytic version Sirt1-H363Y (Figure 2C, 2D). Figure 2 show that the different forms of the human protein in sir2Δ+ and sir2Δ++ cells are expressed at the same level (Figure 2D).
**sir2Δ complementation: ncRNA transcriptional silencing**

*sir2Δ* mutation in *S. cerevisiae* is characterized by a series of typical phenotypes: i) loss of transcriptional silencing at the rDNA locus, telomeres and HM loci [19,20,21]; ii) hyper-recombination at rDNA locus [22]; iii) histone hyperacetylation at silenced loci [23]. We intended to verify whether all or some of these phenotypes are complemented by the introduction and overexpression of *SIRT1* gene into the yeast cells.

In order to assess whether the loss of transcriptional silencing in the *sir2Δ* mutant is rescued when *SIRT1* is expressed, we analyzed the expression profiles of different genes known to be silenced in a Sir2p-dependent manner. RNA was extracted from WT and *sir2Δ* cells transformed with pDGSIRT1 (+) or empty plasmid pYES2 (-), converted into cDNA and analyzed by PCR. Cells were grown in both galactose (*SIRT1* induction) or glucose (*SIRT1* repression) medium. Two transcripts from the NTS (Non Transcribed Spacer) region of the rDNA were studied: *NTS1r* and *NTS2*. These transcripts are synthesized by RNA polymerase II, starting from E-PRO and C-PRO promoters, respectively (map in Figure 3A), [24,25,26]. In WT cells the repression of both transcripts is maintained regardless of the galactose-induction of *SIRT1* gene. Conversely, in *sir2Δ* cells, where *NTS1r* and *NTS2* are expressed, Sir1 induction partially silences *NTS1r* transcription (Figure 3A). In galactose, (gray histograms), the repression is efficient at the E-PRO promoter, while at C-PRO the expression level remains the same; either cells are transformed with empty or *SIRT1*-containing constructs (*NTS1r, sir2Δ+ versus sir2Δ−, *p<5 %). As control we analyzed *NTS1r* transcription level in *sir2Δ* cells grown in glucose because the plasmid with *SIRT1* is repressed (Figure 3A, black histograms).

The same cDNAs were analyzed for telomeric silencing, by studying *IRC7* (sub-telomeric) and *YFR057W* (telomeric) genes (Figure 3, panel B for map details). The *IRC7* gene, not efficiently silenced by Sir2p [27], maintained its expression level in all the analyzed conditions. Conversely, the *YFR057W* gene in *sir2Δ−* cells showed loss of transcriptional silencing which is not rescued by *SIRT1* overexpression in galactose medium (Figure 3B, gray histograms), (*YFR057W* in galactose, *sir2Δ+* versus *sir2Δ−*, p>5 %).

We then analyzed the expression of the *HMLα1* gene (Figure 3C). We chose *HMLα1* transcript as indicator of HM loci derepression. Upon loss of silencing in MATα strain, both HMLα1 and HMLα2 transcripts are subsequently repressed by α1/a1 heterodimer but it has been demonstrated that *HMLα1* transcript remains still detectable [28]. Matacic et al. demonstrated that *HMLα1* expression is an optimal quantitative measure of HM loci expression in *sir2Δ* strain [28].

As reported for telomeric and ribosomal silenced genes, in WT cells *HMLα1* expression did not change after the *SIRT1* expression. In *sir2Δ* cells, however, we observed a mild decrease in *HMLα1* expression when *SIRT1* was induced. In galactose, the reduction between *sir2Δ+* and *sir2Δ−* reaches a statistically significant level (*HMLα1, sir2Δ+ versus sir2Δ−, *p<5 %). However, at *HMLα1* locus there is not a silencing effect during Sir1 expression but only a slight decrease of transcription not comparable to a wild-type repression state (Figure 3C).
We then analyzed HMLα1 transcription in sir2Δ+* control strain. Figure 3C shows that HMLα1 transcription decrease observed in sir2Δ+ is not reverted in sir2Δ+* strain (HMLα1, sir2Δ+* versus sir2Δ+, ** p<1%; sir2Δ+* versus sir2Δ+, ** p<1%; Sirt1/Sirt1-H363Y, sir2Δ+* versus sir2Δ+, ** p<1%; Sirt1/Sirt1-H363Y, sir2Δ+* versus sir2Δ+, p>>5%). It is conceivable to hypothesize that the protein overexpression may have an indirect effect on the transcription of this locus without an involvement of the enzymatic activity of Sirt1.

Another experiment that proves that at HMLα1 there is not an effective repression is the α-factor assay, a powerful test highly sensitive to the degree of chromatin silencing [28]. Alpha-factor is a pheromone that blocks cell growth when HMLα1 is repressed [29]. Thus growth is allowed only when transcriptional silencing on HMLα1 gene is lost. In figure 3D, effective growth is shown for all sir2Δ strain, regardless of SIRT1 galactose-induced expression. This indicates that the
slight transcriptional decrease of \textit{HMLa1} in \textit{sir2Δ} strain, shown by RT-PCR (Figure 3C), is not enough to produce a non-growing phenotype.

Altogether, the data reported in Figure 3 indicate that the human Sirt1 protein rescues silencing phenotypes in \textit{sir2Δ} cells at the ribosomal locus (Figure 3A), while the \textit{HM} loci and the telomeric regions do not present any complementation effect dependent on the catalytic activity of Sirt1 (Figure 3B-C). On the contrary, we demonstrated that the effect on the \textit{rDNA} locus, specifically on the \textit{NTS1r} locus, is strictly connected with the catalytic activity of Sirt1 (Figure 3A). However, the silencing efficiency obtained by \textit{SIRT1} overexpression did not reach WT-level in any the studied loci.

### sir2Δ complementation: Reduction of ERCs formation

Together with the lack of transcriptional silencing at silent loci like HM, telomeres and \textit{rDNA}, \textit{sir2Δ} mutant shows hyperproduction of ERCs. The formation of extrachromosomal \textit{rDNA} circles has been associated with increased recombination activity among ribosomal units, and considered a marker of replicative aging in \textit{S. cerevisiae} [30,31].

In order to evaluate whether \textit{rDNA} recombination, leading to ERCs formation, is reduced upon \textit{SIRT1} expression in \textit{sir2Δ} cells, we compared WT and \textit{sir2Δ} cells transformed with the pDGSIRT1 (+) or the empty plasmid (−) as in the previous section. As further control, we also analyzed the \textit{sir2Δ+*} strain that expresses the Sirt1-H363Y non-catalytic mutant.

DNA was extracted from cells in exponential growth phase (0.5 OD/ml) and subjected to agarose gel electrophoresis. After Southern blotting, the resulting nylon filter was hybridized to \textit{rDNA} intergenic spacer probe (map in Figure 4A), and visualized by autoradiography. In figure 4A, all WT (−,+), samples show a low amount of ERCs, while in \textit{sir2Δ} samples the band corresponding to the ERCs species is evident. However, only in the \textit{sir2Δ} sample transformed with pDGSIRT1 (+) and grown in galactose (\textit{SIRT1} overexpression), the amount of ERCs was reduced to WT-level. In addition, this phenotype is reverted when we checked ERCs level in \textit{sir2Δ+*} control strain. This control demonstrates that, as for \textit{NTS1r} complementation phenotype, also ERCs repression is dependent on Sirt1 catalytic activity (Figure 4A).

Figure 4B reports the quantification of the ERCs species in galactose. (ERCs in galactose medium, \textit{sir2Δ+} versus \textit{sir2Δ−}, **p<1 %; \textit{sir2Δ+*} versus \textit{sir2Δ+}, **p<0.01%).

(D) Alpha factor yeast spot test analysis to evaluate \textit{HMLalpha1} silencing. Five-fold serial dilutions of mutant \textit{sir2Δ} and WT cells transformed with \textit{SIRT1(+)} or empty plasmid (−) were spotted onto minimal medium plates containing alpha-factor.

doi: 10.1371/journal.pone.0083114.g003

**Figure 3.** \textit{SIRT1} overexpression partially restores the transcriptional repression within specific loci in \textit{sir2Δ} mutant. RT-PCR transcriptional analysis in WT and \textit{sir2Δ} strains transformed with empty plasmid (−), \textit{SIRT1} construct (+) or \textit{SIRT1-H363Y} mutant construct (+*) both in repression (glucose) and induction (galactose) conditions. (A) \textit{rDNA} locus: \textit{NTS1r} and \textit{NTS2}; (B) \textit{TEL} VI locus: \textit{YFR057W} and \textit{IRC7}; (C) HM loci: \textit{HMLalpha1}. Histograms indicate averages and Std. Dev. bars from at least three independent biological replicates. Two-tailed t-test was applied for statistical analysis. Asterisks indicate statistically significant differences between \textit{sir2Δ−} and \textit{sir2Δ+} or between \textit{sir2Δ+*} and \textit{sir2Δ+} in galactose medium; α = 0.05. (Percentages of p-value: *p< 5%, **p < 1%, ***p < 0.01%).

(D) Alpha factor yeast spot test analysis to evaluate \textit{HMLalpha1} silencing. Five-fold serial dilutions of mutant \textit{sir2Δ} and WT cells transformed with \textit{SIRT1(+)} or empty plasmid (−) were spotted onto minimal medium plates containing alpha-factor.
specific histone residues. In particular, we studied H3K9, H4K16 and H4K12, all common targets of the deacetylase activity of both yeast Sir2p and human Sirt1 [32,33,34,35].

Although yeast Sir2p has locus-specific roles such as the transcriptional silencing of rDNA, telomeres and HM loci [5,15,36], it is conceivable that Sirt1 has an effect at a global level, especially since the outcome of gene complementation between phylogenetically distant organisms is always unexpected.

In yeast, Sir2p interacts with different protein partners according to the locus to be repressed [15,37,38]. The common mechanism for all targeted loci is the deacetylation of histone tails [23,39]. In fact, evidence shows that sir2Δ mutant is not characterized by high global acetylation levels, indicating that the Sir2p dominant role is played at specific loci [2]. In contrast, the mutation of another yeast-conserved sirtuin, Hst2p, displays high global acetylation levels probably affecting important processes such as the control of the cell cycle [2]. Since histones are highly conserved proteins and nucleosome structure is basically maintained unchanged from yeast to human we expected Sirt1 capable to deacetylate histones as well as Sir2p.

Cells from WT and sir2Δ transformed with the empty plasmid (−), and the sir2Δ strain complemented with the SIRT1 construct (+) were grown in galactose overnight and analyzed by immunoblotting. In Figure 2, panels A and B show that after SIRT1 induction in sir2Δ+ strain, there is a strong decrease in H3K9Ac, (sir2Δ+ versus sir2Δ−, **p<1%), whereas H4K16Ac and H4K12Ac do not show significant changes (H4K16Ac, H4K12Ac, sir2Δ+ versus sir2Δ−, p=10.3% and p=27.2% respectively). The sir2Δ− strain transformed with the empty plasmid showed the same acetylation levels of the WT- strain. This experiment confirms that yeast Sir2p does not alter global acetylation levels, but rather acts in a locus-specific way. In particular, Sirt1 expression has an effect on H3K9Ac whereas it has no effect on H4K16Ac and H4K12Ac (Figure 2B). We also analyzed Sirt1 protein levels after overnight induction in galactose as an expression control (sir2Δ+ versus sir2Δ− or

Figure 4. ERCs level decreases in complemented strain. A) Southern blot analysis of ERCs species in WT and sir2Δ with SIRT1 construct (+), SIRT1-H363Y mutant construct (***), empty plasmid (−) or without plasmid (−) as control. Strains were grown both in SIRT1 repression and induction conditions (glucose or galactose, respectively). DNA was isolated from the specified yeast strains and probed with a radiolabeled rDNA sequence shown in panel (A). ERCs are indicated by an arrow. B) Quantification of ERCs amount in galactose condition: band intensities corresponding to ERCs were normalized to the hybridized bulk rDNA and referred to WT- levels. Histograms indicate averages and Std. Dev. bars from at least 4 biological replicates.

Statistical analysis as in Figure 3.
doi: 10.1371/journal.pone.0083114.g004
WT, ** p<1%) (Figure 2B). No Sirt1 signal was found in WT- or sir2Δ−. All histone acetylation quantifications were normalized to each specific total histone levels (H3K9Ac / H3Ct, H4K16Ac and H4K12Ac / H4Ct) and reported to the WT− strain = 1. We used then the Sirt1 non-catalytic mutant to assess whether the strong H3K9Ac reduction was caused by the protein overexpression or by the Sirt1 enzymatic activity. We analyzed H3K9 acetylation in WT-, sir2Δ−, sir2Δ+ and sir2Δ+*. Acetylation enrichment for H4K16 and H3K9 were normalized to H4 C-terminal and H3 C-terminal, respectively, and referred to sir2Δ- levels =1. Histograms averages and Std. Dev. bars are representative of three technical replicates for at least three biological replicates performed. Statistical analysis as in Figure 3.

doi: 10.1371/journal.pone.0083114.g005

**Figure 5. Sirt1 deacetylates specific loci.** Chlp analysis of H4K16Ac (A) and H3K9Ac (B) at rDNA, HM loci, telomeric and sub telomeric regions in sir2Δ- and sir2Δ+ strains during SIRT1 induction (galactose medium).

(C) Chlp analysis of H4K16Ac and H3K9Ac at E-PRO in WT-, sir2Δ−, sir2Δ+ and sir2Δ+*. Acetylation enrichment for H4K16 and H3K9 were normalized to H4 C-terminal and H3 C-terminal, respectively, and referred to sir2Δ- levels =1. Histograms averages and Std. Dev. bars are representative of three technical replicates for at least three biological replicates performed. Statistical analysis as in Figure 3.

doi: 10.1371/journal.pone.0083114.g005

Histone deacetylation by Sirt1 at specific loci

Since we demonstrated that Sirt1 expression reduces the global acetylation of H3K9 we further analyzed the acetylation of this residue by chromatin IP (Figure 5) within the three silenced yeast loci previously analyzed for specific RNA production (Figure 3).

Cells grown in galactose to exponential phase were treated with formaldehyde, then processed for ChIp analysis using antibody against the acetylated form of H3K9 and H4K16 or the C-terminal region of histones H3 and H4. The analysis was performed on WT-, sir2Δ−, sir2Δ+ and sir2Δ+* strains, all grown in galactose medium to ensure the correct expression of SIRT1.
The immunoprecipitated DNA was amplified by PCR using specific oligonucleotides for the following regions: i) E-PRO and C-PRO (cryptic promoters of transcripts NTS1r and NTS2, respectively, within rDNA); ii) the coding sequence of the HMLα1 transcript; iii) the YFR057W telomeric gene, highly repressed by Sir2p; and iv) the subtelomeric region IRC7, which is normally not silenced by Sir2p [27]. Since sir2Δ-mutants alter nucleosome occupancy within rDNA [26], we normalized H4K16Ac and H3K9Ac signals to those of H4 C-terminal and to H3 C-terminal respectively. All the data have been also normalized to the sir2Δ− strain = 1 (Figure 5).

This analysis revealed that Sir1 expression reduced acetylation of H4K16 in sir2Δ+, to a significant degree only in specific regions. The graph in Figure 5 shows a significant decrease both on E-PRO (sir2Δ+ versus sir2Δ−, *p<5%) and the cryptic promoter C-PRO (sir2Δ+ versus sir2Δ−, *p<5%). However, the acetylation of H4K16 does not decrease in sir2Δ− strain in HM loci as well as in YFR057W telomeric and subtelomeric IRC7 genes.

As for the H3K9 acetylation, the statistical analysis did not reveal any significant changes in the studied loci (H3K9Ac, sir2Δ+ versus sir2Δ−, *p<5%), (Figure 5B).

It is interesting to observe that H3K9Ac and H4K16Ac may have different profiles in the same region; for instance, in rDNA cryptic promoters a general reduction of H4K16Ac prevails, while on the same region H3K9Ac does not exhibit any variation.

In view of these observations we used the sir2Δ−* strain to check if the acetylation decrease at the E-PRO promoter was dependent on the Sir1 enzymatic activity. Figure 5C shows that sir2Δ−* strain, on the E-PRO region, exhibits a reversion of the sir2Δ+ phenotype for H4K16Ac but not for H3K9Ac, (H4K16Ac, sir2Δ+ versus sir2Δ−, *p<5%; sir2Δ+ versus sir2Δ+, *p<5%).

We then analyzed also Sir1 occupancy in the different studied regions (Figure 6). In order to maintain the correct expression of Sir1, we harvested yeast cells in galactose as for the ChIP analysis of acetylated residues and we used sir2Δ− as negative control. Figure 6 shows how Sir1 is more enriched in rDNA locus than the other analyzed regions. In particular, we detected a high enrichment on E-PRO and C-PRO in the rDNA locus both compared to HMLα1, YFR057W, IRC7 in sir2Δ+ and to E-PRO in sir2Δ−, negative control (Figure 6). (Sir1 enrichment, sir2Δ+ E-PRO versus sir2Δ− E-PRO, ** p<1 %).

Here on E-PRO we previously observed three specific regions. The graph in Figure 5 shows a significant variation.

Discussion

Sir2p from S. cerevisiae and human Sirt1 share a consistent amino acid identity [2]. Given the prominent role that Sir1 plays as a master regulator of basic pathways both in healthy and pathological conditions [1,2,3], it would be extremely important to find further chemical regulators of this protein. However, to date, in vivo screening assays evaluating Sir1 histone deacetylase activity are still missing. In view of these considerations, the complementation procedures developed here using S. cerevisiae as a model system could represent a new strategy in the search of chemical regulators of Sir1.

In this work, we studied the capability of human SIRT1 gene to complement the mutation of the ortholog SIR2 in Saccharomyces cerevisiae. In the literature, sir2Δ complementation experiments have been described using the human sirtuin Sir2 that has lower percent identity with yeast Sir2p than Sir1 [2,16,17]. In this study we used Sir1, considered the phylogenetic ortholog of yeast Sir2p and we expressed it under the strong GAL1 inducible promoter to obtain clear-cut results. We also further analyzed the three silent loci where Sir2p normally acts: rDNA, Telomeres and HM loci; we employed techniques for the direct study of RNA silencing (RNA expression profiles were studied), DNA recombination (ERCs production was measured) and the extent of acetylation on specific target regions. This work revealed that SIRT1 complements some of the sir2Δ2 molecular phenotypes at rDNA locus, while there was no recovery at other loci (HM loci and telomeric regions). We also demonstrated that this phenotype depends on the catalytic activity of Sir1 (Figure 3).

Specifically, through transcriptional analysis, we demonstrated that during SIRT1 induction the NTS1r is partially repressed whereas HMLα1, although presents a mild reduction...
is still highly transcribed if compared to wild-type strain (Figure 3A and 3C). Furthermore, we tested, by the transcriptional analysis of the non-catalytic mutant Sirt1-H363Y, if the complementation phenotypes we observed were caused by the enzymatic activity of Sirt1. This analysis revealed that the NTSt1r decrease was attributable to the Sirt1 catalytic activity while as for HMLα1 it seems caused by an indirect effect of the overexpression. In fact in sir2Δ+ strain NTSt1r transcription is restored as in sir2Δ+ while HMLα1 is comparable to sir2Δ+ (Figure 3A and 3C).

In addition the alpha factor growth assay revealed that the sir2Δ+ strain did not arrest its growth compared to the wild-type condition, even if the transcriptional analysis showed a slight reduction of the HMLα1 transcript when compared to the sir2Δ- mutant (Figure 3C and 3D). A conceivable hypothesis is that the slight reduction of the HMLα1 transcript is not sufficient to significantly reduce the expression of the protein and thus inhibit downstream pathways involved in alpha-factor responsiveness, even though the alpha-factor assay is a highly sensitive assay. As far as the telomere YFR057W gene and sub-telomere IRC7 are concerned, despite the overexpression condition employed, Sirt1 protein seemed to have no transcriptional effect on these regions.

In addition this study shows, together with the decrease in NTSt1r, a significant reduction of the production of extrachromosomal rDNA circles. In particular, by Southern blot ERCs analysis, we observed a complete reversion of the sir2Δ mutant phenotype (Figure 4A and 4B). Although the relationship between ERCs production, rDNA intergenic spacer transcription (NTSt1r and NTSt2) and its hyperacetylation has not been elucidated yet, they seem to be closely correlated [40,41].

Our data suggest that Sirt1 triggers the downregulation of the NTSt1r intergenic transcript by deacetylating histones within the rDNA region (Figure 5). We hypothesize that as a consequence, the replication efficiency is reduced as well as the collision events between the replication fork and the transcriptional apparatus, which would lead to a reduced ERCs production (Figure 4). In the literature, the mechanism underlying the relationship between replication activity and ERCs formation has been clearly elucidated [42]. In addition, recent evidence indicates that several mutants show increased amounts of ERCs coupled with increased ncRNA production at rDNA[40].

Our study, by the use of full SIRT1 in sir2Δ complementation, has also shown that the human protein can act on histone residues in a wide scale and not only in a locus-specific manner (Figure 2).

Specifically by Western blot analysis, we determined that Sirt1 is able to significantly reduce the global acetylation of H3K9. Since histones are the most conserved proteins in eukaryotic organisms, the existence of targets shared by both Sirt1 and Sir2p was rather expected and the global effect on histone acetylation in sir2Δ-SIRT1 complementation was quite conceivable.

We also demonstrated, by the use of the control strain sir2Δ +, that the strong reduction of H3K9Ac is caused by Sirt1 catalytic activity indicating that this human protein is able to act on the yeast chromatin (Figure 2C).

Importantly this work decipher the mechanism by which SIRT1 complements the SIR2 mutation in yeast through the modification of histone acetylation. We observed a decreased acetylation of H4K16 residue at rDNA locus (Figure 5) whereas HM loci, telomeric and subtelomeric genes were unaffected by Sir2p overexpression, in fact these regions did not show altered H4K16 or H3K9 acetylation or mRNAs transcription. Interestingly, SIRT1 has a major impact on the global acetylation of H3K9 whereas in the complemented locus (the rDNA) it has effect mainly on H4K16Ac.

The differences in H4K16Ac and H3K9Ac profiles we observed in the studied regions may depend on different kinds of in vivo interactions between Sirt1 and the yeast proteins. In S. cerevisiae at HM loci and telomeres, Sir2p interacts with Sir3p, Sir4p and Rap1p, while at rDNA Sir2p is part of the RENT complex together with Net1p and Cdc14p [43,44,45]. Thus the acetylation pattern within the analyzed regions may depend on how the endogenous partner of Sir2p makes contact with the human protein Sirt1.

To verify Sirt1 enrichment in the different loci analyzed, we performed a chromatin immunoprecipitation experiment upon Sirt1-plasmid induction (Figure 6). This analysis revealed that Sirt1 has a different occupancy on different chromosomal regions studied. In particular the stronger enrichment is present at rDNA locus, here we have demonstrated the strongest complementation phenotype: the ERCs decrease. On the contrary in the HM loci, telomeres and subtelomeres there is not a significant enrichment in Sirt1 occupancy. The ability of SIRT1 to interact in different ways with different regions, despite the overexpression, may reflect in part its different ability to interact with yeast proteins.

In conclusion, we have hereby shown that SIRT1 complementation in sir2Δ mutant cells exhibits heterogeneous profiles in molecular phenotypes, such as histone deacetylation mechanisms, that may trigger the repression effects on both RNA transcription and DNA recombination processes. These phenotypes do not involve all the expected silent loci, being HM loci and telomeres refractory to Sirt1 expression.

Studying the behavior of human Sir1 in S. cerevisiae could be important to shed light on new aspects of this human protein. The study of protein partners of Sir1 within the complemented loci, together with an evolutionary analysis on human homologs of yeast proteins, may bring to light in the future new protein partners of Sir1 in humans.

Finally this work demonstrates that human SIRT1 gene is able to complement different molecular yeast phenotypes of the sir2Δ mutant with different efficiency. These observations indicate that there is a cross-talk between Sirt1 and yeast chromatin and that probably in vivo screenings, focused on H3K9Ac global decrease, would be possible in yeast. Since only in vitro assays are available for the screening of Sirt1 activators and inhibitors, S.cerevisiae, whose chromatin context is highly characterized, would be the first model for in vivo screening of molecules targeting this important protein.
Materials and Methods

Yeast strains, plasmids and oligo sequences

Yeast strains, plasmids and oligo sequences are listed in table S1.

Culture media and conditions

Yeast cells were grown and manipulated according to standard protocols [47]. YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) was used for auxotrophic strains lacking the URA3 gene. For the maintenance of auxotrophic strains complemented with URA3-plasmids, we used minimal SD medium (0.67% Difco yeast nitrogen base without amino acids, 2% dextrose) with Dropout (DO) supplement lacking uridine. SIRT1 induction experiments were carried out in minimal SD medium containing 2% glucose and then shifted in 2% galactose. For solid plates, 2% agar was added to SD medium.

RT-PCR

RNA from logarithmically growing cultures was isolated as previously described [47]. A 1.5-μg amount of DNase I–treated RNA was subjected to cDNA synthesis, starting from 2.5 μM oligo(dT) for evaluation of YFR057W mRNA levels at 55°C. Each cDNA band intensity was normalized to alpha-Tubulin and to the 0h glucose-point before normalization to WT− acetylation levels, WT− = 1 (Figure 2). Sirt1 induction experiments were performed under the following conditions: 95°C for 30 s, 60°C for 30 s, and 88°C for 1 min, with 18 cycles for ACT1, 24 cycles for SIRT1, NTS1r and NTS2, 27 cycles for YFR057W, IRC7 and HMLα1. NTS2 annealing was performed at 55°C.

The resulting cDNAs were amplified by PCR co-amplification using the following primer pairs: SIRT1-F/SIRT1-R, NTS2-F/NTS2-R each with ACT1-450-F/ACT1-450-R; NTS1r-F/NTS1r-R, YFR057W-F/ YFR057W-R, IRC7-F/IRC7-R, HMLα1-F/HMLα1-R each co-amplified with ACT1-182-F/ACT1-182-R.

PCR was performed under the following conditions: 95°C for 30 s, 60°C for 30 s, and 88°C for 1 min, with 18 cycles for ACT1, 24 cycles for SIRT1, NTS1r and NTS2, 27 cycles for YFR057W, IRC7 and HMLα1. NTS2 annealing was performed at 55°C.


taq polymerase (Eppendorf, Cat.No.2200320). [α-32P]dATP (Amersham, GE Healthcare, Cat.No.111-033-144) at 1:40000 dilution; anti-rat IgG-HRP (Santa Cruz Biotechnology, sc-2006) was used at 1:10000 dilution, anti-rabbit IgG-HRP (Jackson ImmunoResearch, Cat.No.111-033-144) at 1:40000 dilution; Anti-Mouse IgG (whole molecule)-Peroxidase (Sigma-Aldrich, Cat.No.A9044) 1:20000.

Detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Prod#34080). The integrated densities of each band were quantified with ImageJ 1.43u (National Institutes of Health). Densitometric analysis of relative histone acetylation levels was normalized to total histone levels (H3K9/H3Ct, H4K16/H4Ct, H4K12/H4Ct) and to WT- acetylation levels, WT− = 1 (Figure 2). Sirt1 Western Blot kinetics on sir2Δ−/ sir2Δ+ (Figure S1) were normalized to alpha-Tubulin and to the 0h glucose-point before cells were shifted to galactose. Normalized values are reported as sir2Δ+/sir2Δ− ratio. Means and error bars refer to at least four independent biological replicates. Student’s t test was applied for statistical analysis; α = 0.05.

Chromatin immunoprecipitation

A 300-ml amount of culture was grown to exponential phase, crosslinked with 1% formaldehyde at room temperature for 15 min, and then incubated with 330 mM glycine for 10 min. Cells were then processed for ChIP as previously described [40].
350-μg amount of chromatin extract was incubated with 2.5 μl (2.5 μg) of antibodies against histone H3 or H4 C-terminal tail, anti-acetyl H4 Lys-16, anti-acetyl H3 Lys-9 (Millipore/Upstate, Cat.No.07-690, 04-858, 17-10101, 07-352), 5 μl (10 μg) of mouse anti-FLAG M1 (Sigma F3040) and mouse anti-IgG (as mock control, mouse a specific IgG Invitrogen 5292). Chromatin–antibody complexes were isolated with protein A–Sepharose beads (Amersham, GE Healthcare, Cat.No. 17-0780-01) for 1.5 h at 4°C. The recovered DNA was resuspended in 200 μl for genomic sample (input) and in 50 μl for immunoprecipitated (IP) and beads only (BO) samples. Different amounts of DNA were used as template for PCR in order to obtain comparable autoradiographic signals (1 μl of a 1:20 dilution for input, and 1 μl for IP and BO). PCR was performed under the following conditions: 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min, with 25 cycles for ACT1, TEL VI genes, HMLα1 and 18 cycles for rDNA sequences. [α-32P]dATP was added to the reaction mixture (0.04 μCi/μl). For each immunoprecipitation three PCR reactions were done. The amplified fragments were separated on a 6% polyacrylamide gel. For quantification ImageJ 1.43u was used. Each set of experiments was repeated at least twice. Quantifications were performed as previously described [40].

Briefly, in Figure 5, acetylation fold enrichment values, for all regions, were calculated as follows: [rDNA(IP)/ACT1(input)]/[rDNA(input)/ACT1(input)]. In figure 5 the acetylation profiles were further corrected for the total amount of the histone H4 or H3. In this case the relative fold enrichment is defined as the ratio (acyetylated histone)/(total histone) for values from the following calculation: [rDNA/IP(ACT1/IP)]/[rDNA/input(ACT1/input)]. The isogenic sir2Δ- (empty plasmid) strain value was then normalized to 1, obtaining the acetylation enrichment shown for the different mutants or conditions. Sirt1 relative fold enrichments in sir2Δ+ strain (Figure 6) for all regions were calculated as: [locus(IP)/ACT1/IP]/[locus/input/ACT1/input]. Sirt1 profiles were reported as sir2Δ+/sir2Δ- with sir2Δ−/sir2Δ+ = 1 ; IP(sir2Δ+)/input(sir2Δ-)/[IP(sir2Δ+)/input(sir2Δ-)]. The graphs show the mean and SD calculated from three technical replicates for at least three independent biological replicas.

Spot-Test assay

Yeast cells were grown and collected at a density of 0.3-0.6 OD / ml. Cells, were then diluted to 4×103 cells/μl. Subsequent fivefold dilutions were made and 5 μl (8000 cells/μl) were spotted onto minimal medium plates containing glucose or galactose as carbon source, incubated at 30°C for 2–4 days and scanned. In the case of plates containing the alpha factor pheromone, this was used at 10 μg/ml final concentration [28].

Supporting Information

Figure S1. SIRT1 Transcriptional and protein levels during galactose induction. (A) RT-PCR to control the induced expression of SIRT1 transcript in galactose (WT+, sir2Δ+, sir2Δ−/sir2Δ+ * versus WT− : *p < 5%). (B) Western blot kinetics in sir2Δ mutant with SIRT1 construct (+), empty plasmid (−) and SIRT1-H363Y (+) to check the presence of the protein during galactose induction. (C) Western blot quantification in sir2Δ- and in sir2Δ+ (Sirt1 levels: sir2Δ+ at hour 9 versus sir2Δ− or WT- in glucose at hour 0; **p < 1%).

(D) Construct for yeast expression with SIRT1 or SIRT1-H363Y under the inducible promoter GAL1 in pYES2 background. (+: SIRT1 construct; −: empty plasmid, +*: SIRT1-H363Y). (E) Yeast spot test analysis of growth phenotypes during plasmid repression and induction conditions (glucose and galactose, respectively). For WT+, sir2Δ-, sir2Δ*, strains five-fold serial dilutions were made and 5 μl were spotted onto minimal medium plates. Histograms (panels A and C) indicate averages and Std. Dev. bars from at least three independent biological replicates. Two–tailed t-test was applied for statistical analysis. Asterisks indicate statistically significant differences between analyzed strains. α = 0.05. (Percentages of p−value: *p< 5%, **p < 1%, ***p < 0.01%).

(TIF)

Table S1. Strains, Plasmids and Oligonucleotides used in this work.

(ODCX)

Acknowledgements

We thank Micaela Caserta, Francesca Di Felice and James Hughes for critical reading, and Monica Brocco for English editing of the manuscript.

Author Contributions

Conceived and designed the experiments: DG AD GC. Performed the experiments: DG AD. Analyzed the data: DG AD GC. Contributed reagents/materials/analysis tools: DG GC. Wrote the manuscript: DG AD GC.

References

1. Saunders LR, Verdin E (2007) Sirtuins critical regulators at the crossroads between cancer and aging. Oncogene 26: 5489-5504. doi: 10.1038/sj.ong.1210616. PubMed: 17694089.
2. Vaquero A (2009) The conserved role of sirtuins in chromatin regulation. Int J Dev Biol 53: 303-322. doi:10.1387/ijdb.082675av. PubMed: 19378253.
3. Li X (2013) SIRT1 and energy metabolism. Acta Biochim Biophys Sin (Shanghai) 45: 51-60. doi:10.1093/abbs/gms108. PubMed: 23257294.
4. Michishita E, McCord RA, Barber E, Kioi M, Padilla-Nash H et al. (2008) SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. Nature 452: 492-496.
5. Sauve AA, Wolberger C, Schramm VL, Boeke JD (2006) The biochemistry of sirtuins. Annu Rev Biochem 75: 435-465. doi:10.1146/annurev.biochem.74.082803.133500. PubMed: 16756498.
6. Barber MF, Michishita-Kioi E, Xi Y, Tasselli L, Kioi M et al. (2012) SIRT7 links H3K18 deacetylation to maintenance of oncogenic transformation. Nature 487: 114-118. PubMed: 22722849.
7. Tanner KG, Landry J, Sternglanz R, Denu JM (2000) Silent information regulator 2 family of NAD- dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. Proc Natl Acad Sci U S A 97: 14178-14182. doi:10.1073/pnas.250422697. PubMed: 11106374.
Stress-dependent regulation of FOXO transcription factors by the transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. Properties of promoters cloned randomly from the Saccharomyces cerevisiae. Cell 66: 1279-1287. doi: 10.1016/0092-8674(91)90049-5. PubMed: 12939617.

Microarray deacetylation maps determine genome-wide functions for removal of Sir2 substrate. Proc Natl Acad Sci U S A 107: 5522-5527. doi:10.1073/pnas.1009361107. PubMed: 20933673.

A cytosolic NAD-dependent deacetylase, Hst2p, can modulate et al. (1999) The conserved core of a human SIR2 homologue. Cell 109: 437-446. doi: 10.1016/S0092-8674(99)00049-4. PubMed: 10193809.

Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 403: 795-800. doi:10.1038/35016122. PubMed: 15186381.

Gartenberg MR (2000) The Sir proteins of Saccharomyces cerevisiae: mediators of transcriptional silencing and much more.Curr Opin Microbiol 3: 132-137. doi:10.1016/S1369-5274(00)00064-3. PubMed: 10744999.

Lieb JD, Liu X, Botstein D, Brown PO (2001) Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association. Nat Genet 29: 100. doi:10.1038/ng0901-100b. PubMed: 22521897.

Huang J, Nicholl MB (2011) Sir2 in malignant transformation: friend or foe? Cancer Lett 306: 10-14. doi:10.1016/j.canlet.2011.02.019. PubMed: 21414177.

Little and silencing functions. Biochim Biophys Acta 1804: 1666-1675. doi:10.1016/j.bbabio.2009.10.022. PubMed: 19879981.

Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 403: 795-800. doi:10.1038/35016122. PubMed: 15186381.