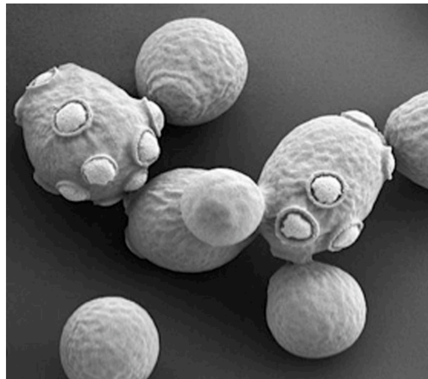
Sapienza University of Rome

“Functional complementation of *sir2Δ* yeast mutation by the human orthologous gene *SIRT1*”



Tutor
Prof. Paola Vittorioso

Supervisor
Prof. Giorgio Camilloni

Coordinator
Prof. Marco Tripodi

Davide Gaglio XXVI cycle
PhD programme in Life Sciences - (Pasteurian Sciences)
Department of Biology and Biotechnology Charles Darwin

Director of doctoral program:

Prof. Marco Tripodi (Professor of Molecular Genetics)
Department of Cellular Biotechnology and Haematology
"Sapienza" University of Rome

Scientific Supervisor:

Prof. Giorgio Camilloni (Professor of Molecular Biology)
Department of Biology and Biotechnology Charles Darwin
"Sapienza" University of Rome

Scientific Tutor:

Prof. Paola Vittorioso (Professor of Molecular Biology of the Plants)
Department of Biology and Biotechnology Charles Darwin
"Sapienza" University of Rome

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GLOSSARY

AROS: Active regulator of *SIRT1*
ARS: Autonomously Replicating Sequence.
ART: Sirtuin ADP-ribosyl-transferase activity
CAR: Cohesin associated region
C-pro: Cryptic RNA Polymerase II promoter located at NTS of rDNA.
CR: Caloric Restriction diet
DAC: Sirtuin dacetylase activity
DBS: Double strand breaks
ERCs: rDNA units excised from chromosome as extrachromosomal circles.
E-pro: Cryptic RNA Pol II promoter located at NTS of rDNA.
H3K56Q: Yeast strain with a mutation of H3 gene that converts lysine 56 into glutamine, mimicking a persistent acetylated state
HAT: Histone acetyl-transferases enzymes
HDAC: Histone deacetyl-transferases enzymes
HM locus: Hidden mating locus of *S. cerevisiae*
IR: Ionizing Radiation
MAT: The mating type locus of *S. cerevisiae*
MSE: Middle sporulation elements
NAD: Nicotinamide adenine dinucleotide, abbreviated NAD+
NAM: Nicotinamide
ncRNAs: non coding RNA group transcribed within NTS region
NHEJ: Non-homologous-end-joining
NTS: The Non Transcribed intergenic rDNA Spacer.
rDNA: Tandemly repeated locus of *S. cerevisiae* encoding for ribosomal RNAs.
RENT: Regulator of Nucleolar silencing and Telophase exit complex
RFB: (Replication fork Barrier). 100bo region located at 3' end of 35S gene
RLS: Replicative Lifespan
RNA Pol-I, II, III: RNA polymerases type I, II or III
SIRT1, SIR2**:** The italic/capital letter nomenclature refers to genes
Sirt1, Sir2p: The lower case nomenclature refers to the proteins

STAC: *SIRT1*-activating compounds
SAHF: Senescence Associated Heterochromatin Foci

INTRODUCTION

1. THE HUMAN SIRTUINS

1.1 HUMAN SIRTUINS: AN OVERVIEW

Sirtuins, class III histone deacetylases (HDAC III), are proteins homologous to the yeast enzyme Sir2p. Enzymes belonging to this family show a strong NAD-dependent activity and are involved in the control of a series of functional and metabolic pathways. These activities have been reported for almost all organisms, from bacteria to human, in which they have been found (Saunders et al., 2007; Vaquero, 2009).

In particular mammalian *SIRT1*, the *S. cerevisiae* orthologue of *SIR2*, has been shown to be involved in important pathways, including energy metabolism, brain functions, inflammation and aging (Saunder et al., 2007; Li 2013) and thus 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) and cell cycle control (prostate cancer), (Saunders and Verdin, 2007; Vaquero, 2009; Li, 2013).

In humans, seven sirtuin family members (*SIRT1-7*) have been described, with different roles and cellular localizations. Human sirtuins are characterized by a shared catalytic core of ≅ 275 amino acids but variable amino- and carboxy-terminal length that regulate their subcellular localizations and catalytic activity, (North and Verdin, 2004).

As far as the enzymatic activity of sirtuins is concerned, Sirt1, Sirt2, Sirt3, Sirt5 and Sirt6 deacetylate proteins at lysine residues (Vaquero, 2009; Michishita et al., 2005; Sauve et al., 2006), while Sirt4, Sirt6 and Sirt7 are the only sirtuins that show a strong ADP-ribosylation reaction, (Figure 1), (Vaquero, 2009; Sauve et al., 2006).

There is recent evidence that also Sirt7 exhibits deacetylase activity (Barber et al., 2006). Deacetylation activity requires the metabolic cofactor NAD+, and the final products of the reaction are the deacetylated protein, nicotinamide (NAM) and O-acetyl-ADP-ribose, (Figure 1), (Tanner et al., 2000). The metabolic cofactor NAD+, which sirtuins activity rely on, directly connects the cellular energy status with the chromatin structure and with the transcriptional repression (Li, 2013).

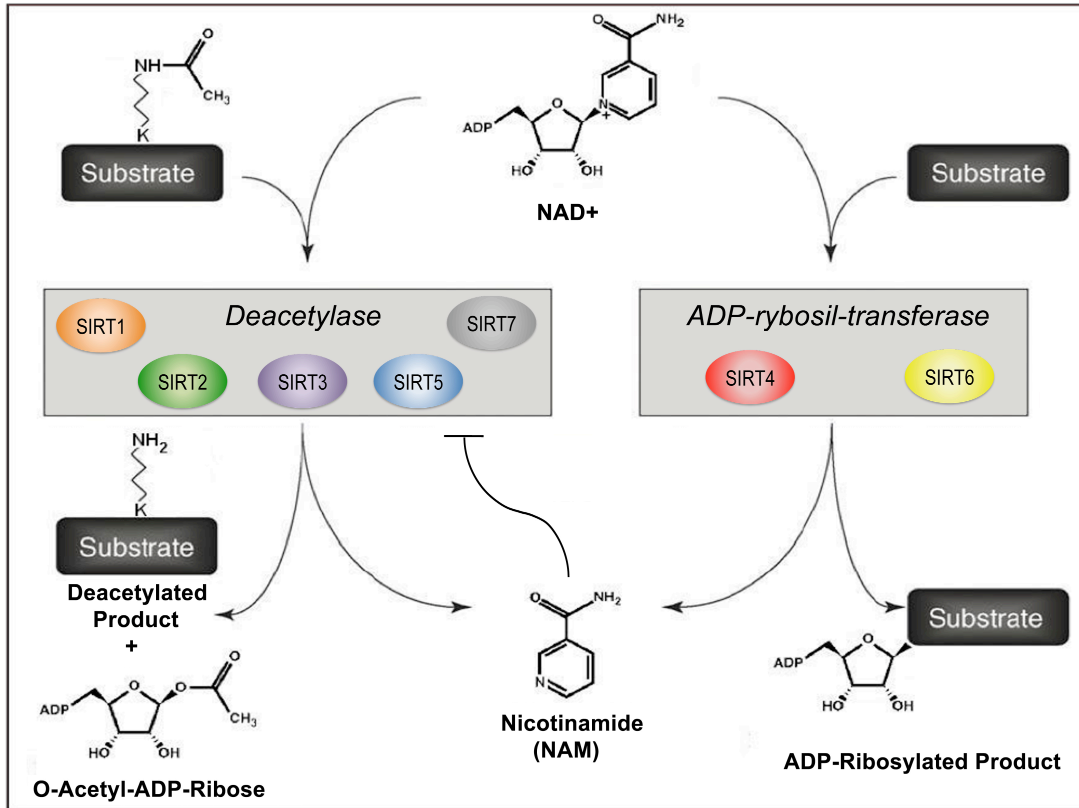

Figure 1: Enzymatic reactions of human sirtuins: Sirt1, -2, -3, -5, -7 have a preponderant deacetylase activity whereas in Sirt4 and Sirt6 prevail an ADP-ribosyl-transferase activity. All sirtuins use NAD+ as cofactor and produce Nicotinamide (NAM). In the deacetylation reaction NAD+ receives the acetyl group from the protein. In ADP-ribosyl transfer, the adenine dinucletide from NAD is linked to the target protein. Both reactions release NAM product that is a potent inhibitor of both sirtuin reaction.

For this reason sirtuins are considered a putative enzymatic system that may adapt genetic programs to the metabolic status of the cell (Vaquero, 2009; Li, 2013). The great attention by the scientific community to this group of highly conserved genes is due to the involvement of sirtuins in the regulation of lifespan, ageing processes and many age-related diseases such as cancer. The recent discovery of molecules activators and inhibitors of sirtuins, such as resveratrol and sirtinol, opened up the possibility of drug testing for therapeutic protocols on ageing-related diseases (Saunders and Verdin, 2007).

In mammalian cells, there are other enzymes with deacetylase activity, the HDAC (histone deacetylase) class I and II that differ from the sirtuins since they use as a cofactor the ion Zn2 + (North and Verdin, 2004; Longo and Kenndy, 2006). Some studies have shown that sirtuins are inhibited by NAM in a non-competitive way respect to NAD (Landry et al., 2000). The peculiarity of this non-competitive inhibition has led these researchers to speculate that NAM binds a new and not characterized site on sirtuins (Avalos et al., 2004 and 2005). The mechanism of inhibition requires that the NAM enters inside an enzymatic pocket structurally conserved, defined C -pocket, adjacent to the binding site of the NAD, (Figure 2). The NAM reacts with the peptide-intermediate of the deacetylation reaction regenerating the NAD. This type of reversibile reaction is very rare in biology, and it is thought to be the main mechanism of sirtuin regulation (Avalos et al., 2004). The request of the NAD + as substrate could be an evidence in favor of the hypothesis that sirtuins have evolved as sensors of the energy status of the cell. In particular, it would seem that sirtuins have evolved to mediate signaling pathways triggered by stress conditions, such as the deprivation of nutrients, in order to produce a metabolic adaptation of the organism for survival, (Figure 2), (Guarente and Picard, 2005; Michan and Sinclair, 2007). Consistent with this hypothesis, it has been observed that extra copies of sirtuins or their overexpression in organisms such as the yeast *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans* have led to a significant increase in life span. In particular, many experiments have now demonstrated the role of this class of genes in mediating the effect of increased longevity due to a caloric restriction, diet that is based on a calorie intake from 20% to 40% less than a normal nutrition (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001; Weiwei Dang et al., 2009).

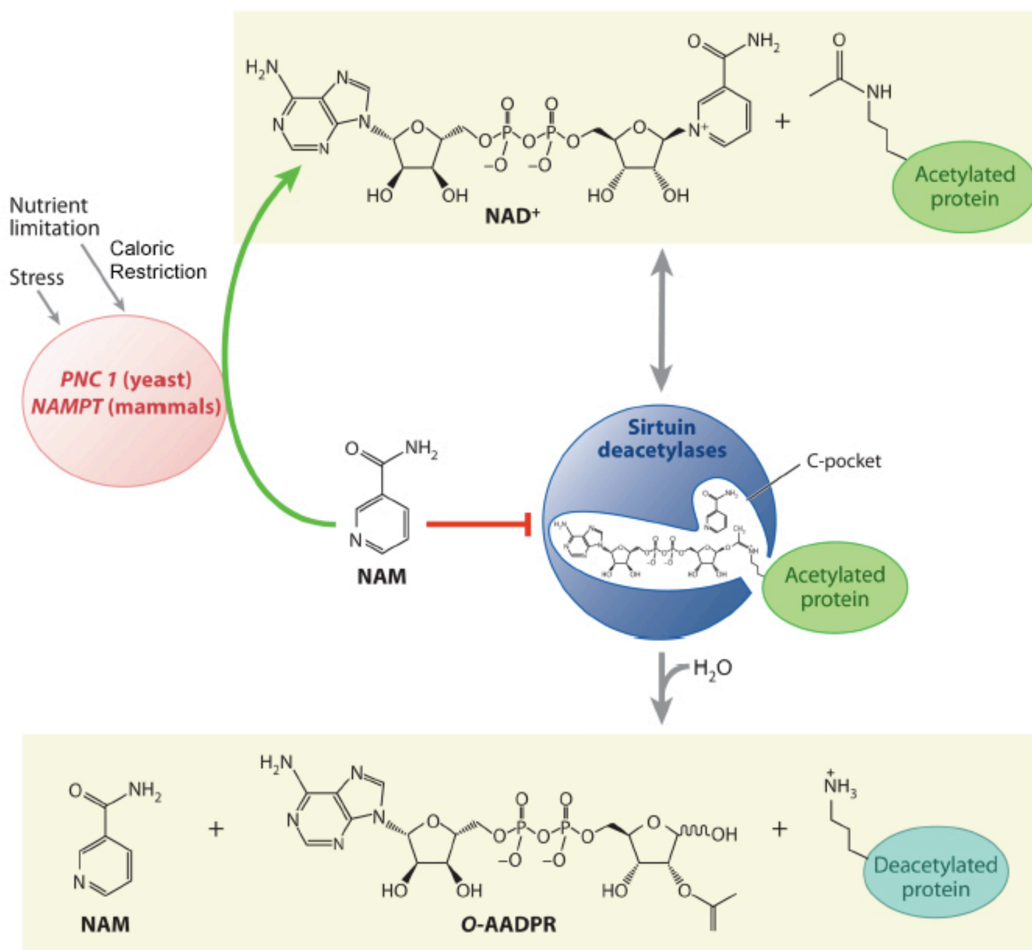

Figure 2: The sirtuin deacetylation reaction and regulation by stress and caloric restriction. Sirtuins catalyze a two-step reaction that consumes nicotinamide adenine dinucleotide (NAD+) and releases nicotinamide (NAM), O-acetyl-ADP-ribose (AADPR), and the deacetylated substrate. The inhibition occurs by the binding of NAM product in the C-pocket thus triggering the reverse reaction. Activation of sirtuins can be facilitated by the NAM conversion to NAD+ by PNC1 (yeast) or NAMPT (mammals and alpha proteobacteria), two genes upregulated by stress and nutrient limitation. From Haigis and Sinclair, 2010.

The life span extension during caloric restriction has been observed in many taxa, also very different between them (Fabrizio et al., 2005; Wierman and Smith, 2013, Smith et al., 2000; Mair et al., 2003; Lakowski and Heikimi, 1998; Ingle et al., 1937; Austad, 1989; Weindruch and Walford, 1982; Loeb and Northrop, 1917), this suggests that the molecular mechanisms triggered during the CR are very ancient and conserved, as indeed also sirtuins , which appear to regulate this process. Although the effects of caloric restriction in humans have not yet been observed, studies on *Macaca mulatta* show that CR leads to several benefits for the organsim, such as reduced fat mass, a decreased concentration of glucose in the blood, lower incidence of diabetes, cardiovascular diseases and cancer. The involvement of sirtuins in CR was observed for the first time in the yeast *sir2Δ* mutant, in which was impaired the elongation of life of 30% induced by low-calorie diet (Schleit et al., 2012; Kaberlein et al., 1999). In more recent studies, however, it has been demonstrated that the involvement of Sir2p in CR depends on the type of caloric diet. In yeast a moderate CR (~ 0.5% Glucose) increases the rate of mitochondrial respiration upregulating the activity of Sir2p and suppressing rDNA recombination (Haigis and Sinclair, 2010). Observations indicate that also the homologues of yeast Sir2p, Hst1p and Hst2p, mediate the same effect of increased replicative lifespan. Lamming et al. in 2005 have demonstrated, however, that the double deletion *sir2Δ, hst2Δ* completely blocks the extension of the replicative life CR-dependent (Lamming et al., 2005). Guarente and colleagues proposed that during the CR the reduction of NADH (an inhibitor of Sir2p) results in an increase in the Sir2p activity, while Sinclair and Smith have proposed that this increase is associated with the upregulation of *PNC1* which reduces NAM levels regenerating the NAD, (figure 2), (Anderson et al., 2003). Interestingly *PCN1* is also positively regulated by stress signals, such as the increase of temperature to 37 ° C or the deprivation of nitrogen, both events that cause an increase in the replicative lifespan, (Figure 2). This mechanism according to many researchers suggests that the CR is a sort of hormetic process that induces a defense response beneficial for the organism (Haigis and Sinclair, 2010). The connection between this class of enzymes and the NAD concentration appeared even more evident when some studies have shown that changes in cellular levels of NAD + and NADH influenced significantly the activity of sirtuins (Yang and Sauve, 2005). Observations indicates that both the metabolism of NAD + and sirtuins contribute to the mechanisms that regulate cell survival under stress conditions (Yang and Sauve, 2006). Recent works have shown that the activity of sirtuins and increased levels of NAD + protect

the nervous system by axonal degeneration in neurons (Yang and Sauve, 2006). In light of these protective effects, both sirtuins and NAD+ metabolism may represent therapeutic targets for the treatment of diseases such as degeneratve conditions of the nervous system. The human nicotinamide phosphoribosyltransferase, Nampt, in human also known as *PBEF*, is an enzyme induced after cellular stress and converts nicotinamide in the mononucleotide form, which, reacting with ATP, regenerates the NAD+, (Figure 2), (Yang and Sauve, 2006). It is observed in primary cells, during replicative aging, a Nampt decrease with consequent inhibition of the sirtuin activity caused by reduced NAD level (Van der Veer et al., 2007). Furthermore, it has been observed that inhibition of Nampt during the first replications of primary cells triggers premature senescence (Van der Veer et al., 2007). This mechanism connects stress pathway with the metabolism of NAD + and then with the activity of sirtuins, essential for directing cell fate toward survival or to apoptosis.

These data, along with many other studies on organisms closest to humans such as rodents, have generated interest in elucidating the role of sirtuins in caloric restriction. So far, studies on human Sirt1 have proved its involvement in mediating many of the positive effects of CR opening the field to drug testing for ageing-related diseases. It has been observed that the activation of sirtuins by resveratrol induces the same effects of increased longevity triggered by caloric restriction, protecting the body from diet-dependent obesity and associated dysfunction in insulin secretion (Jiang, 2008). It has been hypothesized is that the activation of sirtuins by resveratrol could establish in the cell the same molecular pathway triggered by caloric restriction, even though the body is not really in a low caloric diet (Jiang, 2008). The discovery that sirtuins can also be directly modulated by a multitude of small molecules and protein interactors has opened up the possibility to mimic pharmacologically the caloric restriction condition.

Small modulator molecules include inhibitors such as sliptomicin, the sirtinol and the EX - 527 (Solomon et al., 2006; Mai et al., 2005), whereas Sirt1-activating compounds (the STACS) include resveratrol, the SRT1720 and SRT2183. During the last decade over 3500 STACS have been synthesized and analyzed. Most of the studies have been focused on the resveratrol and other polyphenols with a similar structure, such as the fistein and butein. It has been shown that these molecules increase the replicative lifespan of many organisms such as yeast, fly and mouse. Recently, It has also been identified protein modulators, the AROS (active regulator of Sirt1) like the Necdin and DBC1 (the inhibitor deleted in breast cancer 1).

AROS bind to the N-terminal tail of Sirt1, the same region regulated by non-protein activators, revealing a possible control mechanism for all sirtuin modulators (Haigis and Sinclair, 2012).

The cellular localization of sirtuins is regulated by the type of proteins they interact with, for this reason sirtuins display different intracellular compartmentalizations (Figure 3), (Saunders and Verdin, 2007). In particular, the seven human sirtuins are redistributed in the cytoplasm into the mitochondria, in the nucleus and nucleolus (Saunders and Verdin, 2007). Specifically Sirt1 acts in the nucleus deacetylating histones and other non-histone targets such as p53, Ku70 and FOXO, proteins involved in cell cycle regulation, apoptosis, replicative senescence and DNA repair (Saunders and Verdin, 2007).

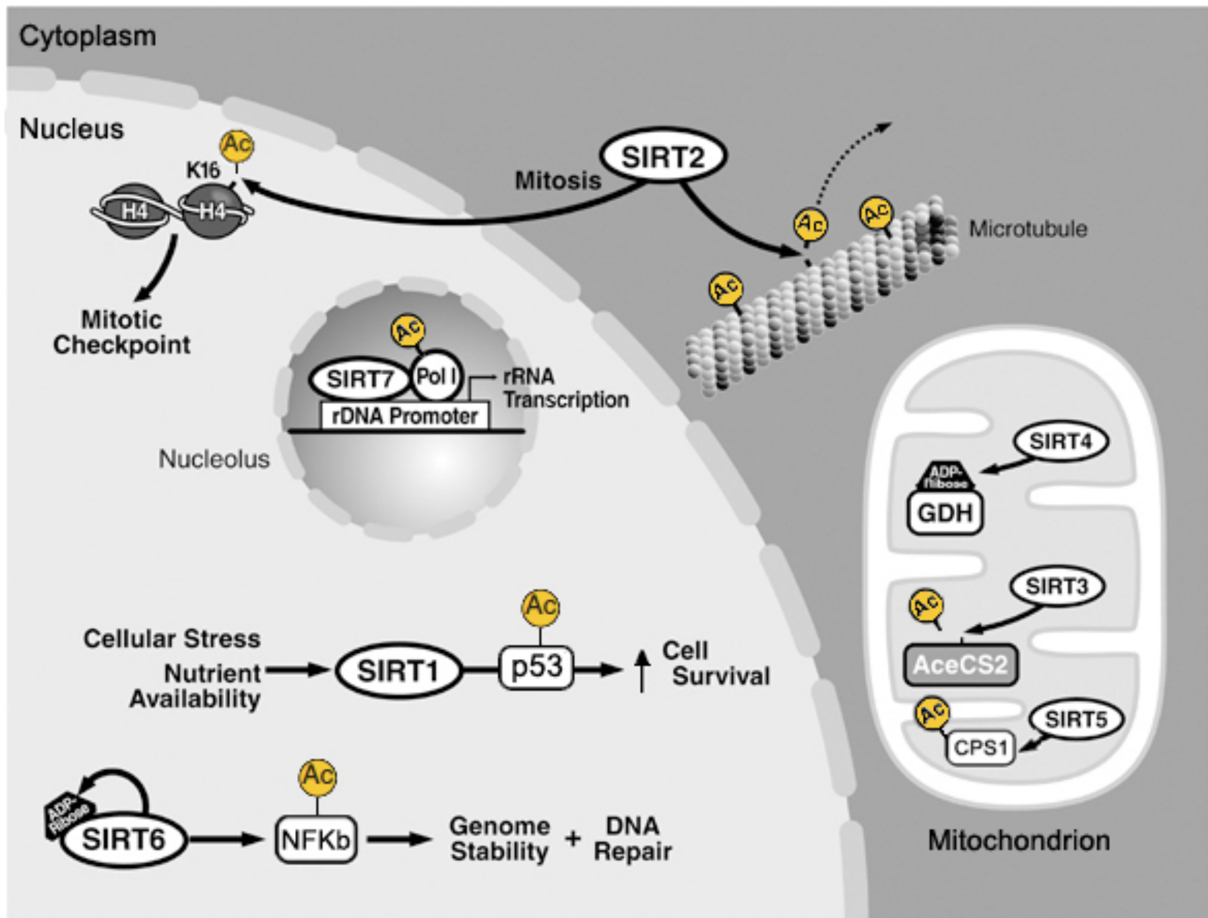

Figure 3: Cellular localization of sirtuins in mammalian cells.

In particular, Sirt1 deacetylates H3K9, H1K26, H4K16 and H4K12 (Vaquero et al., 2004; Imai et al., 2000). Sirt1, although to a lesser extent than the deacetylase activity also displays the mono-ADP-ribosyl-transferase activity and specifically toward histone H1 (Frye, 1999). Sirt1 is one of most controversial sirtuin as it interacts with a very high number of proteins involved in very complex regulatory circuits. Although Sirt1 in some studies it is detected fundamental to trigger replicative senescence at low levels of stress (Chua et al., 2005; Abdelmohsen et al., 2007; Saunders and Verdin, 2007), its expression was found increased in many tumors, in particular those of the skin (Hida et al., 2007). On the contrary, in other tumors, such as in prostate cancer, caused by high levels of acetylation of the androgen receptors, Sirt1 levels are decreased (Fu et al., 2000, 2003, 2006). In experiments on mouse embryonic cells, Sirt1 has been shown to be essential to promote cell survival. To date, we know that Sirt1 is regulated by many factors, both at transcriptional and post-translational, and is the physiologic balance, connected to multiple different causes, that affect Sirt1 in guiding cell fate toward survival or apoptosis. As for Sirt2, this sirtuin is localized in the cytoplasm where deacetylates the α-tubulin, but it was also observed that during mitosis, as a result of its phosphorylation, it is transferred to the nucleus where deacetylates histones, (Figure 3), (Dryden et al., 2003; Inoue et al., 2007; Vaquero et al., 2006). In particular it has been shown that Sirt2 deacetylates lysine 16 of histone H4 and with less efficiency H3K9 (Vaquero et al., 2006). Since the overexpression of *SIRT2* causes a delay in the mitotic exit, it is hypothesized that this protein may function as a cell cycle checkpoint, preventing chromatin condensation, in response to genotoxic stress (Dryden et al., 2003; Inoue et al., 2007; Hiratsuka et al., 2003).
Sirt3, -4 and -5 are the mitochondrial sirtuins, (Figure 3), (Saunders and Verdin, 2007). Since this is the main cell compartment for the production of reactive oxygen species, this organelle is essential to control ageing process and the lifespan extent. Coherently with the mitochondrial localization of sirtuins, studies have shown that 20% of mitochondrial proteins are acetylated (Kim et al., 2006). Sirt3, by deacetylation, activates the acetyl-CoA synthetase -2 bringing about an increase in the production of acetyl-CoA, macromolecule essential for the metabolism of sugars, fatty acids, amino acids and cholesterol (Onyango et al., 2002; Hallows et al., 2006; Schwer et al., 2006).

The expression of *SIRT3* in mammals is very high in brown adipose tissue (Shi et al., 2005), where it appears to be involved in the heat production. Sirt4 ADP-ribosylates and represses the glutamate dehydrogenase (GDH), enzyme responsible of insulin secretion (Haigis et al., 2006). It has been observed that the pancreatic islands of mice *SIRT4* mutant secrete high levels of insulin (Haigis et al., 2006). Some researchers claim that deregulation of *SIRT4* may contribute to the pathogenesis of diabetes by altering GDH functions (Strickler et al., 2001).
Although studies on *SIRT5* are still few, it has recently been discovered that this sirtuin regulates the NH3 levels in the urea cycle. Sirt5 is localized in the mitochondrial matrix and is expressed in all human tissues (Figure 3). It has been proven that Sirt5 has a weak NAD-dependent deacetylase activity and that *SIRT5* mutant mice show a normal development without any kind of metabolic defects (Nakagawa et al., 2009; Lombard et al., 2007). Sirt5 is known to interact with at least two proteins involved in cellular metabolism: the cytochrome C and the carbamoyl-phosphate synthetase 1 (CPS1), (Nakagawa et al., 2009). The CPS1 is involved in the first reaction of the urea cycle and its activity is required for the recover of ammonia resulting from the metabolism of amino acids. Sirt5 by deacetylation of CPS1, stimulates its enzymatic activity. Some researchers have demonstrated that mice *SIRT5* -/-, upon prolonged periods of diet, show high levels of ammonia, suggesting that this enzyme can help the liver to cope with products of the metabolism of amino acids. It has to be proven yet whether the loss of *SIRT5* muation increases the susceptibility to the toxic effects of ammonia (Nakagawa et al., 2009).
Sirt6 is a sirtuin localized only in the nucleus, ADP-ribosylates itself and deacetylates the pleiotropic transcriptional factor NFkB regulating its activity (Liszt et al., 2005). Studies indicate that it may act on proteins involved in BER (base excision repair), hypothesis confirmed by phenotypic analyses of *SIRT6* -/- mutant mice.
In fact *SIRT6* mutant mice show severe signs of premature aging, lymphocyte apoptosis, abnormal curvature of the spine, slow growth, increased sensitivity to genotoxic damage and many other progeroid symptoms (Mostoslavsky et al., 2006). The BER is the main mechanism of protection against damage caused by ROS (reactive oxygen species). Despite the BER represents the main mechanism to repair DNA oxidative damages it has been shown that defects in the BER does not produce premature ageing phenotypes (Wilson and Bhor, 2007; Hasty et al., 2003). This is probably due to the genetic redundancy of the DNA glycosylases, enzymes that break the glycosidic bonds between the damaged base and sugar

(Hasty et al., 2003). Future studies will allow to better understand the involvement of Sirt6 in BER and in deaseses associated with premature aging. Recently it has been observed that Sirt6 has a strong deactylase activity for H3K9Ac and H3K56Ac at the telomeric regions (Michishita et al., 2008). *SIRT6* mutant mice show H3K9 hyperacetylation and telomeric defects including chromosome fusions that lead to premature senescence (Mostoslavsky et al., 2006). In addition Sirt6 is involved in the regulation of the telomeric protein WRN, whose mutation causes Werner syndrome, a rare genetic disease that causes premature aging. This protein is a helicase involved in telomere replication during the S phase and also interacts with Sirt1 (Narala et al., 2008). Sirt7 is localized in the nucleolus, associated with chromosomes during mitosis, it has a deacetylase activity against H3K18Ac and it associates with the RNA polymerase I complex, (figure 3) (North et al., 2003; Ford et al., 2005, 2006). Sirt7 appears to be essential for the transcription of ribosomal RNA genes and coherently it has been found widely expressed in highly proliferating tissues such as the liver, testes and spleen, which require a high protein production and therefore ribosomes (Michishita et al., 2005; Ford et al., 2006). Low levels of Sirt7 were found in non-proliferating tissues such as the heart, brain and skeletal muscle (Michishita et al., 2005). Interestingly, Sirt1 and Sirt7 seem to have an antagonistic role in the nucleolus. In fact, Sirt1 is associated with rDNA copies through interaction with the DNA-methyltransferase Dnmt1 and participates in the silencing of these regions. Sirt1 also inhibits the RNA polymerase I by deacetylating the transcription factor Taf67 (Espada et al., 2007; Muth et al., 2001). Moreover Sirt1 it represses the rDNA copies in association with the NoRC complex containing the histone methyltransferase SUV39 and the repressor protein Nucleomethylin.

Overall, at the biochemical level, the function of sirtuins is to deacetylate and ADP-ribosylate protein target to regulate their biological function. In addition protein regulation by sirtuin deacetylation is highly regulated by metabolic processes and specifically by NAD/NADH ratio. In conclusion this protein class regulate many homeostatic cellular pathways by triggering specific genetic program to promote survival, gene stability or apoptosis.

1.2 *SIRT1*

1.2.1 SIRT1: APOPTOSIS, REPLICATIVE SENESCENCE AND CANCER

SIRT1 human gene is the homologue of *SIR2* in *Saccharomyces cerevisiae*. In mammals *SIRT1* is an important promoter of cell survival in response to specific types of stress, which, otherwise, would trigger apoptosis through the activation of p53 and/or the FOXO transcription factors. The importance of *SIRT1* in mammals is emphasized by experiments on *SIRT1* mutant mice that die before or soon after birth (Cheng et al., 2003; Ford et al., 2005). It has been demonstrated that Sirt1 can interact with p53 that is a tumor suppressor activated by acetylation in response to DNA damages (Barlev et al., 2001). It has been observed that Sirt1 can deacetylate p53 and therefore hypothesized that this event in the cell may reduce the p53-dependent apoptosis (Vaziri et al., 2001; Luo et al., 2001). P53 has many acetylated residues modified both by Sirt1 and other HATs (histones acetylases)/ HDAC (histone deacetylases). It has been observed that this hyperacetylation is required to trigger both the apoptosis and/or cell cycle arrest. Coherently, it has been shown that *SIRT1* overexpression inhibits the activity of p53 and p53-dependent apoptosis in response to genotoxic stress (Vaziri et al., 2001; Luo et al., 2001).
In addition *SIRT1* gene activation, has been directly connected both to p53, which binds two specific sites in its promoter to repress the transcription and also to HIC1, the hypermethylated protein in cancer (Chen et al., 2005). It has been suggested that the HIC1-*SIRT1*-p53 complex acts with a negative feedback mechanism on *SIRT1* transcription. In normal conditions HIC1 represses *SIRT1*, promoting the activity of p53 and thus apoptosis in the presence of oxidative stress. Whereas in cells that are repairing DNA damage, p53 negatively regulates HIC1, inducing the transcription of *SIRT1* and promoting cell survival. Many researchers argue that the hypermethylation of HIC1 during ageing may enhance Sirt1 activity that by p53 deacetylation, would increase the risk for neoplastic transformation (Michan and Sinclair, 2007). In mice thymocytes *SIRT1*-/-, p53 acetylation levels were found significantly increased after exposure to ionizing radiation (IR), this would indicate that Sirt1 plays a role in resistance to stress induced by IR (Chen et al., 2003). Furthermore, p53 and Sirt1 levels have been correlated with replicative senescence. Sirt1, in fact, would seem to promote replicative senescence through a mechanism that involves p19arf, protein that in

mice positively regulates p53 by inhibition of its proteasome degradation (Chua et al., 2005). This behavior in mice appears, however, in total contrast with the function of *SIR2* in yeast, which instead extends the replicative life (Mills et al., 1999; Vaziri et al., 2001; Sinclair and Guarente , 1997).
In replicative young cells has been shown that the high levels of Sirt1 and nicotinamide adenine nucleotide trigger both deacetylation of p53 and histones promoting cell survival (Saunders and Verdin, 2007). On the contrary during ageing process Sirt1 levels decrease and high levels of nicotinamide (NAM) inhibit its activity, as result, p53 is hyperacetylated inducing replicative senescence. Moreover the deacetylation Sirt1-dependent of histone H1 triggers its degradation, contributing to the formation of heterochromatic foci typically associated with senescence, the SAHFs (Senescence Associated Heterochromatin Foci), (Saunders and Verdin, 2007). The Sirt1 inhibition during ageing could promote cell survival after oxidative damages, thus leading to an accumulation of mutations and therefore to an increased risk of tumorigenesis (Saunders and Verdin, 2007).
A second mechanism in response to cellular stress is controlled by the FOXO proteins. Mammals express four conserved genes called FOXO1, FOXO3, FOXO4 and FOXO6. Sirt1 interacts with these transcription factors and in particular deacetylates FOXO1 and FOXO4 (Brunet et al., 2004; Motta et al., 2004). This type of interaction is enhanced after DNA damages and could lead to the arrest of the cell cycle. The importance of FOXO proteins has been highlighted since they are important regulators of cell growth and have been found in new forms of protein fusion after chromosomal translocations in many types of cancers. After oxidative stress, FOXOs proteins are phosphorylated and relocated into the nucleus, where they associate with HATs (histone acetylases) to form transcriptional complexes. The Sirt1-dependent deacetylation of FOXO suppresses the transactivation of proapoptotic proteins such as Bim and Fas, but promotes the expression of Gadd45a and p27kip to induce cell cycle arrest (Brunet et al., 2004; van der Horst et al. 2004). Experiments in rats show that the *SIRT1* overexpression inhibits the FOXO3-induced apoptosis triggering instead the cell cycle arrest (Brunet et al., 2004; Kobayashi et al., 2005). In addition, both Sirt1 and FOXO1 are required on the promoter of the manganese superoxide dismutase (MnSOD). In particular, the deacetylase activity of Sirt1 is necessary for the transactivation of this antioxidant gene, indicating that Sirt1 promotes survival through the protection against oxidative damages (Daitoku et al., 2004; van der Horst et al., 2004). Overall, Sirt1 promotes cell cycle arrest and

DNA repair triggering cell survival rather than apoptosis. This model is consistent with the hypothesis of the connection between the increased longevity of the organisms and the enhanced resistance of the cells to DNA oxidative damages.
Another target of Sirt1 that promotes cell survival, is Ku70, protein mainly localized in the nucleus, where it is involved in the repair of the double-stranded DNA breaks (DBS).
Ku70, in fact, has the important role to protect the free ends of the DNA from degradation until the junctions of these strands are not repaired (Chu, 1997).
On the other hand a small fraction of Ku70 is also localized in the cytoplasm where it regulates apoptosis through sequestration of the proapoptotic protein Bax (Sawada et al., 2003). DNA damages promote the acetylation of Ku70 on multiple lysines inhibiting its association with Bax that, once released, translocates into the mitochondria and triggers apoptosis (Cohen et al., 2004).
The hypothesis is that the balance between the levels of HAT and HDACs/Sirt1 may regulate the acetylation status of Ku70 and p53 determining the ultimate fate of the cell.
It is thought that a combination of HDAC/Sirtuin inhibitors in combination with standard chemotherapeutic agents could promote apoptosis in specific types of tumors (Saunders and Verdin, 2007).
Interestingly Ku70 presents a yeast orthologue protein, yKu70 that interacts with Sir2p regulating telomere silencing and promoting DNA repair.
Recently, an important comparative study between *Saccharomyces cerevisiae* and *Mus musculus* highlighted that the redistribution of Sir2p and Sirt1 on chromatin promotes genomic stability during oxidative stress and alters gene expression during ageing (Oberdoerffer et al., 2008). In yeast, Sir2p normally represses and stabilizes the rDNA repeats. During ageing Sir2p relocalizes in genomic unstable loci, resulting in the derepression of specific genes (such as the HML loci) whose transcription cause infertility, characteristic of the aged yeast. Indeed during ageing the yeast Sir2p translocates into the nucleus in response to the accumulation of ERCs (extrachromosomal circular ribosomal DNA) and is released from HM loci derepressing mating type genes and leading to infertility.
A genomic study on mouse embryonic cells showed that also human Sirt1 represses repetitive DNA sequences and, in response to DNA damages, dissociates from these loci and relocalizes on DNA breaks to promote the repair, causing transcriptional changes that are similar to those that occur in the brains of mice during ageing. From these data it can be assumed that the

Sirt1 redistribution, induced by DNA damages, could be a conserved epigenetic pathway to promote ageing in eukaryotes cells (Oberdoerffer et al., 2008). This theory supports that yeast and mouse ageing could be partially caused by the chromatin reorganization dependent on DNA damages, phenomenon called " RCM " (redistribuition of chromatin modifiers).

1.2.2 *SIRT1 IN THE CIRCADIAN RHYTHM AND ENERGY METABOLISM*

Recent studies have highlighted an important new role for Sirt1 in the cell. These studies revealed a connection between the metabolic functions of Sirt1 and circadian rhythm, controlled by the Clock complex, which if deregulated, could lead to an increased risk for cancer (Jung- Hynes and Ahmad, 2009).
Coherently it has been shown that the decreased production of the hormon melatonin, a known regulator of the circadian rhythm, triggers the circadian cycle deregulation increasing susceptibility to cancer (Jung and Ahmad, 2006; Karasek, 2007).
Many researchers argue that the Sirt1 inhibition may have an antiproliferative effect in ageing-related cancer through the reprogramming of the genetic circuit that regulates the circadian rhythm.
The circadian rhythm in mammals is controlled by genes highly transcribed in the suprachiasmatic nucleus of the brain (CNS), structure that regulates this process. The main genes that are involved in the regulation are: *CLOCK*, *BMAL1*, *PERIOD* and *CRY*. Normally the Clock/Bmal1 complex binds the Per and Cry promoter to induce the transcription through the acetylase activity of Clock. Cry and Per by acting as a negative feedback loop inhibit the transcriptional activity of the complex Clock/Bmal1 (Lee et al., 2001). Nakahata et al. have demonstrated that *SIRT1* is regulated according to a circadian cycle and its expression is correlated with the acetylation of histone H3 and Bmal1 lys537 (Nakahata et al, 2009). This study suggests that Sirt1 transduces signals from cellular metabolites, that reflect the energy status of the cell, directly modulating the machinery of the circadian cycle.
One of the most important research concerning this process is that the oscillations the intracellular concentrations of NAD +, the sirtuin cofactor, follow the circadian rhythm both in yeast and mammals (Richard et al., 1993). Nakahata et al have shown that the Bmal1 complex is associated physically on the promoter of Nampt protein (the limiting enzyme in

NAD recover from NAM) in a time-dependent manner, a phenomenon also confirmed by the protein levels that follow the mRNA trend (Nakahata et al., 2009).
It has been suggested that the circadian rhythm, through the NAMPT-mediated NAD biosynthesis, may change the daily cycles depending on the energy resources of the cell. (Nakahata et al., 2009; Ramsey et al., 2009). These studies show a strong connection between metabolism and circadian rhythm, suggesting a complex interaction between Sirt1 and the Clock complex regulation.
Regarding the involvement of Sirt1 in the metabolism of mammals, it was observed that in *SIRT1* mutant mice the insulin secretion in response to increased levels of blood glucose was significantly lower than the respective wild type (Moynihan et al., 2005; Bordone et al., 2006). This indicates that Sirt1 positively regulates insulin secretion in beta cells of the pancreatic islands (Bordone et al., 2006). Consistent with these experiments, the overexpression of *SIRT1* in transgenic mice show improved glucose tolerance associated with increased insulin secretion (Moynihan et al., 2005). Since Sirt1 is highly involved in the sugar metabolism, some researchers hypothesize that its physiological decrease during ageing may be a factor contributing to the pathogenesis of diabetes and other metabolic disorders related to ageing.
The most important target of Sirt1, as regards the metabolism, is the cofactor PGC- 1α, the main regulator of the biosynthesis of mitochondria (Yamamoto et al., 2007). PGC- 1α is activated by Sirt1 deacetylation (Rodgers et al., 2005; Schwer et al., 2006). It was observed that the activation of PGC- 1α by Sirt1 protects against obesity and metabolic dysfunction (Lagouge et al., 2006). The changes in the NAD+ levels that influences Sirt1 activity, alter in turn PGC- 1α activity concordingly with the energy status of the cell. In this way, PGC- 1α can be coherently regulated to the metabolic needs of the cell and thus also the energy production within mitochondria.
Interestingly, Sirt1, unlike Sir2p in yeast, interacts with many non-histone proteins by regulating their activity. As regards the Sirt1 chromatin regulation, like Sir2p, controls the epigenetic state of rDNA through the histone deacetylation.
In particular, Sirt1 regulates the epigenetic chromatin state of the rDNA units accordingly to the energy status of the cell, mechanism very similar to that showed by Sir2p in yeast. However, Sir2p specifically regulates the transcription of non-coding RNAs in the intergenic spacer regions whereas Sirt1 regulates the transcription of rRNA.

Sirt1 recruited by the NoRC complex inhibits RNA polymerase I activity. The NoRC complex contains the methyltransferase Suv39h1 and the Nucleomethylin, protein that contain a binding domain for the methylated histones. Under glucose starvation, the high increase in NAD/NADH ratio, enhances the Sirt1 activity that deacetylates the transcription factor Taf68 inhibiting its binding to the promoter of the RNA polymerase I.
The NoRC complex with Suv39h1 stimulates histone methylation that in turn recruits the Nucleomethylin that bound Sirt1 and that deacetylates H3K9 inhibiting rDNA transcription, (Murayama et al., 2008).
These studies highlight how human Sirt1 and Sir2p may regulate the same molecular and physiological processes albeit with different mechanisms. These observations underline that during the evolution have been conserved the molecular functions but not the molecular mechanism behind the processes in which these two important enzymes are involved.
Indeed Sir2p and Sirt1 are involved in the same processes such as the energy-dependent transcriptional control, DNA repair and the regulation of the lifespan extent. However Sirt1 and Sir2p protein partners are not always conserved in the evolution, in fact, a lot of aspects of the mechanisms by which they act are different whereas the processes they regulate are very similar.
In many organisms it has been demonstrated that Sirt1 activation by resveratrol mimics the caloric restriction condition protecting from ageing-related phenotypes (Wood et al., 2004). Moreover it has observed that resveratrol during tumorigenesis has chemopreventive properties and it has positive effects on many aspects of metabolism, leading to an increase of life span in all metazoans on which the experiments were carried out.
The mechanisms by which this polyphenol acts on sirtuins and controls the effect of increased longevity still are not very clear. Future experiments will determine whether activators or inhibitors of sirtuins in combination with other drugs may prevent serious diseases associated with aging such as Alzheimer's, Parkinson's and cancer.

2. THE YEAST SIRTUINS

2.1 *SIR2*

The study of *Saccharomyces cerevisiae* has been fundamental to understand the complex regulation of chromatin repression in higher organisms. *S.cerevisiae* is an atypical eukaryote in terms of chromatin structure since it is not repressed in the same way of the other eukaryotes. In yeast only three genomic regions undergo a phenomenon of chromatin silencing: the telomeres, the mating-type loci and the rDNA locus (Gartenberg, 2000). Here the chromatin repression is mainly mediated by histone hypoacetylation and does not require important signals that are present in higher eukaryotes such as the methylation of H3K9 (Buck et al., 2004) but is mainly controlled by histone deacetylases and acetyl-transferases.
Specifically, in yeast the formation of packaged chromatin depends on the catalytic activity of the first sirtuin to be discovered, Sir2p.
Sirtuin family regulate important genetic pathways in every living organism from eubacteria to eukaryiots. The bacterial and archea genomes encode only a single sirtuin although there are exceptions such as *Archaeoglobus fulgidus*, whose genome encodes two sirtuins (Sauve et al., 2006). On the other hand eukaryotes have many sirtuin genes: yeast in addition to Sir2p, contains four sirtuins (*HST1-4*), *Drosophila melanogaster* five sirtuins and human cells contain seven sirtuins (*SIRT1* -7). The presence of more than one sirtuin in eukaryotic genomes requires a careful analysis of the different biological roles of this redundant enzymatic family.

2.1.1 TELOMERES AND HM LOCI

Sir2p was first discovered in studies about the determination of conjugative genes in yeast (mating type genes). The mating type, in *S.cerevisiae*, is determined by a single locus, known as MAT (mating type) that presents two different alleles, MATa and MATα (figure 4). Moreover yeast has on the same chromosome of MAT locus, two additional mating type cassettes known as HMRa and HMLα ("*Hidden Mating Rightward/Leftward"*), which encode the proteins of type "a" and type "α" respectively, (Figure 4). Yeast mating type is

determined by the central and transcribed MAT locus whereas, the lateral cassettes are silent to prevent infertility, (Figure 4), (Sauve et al., 2006).

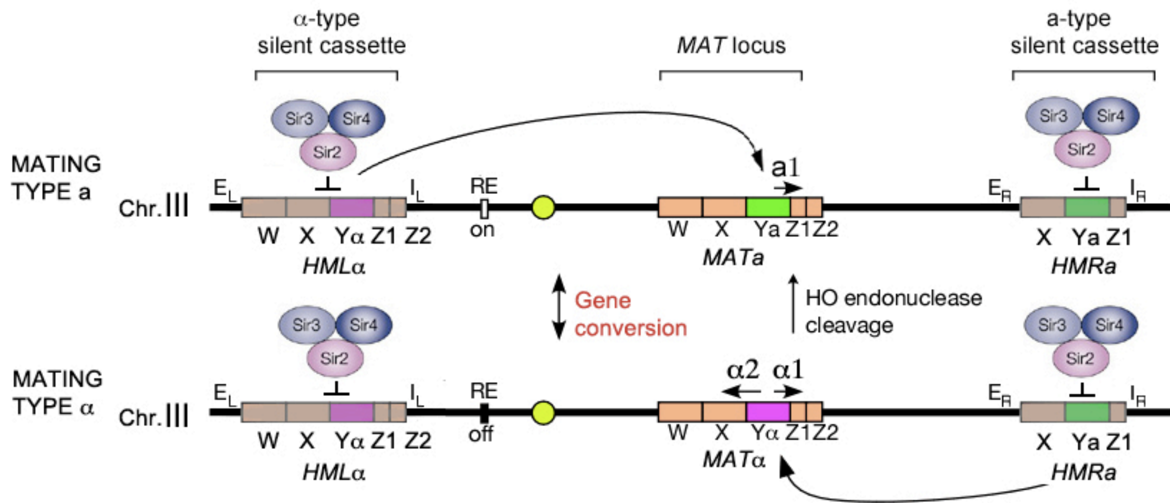

Figure 4: The mating type loci on *S. cerevisiae* chromosome III. MAT is in the middle of the chromosome, HMR and HML silent cassettes are near the right and left end of the chromosome respectively. E and I elements refer to the cis-acting loci that establish heterochromatic chromatin structures at HML and HMR through the binding of the SIR complex. RE refers to the Recombination Enhancer that, when turned on in a cells, stimulates recombination in the left half of chromosome III, thus favouring mating type switching with HML. Y refers to the mating-type-specific genes at each of these loci. (a1, α1 and α2 genes.)

The protein a1 has no effect in α haploid cells but it becomes important for genetic reprogramming in the diploid cells after mating. The "a" or "α" haploid cell-types produce specific proteins to activate genetics pathway that maintain specific molecular features of diploid cells. Upon two different MAT strains coupled, the association of the regulatory proteins a1 and α2 in the same complex trigger the activation of the genetic programme for diploid cell, for example the transcription of genes involved in the gametogenesis and the contemporary repression of genes involved in the vegetative growth. In the haploid population of the same mating type, approximately every cell division the transcribed gene in the MAT locus is excised and replaced by one of the alleles in the close silent loci (HML and HMR), (Figure 4). This phenomenon of gene substitution permits haploid cell to switch mating type thus to mate and produce gametes that enhance genetic diversity in the population (Harashima et al., 1974; Oshima and Takano, 1971). Since the switching process involves the removal of the gene from the transcribed locus MAT and its replacement with another one, this mechanism has been called in molecular biology "gene conversion".

In order to prevent the yeast haploid gene conversion the strains used in molecular biology present the deletion of the endonuclease HO, enzyme responsible for this process.

The silent cassettes, close to the MAT locus are repressed by the deacetylase activity of Sir2p. In order to demonstrate the role of Sir2p in this locus it has been measured the rate of transcription in the repressed locus HMLα in the MATa strain. The loss of silencing in MATa strains allows the expression of α1 and α2 genes from the HML locus. The genes α1 and α2 are subsequently repressed by the action of the heterodimer a1/ α2. However the HMLα1 transcript remains the best transcript detectable in MATa strain lacking Sir2p, and therefore the best indicator of silencing at this locus, (Figure 4), (Matecic et al., 2006).

The *SIR2* gene, was identified for the first time in a mutated yeast strain, originally called mar1-1. Klar and colleagues (Klar et al., 1979) isolated and analyzed many mutants defective in the maintaining the silent state of the HM loci. Similar mutants were isolated by many other groups (Rine et al., 1979; Haber and George, 1979) and these have helped to define the 4 "SIR" genes , which nowadays we know to work in the same complex in order to repress the HM loci and telomeres repression (Bryk et al., 1997; Fritze et al., 1997). The deacetylase Sir2p, is the only SIR protein with a enzymatic activity required for the silencing of all three loci: telomeres, HM loci and rDNA. The other Sir proteins (Sir1p, Sir3p, Sir4), which instead are not sirtuin, are required only for the silencing of the HM loci and telomeres but not for the

rDNA locus, (Figure 4 and 5), (Sauve et al., 2006). The difference between Sir3p/Sir4p/Sir1p and Sir2p localization depends on how these proteins can associate with different partners in the different loci of the genome. The process of the mating-type silencing involves the recruitment of the protein Sir1p by Rap1 and Abf1 within the silencer region (E,I elements) followed by the interaction with Sir3p and the Sir4p-Sir2p dimmer, (Figure 5), (Moazed et al., 1997). Sir3p and Sir4p bind chromatin through the interaction with histone tails of H3 and H4. The recruitment of Sir2p (such as complex Sir4p - Sir2p) results in the deacetylation of the N-terminal domain of H3 and H4 and in particular of H4K16Ac, (Figure 5), (Gartenberg, 2000; Oppikofer et al., 2013). Once recruited the Sir complex, heterochromatin spreads through the binding of Sir3p and Sir4p-Sir2p in H3/H4 hypoacetylated regions. Specifically, in yeast, H4K16 hypoacetylation is considered a marker of Sir2p silencing (Robyr et al., 2002). Conversely, the spread of the SIR complex is inhibited by the hyperacetylation of H3 and H4, indeed the boundaries of heterochromatin are enriched in H4K16Ac (Ekwall, 2005; Kimura et al., 2002; Suka et al., 2002). It has been demonstrated that the acetyltransferase Sas2 creates the H4K16Ac gradient that controls the Sir2p-dependent silencing spread at telomeric regions (Kimura et al., 2002; Suka et al., 2002). Despite Sir2p is involved in the silencing of telomeres, mating type loci and nucleolar rDNA locus, not all SIR proteins partecipate in the repression of this loci. For example the silencing of telomeres involves Sir3p and Sir4p, while rDNA silencing does not require any of these, (Figure 5), (Grunstein, 1997). The telomeres repression can be extended on chromosome anywhere from 2Kb to a maximum of 19Kb, this chromatin feature is important to understand a phenomenon called: “position effect variegation” (PEV) (Aparicio et al., 1991; Lieb et al., 2001). The PEV is a variegated phenotype caused by the inactivation of a gene that occurs only in some cells, this effect depends on the spreading extent of the chromatin repressor complexes along the DNA region where the gene involved in the phenotype is located in. As far as concern telomeric region, Sir4p is responsible for establishing the spread of the SIR complex through the binding of Rap1, yKu70 and yKu80 (Vaquero et al., 2006). After the recruitment of the complex Sir4p-Sir2p, is also recruited Sir3, here the SIR repressor complex spreads along chromatin through a mechanism very similar to the mating-type loci (Figure 5).

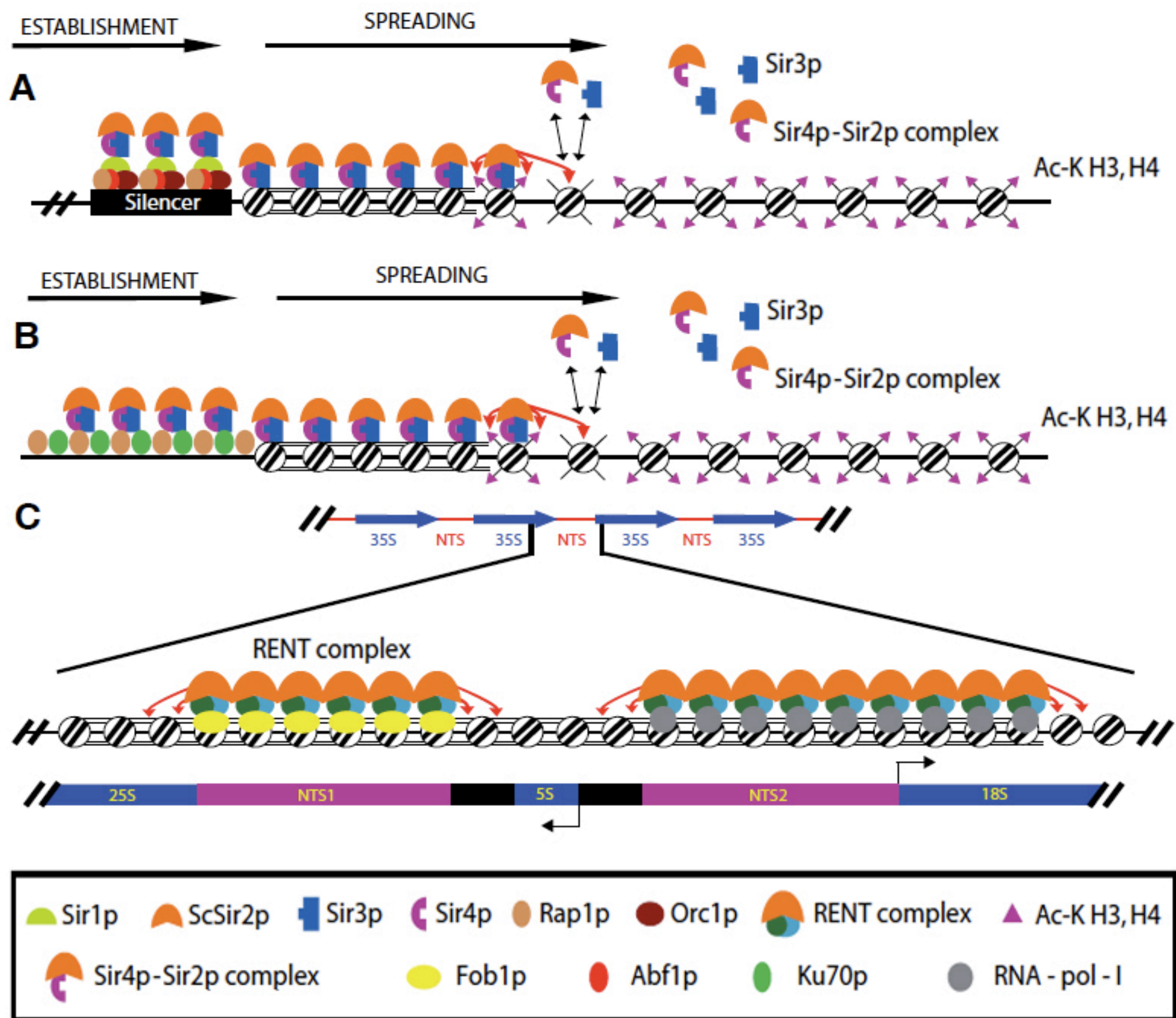

Figure 5: Protein complexes involved in the Sir2p-dependent heterochromatin formation in the A) HM loci, B) telomere regions, C) rDNA locus.

Interestingly Sir2p and Sir3p were found to relocalize from telomeres to DNA damaged sites in order to trigger repair with proteins involved in the non-homologous-end-joining (NHEJ), mechanism activated in the case of DNA double-strand breaks (DBS). It is unknown whether Sir2p is directly involved in DNA repair (Mills et al., 1999) by its catalytic activity.

2.1.2 THE rDNA LOCUS

The third example of heterochromatin locus in *S.cerevisiae*, in which it is fundamental the role of Sir2p is the rDNA locus. The ribosomal DNA (rDNA), is a very complex locus, with an extension of approximately 2Mb, consisting of 100-200 tandem copies of a 9.1 kb repeat, on the chromosome XII, (Figure 6), (Petes, 1979). Each repeats contain the 35S pre-rRNA gene (18S, 5.8S, and 25S) transcribed by RNA polymerase I (RNA Pol-I) and the 5S gene transcribed by the RNA polymerase III, (Figure 6). The rDNA units are interrupted by large regions called NTS (non transcribed spacer) further divided in two regions: the NTS1 and the NTS2 (Figure 6). The NTS region contains specific DNA sequences important for the regulation of the rDNA: the ARS (autonomous replication sequences) for the replication, the RFB site (replication fork barrier) bound by Fob1p protein that stops the replication fork leftward-moving from the ARS and the CAR element required for the proper Cohesin complex recruitment, (Kobayashi, 1996). Two further in-cis sequences have been discovered in the NTS: the E-pro and the C-pro, cryptic promoters that transcribe non-coding RNAs with still unclear function (Figure 6).
Within the NTS1 and NTS2 region have been charachteriezed many non-coding RNAs. E-pro bidirectional promoter generates two different transcripts termed NTS1r and NTS1f, 1Kb and 500 nt in length respectively, (Figure 6). Whereas the cryptic promoter C-pro produces a long non-coding, the NTS2, that can be processed in different sizes from 850bp to 1.6Kb, (Figure 6), (Li et al., 2006; Houseley et al., 2007; Vasiljeva et al., 2008).
In this locus, Sir2p induces epigenetic silencing in the context of a complex called RENT (regulator of nucleolar silencing and telophase exit), where it associates with Net1p and the phosphatase Cdc14 (Straight et al., 1999).
Net1p recruits Sir2p (Straight et al., 1999) and sequesters the protein phosphatase Cdc14 in the nucleolus until the RENT complex disassembles during late anaphase (Shou et al., 1999). The resulting release of Cdc14p permits cells to exit from mitosis, (Shou et al., 1999). Net1p

has been observed to be necessary for the RNA Pol-II silencing reporter genes inserted within the rDNA units and for the recruitment of Sir2p on this locus (Straight et al., 1999).

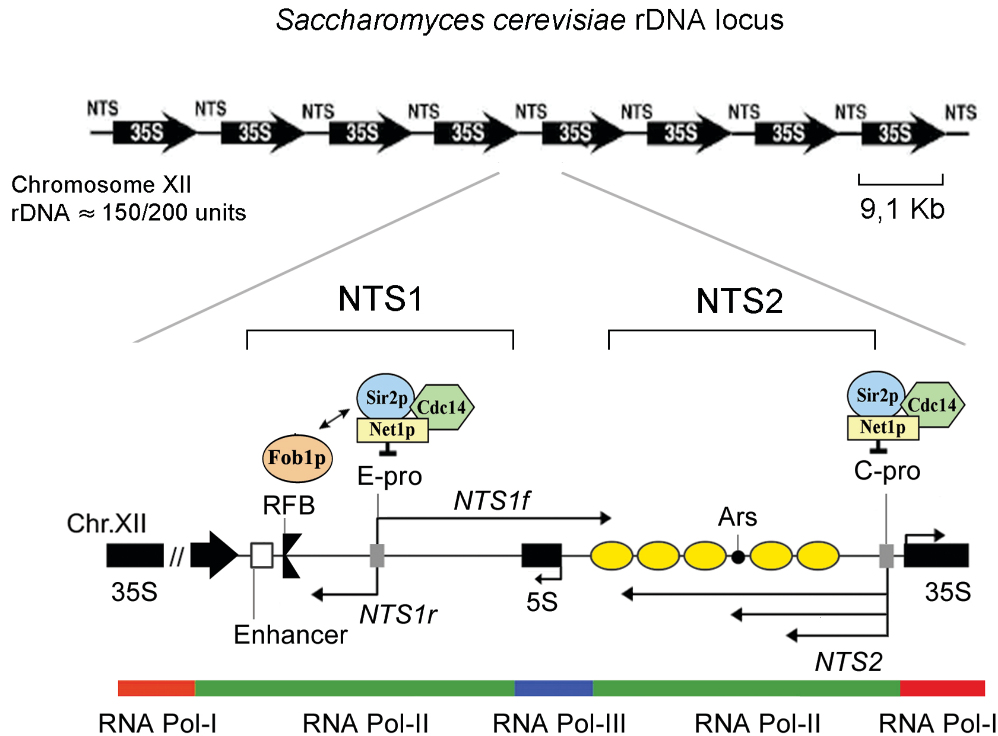

Figure 6: The yeast ribosomal locus (rDNA), 150/200units on chromosome XII. Schematic representation of the rDNA intergenic spacer regions showing ncRNA transcripts, (NTS1r, NTS1f, NTS2); ARS, autonomously replicating sequence; RFB, replication fork barrier interacting with the Fob1p protein; the cryptic promoters E-pro, C-pro repressed by the SIR complex; three different RNA polymerases transcribed regions; coding sequences of the 35S and 5S rRNA.

Chromatin immunoprecipitation (ChIp) experiments show that RENT complex proteins are enriched in three distinct regions within the rDNA: the non-transcribed spacer NTS1, around the RNA Pol-I promoter in the NTS2 and along the 35S rRNA coding region (Figure 6). It has been demonstrated that the binding of the RENT complex to NTS1 region overlaps the replication fork barrier element and require the Fob1p protein (fork-blocking protein) that is able to specifically bind the DNA in this region (Kobayashi et al., 2004). In *fob1Δ* strain, both silencing and the association of the RENT complex are impaired at NTS1 region while the binding near the RNA Pol-I promoter is unaffected (Huang and Moazed, 2003). Interestingly proteins involved in the recruitment of Sir2p and the RENT complex to the NTS2 have not been identified yet even if RNA Pol-I is a good candidate since it physically interacts with RENT. (Huang and Moazed, 2003). Albeit the exact mechanism of rDNA silencing remains still unclear it has been demonstrated that the Sir2p catalytic activity is necessary to repress reporter genes both in NTS1 and NTS2 regions. Observations showed that in *sir2Δ* strain histones H3 and H4 are hyperacetylated and moreover in these regions the micrococcal endonuclease accessibility is enhanced (Cioci et al., 2002). However the *sir2Δ* strain does not repress the rRNA transcription (Buck et al., 2002) but silences non-coding RNAs producted from two cryptic promoters within the NTS1 and NTS2 regions: the E-pro and the C-pro, (Figure 6). The role for this untranslated RNAs is still unknown but some studies showed a high correlation with the recombination rate between rDNA units (Kobayashi and Ganley, 2005). E-pro has been characterized to be a bidirectional promoter activated by the RNA Pol-II, (Figure 6), (Santangelo et al., 1988). Moreover when the cryptic promoter is substituted with the strong galactose-inducible bidirectional promoter it has been shown to highly increase the recombination rate between rDNA units (Kobayashi and Ganley, 2005). Kobayashi and Ganley have also demonstrated that the non-coding RNA produced by the E-pro, the NTS1, is able to disassociate cohesin complex from rDNA (Kobayashi and Ganley, 2005). Cohesin is a big muliproteic complex that keep together sister chromatid until they separate in M phase inhibiting unequal chromatid recombination after double strand breaks formation (Haering and Nasmyth, 2003). Specifically in *sir2Δ* strain it has been found a decrease in the enrichment of the cohesin subunit Mcd1p in the rDNA and a strong increase of NTS1 transcription (Kobayashi and Ganley, 2005; Li et al., 2006). Coherently it has been discovered that Cohesin complex positioning is able to repress transcription during the mitosis

and that this inhbition is removed in the interphase when the complex is removed from rDNA (Glynn et al., 2004). In *sir2Δ* strain the NTS1 transcription from the bidirectional promoter collides during the mitosis with the Cohesin complex and triggers the "unequal recombination" between rDNA cluster in chromatid sister in order to repair DBS close to fork barrier block, the RFB, (Figure 7), (Kobayashi and Ganley, 2005; Li et al., 2006). This process can lead to the variation of the rDNA copy number (rDNA cluster contraction or expansion) characteristic of the yeast strains that alter non-coding transcription in the rDNA.
Sir2p in the nucleolus has two connected phenotype: the non-coding RNAs repression and the inhibition of recombination events that occur within the rDNA units.
In wild type strain, Sir2p, represses E-pro/NTS1 transcription allowing cohesins to associate with NTS and thus triggering equal recombination (Figure 7). In *sir2Δ* strain ncRNAs transcription displace cohesins leading to unequal recombination or intra-chromosomal recombination, (Figure 7), (Kobayashi, 2011). The last one has been connected to the formation of another specific phenotype of *sir2Δ*: the ERCs accumulation (Extrachromosomal rDNA circles), (Figure 7), (Kobayashi, 2006).
The recombination mechanism between rDNA units is activated as response to specific processes that occur within this locus: the double strand breaks formation, probably caused by the collision between the replication machinery and the transcriptional complex (Kobayashi et al., 2003). This process can increase two connected phenotypes, the gene amplification and the ERCs formation, (Figure 7).
The gene amplification appears to be regulated by the E-pro that directs the transcription of NTS1. When yeast displays the rDNA copy number at the wild type level the E-pro is inactivated by Sir2p, (Figure 7), (Kobayashi, 2011). Whereas, when the copy number is reduced, Sir2p, with an uncharacterized mechanism, is released by the E-pro, the non-coding transcription consequently interferes with the function of cohesin complex. As a result of the inability of cohesin to link sister chromatids the unequal sister chromatid recombination is increased allowing gene amplification mechanism to counteract for the loss of rDNA copies (Kobayashi and Ganley, 2005). In fact, the Sir2p mutant strain, beacause of the gene amplification mechanism, reaches around 300 copies of rDNA compared to the 150 rDNA units of the wild type strain. However, so far, it is not known how yeast cells monitor the rDNA copy number and trigger the release of Sir2p from the E-pro.

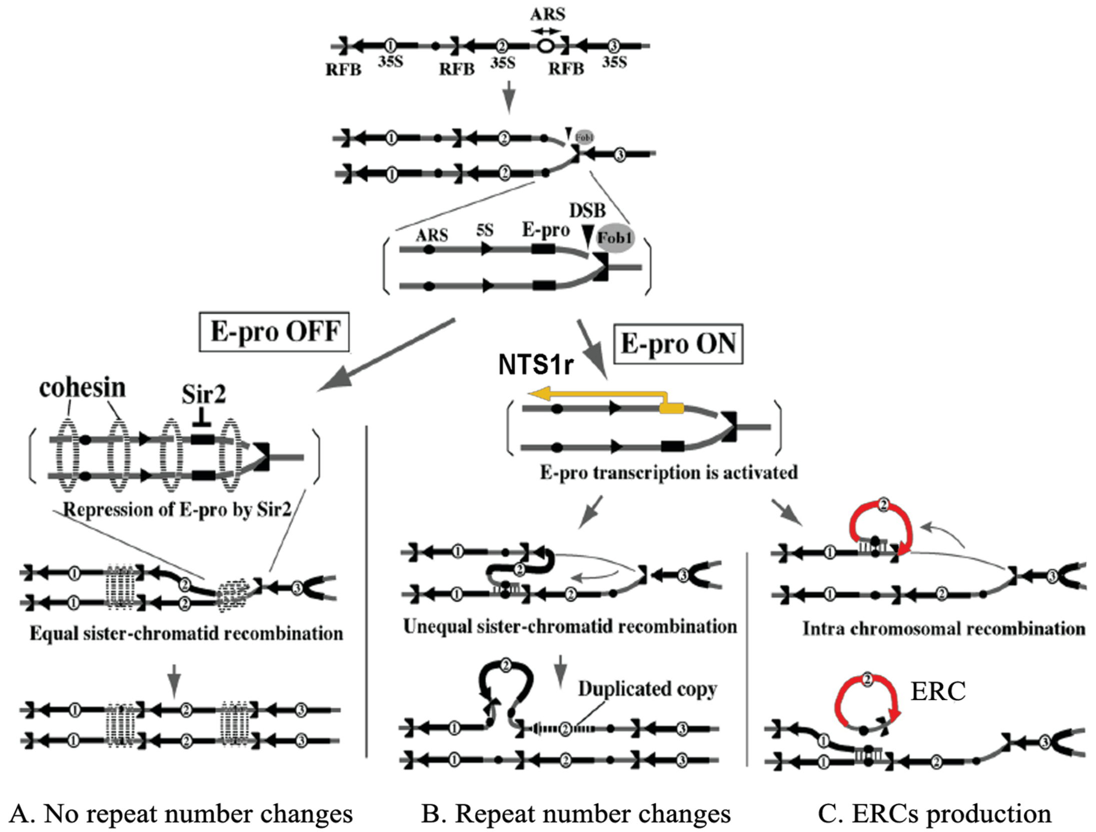

Figure 7: The transcription-induced cohesin dissociation model of rDNA amplification. A) In normal situations, such as wild type rDNA copy number, Sir2p represses E-pro activity, allowing cohesins to associate with NTS. DSBs are repaired by equal sister chromatid recombination, with no change in rDNA copy number. B), C) In Sir2p mutants E-pro is activated and ncRNA transcription displaces cohesins. The lack of cohesion between sister chromatids could lead to unequal sister chromatid recombination (with copy number variation) or to intra chromosomal recombination (with ERCs accumulation). From Kobayashi, 2006.

ERCs production is the phenotype that connects the catalytic activity of Sir2p with the increase of the replicative lifespan in yeast since the accumulation of episomal rDNA has been seen to be associated with the replicative senescence (Gottlieb and Esposito, 1989; Sinclair et al., 1998).
ERCs have been discovered to be asymmetrically inherited and to accumulate preferentially in mother cells characterized by fragmented nucleoli, cessation of cell division, and cellular senescence for this reason it has been proposed that ERCs, excised during S phase, may be the molecular clock that determines the life span of the cell (Sinclair, 1997). Specifically ERCs can't segregate equally since they lack a centromere even though if they can replicate thanks to an autonomous replication origin, rARS. Sir2p deletion in yeast cause a hyper-recombination within the rDNA units increasing the ERCs levels, while the *fob1Δ* deletion reduces ERCs levels as in wild type strain, extending replicative lifespan. The overexpression of Sir2p increases the replicative lifespan but not when combined with the deletion Fob1 suggesting that Sir2p enhances the replicative lifespan thanks to the mechanism of inhibition of recombination of the rDNA unit (Smith et al., 1998, Kennedy et al., 1995; Kennedy et al., 1997; Kobayashi and Horiuchi, 1996).
Sinclair and Guarente in 1997 proposed the hypothesis that the replicative ageing is caused by the progressive accumulation of extrachromosomal rDNA circles in the mother cells, this theory is still on debate because many studies presented controversial results.
Many aspects of ERCs production were experimentally supported such as: they are produced by rDNA units recombination, are self-replicating and inherited in an asymmetrical manner (Sinclair, 1997; Kobayashi et al., 2003; Kobayashi and Ganley, 2005). More controversial is the role that ERCs play in the ageing process: are the ERCs the cause or the effect of ageing in yeast? It has been demonstrated that the introduction of a plasmid carrying one rDNA unit, significantly reduce the lifespan (Falcon and Aris; 2003). However, ERCs could be interpreted also as bio-marker of ageing and not as the cause. On the other hand mutants that affect recombination, replication or DNA repair reduce the duration of life without the accumulation of ERCs (Hoopes et al., 2002; Merker and Klain, 2002; Oakes et al., 1998) .
In some mutants has been observed an increase of ERCs in conjunction with an extension of the lifespan (Kirchman et al., 1999), moreover a Sir2p mutant with increased lifespan was seen to have a normal level of ERCs (Kim et al., 1999). Specifically the effect of Sir2p on the duration of life has been attributed to an altered pattern of gene expression including the

altered transcription of rDNA, which can lead to an imbalance in the synthesis of ribosomes (Jazwinski, 2000). Although many researchers agree that the rDNA locus play a key role in the processes of ageing, the precise role of episomal rDNA circles has long remained obscure. Instead undeniable is the role of the *SIR2* gene in mediating, with a pathway not yet fully elucidated, an increase in the duration of life of the yeast of about 30 % (Guarente, 2000; Kaeberlein et al., 1995; Tissenbaum and Guarente, 2001).
Another interesting observation about ERCs accumulation is the correlation between ARS activation and recombination rate. In fact if the Kobayashi hypothesis is right the more the ARS is efficient in promoting replication during mitosis the higher will be the number of collisions between the transcriptional machinery and the replication fork. In support of this model it has been demonstrated that Sir2p is able to control also the activation of the replication origin within the rDNA (Pasero et al., 2002). Specifically only 50% of the 150 rDNA units are transcribed and moreover only one out of three rARS are activated during mitotic phase (Fangman, 1991). It is conceivable to hypothesize that is the ratio (rDNA units transcribed)/ (active rARS) during the mitotic phase that establish the amount of ERCs level in the cell. Some observations indicate that the acetylation of H4K16 on the ARS region may influence the replication process by the MCM helicase complex recruitment (Weinreich et al., 2004; Chiani et al., 2006). Ganley et al., in 2009 have demonstrated that the ARS efficency is highly correlated with ERCs production, however they did not observe a concomitant reduced lifspan. These results indicate that non-coding production and rARS activation may be two different aspect of rDNA recombination process but that can affect yeast lifespan with different mechanism (Ganley et al., 2009).
In conclusion, the increased ERCs levels and the extension of the lifespan do not always occur together highlighting the fact that there are still many unsolved aspect in this process.
Overall, Sir2p is an enzyme that regulates different molecular pathways such as the telomeres protection, the yeast mating type, the rDNA stability and ageing. The characterization of the ortholog gene *SIRT1* in human, and also in other mammals, is proving the important relevance of this protein in a number of different pathologies such as neurodegenerative diseases diabetes and cancer. The study of this sirtuin in yeast and the elucidation of its molecular pathways are an important starting point to understand the more complex chromatin regulation in humans.

2.2 *HST1-4*

Besides *SIR2* gene, *S. cerevisiae* has four additional sirtuins, named HST (homologues of *SIR Two*, *HST1-4*) that have a high homology sequence with *SIR2* and are involved in the silencing of the mating-type loci and telomeres. *HST1* and *SIR2* have a strong homology sequence: in fact *HST1* is the closest homologue of *SIR2*. Hst1p, as Sir2p, is located in the nucleus and it is a component of two distinct multiprotein complexes (Mead et al., 2007). Hst1p binds the protein Sum1p that in turn interacts with specific DNA sequences through Rfm1 in order to repress middle sporulation genes during vegetative growth (Mead at al., 2007). The middle gene sporulation elements (MSE) are regions located upstream of sporulation genes and are able to act both as activators, during intermediate stages of sporulation, and as repressors during vegetative growth (Pierce et al., 1998). Sum1, Hst1p and Rfm1 are all required for MSE-mediated repression, deletion of Sum1p triggers a strong derepression of sporulation genes, while the deletion of Hst1p results in a modest derepression (Hickman and Rusche, 2007).

Specifically Hst1p is recruited to its targets by the transcription factor Sum1p and like Sir2p induces repression by deacetylating H3 and H4 tails (Xie et al., 1999).

Hst1p, in addition to its role as a transcriptional repressor with Sum1p, also influences DNA replication (Weber et al., 2008). Weber et al. identified many ARS elements whose replication depends on Sum1p and Hst1p. In the absence of Sum1p and Hst1p the acetylation of H4 Lys 5 is dramatically increased at the replication origin. Sum1p recruits histone deacetylase Hst1p on specific replication origins in the yeast genome, and the deacetylation performed by Hst1p on these sequences is required for a proper initiation of replication (Weber et al., 2008). In fact, *sum1Δ* deletion impairs Hst1p localization in ChIP-on-chip experiments (Li et al., 2013). Hst1p has been found also in Set3C complex involved in the repression of early sporulation genes, including meiotic regulator genes such as *IME2* and *NDT80*.

Hst1p, within the Set3C complex, interacts with other six proteins: Set3, Snt1, Sif2, Hos2 (Pijnappel et al., 2001), however it has been seen that Hst1p is not responsible for the activity of the complex (Vaquero et al., 2009).

The strong functional similarities between Hst1p and Sir2p are also confirmed by the observation that these proteins, in particular conditions, are able to substitute each other.

Moreover, unexpectedly, it has been observed that there are genome sites where Sir2p, Hst1p, and Sum1 were co-enriched. These findings suggest that the ability to bind Sum1p exsisted in the original *SIR2/HST1* encoded protein, whereas the specific SIR-mediated heterochromatin establishment evolved after the duplication event. (Li et al., 2013; Vaquero, 2009). Indeed in *hst1Δ* mutants, the Sir2p overexpression partially restores middle sporulation gene repression, on the other hand, Hst1p overexpression can partially restore silencing of the HM loci in the absence of Sir2p (Brachmann et al., 1995; Derbyshire et al., 1996). Unlike Sir2p and Hst1p that have nuclear localization, Hst2p is localized in the cytoplasm. This protein maintains cytoplasmic localization both when expressed at low levels and if it is induced under the control of the strong GAL10 promoter (Perrod et al., 2001). Moreover even when a nuclear localization sequence (NLS) is fused to Hst2p, this protein remains in the cytoplasm until it is not overexpressed at extremely high levels (Perrod et al., 2001). *HST2* is the horthologue gene of human *SIRT2*. Hst2p, like Sirt2, is a histone deacetylase that shows high preference for H4K16Ac and, to a lesser extent for H3K9Ac (Vaquero et al., 2006). It has been demonstrated that Hst2p is not able to restore silencing in *sir2Δ* strains, and its absence has no effect on telomeric and rDNA repression, however its overexpression increases the rDNA silencing but concomitantly derepresses telomeric silencing (Perrod et al., 2001). So far it is not known whether Hst2p is responsible for the drop in H4K16Ac prior to mitosis phase analogously to how Sirt2 acts in human cells.

In *S. cerevisiae* there are two more sirtuins, Hst3p and Hst4p, these are involved in the deacetylation of H3K56, histone mark acetylated by the acetyltransferase Rtt109p. Specifically H3K56 is acetylated before its incorporation into chromatin during S phase, while it is deacetylated by Hst3p and Hst4p, during G2/M. The acetylation of H3K56 is finely regulated during the cell cycle: the expression of Rtt109p reaches a peak during the S phase, and consequently also the K56 acetylation, this modification is subsequently removed from Hst3p and Hst4p during G2 and M (Masumoto et al., 2005; Xu et al., 2007; Celic et al., 2006).

The expression of Hst3p and Hst4p in fact reaches the highest peak during the G2 / M and M/G1 respectively (Maas et al., 2006).

Rtt109p acetylates the histone H3K56 during the assembling of H3/H4 dimers in order to form the nucleosomal core. Once nucleosome is structured, H3K56 is positioned close to the "entry/exit " site of the DNA on nucleosome (Luger et al., 1997), for this reason it has been

supposed that H3K56 may be potential site of regulation in chromatin structure (Yang et al., 2010). Chromatin isolated from cells expressing H3K56Q, which mimics the acetylated state of the lysine, is much more sensitive to digestion by nucleases micrococcus compared to chromatin of wild type cells (Yang et al., 2008). All these findings imply that the acetylated form of H3K56 reflects a more relaxed state of chromatin and that Hst3p and Hst4p may be important regulator of chromatin state in yeast. Furthermore it has been shown that also Sir2p is able to modulate the silencing through the deacetylation of H3K56 (Xu et al., 2007) but the overexpression of Sir2p is not able to restore silencing in mutants *hst3Δ* and *hst4Δ* (Yang et al., 2008).

All these observations show that sirtuins Hst3p and Hst4p play a fundamental and irreplaceable role in the maintenance of the hypoacetylation of H3K56 in silent chromatin. As far as concern the rDNA locus, interestignly Hst2p and Hst4p mutants do not present any chromatin repression defect whereas Hst1p and Hst3p present more similar phenotypes to Sir2p (Cesarini et al., 2012). This effect is quite conceivable for Hst1p, the most similar sirtuin to Sir2p, while it was unexpected for Hst3p. Moreover Hst3p mutant, contrary to Hst4p, showed higher acetylation level on the replication origin and increased extrachromosomal rDNA circles whose accumulation is toxic and seems to be associated with yeast ageing (Sinclair et al., 1998; Cesarini et al., 2012).

It has been demonstrated that also the other yeast sirtuins have a role in the control of the lifespan. Indeed it has been shown that the *SIR2*-independent life span extension by CR is caused by the partial redundancy with *HST2* and *HST1* (Lamming et al., 2005). Concordingly with data showing increased ERCs levels in *hst3Δ* but not in *hst4Δ*, *hst3Δ* mutant has a shortened replicative lifespan like *hst1Δ* or *sir2Δ* (Tsuchiya et al., 2006), while RLS of *hst4Δ* mutant is close to normal, (Cesarini et al., 2012). Moreover *hst3Δ hst4Δ* double mutant has a very short RLS reflective that sirtuins are able to compensate each other only to a certain degree. Genetic studies on sirtuins in yeast have highlighted the major role of sirtuins in gene repression and specifically in telomeres, HM and rDNA loci. All sirtuins, both the HST and Sir2p, even if with different partners, alter the chromatin structure and regulate gene expression. The redundancy of this gene family could be an evolutionary response as a defense mechanism against mutations that can alter their important processes.

The role of sirtuins in ageing processes by the regulation of chromatin is a conserved mechanism. The study of lower organisms such as *S.cerevisiae* in the next future will help us

to understand the more complex processes in higher eukaryiotes. Furthermore the characterization of these mechanisms will be the starting point for the development of epigenetic therapies for diseases associated with ageing.

3. EVOLUTIONARY CONSIDERATIONS ON SIRTUIN FAMILY

The histone deacetylases (HDACs) are proteins that regulate the acetylation of both histone targets and non-histone proteins. These enzymes are responsible for the removal of acetyl groups from the ε- lysine residues and consequently are involved in gene repression and heterochromatin formation. For this reason HDACs are key factors in the epigenetic regulation and in the control of specific spatio-temporal gene expression in many organisms (Ahringer, 2000; Margueron et al., 2005; Vaquero et al., 2003; Verdin et al., 2003). In addition, HDACs have important roles in DNA repair, DNA replication, in cell cycle control and apoptosis (de Ruijter et al., 2003; Kurdistan and Grunstein, 2003).
The acetylation and deacetylation of non-histone proteins has emerged as a new and general mechanism to modulate the function of cellular proteins. To date, data suggest three main levels of sirtuin-dependent regulation of proteins and chromatin: DNA binding, protein stability and enzymatic activity (Glozak et al., 2005).
The HDACs have been divided into 4 phylogenetic groups: Class I –IV, (Figure 8), (Yang and Seto, 2008). Classes I, II and IV are highly correlated both from a structural and functional point of view, while the HDAC class III (sirtuin) are proteins with a different structure and more correlated to metabolic processes and cell survival (Figure 8).
The sirtuin family, or class III HDACs, so defined because of the homology with the yeast silencing factor Sir2p, a NAD-dependent histone deacetylase (Imai et al., 2000; Landry et al., 2000), are involved in processes such as epigenetic silencing DNA repair, replication, recombination, and cell cycle control (Saunders and Verdin, 2007; Vaquero et al., 2007). The conservation between members of the sirtuin family is restricted only to the catalytic domain, a region of about 250 residues that contains two well defined structural domains: a NAD binding domain, known as Rossmann domain and a zinc finger domain with 4 cysteines (Frye, 1999). All members of the *SIR2* family are characterized by their dependence on NAD + (Imai et al., 2000). The NAD dinucleotide is a metabolic cofactor required for the transfer of electrons in a lot of enzymatic reactions of the cell (Sauve and Schramm, 2003).

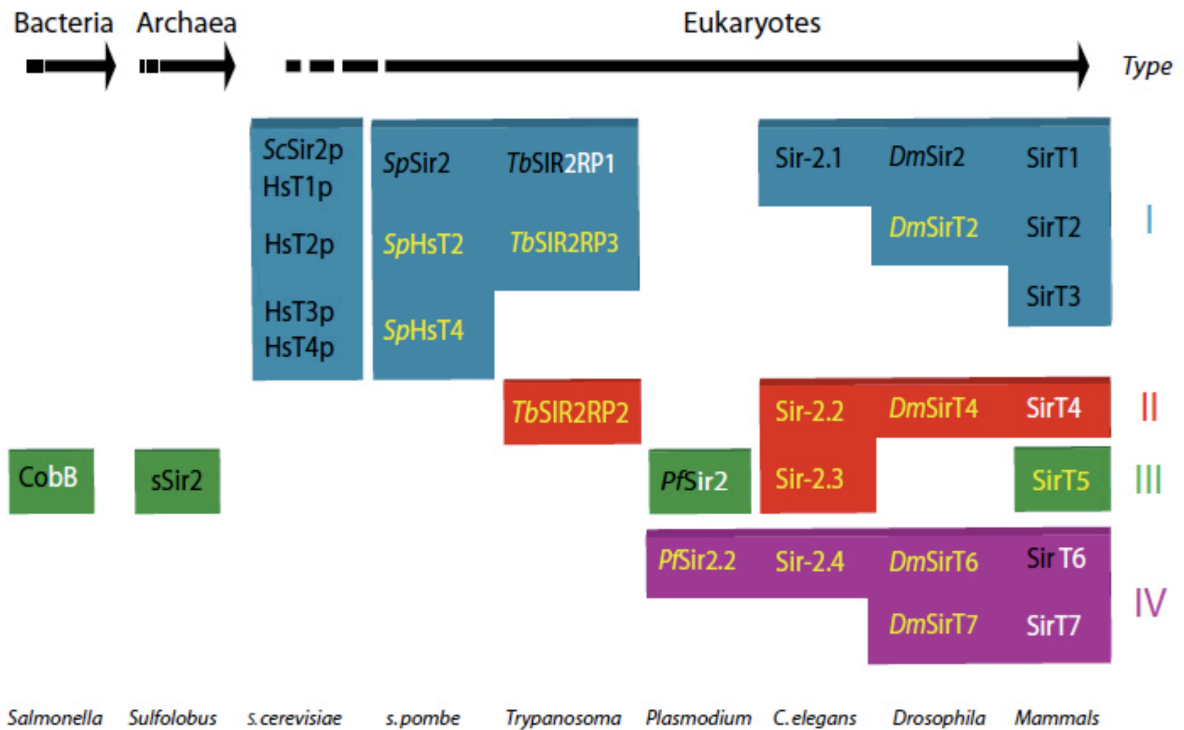

Figure 8: Philogenetic classification of sirtuins in selected organisms from bacteria to humans according to Frye (2000).

In contrast to the other HDAC classes, sirtuin do not release acetyl groups in solution as acetate in fact the acetyl group is transferred to ADP-ribose, releasing the O-acetyl-ADP ribose as a final product (Sauve et al., 2001).
The fact that sirtuins require NAD cofactor it suggests that they can act as sensors of the energy status of the cell (Fulco et al., 2003; Haigis and Guarente, 2006). Coherently sirtuins are capable of triggering changes in metabolism and in chromatin structure through the deacetylation of histones and other proteins.
The catalytic mechanism of the sirtuin family includes not only the deacetylation but also the mono-ADP- ribosylation, activity which is present in almost all sirtuins.
Interestignly the deacetylation reaction is also present in bacteria suggesting that these enzymes are likely to have acquired this function relatively early in the evolution (Starai et al., 2002; Tsang and Escalante-Semerena, 1998).
Interestingly, it is thought that sirtuins are much more ancient of the histone proteins and also of the chromatin structure (Frye, 2000). Indeed sirtuins are found in all three domains of life (eubacteria, archea and eukaryotes) and recent findings suggest that the original role of sirtuins was to regulate metabolism through the regulation of enzymes such as acetyl -CoA synthetase (Vaquero, 2009). Nevertheless, the connection between sirtuins and the chromatin regulation is very old and widespread throughout the animal kingdom from archaea, which have a proto-chromatin genome organization, to mammals that have instead a much more complex chromatin organization (Bell et al., 2002; Kuzmichev et al., 2004; Vaquero et al., 2007).
The sirtuin activity and their chromatin regulation is tightly linked to the deacetylation of histone residues H4K16Ac and H3K9Ac (Vaquero et al., 2007). In particular, the catalytic activity of sirtuins, in contrast to other HDAC, is finely regulated by different mechanisms. One of these, found both in humans and in yeast, is a non-competitive inhibition caused by a high concentration of NAM, metabolic product released after catabolism of NAD + (Bitterman et al., 2002). A second mechanism involves the metabolic intermediate nicotinamide riboside, a precursor involved in the recove of the NAD, molecule that increases the activity of Sir2p in vivo (Belenky et al., 2007). A third mechanism by which sirtuins are regulated is the O-acetyl-ADP-ribose, the major product of the activity of Sir2p, which causes a conformational change of the enzyme and promotes its recruitment on the chromatin (Liou et al., 2005). How O-acetyl-ADP-ribose regulates sirtuins in mammals is not clear yet. An

interesting consequence of the involvement of sirtuins in metabolic pathways is the control the lifespan. This regulation has been demonstrated in many organisms such as *S.cerevisiae*, *C.elegans* and *D.melanogaster,* data suggest that it may also occur in the case of some mammals such as *Mus musculus* or *Macac mulatta* (Boily et al., 2008; Longo and Kennedy, 2006).
The number of sirtuin in the different organisms is generally correlated with the complexity of the organisms (Figure 8). For example prokaryotes generally have from one to two sirtuins, the yeast has from three to five, insects and mammals have from five to seven sirtuins (Figure 8). However the plants are an exception to this rule: *Arabidopsis thaliana* and rice have only 2 sirtuins (Frye, 2000).
The sirtuins have been divided into 5 types: I to IV and type U, (Figure 8), (Frye, 2000). Type-I sirtuins are clearly related to chromatin regulation and show a strong deacetylase activity on histones, this class include all the yeast and human sirtuins *SIRT1-3,* (Figure 8). Type-III sirtuins include prokaryotic sirtuins that seem to be involved only in the control of metabolism and most of eubacteria and archea sirtuins (Figure 8).
These sirtuins appear to have mainly an ADP-ribosiltransferase activity and are localized in the mitochondria. The eukaryotic sirtuins are: Type-II, localized in the mitochondria (Sirt4 and Sirt5). Type IV which have an ADP- ribosyl-transferase activity (Sirt6 and Sirt7), recent observations have shown the ability of Sirt6 and Sirt7 to deacetylate H3K9Ac and H3K18 respectively, (Figure 8), (Michishita et al., 2008). The U-type includes the prokaryotic sirtuins that have not yet been characterized. Throughout the evolution the more conserved sirtuins in organisms are those of type II and type IV. In the next future, further studies will be required to elucidate and determine how sirtuins have evolved in different phylogenetic branches.
Despite the recent explosion in the number of publications concerning the evolutionary analysis of sirtuins, there are still many issues to be solved. Since these enzymes are very ancient and conserved, the functional study of sirtuins in simpler organisms could bring a big contribution to our knowledge about many cellular mechanisms in higher eukaryotes. In many cases during the evolution there has been a selective and extensive loss of these enzymes, especially in insects and plants. For this reason, it is conceivable to think that this loss may have been compensated by the redundant function of the others.
This concept may explain the relative weakness of the phenotypes caused by mutations in single sirtuins. For example, in *Mus musculus*, except for *SIRT1* and *SIRT6*, mutations in

other sirtuins do not lead to significant phenotypes do not altering the proper development of the organism. For this reason, in order to better elucidate the functions of this class of enzymes is required the use of organisms easily to be manipulated genetically such as *Saccharomyces cerevisiae*. Yeast Sir2p is part of different complexes that act in different loci of the genome (Aparicio et al., 1991; Shou et al., 1999; Straight et al., 1999). It would be interesting to see whether this type of regulation is a common denominator in other organisms. A phylogenetic approach to this type of problem could potentially shed light to new protein partners of sirtuins and may help to understand many processes in which sirtuin are involved in. For example, while the conservation of the catalytic core of sirtuins is very high, N- terminal and C -terminal tails are very different among organisms. Recently studies have showed that these domains are involved in the functional differences between the sirtuins by recruiting different factors (Vaquero, 2009). As far as concerns the comparison between human *SIRT1* and yeast *SIR2*, studied in this thesis, they show a high percent identity (41%), (Vaquero, 2009). In particular the catalytic core of these two enzymes show a percent identity of 85% with both an identical nucleotide binding site and cysteine zync finger domain. However, the human Sirt1 is bigger than Sir2p (747aa versus 562aa) with a longer C-terminal. This difference in the C-terminal may increase in human the number of interaction and regulatory pathways in which Sirt1 is involved in. Moreover it has been demonstrated that the Sirt1 C-terminal domain contributes significantly to the K(m) for NAD(+) even if is the N-terminal domain that contributes predominantly to catalytic rate (Min Pan, 2012).
In addition, human Sirt1 and yeast Sir2p have a partially overlapping specificity for histone residues, specifically for H4K16Ac and H3K9Ac (Vaquero, 2009).
However, Sir2p deacetylates only histones whereas Sirt1 also deacetylates transcription factors, triggering their activation or inactivation, with relevant consequences on gene expression (Fang and Nicholl, 2011). The relevance of the sirtuin family is supported by the ever-growing number of studies in the literature and the discovery of a series of molecules that inhibit or activate sirtuins has had a strong impact on biological and biomedical research (Fang and Nicholl, 2011; Grubisha et al., 2005).
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, Sirt1 activity assays are based on in vitro deacetylation reaction with peptide substrates (Howitz et al., 2003). We therefore decided to express the *SIRT1* gene in *S. cerevisiae* since the yeast model presents many advantages (Forsburg, 2001; Grunstein, 1997), i.e. the availability of specific mutants, the highly characterized genetic environment and the easy genetic manipulation.

Moreover an extensive study by computer modelling approach of each class of sirtuins, combined with structural studies, may identify conserved residues on the surface that are critical for specific interaction with different protein partners. In light of the involvement of sirtuins in ageing, neurodegenerative diseases and in specific type of cancers, the development of compounds that specifically activate or inhibit individual members of sirtuins could help to establish new drug therapies. To this purpose the comparative study of sirtuins between different organisms may be a powerful tool to answer many questions that concern this interesting class of enzymes.

AIM OF THE THESIS

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 ageing through its deacetylase activity, acting on both histone and non-histone substrates. The recent discovery of molecules that target this enzyme class and the elucidation of their molecular mechanisms is the starting point for the development of pharmacological protocols. However Sirt1 activity assays are only based on *in vitro* deacetylation reaction with peptide substrates (Howitz et al., 2003) and drug candidates have also often tended not to have drug-like properties because of poor physicochemical properties. A possible approach to circumvent this problem is the use of *in vivo* models in the discovery phase to identify molecules with desired biological profiles eliminating those compounds with poor pharmacokinetic properties. We therefore decided to express the *SIRT1* gene in S. *cerevisiae* since the yeast model presents many advantages i.e. the availability of specific mutants, the highly characterized chromatin environment and the easy genetic manipulation (Forsburg, 2001; Grunstein, 1997).
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 aim of this work is: i) To check if *SIRT1* overexpression is toxic in *sir2Δ* yeast cells, ii) Analyze if *SIRT1* expression is able to rescue *sir2Δ* molecular phenotypes by its catalytic activity iii) Characterize how the complementation occurs in order to exploit *S.cerevisiae* for in vivo chemical screening. The structure of chromatin is basically maintained from yeast to human. Thus, yeast chromatin would be a favourable environment to evaluate, inhibit or activate an ectopic histone deacetylase activity in an *in vivo* substrate.

RESULTS

1. *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 (Brunet et al., 2004) into the pYES2 plasmid to yield pDGSIRT1 (Figure 9A; 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 9B). 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 9B).

We also cloned the non-catalytic version of Sirt1, (Sirt1-H363Y), into the pYES2 plasmid to check which complemented phenotypes are due to the catalytic activity of the human protein (Figure 9A). The *SIRT1-H363Y* containing plasmid, pDGSIRT1-H363Y, has been used to transform *sir2Δ* cells in order to yeld the control strain *sir2Δ*+*. We further tested the toxicity of this non-catalytic Sirt1 version by spot assay (Figure 9B). The *sir*2Δ- (empty vector), *sir2*Δ+ (catalytic Sirt1) and *sir2*Δ+* (non-catalytic Sirt1 version) strains did not exhibit growth defects when plated on glucose medium or in galactose medium (Figure 9B).

Taken together, these data suggest that Sirt1 and the non-catalytic mutant Sirt1-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 Sirt1, (Figure 9B).

In order to verify the correct mRNA expression of *SIRT1* and *SIRT1-H363Y*, we analyzed their transcripts by reverse transcription PCR (Figure 9C). Moreover the presence of Sirt1 protein in strains transformed with empty plasmid (-), *SIRT1* gene-containing vectors (+) or *SIRT1-H363Y* gene (+*) was evaluated by Western blot (Figure 9D).

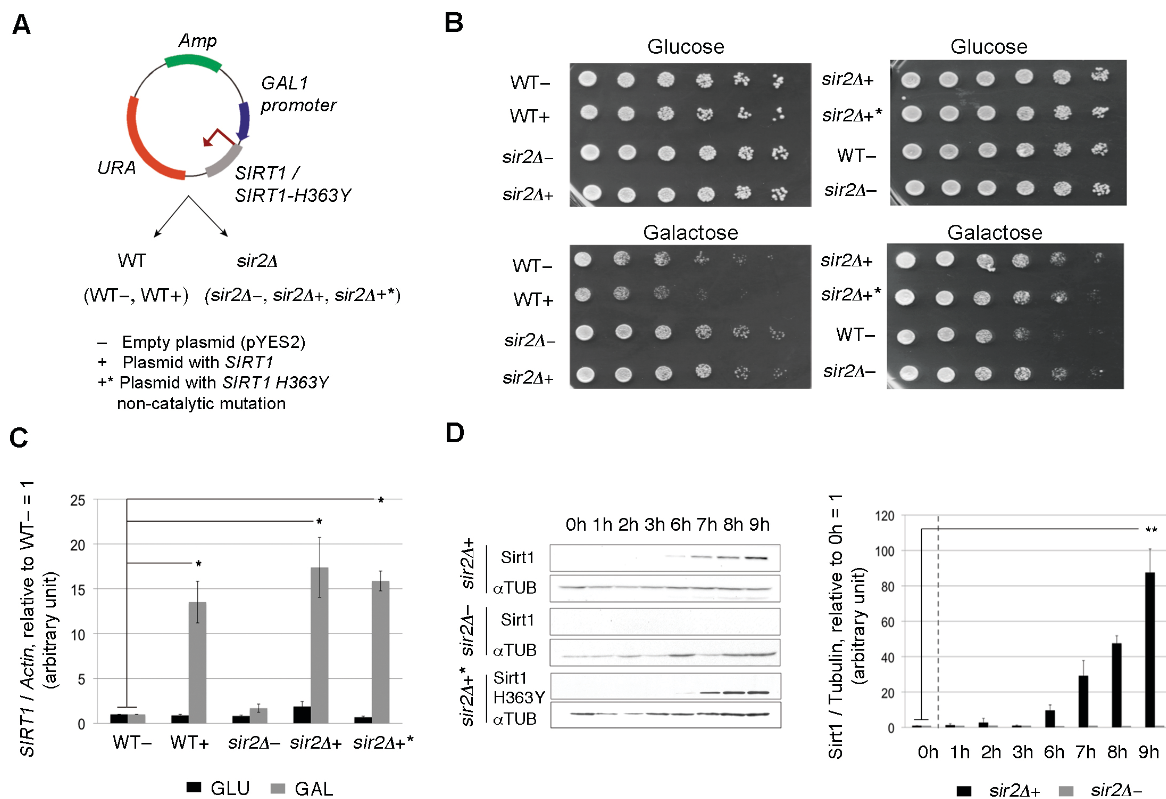

Figure 9: (A) Construct for yeast expression with *SIRT1* or *SIRT1-H363Y* under the inducible promoter GAL1 in pYES2 background. (+: *SIRT1* construct; −: empty plasmid, +*: *SIRT1-H363Y*). (B) Yeast spot test analysis of growth phenotypes during plasmid repression and induction conditions (glucose and galactose, respectively). For each strain five-fold serial dilutions were made and 5 μl were spotted onto minimal medium plates. (C) RT-PCR to control the induced expression of *SIRT1* transcript in galactose (WT+, *sir2Δ*+, *sir2Δ*+ * versus WT− : *p < 5%). (D) Western blot kinetics in *sir2Δ* mutant with *SIRT1* construct (+), empty plasmid (−) and *SIRT1-H363Y* (+*) to check the presence of the protein during galactose induction. Western blot quantification in *sir2Δ*− and in *sir2Δ*+, (Sirt1 levels: *sir2*Δ+ at hour 9 versus *sir2Δ*− or WT− in glucose at hour 0; **p < 1%). Asterisks indicate statistically significant differences; α = 0.05. (Percentages of p−value: *p< 5%, **p < 1%, ***p < 0.01%).

Figure 9 panel D show a time-course analysis of Sirt1 expression after switching the carbon source from glucose (GLU) to galactose (GAL) in the growth medium. Sirt1 expression was evident between 6 and 9 hours of GAL induction both in *sir2*Δ+ and *sir2*Δ+* cells. However, after overnight induction the protein was still abundantly present (Figure 12A). In order to analyze complementation phenotypes we also checked the protein expression level of the human Sirt1 and the non-catalytic version Sirt1-H363Y (Figure 12, C and D). Figure 12 show that the different forms of the human protein in *sir2*Δ+ and *sir2*Δ+* cells are expressed at the same level (Figure 12D).

2. *SIRT1-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 (Aparicio et al., 1991; Bryk et al., 1997; Smith and Shilatifard , 2007); ii) hyper-recombination at rDNA locus (Gottlieb and Esposito, 1989); iii) histone hyperacetylation at silenced loci (Robyr et al., 2002). 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 10A or Figure 6), (Santangelo et al., 1988; Kobayashi and Ganley, 2005; Li et al., 2006). 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, Sirt1 induction partially silences *NTS1r* transcription (Figure 10A).

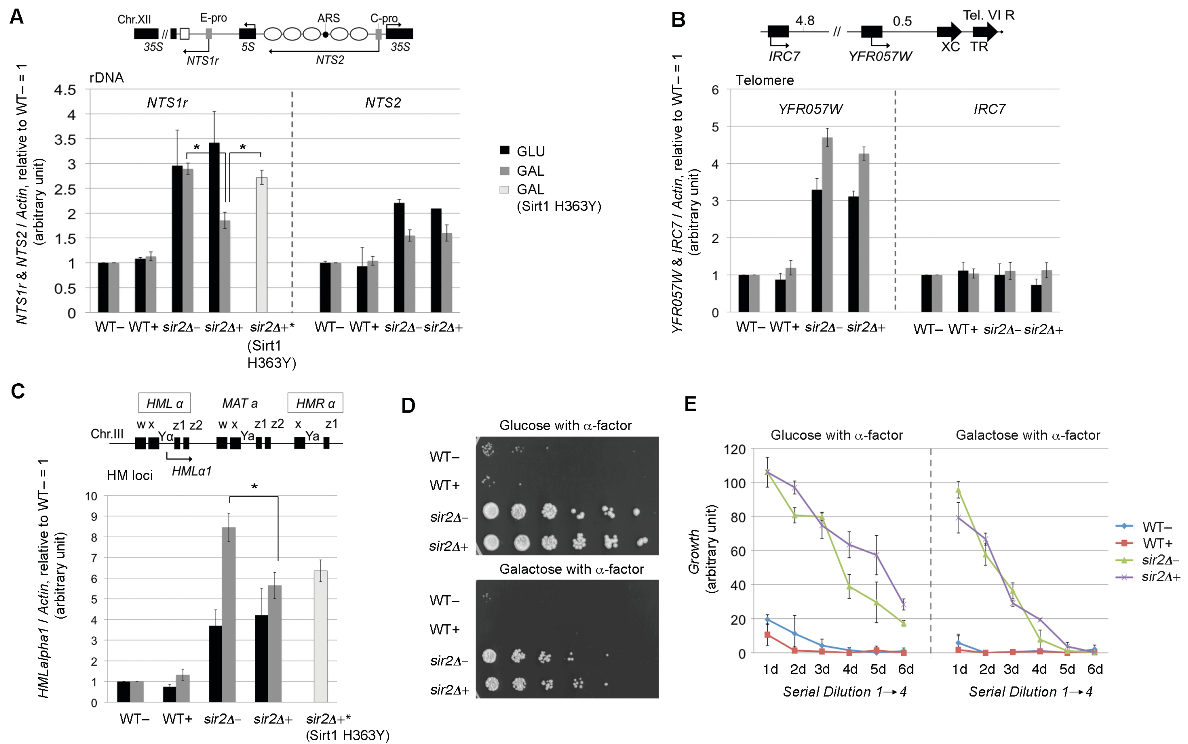

Figure 10: RT-PCR transcriptional analysis in WT and *sir2Δ* strains transformed with empty plasmid (-), *SIRT1* construct (+) or *SIRT1-H363Y* mutant construct (+*) both in repression (glucose) and induction (galactose) conditions. (A) rDNA locus: *NTS1r* and *NTS2*; (B) TEL VI locus: *YFR057W* and *IRC7;* (C) HM loci: *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 *sir2Δ*− and *sir2Δ*+ or between *sir2Δ*+* and *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 *HMLalpha1* silencing. Five-fold serial dilutions of mutant *sir2Δ* and WT cells transformed with *SIRT1*(+) or empty plasmid (−) were spotted onto minimal medium plates containing alpha-factor. (E) Quantification of yeast spot test in (D).

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+** strain that expresses the non-catalytic form of Sirt1. Figure 10A shows that in *sir2Δ+** strain, *NTS1r* transcription is maintained as in *sir2*Δ- strain, this experiment demonstrates that *NTS1r* RNA repression is strictly dependent on the catalytic activity of Sirt1 (*NTS1r*, *sir2Δ+* versus sir2Δ+*, * p<5 %). Moreover the silencing of *NTS1r* does not occur in *sir2Δ+* cells grown in glucose because the plasmid with *SIRT1* is repressed (Figure 10A, black histograms).

The same cDNAs were analyzed for telomeric silencing, by studying *IRC7* (sub-telomeric) and *YFR057W* (telomeric) genes (Figure 10, panel B for map details). The *IRC7* gene, not efficiently silenced by Sir2p (Ehrentraut et al., 2010), 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 10B, gray histograms), (*YFR057W* in galactose, *sir2Δ+* versus *sir2Δ-,* p>5 %).

We then analyzed the expression of the *HMLα1* gene (Figure 10C). We chose *HMLα1* transcript as indicator of HM loci derepression. Upon loss of silencing in *MATa* strain, both *HMLα1* and *HMLα2* transcripts are subsequently repressed by *α1* /a1 heterodimer but it has been demonstrated that *HMLα1* transcript remains still detectable (Matecic et al., 2006). Matecic et al. demonstrated that *HMLα1* expression is an optimal quantitative measure of HM loci expression in *sir2Δ* strain (Matecic et al., 2006).

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 Sirt1 expression but only a slight decrease of transcription not comparable to a wild-type repression state (Figure 10C).

We then analyzed *HMLα1* transcription in *sir2Δ+** control strain. Figure 10, panel C shows that *HMLα1* transcription decrease observed in *sir2Δ+* is not reverted in *sir2Δ+** strain (*HMLα1*, *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 (Matecic et al., 2006).

Alpha-factor is a pheromone that blocks cell growth when *HMLα1* is repressed (Harashima et al., 1989). Thus growth is allowed only when transcriptional silencing on *HMLα1* gene is lost. In figure 10D, effective growth is shown for all *sir2Δ* strain, regardless of *SIRT1* galactose-induced expression. In figure 10, panel E, are shown the quantifications of biological replicates of panel D. In galactose condition, the *sir2Δ*+ strain (violet line), presents high growth rate for each serial dilutions if compared to the WT (-,+) strains (blue or red line). Moreover there are not differences in the growth efficiency between *sir2Δ*+and *sir2Δ-,* indicating a repressed chromatin state of the HM loci in both strains.

This experiment indicates that the slight transcriptional decrease of *HMLα1* in *sir2Δ*+ strain, shown by RT-PCR (Figure 10C), is not enough to produce a non-growing phenotype in the *α*-factor assay (Figure 10 D and E).

Altogether, the data reported in figure 10 indicate that the human Sirt1 protein rescues silencing phenotypes in *sir2Δ* cells at the ribosomal locus (Figure 10A), while the HM loci and the telomeric regions do not present any complementation effect dependent on the catalytic activity of Sirt1 (Figure 10, B and C). On the contrary we demonstrated that the effect on the rDNA locus, specifically on the *NTS1r* locus*,* is strictly connected with the catalytic activity of Sirt1 (Figure 10A). However, the silencing efficiency obtained by *SIRT1* overexpression did not reach WT- levels in any the studied loci.

3. *SIRT1-sir2Δ* complementation: Reduction of ERCs formation

Together with the lack of transcriptional silencing at silent loci like HM, telomeres and rDNA, *sir2Δ* mutant shows hyperproduction of ERCs. The formation of extrachromosomal rDNA circles has been associated with increased recombination activity among ribosomal units, and considered a marker of replicative aging in *S. cerevisiae* (Park et al., 1999; Sinclair and Guarente, 1997).

In order to evaluate whether rDNA recombination, leading to ERCs formation, is reduced upon *SIRT1* expression in *sir2Δ* cells, we compared WT and *sir2Δ* cells transformed with the pDGSIRT1 (+) or the empty plasmid (−) as in the previous section. As further control, we also analyzed the *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 an rDNA intergenic spacer probe (map in Figure 11A), and visualized by autoradiography. In figure 11A, all WT(−,+) samples show a low amount of ERCs, while in *sir2Δ* samples the band corresponding to the ERCs species is evident. However, only in the *sir2Δ* sample transformed with pDGSIRT1(+) and grown in galactose (*SIRT1* overexpression), the amount of ERCs was reduced to WT− level. In addition this phenotype is reverted when we checked ERCs level in *sir2Δ*+* control strain. This control demonstrates that, as for *NTS1r* complementation phenotype, also ERCs repression is dependent on Sirt1 catalytic activity (Figure 11A).
Figure 11B reports the quantification of the ERCs species in galactose. (ERCs in galactose medium, *sir2Δ*+ *versus sir2Δ*-, ** $p<1$ %; *sir2Δ*+* *versus sir2Δ*+, ** $p<1$ %).
Data shown in figure 11 demonstrate that the overexpression of human *SIRT1* in *S.cerevisiae* reduces ERCs production in *sir2Δ* strain and that this reduction is dependent on its catalytic activity.

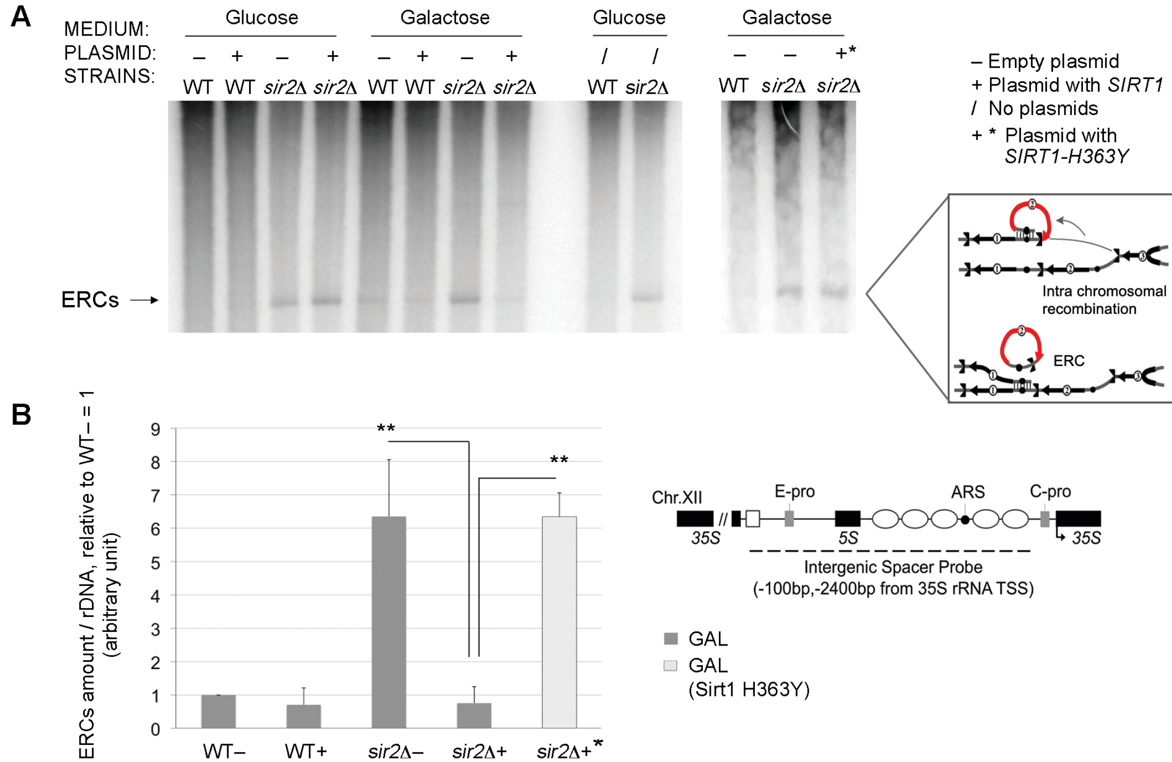

Figure 11: A) Southern blot analysis of ERCs species in WT and *sir2Δ* with *SIRT1* construct (+), *SIRT1-H363Y* mutant construct (+*), empty plasmid (−) or without plasmid (/) as control. Strains were grown both in *SIRT1* repression and induction conditions (glucose or galactose, respectively).DNA 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 10.

4. H3 lysine 9 acetylation is influenced by *SIRT1*

In order to analyze the *in vivo* role of Sirt1 in yeast cells, we performed a global acetylation analysis by quantitative immunoblotting. In these experiments we measured the effect of human *SIRT1* overexpression on the acetylation level of 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 (Nakagawa and Guarente, 2011; Vaquero et al., 2004; Zhang and Kraus, 2010; Imai et al., 2000).
Although yeast Sir2p has locus-specific roles such as the transcriptional silencing of rDNA, telomeres and HM loci (Sauve et al., 2006; Grunstein, 1997; Gartenberg, 2000), 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 (Grunstein, 1997; Lieb et al., 2001; Huang and Moazed, 2003). The common mechanism for all targeted loci is the deacetylation of histone tails (Robyr et al., 2002; Kurdistani et al., 2004). 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 (Vaquero, 2009). 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 (Vaquero, 2009). 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 12, 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 12B).

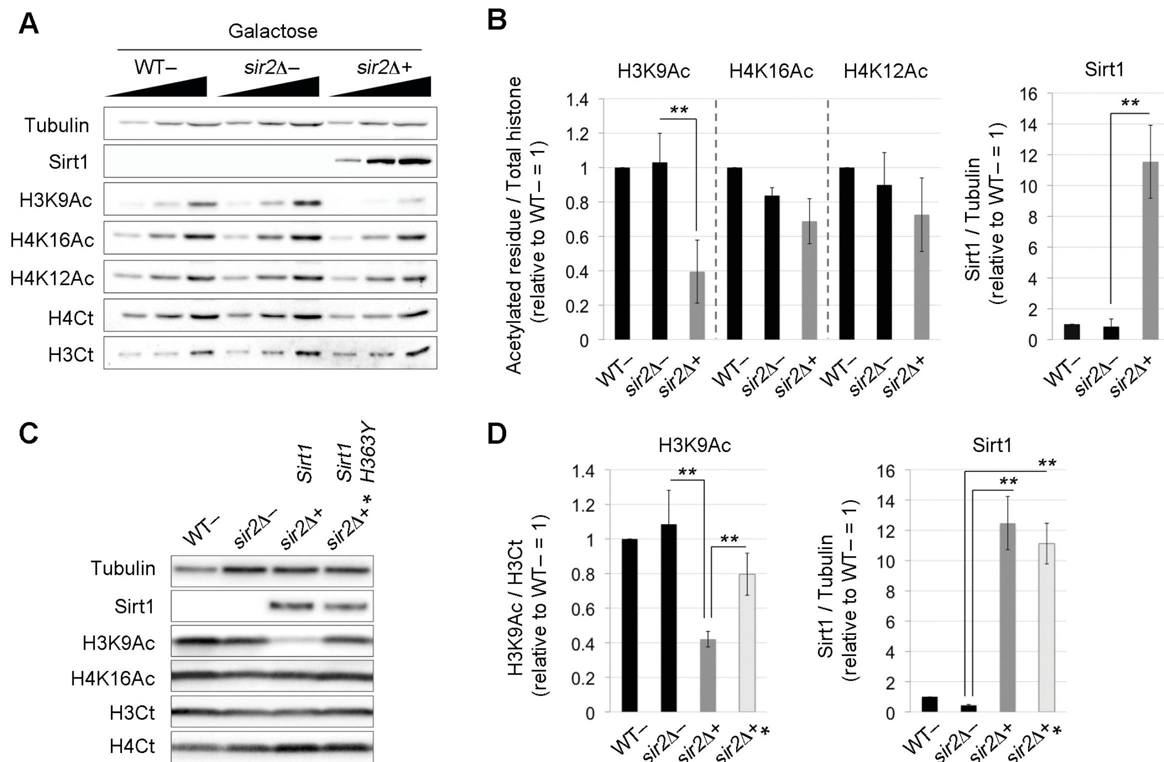

Figure 12: (A) Western blot analysis of H3K9, H4K16, H4K12 global acetylation after *SIRT1* overnight induction. *SIRT1*, H3Ct, H4Ct and α*TUB* hybridization were performed as induction or loading controls. (B) Western blot quantification of panel A. Histograms indicate averages and Std. Dev. bars from 4 biological replicates (H3K9Ac **p < 1%, H4K16Ac p = 10.3%, H4K12Ac p = 27.2%). (C) Western blot analysis of H3K9Ac on the same strains that in panels A and B but also with the strain *sir2Δ+** containing the non catalytic mutant *SIRT1-H363Y* as control. (D) Western blot quantification of panel C. Histograms indicate averages and Std. Dev. bars from 4 biological replicates, (H3K9Ac, *sir2Δ- versus sir2Δ*+, ** p<1%; *sir2Δ*+* *versus sir2Δ*+, ** p<1%; Sirt1/Sirt1-H363Y, *sir2Δ- versus sir2Δ*+ or *sir2Δ*+ *, ** p<1%; Sirt1/Sirt1-H363Y, *sir2Δ*+* *versus sir2Δ*+, p>>5%). Statistical analysis as in figure 10.

We also analyzed Sirt1 protein levels after overnight induction in galactose as an expression control (*sir2Δ+* versus *sir2Δ–* or WT–, ** p<1%) (Figure 12B). 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*+*, all grown in galactose medium. In figure 12, panel C and D show that H3K9Ac strong decrease, observed in *sir2Δ*+, is reverted in *sir2Δ*+* strain (H3K9Ac, *sir2Δ- versus sir2Δ*+, ** p<1%; *sir2Δ*+* *versus sir2Δ*+, ** p<1%). Moreover H3K9Ac decrease is properly caused by the enzymatic activity and not by the different levels of Sirt1 in the analyzed strains since *sir2Δ*+ and *sir2Δ*+* present the same expression level of the human protein (Sirt1/Sirt1-H363Y, *sir2Δ*+* *versus sir2Δ*+, p>>5%), (Figure 12D).

5. Histone deacetylation by Sirt1 at specific loci

Since we demonstrated that Sirt1 expression reduces the global acetylation level of H3K9 we further analyzed the acetylation of this residue by chromatin IP (Figure 13) within the three silenced yeast loci previously analyzed for specific RNA production (Figure 10).
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 (Ehrentraut et al., 2010).

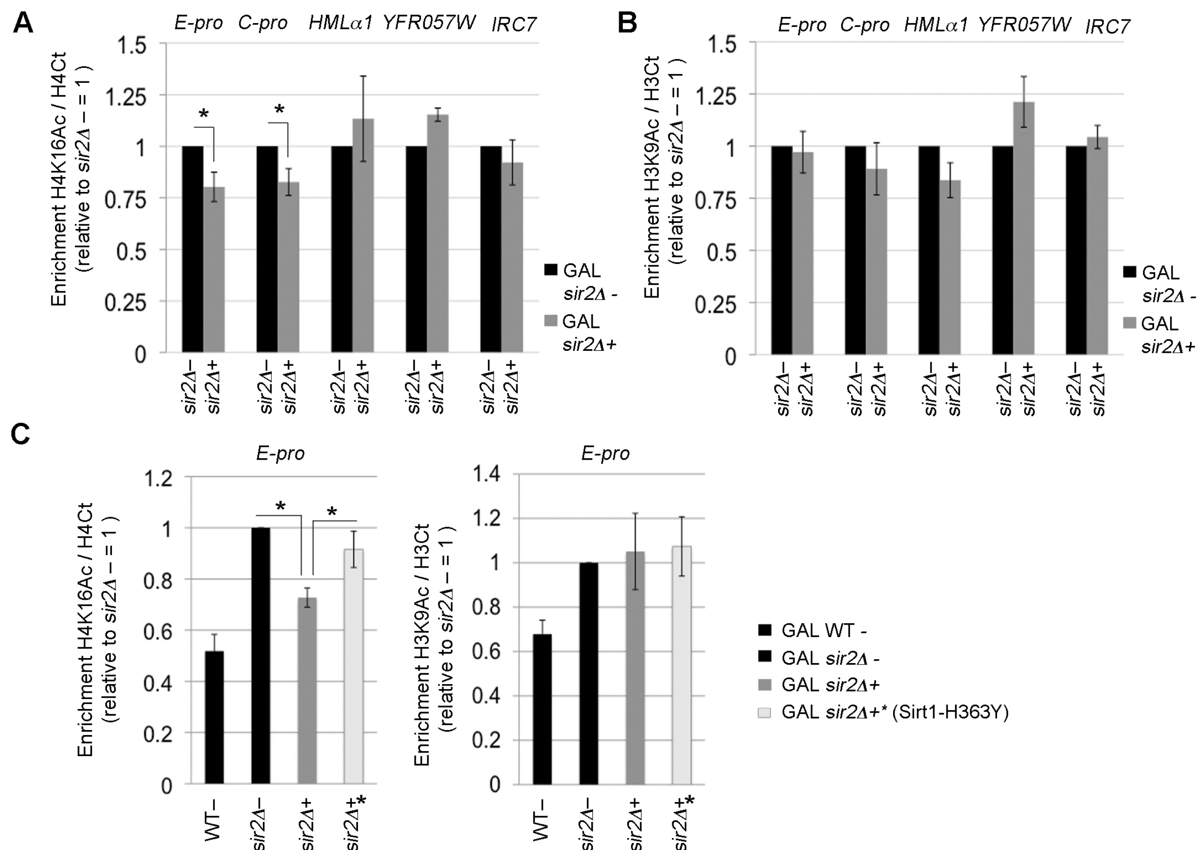

Figure 13: ChIp analysis of H4K16Ac (A) and H3K9Ac (B) at rDNA, HM loci, telomeric and sub telomeric regions in *sir2Δ*− and *sir2Δ*+ strains during *SIRT1* induction (galactose medium).

(C) ChIp 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 10.

Since *sir2Δ−* mutants alter nucleosome occupancy within rDNA (Li et al., 2006), 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 13).
This analysis revealed that Sirt1 expression reduced acetylation of H4K16 in *sir2Δ+,* to a significant degree only in specific regions. The graph in figure 13 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 13B).
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 (Figure 13).
In view of these observations we used the *sir2*Δ+* strain to check if the acetylation decrease at the E-PRO promoter was dependent on the Sirt1 enzymatic activity. Figure 13 shows that *sir2*Δ+* strain, on the E-PRO region, exhibits a reversion of the *sir2*Δ+ phenotype for H4K16Ac but not for H3K9Ac, (H4K16Ac, *sir2Δ+* versus *sir2Δ−,* *p<5%; *sir2Δ+** versus *sir2Δ+,* *p<5%)
We then analyzed also Sirt1 occupancy in the different studied regions (Figure 14A). In order to maintain the correct expression of Sirt1, we harvested yeast cells in galactose as for the ChIP analysis of acetylated residues and we used *sir2Δ-* as negative control. Figure 14, panel A, shows how Sirt1 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 14A), (Sirt1 enrichment, *sir2Δ*+ E-PRO versus *sir2Δ-* E-PRO, ** p<1 %). Here on E-PRO we previously observed three specific phenotypes: silencing of NTS1r (Figure 10A), reduction of ERCs formation (Figure 11, A and B) and acetylation decrease in H4K16 (Figure 13A). This experiment shows that Sirt1 is more enriched in this locus where the effective complementation occurs.
Experiments in the literature, showed a specific Sir2p positioning at the rDNA locus, (Figure 14B) (Huanh and Moazed, 2003; Huang et al., 2006).

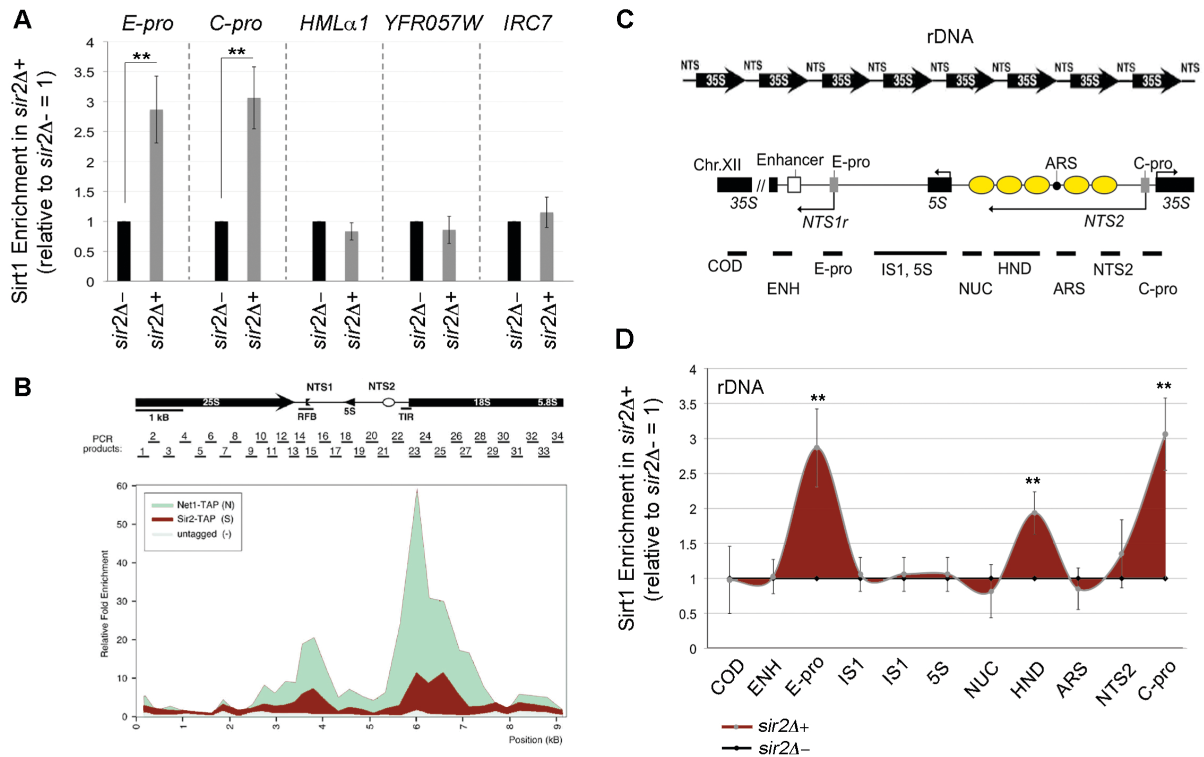

Figure 14: (A) ChIp analysis of Sirt1 enrichment at rDNA, HM loci, telomeric and sub telomeric regions in *sir2Δ*+ and *sir2Δ*- strains during *SIRT1* induction (galactose medium). Sirt1 enrichment for *sir2Δ*+ was referred to *sir2Δ*– levels =1. Histograms averages and Std. Dev. bars are representative of three technical replicates for at least three biological replicates performed. (B) Sir2p and Net1p distribution at the rDNA locus accoriding to Huang and Moazed study (2003). (C) Schematic map showing PCR-amplificated regions in ChIp analysis of Sirt1 positioning in the rDNA. (D) ChIp analysis of Sirt1 enrichment at rDNA intergenic spacer regions in *sir2Δ*+ and *sir2Δ*- strains during *SIRT1* induction (galactose medium). Sirt1 enrichment for *sir2Δ*+ (in red) was referred to *sir2Δ*– levels =1 (in black). Statistical analysis as in figure 10.

These experiments demonstrated that Sir2p enrichment occur in regions within the rDNA where the proteins Net1p and Fob1p are specifically associated (Figure 14B). In order to check human Sirt1 distribution on the rDNA locus, it has been performed Sirt1 chromatin immunoprecipitation all over the intergenic spacer region (map details in figure 14C). Figure 14D represents Sirt1 distribution within the rDNA. In addition to the previous experiment where it has been identified Sirt1 enrichment peaks at the E-pro and C-pro (Figure 14A), we discovered a new region, between the ARS and the 5S gene, where Sirt1 is associated (Figure 14D). This sequence that we called HND (High Density Nucleosome region) has been characterized in the literature to have high nucleosome occupancy, (map details in figure 14C), (Li et al., 2006). In figure 14D is shown Sirt1 profile along the intergenic spacer region, the red profile represents the Sirt1 occupancy in *sir2Δ*+ strain normalized to *sir2Δ*- (black profile). Statistics analysis revealed a significant enrichment for Sirt1 in the HND region, (Sirt1 enrichment, *sir2Δ*+ HND versus *sir2Δ*- HND, ** p<1 %). Identified the Sirt1 enrichment also in the HND region, we analyzed acetylation levels of H4K16 and H3K9 as for E-pro and C-pro cryptic promoters in figure 13. Figure 15, panel A, shows that also in this region H4K16Ac decreases upon Sirt1 induction in *sir2Δ*+ if compared to *sir2Δ*- (H4K16Ac, *sir2Δ+* versus *sir2Δ−,* **p<1%). Moreover the analysis of the strain *sir2Δ+** revealed that this decrease is dependent on the activity of the human protein, (Figure 15A), (H4K16Ac *sir2Δ+** versus *sir2Δ*+*,* *p<5%). However also in this experiment the histone mark H3K9Ac did not show any variation among the analyzed strains (*sir2Δ*-, *sir2Δ+, sir2Δ+**)*,* (Figure 15A). As negative control we analyzed H4K16Ac in the regions that in the ChIp analysis did not present a strong enrichment of Sirt1: the enhancer (ENH) and the autonomously replicating sequence (ARS), (Figure 14D). Coherently with Sirt1 occupancy the H4K16Ac levels in these rDNA regions were not affected by Sirt1 induction, (Figure 15B), (H4K16Ac, *sir2Δ+* versus *sir2Δ−,* p>>5%).
These experiments highlight that the acetylation profile at the intergenic spacer region, within the rDNA, matches the Sirt1 occupancy. Moreover, data on the non-catalytic mutant strain *sir2Δ+** further confirm the relationship between the H4K16 acetylation state in the rDNA and the activity of the human protein Sirt1 in this locus (Figure 13C and 15A).
Taken together, these data further indicate that Sirt1 is able to act on the *S.cerevisiae* chromatin environment. However, four main points should be underlined: i) histone acetylation decreases only at the ribosomal locus; ii) At this locus Sirt1 is more enriched

compared with the other regions iii) Sirt1 histone deacetylation differs from that of Sir2p in a region-dependent manner; vi) the locus where H4K16 acetylation is decreased, the rDNA, is the only region in which silencing is restored almost to a wild type level.

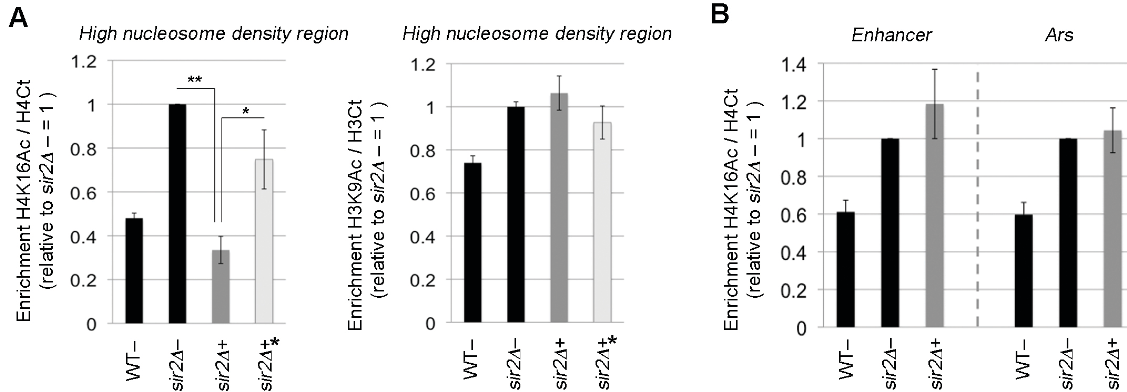

Figure 15: (A) ChIp analysis of H4K16Ac and H3K9Ac at the high nucleosome density region (HND) in WT−, *sir2Δ*−, *sir2Δ*+ and *sir2Δ*+*. (B) ChIp analysis of H4K16Ac and H3K9Ac at the enhancer and Ars regions in WT−, *sir2Δ*−, *sir2Δ*+. Acetylation enrichment for H4K16 and H3K9 were normalized to H4 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 10.

DISCUSSION

S. cerevisiae Sir2p and human Sirt1 share a consistent amino acid identity (Vaquero, 2009). Given the prominent role that Sirt1 plays as a master regulator of basic pathways both in healthy and pathological conditions (Saunders and Verdin, 2007; Vaquero, 2009; Li, 2013), it would be extremely important to find further chemical regulators of this protein. However, to date, *in vivo* screening assays evaluating Sirt1 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 Sirt1. 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 Sirt2 that has lower percent identity with yeast Sir2p than Sirt1 (Vaquero, 2009; Sherman et al., 1999; Perrod et al., 2001). Sherman et al. described the molecular cloning of a human sirtuin in *S. cerevisiae* sir2Δ mutant (Sherman et al., 1999). Specifically they did not express Sirt1 but hSir2Ap, also known today as Sirt2 (Sherman et al., 1999; Perrod et al., 2001). They discovered that only a chimera with N/C-terminal of yeast Sir2p and the core portion of hSir2A (human Sirt2) was able to replace Sir2p activity on a subset of genetic loci. Actually, Sirt1 shows a higher percent identity with yeast Sir2p than Sirt2 and is considered the phylogenetic ortholog of yeast Sir2p (Vaquero, 2009). Although Sirt2 still shows a high similarity with the yeast protein Sir2p, it is considered more similar to another yeast sirtuin, Hst2p (Vaquero, 2009).

In this study we used Sirt1, 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 *sir2Δ* 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 Sirt1 (Figure 10A).

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 10A and 10C). Furthermore, we tested, by the transcriptional analysis of the non-catalytic mutant Sirt1-H363Y, whether the complementation phenotypes we observed were caused by the enzymatic activity of Sirt1. This analysis revealed that the *NTS1r* 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 *NTS1r* transcription is restored as in *sir2Δ-,* while *HMLα1* is comparable to *sir2Δ+* (Figure 10A and 10C).
In addition the alpha factor growth assay revealed that the *sir2Δ+* strain did not decrease 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 10C and 10D,E). 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 α-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 (Figure 10B).
In addition this study shows, together with the decrease in *NTS1r*, 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 11A and 11B). Although the relationship between ERCs production, rDNA intergenic spacer transcription (*NTS1r* and *NTS2*) and its hyperacetylation has not been elucidated yet, they seem to be closely correlated (Cesarini et al., 2012; Pasero et al., 2002).
Our data suggest that Sirt1 triggers the downregulation of the *NTS1r* intergenic transcript by deacetylating histones within the rDNA region (Figure 13C). 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 11). In the literature, the mechanism underlying the relationship between replication activity and ERCs formation has been clearly elucidated (Ganley et al., 2009). In addition, recent evidence indicates that several mutants show increased amounts of ERCs coupled with increased ncRNA production at rDNA (Cesarini et al., 2012).

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 12). Specifically by Western blot analysis, we determined that Sirt1 is able to significantly reduce the global acetylation of H3K9 (Figure 12). 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 (Figure 12C).
The H3K9 acetylation decrease is dependent on the catalytic activity of Sirt1 but it appears to not affect the studied loci: HM loci, telomeres and rDNA locus (Figure 13B and 15A). We hypothesize that Sirt1 may regulates, through deacetylation, the activity of a yeast acetyltransferase specific for H3K9 that act on different regions. This hypothesis would explain why this effect is dependent on the Sirt1 activity even if we did not observed a decrease in H3K9Ac on the analyzed regions. In support of this hypothesis there are studies that demonstrated how the HATs activity is highly regulated from lysine acetylation state (Brittany et al., 2011).
Importantly this work deciphers 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 13A) whereas HM loci, telomeric and subtelomeric genes were unaffected by Sirt1 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 (Straight et al., 1999; Marston et al., 2003). 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 14). 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 observed 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 (Figure 14 A). Moreover we characterized Sirt1 occupancy all over the intergenic spacer regions within the rDNA locus where we observed the strongest complementation phenotype (Figure 14C, 14D). Interestingly Sirt1 appears to have a very specific distribution in this region (Figure 14 D). In particular, the enrichment peaks were identified at the E-pro, C-pro and at the high nucleosome density region, the HND (Figure 14D). Here we observed a decrease in H4K16Ac that is efficiently restored in the non-catalytic mutant *sir2Δ+** strain (Figure 13 and 15). 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. Sirt1 in fact presents a very specific profile that could shed light to new protein partners both in yeast and human. The analysis of the difference between Sir2p occupancy on the rDNA (literature data, Figure 14B) and our experiments on Sirt1 indicate that these proteins may share protein partners in this locus. Specifically Sir2p it is recruited by Net1p in the RENT complex at the E-pro and with a not characterized mechanism in the region shared by the C-pro and the promoter of the RNA polymerase I (Straight et al., 1999; Huang and Moazed, 2003; Huang et al., 2008). Moreover the protein Fob1p appears to be necessary for the recruitment of the RENT complex in the E-pro region but not on the promoter of the 35S (Huang and Moazed, 2003; Huang et al., 2008). This data underline the existence of another mechanism of recruitment of the RENT complex on the rDNA locus. Interestingly Sirt1, in human cells, interact with the promoter of the RNA polymerase I recruited by the Nucleomethylin protein, orthologue gene of the yeast Rrp8, transcriptional factor involved in the processing of the rRNA (Murayama et al., 2008). It is not clear if the human RNA polymerase-I is able to interact directly with Sirt1. Since the RNA polymerase I is one of the most conserved protein in the evolution it is easy to speculate that human Sirt1 may be able to interact directly with the yeast RNA Pol-I in the C-pro sequence. Overall our data also confirmed partially the Kobayashi model about the connection between the non-coding RNAs transcription and the ERCs production, (Figure 7), (Kobayashi, 2001). In fact we observed, upon Sirt1 induction in *sir2Δ* strain, a decrease of the *NTS1r* and reduced ERCs level (Figure 10A and 11B). In addition we characterized a reduced H4K16Ac levels in all the Sirt1-associated regions: E-

pro, C-pro and the HND region. Interestingly it has been demonstrated that the hyperacetylation of rARS sequences is necessary for the replication process within the rDNA (Chiani et al., 2006; Weinreich et al., 2004; Vogelauer et al., 2002). Moreover the increased frequency of the activation of the rARS has been linked with high rDNA intrachromosomal recombination rate and enhanced ERCs production (Ganley et al., 2009). Then, we hypothesized that the Sirt1 enrichment in the HND region could affect the acetylation level also in the near ARS sequence (Figura 14D) altering ERCs production. However we did not observed H4K16 acetylation decrease in this region (Figure 15B). This experiment indicates that the ERCs reduction we observed, upon Sirt1 induction, is attributable only to the *NTS1r* repression (Figure 10 A) confirming Kobayashi model in figure 7. In the next future would be interesting to analyze the *sir2Δ*-Sirt1 complementation in yeast mutants for the endogenous Sir2p recruiter to check if the complementation still occurs. Co-immunoprecipitation experiments will be also necessary to identify which yeast proteins interact with human Sirt1. In conclusion, we have hereby shown that *SIRT1* complementation in *sir2Δ* mutant cells exhibits heterogeneous molecular phenotypes, such as histone deacetylation, 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 Sirt1 in *S. cerevisiae* could be important to shed light on new aspects of this human protein. The study of protein partners of Sirt1 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 Sirt1 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 specific cross-talk between Sirt1 and yeast chromatin and that probably *in vivo* screenings, focused on H3K9Ac global decrease or ERCs decrease, would be possible in yeast. Moreover a recent study produced a viable strain with all the yeast sirtuins deleted: *sir2Δ, hst1-4Δ* (Chou et al., 2008). Since the main problem to find specific chemicals is the sirtuin redundancy, the *sir2Δ hst1-4Δ* strain, in light of the *sir2Δ-SIRT1* complementation, would be a powerful tool for future *in vivo* drugs screening. To date are available only *in vitro* assays 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.

CONCLUSION

The scientific importance of sirtuins in the biomedical field is highlighted by their involvement in many molecular processes such as in the pathogenesis and development of many diseases. In addition to the discovery of their involvement in mediating the effects of caloric restriction on the increased lifespan has opened up the drug testing on ageing related-diseases. The recent discovery of molecules that target this enzyme class and the elucidation of their molecular mechanisms is the starting point for the development of pharmacological protocols. Although the growing number of studies on this enzymes, drugs that target specifically each sirtuin are still missing. The redundancy of this gene family within the organisms and the involvement of each sirtuin in more processes it makes difficult to develop targeted and efficacy therapies.

Nowadays, synthetic combinatorial methods, combined with rapid screening assays, have advanced the ability to synthesize large numbers of compounds. Drug candidates resulting from many combinatorial approaches have also often tended not to have drug-like properties because of poor physicochemical properties (Carroll et al., 2009). One approach to circumvent this problem would be to use *in vivo* models directly in the discovery phase to identify molecules with desired biological profiles eliminating those compounds with poor absorption, distribution, metabolism and pharmacokinetic properties. At present, Sirt1 activity assays are based on *in vitro* deacetylation reaction with peptide substrates (Howitz et al., 2003). We therefore decided to express the *SIRT1* gene in *S. cerevisiae* since the yeast model presents many advantages (Forsburg, 2001; Grunstein, 1997), i.e. the availability of specific mutants, the highly characterized genetic environment and the easy genetic manipulation. In order to study human Sirt1 in yeast we studied the complementation phenotype in the mutated yeast strain for the orthologous gene *SIR2*. The yeast *sir2Δ* mutant shows a series of different phenotypes, all depending on the deacetylase activity of Sir2p. We analyzed the three silent loci where normally Sir2p acts: ribosomal DNA, telomeres and the mating type loci. By strong *SIRT1* overexpression in *sirΔ* 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 (Figure 16). The complementation at the other studied loci: HM loci, telomere and sub-telomere does not occur (Figure 16). The analysis of the non-catalytic mutant Sirt1-H363Y revealed that both NTS1r and ERCs decrease was attributable to the Sirt1 catalytic activity. Coherently it appears that the complementation mechanism occurs by histone acetylation modificiation. We observed a decrease in H4K16Ac within specific regions at rDNA locus: E-pro, C-pro, High density nucleosome region (Figure 13 and 15). In addition Sirt1 is more enriched in the rDNA locus where the effective complementation occurs and is not detected on the other regions where the endogenous yeast Sir2p is recruited. The specific Sirt1 distribution on the rDNA indicates that the human Sirt1 may interact with endogenous yeast proteins in this region. On the rDNA Sirt1 presents a very specific profile that could shed light to new protein partners both in yeast and human. Overall, our observations indicate that: i) there is a specific cross-talk between Sirt1 and yeast chromatin and that, indeed Sirt1 is able to complement specific molecular phenotypes of the *sir2Δ* mutant ii) the specific phenotypes that Sirt1 displays in *S.cerevisiae* highlight that Sirt1 is able to perform its deacetylas activity on the yeast chromatin and thus in our opinion the *in vivo* screening of Sirt1 activity would be possible in this organism. Moreover the study of protein partners of Sirt1 within the complemented loci, may bring to light in the future new protein partners both for Sirt1 in humans and for Sir2p in *S.cerevisiae* elucidiating novel regulatory mechanism useful to develop specific epigenetic therapies.

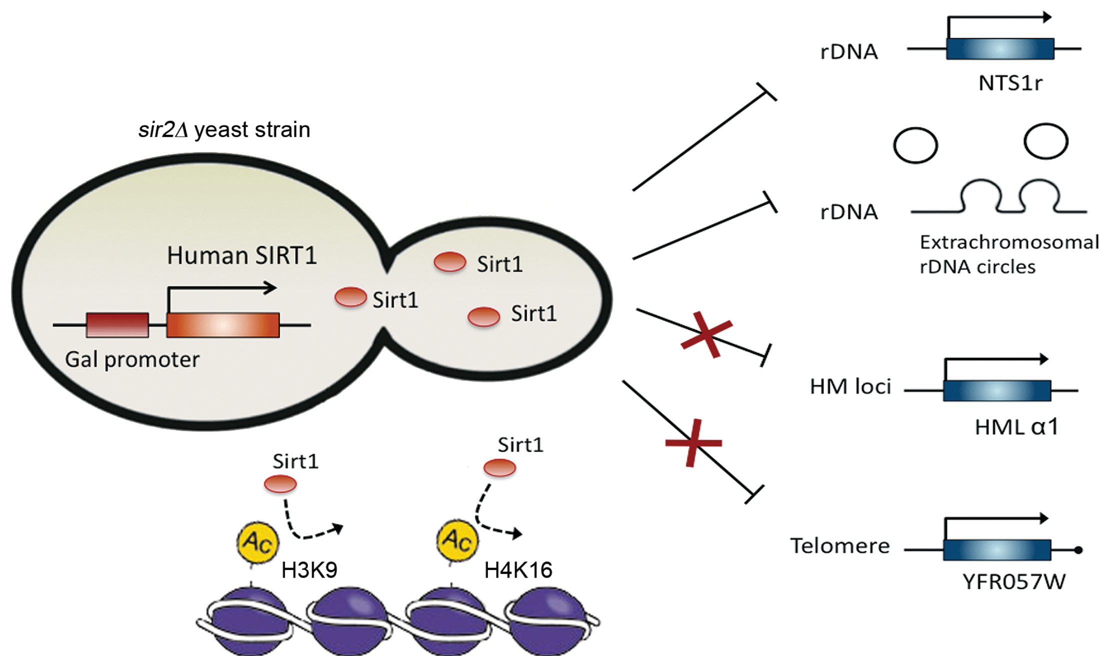

Figure 16: Human Sirt1 complementation of *sir2Δ* mutant phenotypes: transcriptional silencing at rDNA is restored, extrachromosomal rDNA circles repressed and histone acetylation at H3K9 and H4K16 decreased. The complementation at the other studied loci: HM loci, telomere and sub-telomere does not occur.

MATERIALS AND METHODS

1. Yeast strains

WT (W303-1a) (Mat a; ade2-1; ura3-11; trp1-1; leu2-3.112; Can1-100).
WT – (WT strain, W303-1a, with pYES2 Plasmid.), (Mat a; ade2-1; ura3-11; trp1-1; leu2-3.112; Can1-100; pYES2).
WT + (WT, W303-1a, strain with pDGS1 Plasmid.), (Mat a; ade2-1; ura3-11; trp1-1; leu2-3.112; Can1-100; pDGS1).
sir2Δ (W303-1a (NOY1045, kindly provided by M. Nomura), (Mat a; ade2-1; ura3-11; trp1-1; leu2-3.112; Can1-100; *sir2*::LEU2).
sir2Δ – (*sir2Δ*, W303-1a, strain with pYES2 plasmid.), (Mat a; ade2-1; ura3-11; trp1-1; leu2-3.112; Can1-100; sir2::LEU2; pYES2).
sir2Δ + (*sir2Δ*, W303-1a, strain with pDGS1 Plasmid.), (Mat a; ade2-1; ura3-11; trp1-1; leu2-3.112; Can1-100; sir2::LEU2; pDGS1).
sir2Δ +* (*sir2Δ* (W303-1a) strain with pYES2 plasmid with pDGS1-H363Y Plasmid.), (Mat a; ade2-1; ura3-11; trp1-1; leu2-3.112; Can1-100; sir2::LEU2; pDGS1-H363Y).

– = empty plasmid pYES2
+ = pYES2 backbone with FLAG-*SIRT1* insert (called pDGS1)
+* = pYES2 backbone with FLAG-*SIRT1-H363Y* insert (called pDGS1-H363Y)

2. Plasmids

Addgene plasmid 1791: pECE mammalian expression vector; *SIRT1*-FLAG. (Brunet et al.; Science, 2004)
pYES2: 2μ, URA3, AMP, GAL1 promoter, Poli-Linker CYC1 terminator. (T.Rinaldi et al.; Mol Biol Cell. 1998)
pDGS1: 2μ, URA3, AMP, GAL1 promoter, Poli-Linker, CYC1 terminator, h*SIRT1*-FLAG.
pYMG1: Centromeric CEN6/ARS4. pRS316 backbone + 2212bp yeast rDNA fragment. (F.Cioci et al.; J. Mol. Biol. 2002)

pDGS1-H363Y: 2µ, URA3, AMP, GAL1 promoter, Poli-Linker, CYC1 terminator, hSIRT1-H363Y-FLAG.
Addgene plasmid 1791 SIRT1-H363Y: pECE mammalian expression vector; SIRT1-H363Y-FLAG. Sirt1-H363Y,deacetylase domain mutation. (Brunet et al., Science, 2004)

3. Oligonucleotide sequences

Oligonucleotide	*Oligonucleotide sequence (5' to 3' direction)*
ACT1-182-F	ACGTTCCAGCCTTCTACGTTTCCA
ACT1-182-R	AGTCAGTCAAATCTCTACCGGCCA
ACT1-450-F	GGTATTGTCACCAACTGGGACGAT
ACT1-450-R	GAAGTCCAAGGCGACGTAACATAG
SIRT1-F	GACAACTTGTACGACGAAGACGAC
SIRT1-R	GGAGTCCAGTCACTAGAGCTTGCA
YFR057W-F	CTATAGTAAGTGCTCGGCCAAGTC
YFR057W-R	CTCTTCTGAGACGAAGTCGTTGCT
NTS1r-F	GCACCATCAGAGCGGCAAAC
NTS1r-R	CGCTGCCTCTCTGGAAC
HMLα1-F	CTTCCCAATATCCGTCACCACGTA
HMLα1-R	TCCAGATTCCTGTTCCTTCCTCTC
IRC7-F	CAACCGTCATTTTCCTCGAAAGCC
IRC7-R	GCAATGCTAATTGACAGTCCTCGG
EPRO-F	TGTTAGTGCAGGAAAGCGGGAAGGA
EPRO-R	GCACTATCCAGCTGCACTCTTCTTC
CPRO-F	AATAGTGAGGAACTGGGTTACCCG
CPRO-R	TTGTACTCCATGACTAAACCCCCC
NTS2-F	ATGTTCAGTAGGTGGGAGTGAGAG
NTS2-R	CATCCGGTGCCGTAAATGCAAAAC
HND-F	ATGTTCAGTAGGTGGGAGTGAGAG
HND-R	GTGACGGAAATACGCTTCAGAGAC

ENH F	CTCTGATGGTGCGGAAAAAACTGC
ENH R	GGGGCCTAGTTTAGAGAGAAGTAG
NUC-F	ATGTTCAGTAGGTGGGAGTGAGAG
NUC-R	CATCCGGTGCCGTAAAT GCAAAAC
COD-F	GGGCTCATGGA GAACAGAAATCTC
COD-R	CCGAATGAACTGTTCCTCTCGTAC
ARS-F	AAAGTGGACAGAGGAAAAAGGTGCG
ARS-R	GTGACGGAAATACGCTTCAGAGAC
IS15S-F	TGTTAGTGCAGGAAAGCGGGAAGGA
IS15S-R	CATCCCGGTGCCGTAAATGCAAAAC

4. Culture media and conditions

Yeast cells were grown and manipulated according to standard protocols (Verdone et al., 1996; Sherman et al., 1983). 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. Restriction enzymes were purchased from Roche and New England Biolabs; Taq polymerase was from Eppendorf; radiochemicals were from Amersham.

***5. SIRT1* molecular sub-cloning**

Given the presence of a HindIII restriction site at the N-terminal of FLAG-h*SIRT1 (or FLAG-hSIRT1-H363Y)* in addition to that inside the gene, we performed a controlled kinetics restriction in order to isolate the complete insert from the plasmid addgene 1791. First, 1791

was digested with XbaI: 390 ng/μl plasmid, cut Buffer H (0.05 Tris-HCl M, 0.1 M NaCl, 0.01 M MgCl2, 1mM dithiothreitol pH 7.5 at 37°C), 1 U/μl XbaI (Roche) at 37°C for 3h. Then the restricted plasmid was digested with HindIII: 390 ng/μl plasmide, cut Buffer B (10 Tris-HCl mM, 0.1 M NaCl, 5 mM MgCl2, 1 mM 2-mercaptoethanol pH 8 at 37°C), 0.2 U/μl di HindIII (Roche) performer at 37°C. We performed three samples (0.5h, 1h, 1.5h) to check the kinetics point where M2-FLAG-h*SIRT1* was not cut inside the coding sequence. The pYES2 plasmid was digested as 1791 palsmid, but the HindIII reaction was performed, without sampling during the kinetics.
The pYES2 plasmid backbone was subjected to dephosporilation reaction as follows: 80 ng/μl of restricted pYES2, dephosphorilation buffer 1X (0.05 M Tris-HCl, 0.1 mM EDTA, pH8.5 at 20°C), 0.06 U/μl of alkaline phosphatase (Roche), at 37°C for 30 minutes. DNA ligase reaction between dephosphorilated pYES2 backbone and FLAG-h*SIRT1* insert, both HindIII-XbaI digested, was carried out in these conditions: 2 ng/μl pYES2 plasmid, 2 ng/μl FLAG-h*SIRT1* insert, Buffer T4 DNA ligase (50 mM Tris-HCl, 10 mM MgCl2, 10 mM DTT, 1 mM ATP), 0.1 U/μl T4 DNA ligase. Ligase reaction was performed at room temperature for 4 hours. Cell transformation was performed with 5 μl of ligase reaction and 50 μl of HIT-DH5α competent cells from Invitrogen. Cells were vortexed, placed in ice for 20 minutes and then at 42°C for 1 minute. Positive clones were selected on LB plates selective for ampiciliin resistance (1% Bacto peptone, 0.5% yeast extract, 0.5% NaCl, 2% Agar, 0.05 mg/ml ampicillin). Cells were grown at 37°C for 15 hours. Clones were controlled by enzymatic restriction and PCR for their correct integration in the backbone plasmid.

6. RT-PCR

RNA from logarithmically growing cultures was isolated as previously described (Verdone et al., 1996). 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 *SIRT1, NTS1r, YFR057W, IRC7* and *HMLα1* mRNA expression levels (50ng/μl Random hexamers at 25°C 10min for *NTS2*), by incubation with 30 U of SuperScript III Reverse Transcriptase (Invitrogen, Cat.No. 18080-093) for 30 min at 50°C, followed by heating inactivation at 85°C for 5 min.
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, HML1α-F/ HML1α-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 68°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.PB10204) was added to the reaction mixture (0.04 μCi/μl). Template titration for each sample was performed to evaluate the linear range of amplification. The amplified fragments were separated on a 6% polyacrylamide gel. For quantification ImageJ 1.43u was used. Each cDNA band intensity was normalized to *ACT1*. For mRNAs expression analysis data, average (with standard deviations, SD) refers to at least three independent biological replicas: (WT+; *sir2Δ*−; *sir2Δ*+)/(WT−). Student's t test was applied for statistical analysis; α = 0.05.

7. ERCs analysis

Yeast cells grown to exponential phase (OD600 0.5/ml) were lysed with lysis buffer (1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA) and glass beads (Sigma-Aldrich, G9268-500G) by vigorous shaking for 1 hour at 4°C. The DNA was then purified by three phenol–chloroform–isoamylic alcohol (24:24:1 [vol/vol/vol]) extractions followed by ethanol precipitation. Proteinase K (0.2 μg/μl) and RNase A (0.15 μg/μl) treatments were also performed. A 5-μg amount of the recovered DNA was run in 0.8% agarose gel electrophoresis (1.75 V/cm) and transferred onto a nylon membrane (GE Healthcare, Cat.No. RPN203B). Hybridization was performed with an rDNA probe annealing from −100 to −2400 base pairs upstream of the RNA polymerase I transcription start site. For image acquisition the Typhoon 9410 Variable Mode Imager (GE Healthcare) was used. The band intensities corresponding to ERCs were measured with ImageJ 1.42q (National Institutes of Health), and normalized to the hybridized bulk rDNA (loading control). ERC values were then divided by the calculated WT− level. Means and error bars refer to five independent biological replicates: (WT+; *sir2Δ*−; *sir2Δ*+)/(WT−). Student's t test was applied for statistical analysis; α = 0.05 (Cesarini et al., 2012).

8. Western blot

Yeast cells were grown to the exponential phase and lysed with NP40 buffer (0.2% NP40, 200 mM NaCl, 50 mM Tris pH 7.5, 1 mM PMSF, and protease inhibitors) and glass beads (Sigma-Aldrich, G9268-500G) by vigorous shaking for 1 h at 4°C. A 40-µg amount of protein extract was separated by a 13% SDS-polyacrylamide gel followed by immunoblotting. The PVDF membranes (Millipore, Cat.No.ISEQ20200) were incubated overnight at 4°C with primary antibodies: rat anti-Tubulin (Santa Cruz Biotechnology, sc-53030) was used at 1:5000 dilution; rabbit anti-acetyl H3K9, H4K12 at 1:7000 dilution (Upstate/Millipore, Cat.No.07-352 and 07-595); rabbit anti-acetyl H4K16 at 1:6000 (Santa Cruz Biotechnology, sc-8662-R); rabbit anti-acetyl H4Ct at 1:500 (Santa Cruz Biotechnology, sc-8658-R); rabbit anti-acetyl H3Ct at 1:500 (Santa Cruz Biotechnology, sc-10809); mouse anti-Flag 1:3000 (Sigma-Aldrich, Cat.No.F3165). Secondary antibodies: 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 were normalized to total histone levels (H3K9/H3Ct, H4K16/H4Ct, H4K12/H4Ct) and to WT− acetylation levels, WT− = 1 (Figure 12). Sirt1 Western Blot kinetics on *sir2Δ(*−,*+,+*)* strain (Figure 9) 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 replicas. Student's t test was applied for statistical analysis; $\alpha = 0.05$.

9. 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 (Cesarini et al., 2012). A 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 aspecific 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, *HMLalpha1* 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 (Cesarini et al., 2012).

Briefly, in figure 13 and 15, acetylation fold enrichment values, for all regions, were calculated as follows: [rDNA(IP)/*ACT1*(IP)]/[rDNA(input)/ *ACT1*(input)]. In figure 13 and 15 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 (acetylated histone)/(total histone) for values from the following calculation: [rDNA(IP)/*ACT1*(IP)]/[rDNA(input)/ *ACT1*(input)]. The isogenic *sir2Δ–* (with 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 14) for all regions were calculated as: [locus(IP)/*ACT1*(IP)]/[locus(input)/ *ACT1*(input)]. Sirt1 profiles were reported as *sir2Δ+/sir2Δ-* with *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.

10. 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.

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