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The Roles of SIR2 in Yeast Cellular Aging

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

Yijin Liu

Committee in charge:

Professor Nan Hao, Chair
Professor Lin Chao, Co-Chair
Professor Gen-sheng Feng

2021

The Thesis of Yijin Liu is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

EPIGRAPH

Instead of being the biological center of the Universe, I believe our planet is just an assembly station, but one with a major advantage over most other places. The constant presence of liquid water almost everywhere on the Earth is a huge advantage for life, especially for assembling life into complex forms by the process we call 'evolution.'

FRED HOYLE

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LIST OF ABBREVIATIONS

NAD	Nicotinamide Adenine Dinucleotide
NA	Nicotinamide
NaMN	Nicotinic Acid Mononucleotide
DMB	Dimethyl Benzimidazole
RENT	regulator of nucleolar silencing and telophase exit
NTS1	non-transcribed spacer I
HML	Homothallic Mating Left
HMR	Homothallic Mating Right
ERCs	extrachromosomal rDNA circles
RACF	retrograde actin cable flow
ROS	reactive oxygen species
Mca1p	type I metacaspase
SQC	spatial protein quality control compartments
IPOD	Insoluble Protein Deposit
JUNQ	Juxta Nuclear Quality control compartment

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ABSTRACT OF THE THESIS

The Roles of SIR2 in Yeast Cellular Aging

by

Yijin Liu

Master of Science in Biology

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Professor Nan Hao, Chair
Professor Lin Chao, Co-Chair

Aging is defined by a progressive decline in cellular functions, and problems within cell accumulate over time leading to death. The yeast Saccharomyces cerevisiae is a powerful model system for human to study and understand the aging at molecular basis. As a longevity gene that has been studied over years, Sir2 is believed to plays a crucial role in modulating cellular senescence. Here we review how Sir2 increases the yeast cell lifespan by strictly regulating the rDNA stability, oxidative stress, and asymmetric cell division. After that, we provide new sight that possibly link the Sir2 activity with cellular protein aggregation, which is potentially another factor that affect the yeast cellular aging.

Introduction

Aging and longevity have always been a hot topic since they are directly related to many known human diseases. Focusing on the molecular level, the silent information regulator 2 (Sir2) is currently the best studied yeast longevity gene, and it functions within a wide range of organisms from yeast to human. As an NAD-dependent protein deacetylase, Sir2 functions at different locations and participates within multiple pathways in the yeast cell. As a histone deacetylase, it is an essential regulator of yeast chromatin structure. One of the regions where Sir2 regulates chromatin silencing is in the rDNA region—a highly repetitive and highly unstable region of DNA within the yeast genome. As Sir2 constantly regulate the rDNA stability, it helps to prevent the formation of ERCs, whose accumulation leads to lose of nuclear homeostasis. Sir2 also involved in the pathway of nutrient stress, including the TORC1 inhibition and Calorie Restriction. In addition, instead of regulating rDNA silencing directly through its deacetylase activity, Sir2 is also involved in pathways that dealing with environmental stress, such as oxidative stress. During cellular aging, as the ROSs continuously accumulate in the yeast cell, Sir2 works actively to protect the cell from oxidative damages by either dealing with cellular stress or providing help to the asymmetric cell division.

Each of the aspects mentioned above can contribute to regulating cellular protein homeostasis. Given Sir2 activity and effects, we propose that one of the major ways Sir2 promotes longevity is by helping cells maintain cellular protein homeostasis.

Sir2 and Its Basic Cellular Functions

Sir2 as a NAD-dependent Protein Deacetylase

Gene silencing can be seen as the inactivation of gene expression, which packs the DNA into an inaccessible chromatin structure. For the budding yeast Saccharomyces cerevisiae, the cell uses transcription silencing to regulate multiple cellular functions and achieve a different purpose. For example, the haploid cell regulates the cellular identity via silencing its DNA domains at the mating-type loci [1]. In addition, the telomeric region in yeast is also packed into a silenced structure [2]. On the other hand, Sir2 works together with other proteins and enzymes to regulate the chromatin silencing at mating-type loci, telomeres, and rDNA region, which are all repeated DNA sequences that need tight regulation to maintain their proper functions [1-4]. Therefore, Sir2 is believed to carry some specific enzymatic activity that provides it such a special and important role within the cell.

Recent studies have shown that the conserved core domain in Sir2-like proteins carries enzymatic activity which might be able to transfer the ADP-ribose from nicotinamide adenine dinucleotide (NAD) to protein substrates [5]. A study on the Salmonella typhimurium Sir2-like protein CobB shows that this gene can compensate for another gene, CobT, in the pathway of cobalamin synthesis [6]. CobT is known as a protein enzyme called nicotinate mononucleotide that transferred ribose–phosphate from nicotinic acid mononucleotide (NaMN) to dimethyl benzimidazole (DMB). This evidence provides a possibility that Sir2, similar to CobB, might contain the activity to catalyze a similar reaction, as CobT, on the NaMN. After tested multiple pyridine nucleotide derivatives as the donor, Frye finally proved that Sir2-like proteins from E. coli (CobB) and humans (Sir2T) can transfer the ribose–phosphate from only NAD to other protein carriers in vitro [5] (Fig. 1a). Therefore, it was suggested that Sir2-like proteins carry the activity of ADP-

ribosyltransferases by transferring the 32P from NAD to a protein carrier. However, the biological function of neither CobB nor Sir2T is known. Therefore, using Sir2 from budding yeast and NAD as a cofactor, Tanny tested yeast Sir2's enzymatic activity of ribosyltransferase. The result shows that Sir2 carries only weakly ribosyltransferase activity but is essential for its silencing function in vivo with NAD as its cofactor [7] (Fig. 1b).

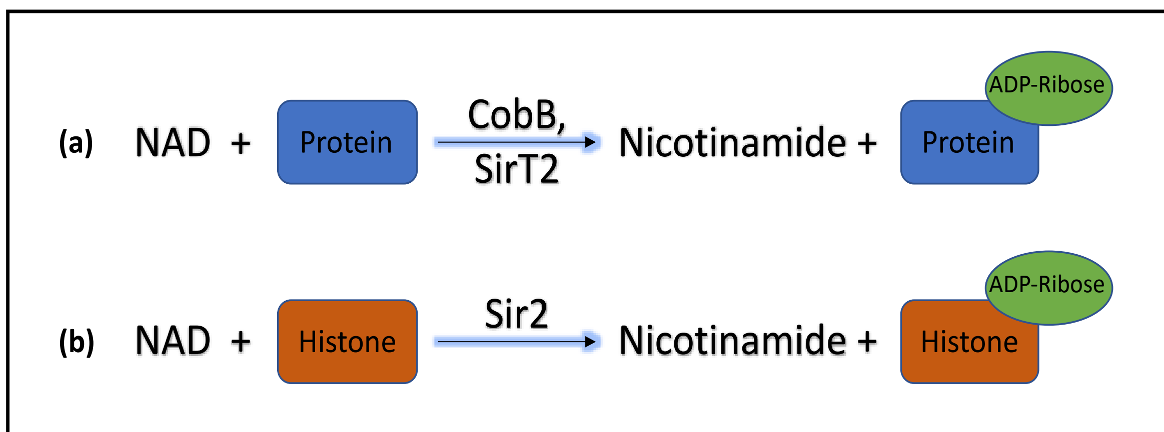

Figure 1: Mechanism of Phosphoribosyltransferase Activity of Sir2 and Sir2-like Proteins. a) CobB in E.coli and SirT2 in Human carry ADP-ribosyltransferase activity. They use and can only use NAD as donor and transfer ADP-ribose to other protein substrates. b) Sir2 in the yeast carries weak ADP-ribosyltransferase activity. It uses NAD as donor and transfer ADP-ribose to histone.

Since yeast Sir2 uses NAD as a cofactor in the ADP-ribosylation reaction, a later study tested whether NAD could be a cofactor for deacetylase activity. On histone, both H3 and H4 can accept 32P from NAD, but only in an acetylated state. Therefore, using purified recombinant Sir2 in a reaction with NAD and a peptide of the histone H3 N-terminal tail di-acetylated at lysines 9 and 14, 23-27% of the acetyl lysines at each position of lysines 9 and 14 were deacetylated by Sir2 in the presence of NAD. In contrast, NADH, NADP, and NADPH did not promote a significant level of deacetylation by Sir2, and neither NADH nor NADP inhibited the activity of NAD in this reaction. Therefore, Sir2 was proved to carry the NAD-dependent histone deacetylase activity that can deacetylate either Lys 9 or Lys 14 on the H3 N-terminal tail [8]. Based on this conclusion, Imai kept testing whether the histone deacetylase activity of Sir2 is required for in vivo silencing functions. In that case, the Sir2

gene with two separate point mutations (Gly270 and Asn345) was introduced into yeast cells with the Sir2 gene deleted. Results showed that the mutation at Asn345 causes the Sir2 protein to completely lose its silencing function [8]. Comparing Sir2's activity of ADP-ribosyltransferase to its deacetylase activity, the Gly 270 mutant leads to highly defective ADP-ribosyltransferase but retains 80% of the wild type of deacetylase activity. Therefore, we can conclude that the NAD-dependent deacetylase activity of Sir2 is more essential for its in vivo silencing. The ADP-ribosyltransferase activity of Sir2 might function in other places such as DNA repair [8]. Sir2 had been suggested as the histone deacetylase before since overexpression of Sir2 resulted in the general histone hypoacetylation in yeast [9].

The activity of being a protein deacetylase offers Sir2 a variety of functions. Therefore, it becomes more necessary for us to understand the mechanism of how is Sir2 regulated within the yeast cell. Being as the co-substrate of Sir2 activation, the intracellular NAD+ concentration or the NAD+/NADH ratio are thought to affect the Sir2 activity level [10]. Under aerobic conditions, the cellular concentration for NAD+ and NADH are reported to be 4 mM and 0.2 mM, making the NAD+/NADH ratio to be around 20 [11]. Under the caloric restriction (CR) condition which should increase the Sir2 activity, the NAD+/NADH ratio fluctuated within 2-fold only because of the change in NADH concentration [11]. Although NADH is reported as a competitive inhibitor of Sir2 in vitro, the high value of NADH's IC50, which ranges from 11 to 28mM, makes it less possible to inhibit the Sir2 activity in vivo. Therefore, with the Km value range from 10 to 100uM, changes in NAD+ concentration are more likely to be the essential factor of Sir2 activity regulation [11]. In that case, the activation of NAD+ consuming enzymes elsewhere in the cell can decrease the intracellular NAD+ concentration and, thus, negatively regulate the Sir2 activity. On the other hand, Nicotinamide, the product from the Sir2 deacetylation reaction,

is believed to be the physiological inhibitor of Sir2 with an in vitro IC50 around 120uM [11]. As the intracellular Nicotinamide level increased, it can effectively reverse the Sir2 deacetylation reaction and, as a result, inhibit the Sir2 activity [12]. The experiment has shown that the increased Nicotinamide in vivo results in decreased chromatin silencing and more active rDNA recombination rate, which further leads to decreased lifespan [13]. In addition, research has shown that the overexpression of Pnc1, a nicotinamidase, enhances the chromatin silencing at telomeres and rDNA regions under the condition with a high level of Nicotinamide, while the deletion of Pnc1 has the opposite effect [13]. Therefore, we can conclude that the Sir2 activity in vivo is positively regulated by the present amount of NAD+, while the Nicotinamide acts as a non-competitive inhibitor for Sir2 and negatively impact the yeast cellular aging.

Sir2 Largely Involved in Transcriptional Silencing

For all living organisms, it is important for them to keep a tight regulation on their chromatin structure and express their genes at the proper time during the cell cycle. The structure of chromatin is regulated by multiple different enzymes to alternate the chromatin states in between Euchromatin (active) and Heterochromatin (inactive). One way to regulate chromatin silencing is through acetylation and deacetylation on the core histone within the chromatin. In budding yeast, the silent mating-type loci (HML and HMR) and the telomeric DNA regions are normally considered as the heterochromatin equivalents [14]. In addition, the ribosomal DNA (rDNA) region, which is also highly repetitive, is regulated within the heterochromatin structure as well.

With its NAD-dependent protein deacetylase activity, Sir2 functions in transcriptional silencing at HML, HMR, and telomeres regions together with other silencing

protein factors (Sir3 and Sir4) to form a complex called SIR holocomplex [15]. This SIR holocomplex carries the function of creating, spreading, and regulating the silent chromatin area through histone deacetylation [16] (Fig. 2a). On the other hand, Sir2 combined with Net1and Cdc14 to form a complex called regulator of nucleolar silencing and telophase exit (RENT) to further regulate the chromatin silencing at the rDNA region [4]. The RENT complex is recruited by Fob1, a gene that binds the rDNA replication fork barrier, to the non-transcribed spacer I (NTS1) region of rDNA together with Tof2, Lrs4, and Csm1 [17] (Fig. 2b).

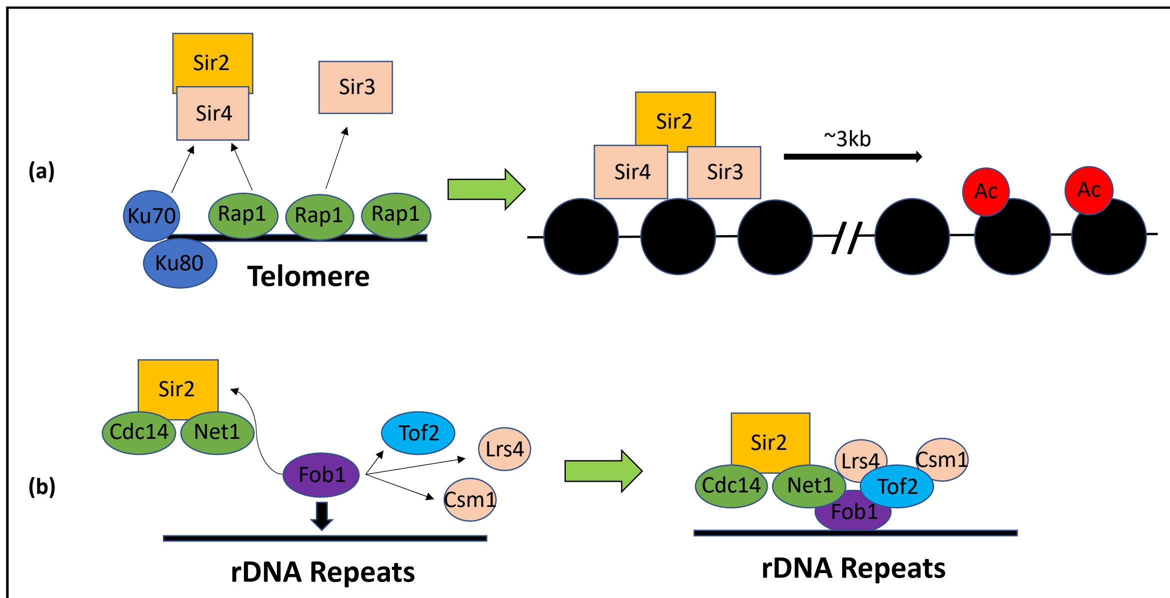

Figure 2: Sir2 Containing Complex involved in the Chromatin Silencing in Budding Yeast. a) The model of SIR complex (Sir2/3/4) recruited by Ku proteins and Rap1 to the telomeric region in yeast. The enzymatic activity of Sir2 deacetylates histones and change the telomere into silencing state. The SIR complex spanning approximately 3 kb from the telomere end. Ac stands for acetylated head on the histone. b) Sir2 in the RENT complex is recruited and binds to the Fob1 molecule bound to the non-transcribed spacer on the rDNA repeat region in yeast. In the meanwhile, Fob1 also recruits Tof2, Lrs4, and Csm1 to bind to the same place.

In yeast, the rDNA is a highly repetitive region consists of a 9.1 kb unit with tandemly repeated 100–200 times on chromosome XII. Each unit contains genes encoding for the 35S rRNA (which later processed into 5.8S, 18S, and 25S rRNA species) and the 5S rRNA, separated by a non-transcribed spacer (NTS) [18]. This highly repetitive feature of rDNA

makes it the most unstable region within the yeast genome since it can constantly lose copies via the homologous recombination process [19]. Despite the fact of being unstable, the rDNA region can recovery from the rDNA pieces lost with the help of the gene amplification system [19]. This gene amplification starts when Fob1, a replication fork-blocking protein, binds at a specific site in the rDNA called the replication fork barrier (RFB) and result in DNA double-strand breaks (DSBs) [20]. This DSB further leads to unequal sister chromatid recombination (USCR) [21]. On the other side, when the mRNA level reaches its normal value, the Sir2p binds and silences a non-coding bidirectional promoter E-pro in the rDNA region. The E-pro transcription functions to release the cohesin complex that normally holds sister chromatids together. This allows the DSB ends to pair and complete the USCR [22]. There is research suggests that, in a Sir2 mutant cell, the E-pro transcription is highly activated, which leads to a high level of rDNA copy number and instability [21]. Furthermore, a study has shown that Sir2 is required for transcriptional silencing of reporter genes integrated at the rDNA region [23]. In addition, detected by immunofluorescence, most of the cellular Sir2 proteins are located in the nucleolus, where synthesized rRNA is located [24]. In yeast, Sir2 tightly regulates the stability and homologous recombination of those rDNA repeat regions, as the frequency of the rDNA recombination rate increases about 10-fold in sir2 mutants [25]. Thus, Sir2 now is broadly believed as the essential factor for regulating the rDNA silencing, and its regulation on the rDNA stability is one of the major ways of promoting the yeast replicative life span (RLS).

Sir2 Promotes Yeast Lifespan by Regulating rDNA Stability

It has been well-accepted that Sir2 plays important role in preventing yeast cellular aging. The lifespan of yeast greatly decreased when Sir2 is deleted; conversely, when introducing an extra copy of the SIR2 gene, the lifespan increased [26]. Sir2 is believed to increase the yeast RLS by decreasing the production of the extrachromosomal rDNA circles (ERCs) while regulating the silence on the rDNA region [27]. ERCs have been seen as one of the toxic elements that contribute to yeast cellular aging. ERCs are formed from the rDNA recombination process. It acts as an autonomously replicating sequence (ARS) containing plasmid and asymmetrically segregates into mother cells during mitosis [28]. The formation of ERCs is favored with the presence of Fob1, which provides a great chance for double strand breaks and thus increases the rDNA recombination events. ERC has been hypothetically toxic to the cell by negatively influencing the rDNA-specific RNA polymerase I [29]. To test how ERC accumulation is detrimental to yeast cells, Morlot first tagged Rpa190, the largest subunit of Pol I in yeast, with GFP and developed a fluorescence in situ hybridization protocol to measure total pre-rRNA levels in cells. Surprisingly, results from the fluorescence measurement and RNA-FISH together showed that, as ERCs exponentially accumulate within the nucleolus, it actively increases the amount of functional Pol I up to 10-fold compared with the new-born cell and, as a result, increases the levels of pre-rRNA before entering the senescence [29]. To test whether this increase in pre-RNA level is coupled with elevated ribosome biogenesis, Morlot tagged Nog2 and Rpl13a with GFP to further detect steps associated with pre-ribosome nuclear export. Nog2 is required for the nucleoplasm steps of pre-60S maturation, and Rpl13a represents a component of the 60S ribosomal subunit. Results together indicate that the increased level of pre-RNA doesn’t lead to increased ribosome biogenesis [29]. Since ribosomal biogenesis is essential for protein

production and cell growth, uncoordinated rDNA transcription and ribosome biogenesis can hurt cell growth and further lead to the loss of nuclear homeostasis and drive the decline in cellular physiological functions [29] (Fig. 3). Taking those results altogether, the exponential growth of ERC increases the pre-RNA level only but not the nuclear ribosomal biogenesis, and this further leads to a loss in nuclear homeostasis characterized by the enlarged nucleoplasm and the accumulation of nuclear proteins.

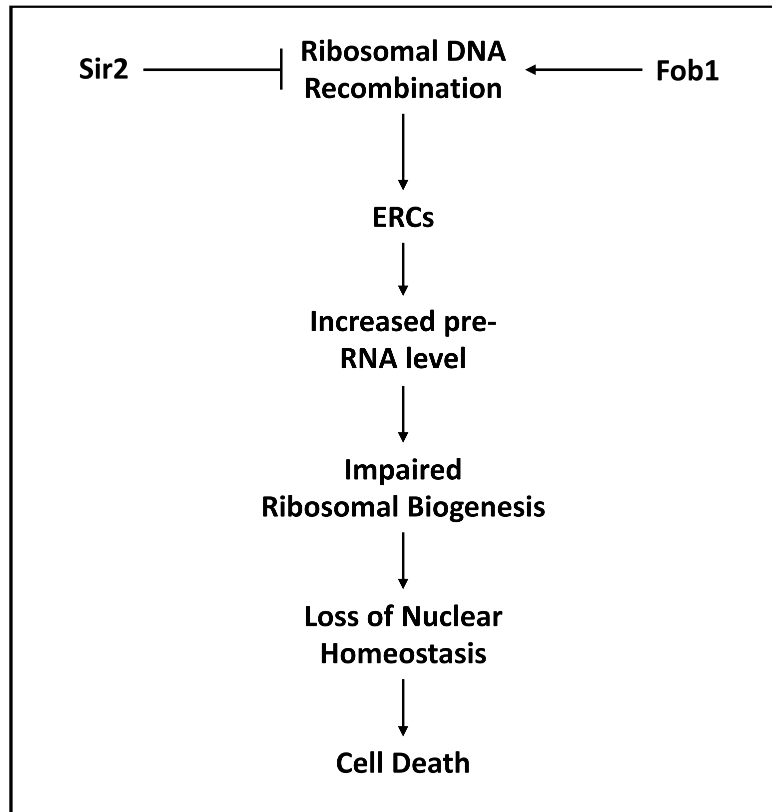

Figure 3: Mechanism of ERCs leading to cell death. Sir2 promotes the longevity of yeast cells by binding to the rDNA region and inhibiting rDNA recombination, which further leads to the production of ERCs. Arrows stands for positive relations; flat arrow denotes for inhibition.

As the stability of the rDNA region is tightly regulated by Sir2, deleting Sir2 results in hyper-recombination at the rDNA region, which promotes the rDNA instability and ERCs formation [30]. Therefore, we can claim that as the Sir2 activity is gradually lost during the yeast cellular aging, it accelerates the formation of ERCs and cell death. In addition, since the ribosomal biogenesis is uncoupled cellular growth within the aged cell, it can largely

affect the alinement of transcription and translation of genes involved in protein biogenesis in the aging cell at the same time [31]. This can further lead to unsuccessful protein folding issues and protein aggregation as a result. On the other hand, mentioned by Morlot, the excess formed ERCs and pre-RNA build-up can possibly block the nuclear pores and thus lead to a defective protein shuttling across the nucleus. As more protein being forced to stay within the nucleus, it offers a higher possibility for protein aggregation to take place.

In addition to the ERC theory, recent data has pointed out that the rDNA instability itself is enough for limiting the yeast lifespan regardless of how much ERCs are produced [32]. Based on Ganley, the non-functional DNA repair proteins could be distributed to the aged mother cell during the asymmetrical segregation and further damage the rDNA region in the mother cell [32]. As those damages accumulated, it could result in the decreased functioning of ribosomes generated within the mother cell and lead to protein folding issues. To sum up, Sir2 can greatly protect the yeast cell from getting aged by tightly inhibiting the rDNA instability, which is the major factor leading to uncoupled ribosomal biogenesis and possibly protein aggregation within the nucleus.

Although Sir2 has direct regulation on the rDNA region within yeast, it also involves other pathways that connect rDNA with cellular aging. TOR kinase (Target of Rapamycin) is a nutrient-responsive phosphatidylinositol kinase-related protein kinase, which carries essential functions, including regulating transcription, translation, and ribosome biogenesis, in cell growth [33-34]. Normally, within yeast, TOR kinase presents in two different protein complexes, TOR complex1 (TORC1) and TOR complex2 (TORC2) [35]. Rapamycin, an anticancer drug, can specifically inhibit the function of TORC1 by negatively regulating the transcription of all three RNA Polymerases. As a result, the inhibition of the TORC1 complex leads to a sharp decrease in ribosome biogenesis [36]. Surprisingly, the inhibition of TORC1

signaling can increase the total lifespan in yeast [37]. Research has shown that the inhibition of TORC1 can activate the Sir2 by upregulating the expression of PNC1, which encodes for nicotinamidase that consumes the yeast cellular nicotinamide [38]. The nicotinamide is a cellular repressor for Sir2. As a result, as the nicotinamide level drops due to the inhibition of TORC1, the Sir2p becomes more activated and thus better regulates the rDNA stability and limits the formation of ERCs. In addition, when Pnc1 and Net1 are present, the inhibition of TORC1 increases the association between Sir2 and rDNA [39]. Sir2, in that case, promotes the transcriptional silencing of Pol II-transcribed genes at the rDNA region and applies more histone deacetylation on the rDNA. Therefore, we can conclude that Sir2 further increased the rDNA silencing and rDNA stability under the condition of TORC1 inhibition and, as a result, extend the lifespan of yeast.

Sir2 and Oxidation Stress

Free radicals have been identified and suggested to be involved in the cellular aging process for years. They are reactive atoms or molecules with more than one unpaired electron in their external shell. Those free radicals are produced within the cell when oxygen reacts with certain molecules and becomes either oxidants or reductants. The Reactive Oxygen Species (ROS), in addition, is a group combined with free radical and non-free radical oxygen-containing molecules, such as the superoxide anion (O2-), hydrogen peroxide (H2O2), and the hydroxyl radical (OH-).

Although ROS can be generated via multiple pathways within the cell, one of the most fundamental physiological sources is cellular respiration. During cellular respiration,

most electrons pass along four protein complexes on the Electron Transport Chain (ETC) and fully reduce oxygen to water. However, sometimes a small number of electrons can escape from the ETC. This will lead to the incomplete reduction of oxygen and produce superoxide anion (O2-) [40]. The O2- can be detoxified into O2 and H2O2 via a spontaneous reaction, which can be catalyzed by the superoxide dismutase (SOD) [41]. The other two ROS mentioned above, the hydrogen peroxide and the hydroxyl radical, are located downstream of the O2- detoxification pathway. The hydroxyl radical, however, is the most reactive molecule among all those three ROS, and it can broadly oxidize lipids, nucleic acids, and amino acids [42].

Although how oxidative stress leads to cellular function decline and aging is still not fully understood, there are shreds of evidence that strongly support that the ROS damage to cellular functions is involved in various aging-related diseases, such as atherosclerosis, pulmonary dysfunction, and many kinds of neurological disorders [43]. Even though there is a debate going on regarding the machinery between ROS damage and cellular aging, no one can ignore the importance of damages that ROS brought onto the nucleic acid and lipids. By measuring the in vivo production of modified purine and pyrimidine base, research shows that ROS are responsible for modifying over 1000 DNA base pairs per day [44]. This is such as a huge number of damages that exceed the number of repairments provided by the DNA repair mechanism. The accumulation of those damaged DNA highly likely leads to the age-related loss of physiological functions. In addition, ROS can also interact with lipid. Therefore, the oxygen-free radicals can further lead to the decline of membrane integrity by mediating the oxidations of lipids in the cell membrane.

On the other hand, not until recent years, people start to pay attention that the protein aggregation caused by ROS damage might also involve in the cellular aging progress.

Normally, the damaged proteins will not lead to cellular aging because the protein turnover system can constantly degrade the damaged protein and prevent them from aggregating. However, this can become an issue when either the turnover system has a decreased efficiency, or the production rate of damaged proteins exceeds its degradation rate. Therefore, as more of the damaged proteins oxidized to carbonyl derivatives accumulate together, the cellular protein homeostasis is disrupted and further leads to aging.

Since it was wildly accepted that oxidative stress increased throughout the aging process, the SIR family proteins, which are believed to carry the potential of mediating oxidative stress during cellular aging, have attracted great attention. This type of protein functions to catalyze the removal of the acetyl group from the amino group of lysine residues [45]. Among those SIR proteins, Sir2 is the longevity gene that has been studied the most. Most of the evidence that links the SIR family protein with the oxidative stress used Sir2 in eukaryotic model organisms such as S. cerevisiae and C. elegans. Evidence has shown that overexpression of Sir2 in the cell can greatly extend the shortened lifespan when cells were treated with hydrogen peroxide [46]. In addition, the deletion of Sir2 also diminished the extension of lifespan observed when yeast cells were treated with the caloric restriction condition [46]. This suggests that Sir2 might protect the cell from getting aged by reducing the cellular oxidative stress.

As previously mentioned, oxidative damage increases throughout the aging process. However, the Caloric Restriction (CR), which is suggested to weaken that damage, can increase the lifespan of several model organisms. Recently, there are genetic and molecular studies that suppose that CR might be regulated by machinery where the SIR2 gene is largely involved. First, when Sir2 is deleted, the CR condition could not extend the lifespan anymore [47]. Second, CR improves the silencing activity of Sir2, so that the rDNA region will be

even more tightly regulated [48]. Third, an activator for the Sir2 enzyme called resveratrol was identified during compound screening and was proved to increase the yeast replicative lifespan [49]. However, it could not further extend the lifespan when combined with CR condition, which suggests that the resveratrol and CR might work in the same regulating pathway. Since the resveratrol acts as an activator for Sir2, there must be a tight linkage between CR and Sir2 as well.

Lin's research brought us new sight for establishing the internal connection between Sir2 and CR [48]. When the glucose level is limited, the cell prefers to do cellular respiration rather than fermentation to produce more ATPs. Under such conditions, carbon molecules are sent to the mitochondria, and electron transportation and respiration increase as a result. Evidence shows that when the gene Cyt1, which codes for the cytochrome c1 involved in the electron transportation, is deleted, CR failed to extend the lifespan [48]. This indicates that the metabolic shift to cellular respiration is required for CR to have the life-extension effect. The increased respiration activity brought by CR leads to a higher rate of electron transportation. This further increases the oxidation of NADH into NAD and causes the NADH/NAD ratio to decrease. Since the NADH is a competitive inhibitor for the Sir2 enzyme, the Sir2 activity will be upregulated when the amount of NADH decreases in mitochondria and cytosol [50]. This is also supported by evidence that overexpressing the NADH dehydrogenase, an enzyme that takes an electron from NADH to the electron transport chain (ETC), can mimic the CR effect when growing cells in high glucose media [50]. Although currently more findings are suggesting that there might be more than one pathway that can mediate CR in different yeast models, Sir2 is still believed to play an important role in connecting CR to increased cellular lifespan.

Since Caloric Restriction is defined as putting yeast cells within an environment with

less glucose level, CR is considered as nutrient stress. Just like other nutrient signaling pathways inhibition, such as the TORC1 inhibition mentioned above, CR also results in upregulation of Pnc1 [51]. Being as a nicotinamidase present in the NAD+ salvage pathway, Pnc1 plays a role to produce

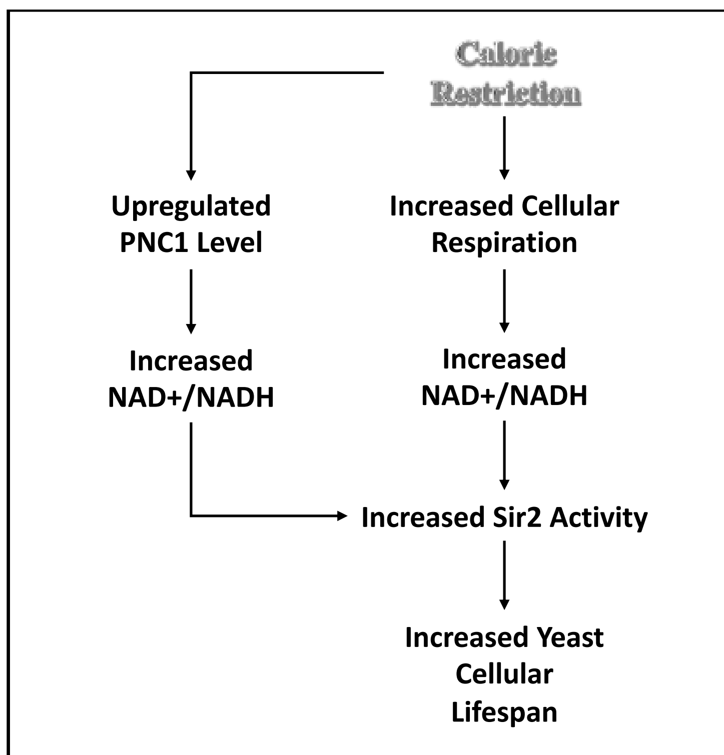

Figure 4: Mechanism of CR Pathway. The Calorie Restriction depends on Sir2 to increase yeast cellular lifespan. The pathways are described in text.

NAD+ from its cleaved products, Nicotinamide (NA) and ADP- ribose. Since NAD+ acts as the activator for Sir2 and Nicotinamide plays the opposite role, the upregulation of Pnc1 increases the activation of Sir2. Therefore, we can conclude that the CR condition increases the activation of Sir2 via the upregulation of Pnc1, which reduces the intracellular NAM concentration and/or increases the NAD+ level. Since we know that the CR can result in an elevation of cellular respiration and electron transportation rate, we can draw a tighter connection between Sir2 activity and the cellular oxidative stress (Fig. 4).

Sir2 Involves in the Asymmetric Cell Division

As mentioned earlier, the accumulation of oxidatively damaged protein as cells get aged can negatively impact the Replicative life span (RLS) of yeast. Therefore, yeast has developed a method called asymmetric cell division, in which case cells filter all harmful components that negatively affect the cell lifespan and keep them within the mother cell. This allows the daughter cell to maintain full replication potential while leads to the cell aging for the mother cell [52].

During the process of cell division in Saccharomyces cerevisiae, a budding site will be selected on the cell surface that the cytoskeleton will be polarized toward, which leads to the transportation of cellular constituents from the mother cell to the daughter cell. The cytoskeleton also acts as a filter to prevent asymmetric inheritance aggregations from daughter cells. The cortical tags are also involved in the selection process and activate a GTPase-dependent signaling cascade to assemble the cytoskeleton, further establishing the polarity. In GTPase-dependent signaling cascade, Cdc24p, Bud1p/Rsr1p, and Bud2p, small GTPases, play important roles to establish the specialized cytoskeletal structures. Actin patches and actin cables, the F-actin-containing structures, are also maintained in the cell cycle. Actin patches help recycle the membrane constituents while polarization. Actin cables serve as a dynamic track for the retrograde cargo transportation to play a role in aggregate retention, which are called retrograde actin cable flow (RACF) [53]. Meanwhile, aggregate inheritance relies on an Hsp104-dependent control that can retain aging determinants in the mother cell. Additionally, Sir2 is required for the asymmetric division of toxin aggregates [54-55]. Both Hsp104 and Sir2 are related to the retrograde actin cable flow (RACF) and the polarisome [56]. As mentioned, actin cables contribute to asymmetric inheritance aggregations retention. Many studies point out that their physical association with the actin

cytoskeleton prevents their free diffusion into the daughter cells [57-59]. Also, many studies showed that the deficiency of Sir2 reduces actin cable abundance, cytoskeletal functions, and the velocity of retrograde actin flow from the polarized region [60-61]. Thus, Sir2 is linked to actin cable function, and it affects the rate of actin folding and further influences the retention of asymmetric inheritance aggregations.

Some components in asymmetric inheritance aggregations contribute to cell aging, such as including oxidatively/damaged protein aggregates, mitochondria, vacuoles, and extra-chromosomal rDNA circles (ERCs). We also call them the aging determinants. Recently, many new studies have been linked the asymmetric distribution of protein aggregates and mitochondria to aging. Specifically, oxidatively damaged protein aggregates and oxidatively damaged mitochondria will stay in the mother cell while there is a lower level of reactive oxygen species (ROS) in the daughter cells [62-64]. Theoretically, the addition of antioxidants or overexpression of radical metabolizing enzymes will increase lifespan by decreasing the level of reactive oxygen species (ROS). However, studies have shown that the observation was weaker than the theory [62-64]. Surprisingly, the daughter cells have a lower level of ROS, more fragmented mitochondria, and a shorter life span when Sir2 is deleted [65]. On the other hand, the asymmetric aggregates of damaged protein are another factor that plays a role in senescence. Removing the damaged protein aggregates in the daughter cell and sequestering the damaged protein aggregates in the mother cell are the two mechanisms for asymmetric inheritance. For removing the damaged protein aggregates in the daughter cells, the expression of type I metacaspase (Mca1p) will be elevated to promote the clearance. For sequestering the damaged protein aggregates in the mother cells, damaged proteins and aggregates will go to two spatial protein quality control compartments (SQC), which are the Insoluble Protein Deposit (IPOD) and Juxta Nuclear Quality control

compartment (JUNQ). Hsp104 chaperones, which have been localized to IPODs, will bind to the misfolded proteins and facilitate the refolding and degradation process [66-68]. When deleting Hsp104, the refolding and degradation could not be processed while the actin cable is destabilized, which also results in loss of the asymmetric segregation of damaged protein aggregates [69]. Furthermore, the protein carbonylation and premature protein aggregation increased in cells and damaged proteins fail to retain within the mother cell when Sir2 is deleted [69-70]. Consistent with this, deletion of Sir2p destabilizes actin cables and inhibits RACF [69,71]. Therefore, it provides the possibility that Sir2p is functional in the asymmetric inheritance of protein aggregates via the interaction with actin cytoskeleton and RACF.

The oxidative stress accumulated through the yeast aging process and the mechanism of asymmetric inheritance together led to the protein aggregation within the yeast mother cell. As mentioned above, oxidative stress can produce oxidatively damaged protein via the process of protein carbonylation, which is irreversible protein damage. And those damaged proteins can lose their functions and become easily aggregated. However, the asymmetric inheritance mechanism helps yeast cells to filter those damaged proteins into the mother cell and thus protect the daughter cell from the accumulated oxidative stress. An experiment has shown that "carbonylation damage per total protein in the first-generation daughter cells" is six-fold lower than in the mother cell after the mother cell was treated with paraquat to increase the level of carbonylated proteins by two-fold [72]. In addition, compared with wild-type daughter cells, the first-generation daughter of Sir2 deleted cells inherits an extra load of oxidatively damage after paraquat treatment, and the protein carbonyls are evenly distributed between mother and daughter cells [72].

Therefore, the oxidatively damaged proteins are asymmetrically distributed during the

yeast cell division, and this process is Sir2 dependent. By limiting most of the age-contributed factor within the mother, Sir2 protect the daughter cell from oxidative damage and make sure its newborn vitality. In the meanwhile, Sir2 also prevents protein aggregation within the daughter cell by segregate the carbonyl protein aggregation into the old mother cell. Since both oxidative stress and protein aggregation are considered as factors that contribute to cellular aging, we can conclude that Sir2 protects the daughter cell from getting aged through the process of asymmetric cell division.

Discussion

As a longevity gene that has been studied for years, Sir2, a NAD-dependent deacetylase, is broadly accepted to be the most important factor that regulates the lifespan from yeast to human. So far, we have introduced three major ways, where Sir2 is largely involved, protecting the cell from aging. The combined effects of Sir2 regulation of rDNA silencing, cellular oxidation, and asymmetric division of protein aggregates all suggest that Sir2 plays a major role in regulating cellular protein homeostasis.

To begin with, regulation of the rDNA stability can be seen as the most important role for Sir2 in promoting yeast cell RLS. As mentioned earlier, the rDNA region is the most unstable region within the yeast genome, and its stability is strongly associated with the yeast life span [73]. The formation of ERCs due to the rDNA instability takes most charges for the yeast aging effect and the protein aggregation in the meantime. As ERCs being produced and accumulating within the nucleus, it further upregulates the expression of RNA polymerase I, and greatly increases the amount of pre-rRNA transcribed as a result. However, this raised pre-rRNA doesn't couple with the ribosomal biogenesis process. Therefore, throughout the yeast lifespan, as the amount of ERCs and pre-RNA accumulate within the nucleus, the ribosome level stays the same. Since ribosome responses for protein production and is essential for cell growth, uncoupled ribosomal biogenesis will result in decreased protein production rate compared to cell growth speed. This can further lead to the loss of nuclear homeostasis since more physiological material can aggregate within the nucleus and even block other proteins from exit the nucleus via the nuclear pores. Therefore, by tightly regulating the stability of rDNA and limiting the formation of ERCs, Sir2 can slow down the yeast aging process and better prevent protein aggregation within the yeast nucleus.

On the other hand, the highly repetitive and unstable features of the rDNA further led to the development of the "rDNA theory" of aging from Kobayashi in 2008 [74]. This theory predicts that the cell lifespan is tightly correlated with the rDNA stability, and the age-related damage on rDNA will be more effective on cellular aging than other regions on the genome [74]. In contrast, the "ERC theory "of aging states that the accumulation of ERCs is the major cause of aging, while the "rDNA theory" points out that the formation of ERCs is just the by-products of rDNA instability, which can be seen as the marker and measurement of rDNA instability. As the damage accumulates through aging, the increased rDNA instability keeps lengthening the cell cycle via an unknown signal pathway and eventually leads to senescence [75]. This rDNA theory is strongly supported by the CR (the dietary restriction) and the TOR inhibition pathway. Those two pathways both promote the yeast cell longevity by increasing the interaction between the Sir2 and the rDNA, thus increase the rDNA stability. Therefore, the rDNA instability can be seen as the major cause of yeast cellular aging.

While being the major cause of yeast cellular aging, the instability of rDNA is strongly correlated with protein aggregation as well. First, since rDNA is the direct source for the cell to make ribosomes, as the age-related damage accumulated on the rDNA region, it might affect the normal function of the ribosome and further cause faulty translation from mRNA to polypeptide. Second, being the by-product of rDNA instability, the ERCs exponentially accumulate within the nucleolus throughout the entire lifespan. The accumulation of ERC will further lead to an increased amount of functional Pol I, which, as a result, increases the total amount of rRNA being transcribed [24]. However, this action doesn't correlate with the increase of ribosome biogenesis. This uncoordinated rDNA transcription and ribosome biogenesis further lead to the loss of nuclear homeostasis and

highly likely the loss of protein homeostasis within the nucleolus. Both aspects mentioned above could tie the rDNA instability to the protein aggregation issue. To carry proper functions, the majority of proteins fold specifically into a three-dimensional structure. The protein folding process starts since the mRNA binds to the ribosome. However, not all of the proteins can fold properly. Those folding intermediates, including misfolded proteins, easily expose their hydrophobic protein residues that are normally protected within protein internal structures. Those exposed hydrophobic surfaces are called aggregation-prone regions (APR), which is one of the major reasons that trigger protein aggregation [76]. In addition, sometimes the folding error isn't because of the incorrect folding process. Instead, the translational errors accumulated during the aging process can also lead to misfolded proteins [77]. This can disrupt the protein's natural folding process and expose its hydrophobic region leading to aggregation. Since Sir2 tightly regulates the rDNA stability, when Sir2 becomes less active during cellular aging, the increased rDNA instability can hurt the protein translation process and eventually lead to protein aggregation.

On the other hand, even though internal folding error can lead to protein aggregation, environmental change like oxidative stress is one of the major reasons that lead to protein aggregation as well. As mentioned above, Reactive oxygen species (ROS) are mostly by-products generated from cellular reaction with oxygen. Normally, the intracellular ROS level remains low, which is just enough for carrying specific redox reactions within the cell. Oxidative stress, on the other hand, takes place when the ROS concentration exceeds the antioxidant capacities of the cell, leading to protein damage and carbonylated proteins. Carbonylated proteins are formed by an irreversible metal-catalyzed oxidative (MCO) attack on specific amino acid side chains that add a carbonyl group (CO) onto proteins [78]. The carbonylation of protein generated by ROS mainly affects lysine, arginine, threonine, and

proline residues on the protein, and this modification always largely reduces the target protein activity [79]. It has been shown that excessive carbonylation leads to protein aggregation, especially under the condition of losing proteasome activity [80]. As a factor that prevents cells from becoming overly damaged by oxidative stress, the evidence suggests that Sir2 involves in pathways that regulate cellular protein homeostasis.

Lastly, during the asymmetric cell division, yeast cells keep the oxidatively damaged proteins within the mother cell by using the mechanism with Sir2 as an essential component to keep its functioning. As Sir2 function is inhibited, more oxidative damaged proteins will be passed to the daughter cell and thus destroy the daughter cell's "newborn" status. As more damaged proteins accumulate within the daughter cell, there will be a greater chance for the daughter cell to get into the senescence phase earlier, and the lifespan of the daughter cell will highly likely be shortened. Therefore, by properly managing the asymmetric cell division, Sir2 protects the newborn daughter cell from over-accepting oxidative damaged protein aggregation from the mother cell and maintaining its original functions.

As mentioned above, either misfolded proteins or the carbonylated proteins can become toxic to cells by eliminating key cellular functions or by inhibiting the cell's protein quality control (PQC) system, which evolved to regulate the cellular protein homeostasis [81]. Also, cell under stress appears to have a reduced threshold for protein aggregation, leading to further loss of physicochemical properties [82-83]. Therefore, to conclude, all three aspects that Sir2 is involved in together provide us a new sight that Sir2 can participate in the pathway of preventing the protein aggregation and, as a result, promote the yeast cell RLS.

Conclusion

The discovery of Sir2 being an NAD+-dependent histone deacetylase brought new sight for people to better understand the mechanism of how it functions to protect the yeast cell from aging. After that, most research starts to focus on how Sir2's enzyme activity functions to promote yeast lifespan. Here, we mentioned three different aspects for Sir2 contributing to lengthening the yeast RLS, including the Sir2 regulation of rDNA silencing, cellular oxidation, and asymmetric division of protein aggregates. Those studies together provide us reliable evidence for Sir2 being an essential regulator for regulating the protein homeostasis within the yeast cell during aging. Since many human diseases are related to protein aggregation, such as Alzheimer's, Parkinson's, and Huntington's diseases, this finding can be very important for Sir2 and medical research in the future.

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