



# The role of NAD and NAD precursors on longevity and lifespan modulation in the budding yeast, *Saccharomyces cerevisiae*

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**Abstract** Molecular causes of aging and longevity interventions have witnessed an upsurge in the last decade. The resurgent interests in the application of small molecules as potential geroprotectors and/or pharmacogenomics point to nicotinamide adenine dinucleotide (NAD) and its precursors, nicotinamide riboside, nicotinamide mononucleotide, nicotinamide, and nicotinic acid as potentially intriguing molecules. Upon supplementation, these compounds have shown to ameliorate aging related conditions and possibly prevent death in model organisms. Besides being a molecule essential in all living cells, our understanding of the mechanism of NAD metabolism and its

regulation remain incomplete owing to its omnipresent nature. Here we discuss recent advances and techniques in the study of chronological lifespan (CLS) and replicative lifespan (RLS) in the model unicellular organism *Saccharomyces cerevisiae*. We then follow with the mechanism and biology of NAD precursors and their roles in aging and longevity. Finally, we review potential biotechnological applications through engineering of microbial lifespan, and laid perspective on the promising candidature of alternative redox compounds for extending lifespan.

**Keywords** Aging · NAD · Biosynthesis · *Saccharomyces cerevisiae* · Lifespan · Cell factory

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## Introduction

Aging is the inevitable biological process characterized by a persistent decline in cellular fitness owing, in part, to deterioration and accumulation of damaging molecules (Gómez 2010; McCleary and Rine 2017). Degenerative physiological damage can be initiated and propagated by cellular programs leading to the onset of diseases and cell death (López-Otín et al. 2013; Belenky et al. 2007). These damaging cellular programs include genetic instability, metabolic disorders, environmental changes, and stress, among others (López-Otín et al. 2013; Skoneczna et al. 2015; Taylor and Hetz 2020). During aging, cells gradually lose ability to

maintain homeostasis; this condition heightens the likelihood of cell death (Guo et al. 2020; Rose et al. 2012). Interestingly, there is interplay between aging progression and secretion of secondary metabolites. These metabolites modulate the rate of aging, and may include sphingolipids (Ren and Hannun 2016), NADPH (Mohammad et al. 2020), hydrogen peroxide (Dakik and Titorenko et al. 2016), ethanol (Mohammad et al. 2018), acetic acid (Baroni et al. 2020), and hydrogen sulfide (Huang et al. 2017), among others.

The budding yeast *S. cerevisiae* is an evolutionary conserved unicellular organism. It gets its name from the fact that it undergoes budding as the mechanism of cell division (referred as division or budding henceforth). It is a model organism of choice due to its relatively short lifespan, well-characterized genome, and easy genetic engineering with myriad protocols readily available (Dakik et al. 2020; Kaeberlein 2010). *S. cerevisiae* has become the most extensively used eukaryote in the study of aging (Arlia-Ciommo et al. 2014; Bilinski et al. 2017), cell cycle (Musa, et al. 2018; Leonov et al. 2017), gene expression (Postnikoff et al. 2017; Ksiazek 2010), metabolism (Baccolo et al. 2018; Croft et al. 2020), signal transduction (Hohmann et al. 2007; Babazadeh et al. 2014), and apoptosis (Baroni et al. 2020; Owsianowski et al. 2008). Hence, to understand the complexity of organismal lifespan in a model organism (e.g., yeast *S. cerevisiae*), aging studies are often designed to; (i) identify underlying causes of aging and in extension the associated physiological cellular damage it causes (ii) to devise strategies for the delay and possible reversal of aging development, (iii) and thirdly, to limit cell vulnerability to a wide range of aging inducing conditions.

Accordingly, there are two classical lifespan study models in budding yeast. The first is the replicative lifespan (RLS); which measures the number of daughter cells an individual yeast mother cell produces prior to senescence (Mortimer and Johnston 1959; Steffen et al. 2009; Boehm et al. 2016; Avelar-Rivas et al. 2020; Rallis et al. 2021). The second is the chronological lifespan (CLS), which is defined as the maximum length of time a cell remains viable in a quiescent state (Murakami and Kaeberlein 2009; Orlandi et al. 2018; Mukai et al. 2019). These models represent strategies for the analysis of proliferating (mitotically active) and non-proliferating tissues (post-mitotic)

in higher eukaryotes (He et al. 2018; Longo et al. 2012; Kaeberlein 2010). The latter, CLS, is traditionally studied by culturing yeast cells to a stationary phase in a synthetic defined medium and monitoring survival as a function of time. Typically, CLS determines cell viability by measuring the number of cells viable in growth media by quantifying cells capable of returning to vegetative growth upon nutrient replenishment (Fabrizio and Longo 2007). The CLS study model holds the advantage of being compatible to several high-throughput techniques such as spotting assays (Teng and Hardwick 2013), flow cytometry (Carmona-Gutierrez et al. 2019) and outgrowth data evaluation (Murakami et al. 2008). Likewise, RLS is also attuned to high throughput microfluidic assessment (Yu et al. 2018a). Each of these tools and techniques provide a platform for molecular analysis of yeast cells for antiaging compounds, screening for longevity associated genes and evaluate glucose and other substrate effects (e.g., calorie restriction, standard and/or calorie excess) according to the study design. As nutrient content progressively diminishes during growth in the liquid medium during CLS, the cell exits the metabolically active to non-dividing state usually in a free-float, leading to the accumulation of stored carbohydrate, cell wall thickening and an overall decline in protein synthesis (Garay et al. 2014; Burtner et al. 2009). These conditions predispose the cell to a distress biological state, accumulate toxic macromolecules and also reduce cellular fitness (Pomatto and Davies 2017).

Many studies focus the search for compounds with properties of improving (extending) lifespan. These led to advances in the application of both natural and non-natural compounds such as plant base derivatives i.e., phenol & flavonoid (Carmona-Gutierrez et al. 2019; Lutchman et al. 2016), anti-fungal/anti-bacterial agents (Choi et al. 2013), ester (Hibi et al. 2018), metal-based compounds (Ribeiro, et al. 2017), and DNA damaging agents (Ross and Maxwell 2018) on lifespan. One that is particularly promising amongst these geropotential alternatives are derivatives of the NAD cofactor (elaborated in subsequent sections) (Mitchell et al. 2018; Rajman et al. 2018; Yoshino et al. 2018) and resveratrol; a natural polyamine spermidine whose role as an anti-aging, antioxidant and anti-inflammatory agent has been well established (Jang et al. 1997; Berman et al. 2017; Pan et al.

2017). Even though studies on resveratrol have been marked with much controversies (Gehm et al. 1997; Pacholec et al. 2010; Miller et al. 2011; Kjaer et al. 2017), its position as antiaging compound has been elucidated. A mounting body of research has established that this compound increases lifespan through a number of mechanistic and molecular targets (Cao et al. 2009; Pezzuto 2011; Szklarczyk et al. 2017; Pan et al. 2017; Kumar et al. 2018). In other words, resveratrol does not abide by the concept of ‘one drug, one target’; thus, suggesting it as promiscuous compound whose effect is dependent on interaction with a host of targets or protein – protein association (Pezzuto 2011, 2019). Consequently, excellent review studies exist in the compound base lifespan extension as demonstrated in the work of Zimmermann et al. (2018), whose study laid guideline for the identification of anti-aging compounds; Hibi et al. (2018) and Dakik et al. (2020) who reported the discovery of new geroprotective compounds and Orlandi et al. (2020) whose work elaborated emerging roles of biosynthetic redox cofactors on yeast RLS and CLS aging.

Genetic and metabolic engineering approaches identified factors that delay the onset of aging and cell death in many model species (Stepień et al. 2020; Gómez-Linton et al. 2019). Early studies revealed the Sirtuins and TOR (Target of Rapamycin) signaling as longevity regulators (Longo and Kennedy 2006; Powers et al. 2006; Wierman and Smith 2014). These proteins (the Sirtuins and TOR factors) provide insight on and have revolutionized aging biology (Deprez et al. 2018; Devare et al. 2020). Besides playing a key role in identifying over 1000 genes through the CLS paradigm in *S. cerevisiae* (Jung et al. 2015), genetic techniques have revealed several pathways and regulatory networks that intersect to modulate lifespan. These pathways promote autophagy, enhance mitochondrial functions, activate stress response, and function as translation factors (Dahiya et al. 2020; Garay et al. 2014; Bjedov and Rallis 2020).

In this review, we focus first on the recent advances, techniques and applications in the study of CLS and RLS – especially analytical tools and high throughput approaches that have transformed molecular lifespan study in yeast. Then we elaborate on the mechanisms, application and biology of

nicotinamide adenine dinucleotide (NAD) and its biosynthetic precursors on aging lifespan. Lastly, we review opportunities for biochemical production via engineering of the yeast lifespan, with future perspectives on the possible application of NAD mimics and analogues for lifespan modulation.

### Aging in yeast: recent techniques and applications

In the last few years, research on the molecular mechanisms of aging has progressed tremendously. It has seen the continuous birth of new technologies and techniques for advanced quantitative and complex molecular study and analysis of biological systems. These tools include cutting-edge imaging techniques (Liu et al. 2018), high precision genome editing tools (Frieda et al. 2017), development of sequencing protocols (Macosko et al. 2015), and design of microfluidics devices (Zhang et al. 2019) have aided in the generation of the mechanisms underlying aging (O’Laughlin et al. 2020). Most notably, geroscience seeks to unravel the basic mechanisms of aging and how its knowledge could be applied in addressing human diseases while also promoting healthy lifespan (Kaerberlein et al. 2017).

With tremendous progress made in the study of aging using budding yeast, recent advances are poised to illuminate single-cell analysis in *S. cerevisiae*. New RLS high-throughput techniques – including microfluidics – represent efficient methods established to overcome the laborious and time-consuming manual separation of budded yeast during lifespan screening (O’Laughlin et al. 2020; Jo et al. 2015). Here, a number of polydimethylsiloxane (PDMS) based devices are designed to address difficulties and challenges of traditional microdissection by automating the separation of mother and daughter cells during aging. This allows for RLS measurements to be interfaced with time-lapse fluorescence microscopy. This technology, though not without limitations, is considered advantageous in yeast lifespan study as it measures the dynamics of aging process in a single cell (Table 1). This is achieved by permitting the tracking and quantification of various molecular and cellular processes at the molecular level (Zou et al. 2020; Yu et al. 2018b; Jo et al. 2015). Microfluidic-based single-cell analysis has recently been harnessed for ribosome profiling and RNA-seq screening to

**Table 1** Advantages and disadvantages of microfluidic and plates readers technologies for lifespan study

High throughput technologies	Advantage	Disadvantages	References
Microfluidic devices for RLS assay	Flexibly designed and relatively easy to implement in a diverse single-cell manipulation and analysis Overcome laborious and time-consuming encounter in traditional microdissection of budded yeast Allows for automated separation of daughter cell. This prevents contamination and errors introduced by manual separation Design to interface with time-lapse fluorescence microscopy, fluorometry and mass spectroscopy for single-cell analysis during RLS measurements Permit the study of aging process in a single yeast cells Utilize very small volume of sample. This help to reduce sample loss and also resulting in highly sensitive detections Microfluidics technology provides the advantage of scalability, precise, and attractive for long-term culture in a controlled environment Overcomes technical challenges of low-throughput yeast lifespan analysis by providing a fast, high-throughput, and accurate analytical method at the single-cell level	Microfluidics has challenge of mass-production for commercialization due to high fabrication cost and choice of materials During yeast cells aging microfluidic channels get clogged and lose the tracked cells, resulting in low efficiency The micropads trapping of cells depend on the size differentials of both the mother and daughter Old cells become exponentially diluted relative to the younger cells as the population grows Masking single-cell heterogeneity during aging Multiple cells can be trapped underneath one micropad, whereas no cells are trapped under others Immobilized of mother cells underneath soft elastomer PDMS micropads. This limits the number of usable cells in the lifespan calculation	Mi et al. (2016), Yu et al. (2018b) Liu et al. (2016), Liu et al. (2015) O’Laughlin et al. (2020), Jo et al. (2015) Mu et al. (2012) Tao Luo et al. (2019) Lee et al. (2012a, b) Jo et al. (2015) Zhang et al. (2012), Xie et al. (2012)
Plates readers for CLS assay	Use to monitor various aging conditions, screen for longevity genes and mutants strain An automated process with a minimum resource requirement and substantially less cumbersome Data generated from plate readers are compactable to a number of cell survival analysis tools and software for CLS analyses e.g., YODA, Gathode/Cathode, AUDIT and SPOCK	Plate readers machine are relatively expensive, and so has high purchasing cost The Honeycomb plates used in (Bioscreen C MBR machine) which has 100-well plates, are not easily adapted for robotic 96 or 384-well assays There are bottlenecks associated with manual data analysis for doubling time and survival percentage calculation	Murakami et al. (2008), Powers et al. (2006) Murakami et al. (2008) Small et al. (2020), Jung et al. (2015)

**Table 1** (continued)

High throughput technologies	Advantage	Disadvantages	References
	Effective method for quantifying aging of large numbers of strains with high accuracy and precision Plate readers allow the user to measure OD at a defined wavelength over time, without manual intervention, and without disturbance of the growth culture	The OD measurements outside of the linear range vary proportionally to the actual culture density There is complexity such as random scattering of transmitted light, flocculation and deflocculation of yeast cell. These create substantial degree of noise in the lag phase	Olsen et al. (2010), Coutin et al. (2020) Murakami et al. (2008)

identify mechanisms by which calorie restriction (CR) can extend yeast RLS (Zou et al. 2020). It also holds the advantage of being relatively easy to implement (Yu et al. 2018b). Elsewhere, the mechanism by which aging is regulated by an evolutionarily conserved enzyme and its molecular pathway has been elucidated (Jo et al. 2015). Cabrera et al., (2017), also explored this tool in the screening of replicative age-dependent intracellular changes, such as protein abundance and its localization.

Lifespan is traditionally studied using a low-throughput clonogenic assay where yeast cells are cultured in enriched media (YPD) plates. This protocol has since transitioned to a high-throughput process with capacity to determine microbial response via outgrowth generated readout. The outgrowth-generated data are values of optical density of an aging culture obtained from plate readers such as a Bioscreen MBR, Biotek Synergy, VICTOR Nivo, Tecan M200 Infinite Pro etc. after hours of growth on fresh media (Small et al. 2020). These machines measure the OD at a particular wavelength over time without manual intervention. Accordingly, this technique has been used to monitor osmolarity effects on yeast aging (Murakami et al. 2008), and screen for longevity associated genes among mutant strains (Powers et al. 2006). Aside from being an automated process that requires minimum resources, it also presents a huge bottleneck in the area of data analysis (Table 1). Consequently, a number of cell survival analysis toolboxes in the CLS model have added to the exploration of genes, aging mechanisms, and underlying cellular damages in budding yeast. Software packages such as Yeast Outgrowth Data Analyzer (YODA) (Olsen et al. 2010), Gathode/Cathode (Jung et al. 2015), Automated Usher for Data Inspection and Tidying (AUDIT) (Coutin et al. 2020), and lately, Survival Percentage and Outgrowth Collection Kit (SPOCK) (Small et al. 2020) are all designed for CLS evaluation. Each of these tools can carry out an automated CLS analysis by calculating doubling time and survival percentage on Hypertext Markup Language or Hypertext Preprocessor (HTML/PHP), on Python and R language respectively. SPOCK presents a number of sanity checks on usage as it protects against possible experimental errors. It can also be applicable in phenotypic analysis of mutant strains. These packages greatly mitigate some of the bottlenecks associated

with traditional yeast clonogenic techniques, and in the calculation of doubling time and survival analysis from the plate reader outgrowth kinetics data (Small et al. 2020). Data obtained from these analytical tools are precise and provide insight on aging and lifespan of the biological systems—in this case yeast *S. cerevisiae*.

Also, microcolony formations which function in principle with clonogenicity is considered a high-throughput technique because of its faster readout (Zimmermann et al. 2018). In this case, aliquots of an aging culture are patched on agar plates and colony growth monitored with a BioSpot® Analyzer which distinguishes cells with a 25 µm diameter (Teng and Hardwick 2013). In the search for drug or antiaging compound, this technique could be applicable to screen for aging yeast culture in well plates and in shaking incubator. At different age points, samples of the aging culture are collected either manually or by automated liquid-handling workstations (e.g. QIAGEN BioRobot) then spotted, and examined for microcolonies after approximately 18 h (Zimmermann et al. 2018). Consequently, flow cytometry measures cell viability through which cell physiological states are determined by staining dyes (FUN-1 or AlamarBlue®). Cell viability is differentiated by varying coloration to determine cell metabolic activity. At this point, the metabolized FUN-1 shifts from green to red fluorescence (Carmona-Gutierrez et al. 2018). Flow cytometric and/or photometric analysis of cell death by membrane integrity are also evaluated using Propidium Iodide dye. Cellular reactive oxygen species (ROS) levels, an important parameter for yeast lifespan could be established by differentially quantifying ROS-dependent conversion of dihydroethidium to hydroxyethidium and ethidium respectively using flow cytometric or photometric analysis (Pan 2011; Kainz et al. 2017).

### Overview of NAD: an essential cofactor in biological system

NAD is an important coenzyme and pyridine nucleotide extant in all living cells. It is central in cellular energy generation and cell metabolism. As a substrate to a number of cellular processes, NAD participates actively in biological functions ranging from energy generation, DNA repair, signaling, gene expression,

and longevity (Braidly and Liu 2020). In addition to being a competent cofactor for many oxidation and reduction reactions (redox) by functioning as an electron donor and acceptor; it also participates in the numerous metabolic pathways including: glycolysis, the tricarboxylic acid cycle (TCA), oxidative phosphorylation and  $\beta$ -oxidation (Cantó et al. 2015). NAD was first reported to play a role in metabolic regulation in yeast cell extracts, and thereafter, as the main hydride acceptor in redox reactions (Covarrubias et al. 2021). Its outstanding features i.e., ability to accept hydride ions, and the capacity of forming a reduced version, NADH, places it (NAD) on a fulcrum critical for metabolic reactions in all living organisms. In addition, it modulates several catabolic pathways, such as glutaminolysis and fatty acid oxidation, through the regulation of dehydrogenase activity. NAD also phosphorylates to form NADP<sup>+</sup>; a hydride acceptor for the formation of NADPH that inhibits oxidative stress and a reducing power in anabolic pathways (Covarrubias et al. 2021).

One of the earliest studies that laid credence to the role of NAD was its discovery in pellagra disease. Pellagra is a dermatitis, diarrhea, and dementia related disease that occurs as a result of dietary deficiency of niacin and vitamin B3 compounds (Orlandi et al. 2017; Sahar et al. 2011). There have been elaborate studies on how this molecule relates to aging (Braidly and Liu 2020; Yoshino et al. 2018; Depaix and Kowalska 2019), thus positioning it as a major universally conserved age-dependent biomolecule. Upon decline of NAD, cell aging ensues owing to the damaging effects of accumulated toxic molecules. This detrimental and life threatening conditions are abrogated by maintaining optimum cellular NAD levels and increasing intracellular adenosine triphosphate (ATP) generation (Poljsak et al. 2020; Chini et al. 2017; Yoshino et al. 2018), usually through supplementation of NAD or its precursors.

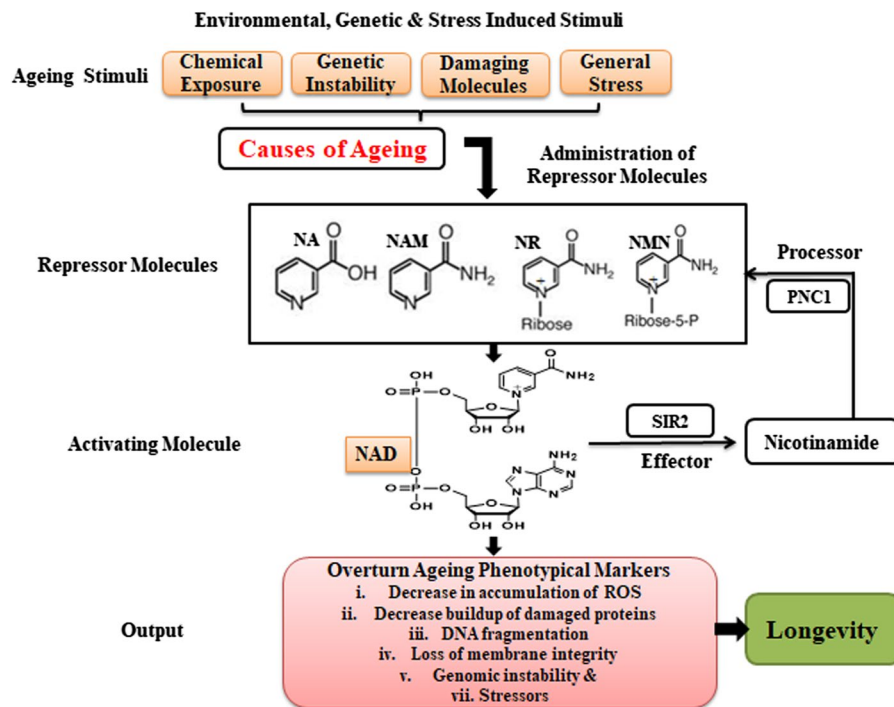
Within the last few years, research on NAD biology and how it applies to aging and longevity in model organisms has gained much momentum (Yoshino et al. 2018; Braidly and Liu 2020; Depaix and Kowalska 2019; Sedlackova and Korolchuk 2020). Some of the notable NAD boosting precursors include: NR, NA and NMN (Covarrubias et al. 2021; Belenky et al. 2007; Orlandi et al. 2020). Even though the metabolic processes of how these compounds increase health span have not been fully understood,

exogenous supplementation has shown preventive and therapeutic effects in ameliorating age-related physiologies and cell dysfunction (Yoshino et al. 2018; Rajman et al. 2018; Lautrup et al. 2019). NR, the most promising geroprotective compound has been classified in pharmacogenomics in the recently launched multi-omics dataset for aging biology (Aging Atlas Consortium 2021). In general, NAD homeostasis is dependent on the dynamic and flexible nature of its precursor molecules. This could be seen in NAM which functions as a precursor to – and an inhibitor of – NAD-dependent enzymes (Jackson et al. 2003; Burtner et al. 2011). Sirtuins are an NAD dependent and evolutionarily conserved family of type III deacetylases that modulate organismal NAD level (Kosciuk et al. 2019). The enzymes (sirtuins) is controlled by the changes in the cellular NAD levels and functions as a link between energy metabolism and longevity regulation (Lee et al. 2019).

**NAD overturns detrimental cellular effect**

Throughout their life cells are exposed to a number of harmful conditions considered detrimental for their survival. These conditions termed “Aging Stimuli” emanate from either the changing environmental state (e.g., exposure to chemical agents and general stress) or genetic influences (e.g., genetic instability) (Fig. 1). These effects are largely dependent on the severity of the stimuli and/or exposure time. Accordingly, they trigger the development of adaptive stress responses (Musa et al. 2018).

To ameliorate these conditions, repressor molecules such as NAD precursors (NA, NAM, NR and NMN), have remained the most promising compounds with the capacity to overturn and reverse these detrimental cellular effects (Croft et al. 2018 2020; Aging Atlas Consortium 2021). Detailed studies have elaborated NAD metabolism



**Fig. 1** Overview of cell response to aging stimuli: cells are exposed to a number of aging stimuli such as chemical agents, damaging molecules accumulation, genetic instability and a number of cellular stresses. These effects along with the corresponding decrease in NAD level predispose the cell to aging conditions and also death. Supplementation of repressor molecules (NA, NAM, NR, NMN) cushion aging associated effects

by producing NAD which is termed activator molecule. Effector and processor proteins (SIR2 & PCN1) also play a key role via the NAD salvage pathways for increased NAD biosynthesis. Through these mechanisms, the intracellular NAD level increases thus suppressing the causes of aging and promoting longevity

and the molecular pathways underlying NAD precursors and their effect upon application in model organisms. These mechanisms are complex owing to dynamic flexibility of precursors used to generate NAD (Croft et al. 2020). For instance, NAM could function as both NAD precursor and an inhibitor of NAD dependent enzymes, such as the sirtuins (Jackson et al. 2003; Bitterman et al. 2002).

The Sir2 protein in *S. cerevisiae* is the founding member of the sirtuin family. It is characterized as a histone deacetylase and functions in transcriptional silencing of heterochromatin and serves as a pro longevity factor (Wierman and Smith 2014). First reported in 1999 to increase lifespan in yeast cell, Kaerberlein and coworkers demonstrated that extra copies of *SIR2* extend by 30% the lifespan of *S. cerevisiae*, in part by inhibiting the formation of extrachromosomal DNA circles (Kaerberlein et al. 1999). Ever since, there have been increased studies on *SIR2* and its associated family members (Anderson et al. 2003; Arlia-Ciommo et al. 2014; Leonov and Titorenko et al. 2013; Ondracek et al. 2017; Dix et al. 2020), as illustrated in Fig. 1 where Sir2 and Pcn1 function as effector and processor proteins for increased NAD biosynthesis.

The biosynthesis of NAD is maintained by three main pathways: de novo synthesis, NAM/NA salvage, and NR salvage (Fig. 2). The NAD molecule along with the biosynthesis and degradation enzymes are compartmentalized in the cell cytoplasm and also regulated within other cellular organelles (mitochondria and nucleus) (Cambronne and Kraus 2020; Verdin 2015). In these regions, NAD is utilized as a cofactor by NAD-consuming enzymes to generate nicotinamide (NAM) as a by-product. NAD utilizing enzymes include protein deacylase family of sirtuins, PARPs (poly adenosine diphosphate-ribose polymerase) and NADases (CD38, CD157 and SARM1) known as NAD glycohydrolases. Because NAD is a critical coenzyme for multiple metabolic processes, energy balance, and cell redox state, the NAM (by-product) are recycled back to NAD via the NAM salvage pathway. These mechanisms enhance cellular energy generation by ensuring constant synthesis, catabolism and recycling of NAD in the cell. This event increases the intracellular NAD concentration thus, activating processes that suppress and reverse aging conditions (Fig. 1).

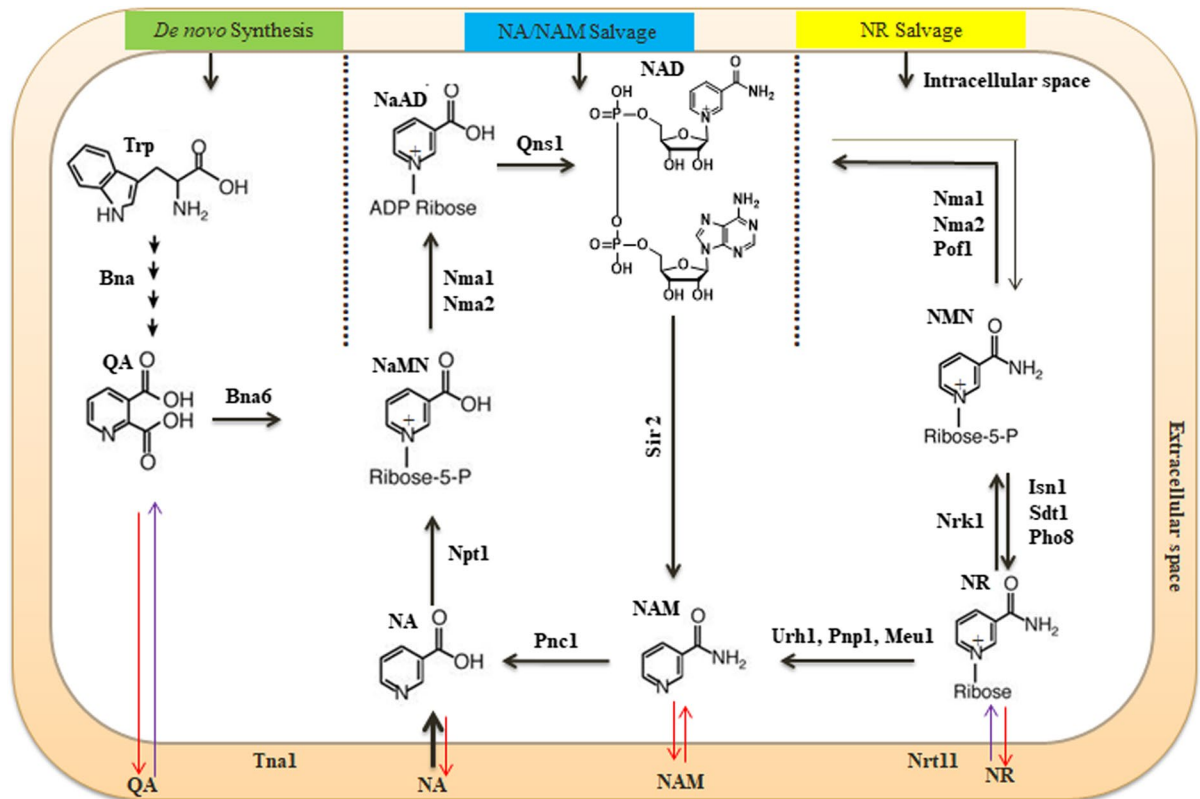
Earlier studies have suggested that NAD salvage pathways extend replicative lifespan by mimicking a calorie restriction effect in yeast (Anderson et al. 2002). During this cellular program, *PNC1* gene, a rate-limiting step of NAM encodes nicotinamidase in the yeast NAD salvage pathway (Anderson et al. 2002). As a result, *PNC1* gene gets activated by stress leading to increased Sir2 activity (Bonkowski and Sinclair 2016). As a histone deacetylase; Sir2 protein also requires NAD, a cofactor for redox reactions and whose levels change in response to nutrients and stress conditions (Imai et al. 2000). These illustrate the intriguing mechanisms by which yeast Sir2 and Pcn1 act as nutrient and metabolic sensors to modulate cellular NAD ratio and alter both transcription and genome stability (Bonkowski and Sinclair 2016; Imai et al. 2000; Anderson et al. 2002). It has also been reported that decrease in NAD level could sufficiently inhibit Sir2 function in transcriptional silencing and shorten RLS (Smith et al. 2000; Sandmeier et al. 2002). Conclusively, Sir2 suppresses cellular senescence by modulating the delay of age-related telomere attrition, promote repair of damaged DNA, and enhanced genome integrity (Lee et al. 2019). They also play a key role in organismal lifespan by interacting with numerous lifespan signaling and pathways e.g., insulin signaling pathway, AMP-activated protein kinase (Lee et al. 2019). Therefore, administration of NAD precursors is considered a promising strategy of promoting longevity (Fig. 1).

## NAD biology and its metabolism

NAD: a biomolecule and energy currency for biological processes

NAD is a major player in oxidative phosphorylation, ATP synthesis, metabolism, and in lifespan (Chini et al. 2017; Yaku et al. 2018b; Braidly et al. 2019). It regulates the activities of sirtuin mediated protein deacetylation and ADP-ribosylation through biosynthetic pathways and cells product degradation (Croft et al. 2020). It also serves as an energy currency for redox reactions (Yin et al. 2014). During the catabolic process, cells break down larger molecules to smaller building blocks and are subsequently used for energy generation (Shi et al. 2005; Croft et al. 2020). Also, high-energy electrons





**Fig. 2** Pathways for NAD biosynthesis in yeast cell. NAD is synthesis through three basic pathways **a** tryptophan de novo synthesis, **b** NA/NAM salvage pathways and **c** NR-mediated synthesis. In tryptophan de novo synthesis, biosynthesis of nicotinic acid proteins (Bna 2, 7, 4, 5, 1) participate in the spontaneous cyclization leading to the formation of QA and subsequently NaMN by biosynthesis of nicotinic acid 6. On sufficient abundance of NAD, this pathway ostensibly remains inactivated. In the NA/NAM salvage pathway, NaMN is also produced upon conversion of NA; this leads to the formation of NaAD, which metabolizes to NAD using Nma1, Nma2 and Qns1, respectively. The NR-mediated NAD biosynthesis converts NR to NMN by Nrk1, which helps to synthesize NAD using Nma1, Nma2 and Pof1. In other words, NR are assimilated through the Nrk1-dependent and Urh1/Pnp1-mediated

routes responsible for the utilization of exogenous NR generated from NMN by the nucleotidase activities of Isn1 and Sdt1 in the cytosol. The thick dark arrow illustrates exogenous uptake of NA from yeast growth media. Light purple arrow shows transporters of QA and NA using the Tna1 transporter and that of NR by Nrt11. The light red arrows represent some of the poorly understood mechanisms involving the leakage of molecules into the extracellular space. The left panel demonstrates the initialization of *de novo* synthesis from tryptophan, the middle is the NA/NAM salvage pathways while the left panel illustrates NAD synthesis through NR salvage pathways. These panels are also demarcated by broken lines. White background shows the intracellular space while the pink shaded part stands for the extracellular space

are stored temporarily as NADH and donated to the electron transport chain during respiration to produce ATP (Friedkin and Lehninger 1948). Its phosphorylated form (NADP) is vital for the reductive biosynthesis of molecules and protection of cells against reactive oxygen species.

The dynamism and homeostasis of NAD is dependent on its supplying precursors. In *S. cerevisiae*, NAD is produce first, through the de novo synthesis pathways which catalyze tryptophan to

generate NAD from quinolinic acid (QA) and then to nicotinic acid mononucleotide (NaMN) using biosynthesis of nicotinic acid protein (Bitterman et al. 2003; Misiak et al. 2017). Then secondly, from the salvage pathways where precursor molecules (NA, NAM and NR) are harnessed in the form of NA/NAM salvage and NR salvage pathways to generate NAD (Kato and Lin 2014). In yeast cells, two cytosolic NAD kinases exist. These include NAD substrate producing NADP (Shi et al. 2005) and mitochondrial NAD kinase

which prefers NADH substrate producing NADPH (Strand et al. 2003). Constitutively, amino acids, triacylglycerols, steroids and nucleotides and phospholipids are produced using NADPH. Aside being required for anti-oxidative defense systems, NADPH also activates glutathione and thioredoxin reductases by direct reduction of inactive glutathione and thioredoxin (Jamieson 1998; Inoue et al. 1999; Minard and McAlister-Henn 2001).

As a vital molecule in biological systems, diseases such as cancer, neuron degeneration and diabetes are linked to aberrant NAD metabolism (Canto et al. 2015; Nikiforov et al. 2015; Verdin 2015; Chini et al. 2017). Supplementation of NAD<sup>+</sup> precursors increase NAD levels and ameliorate health deficiencies in model organisms (Belenky et al. 2007; Brown et al. 2014; Williams et al. 2017). Very recently, studies on the regulatory mechanisms of NAD in unicellular eukaryotes suggest a systemic decline of NAD that coincides with, decrease decline in cellular function, and reduced cell metabolism in aging cells (Orlandi et al. 2020; Croft et al. 2020). These however, have laid solid foundations for more studies moving forward.

#### De Novo synthesis and NA/NAM salvage biosynthesis pathways

The spontaneous cyclization activity of the enzymatic reaction involving biosynthesis of nicotinic acid proteins, an enzymes that consume the amino acid tryptophan for de novo biosynthesis, has suggest de novo to be the least preferred NAD biosynthesis pathway in the yeast cell (di Luccio and Wilson 2008; Beas et al. 2020). Here, phosphoribose moiety of phosphoribosyl pyrophosphate (PRPP) is transferred from biosynthesis of nicotinic acid to tryptophan-derived QA or to nicotinic acid (NA) is catalyzed by biosynthesis of nicotinic acid 6 (Bna6) and NA phosphoribosyltransferase (phosphoribosyl transferases Npt1) respectively (Cantó et al. 2015; Covarrubias et al. 2021). In the QA driven amino acid tryptophan reaction, five essential proteins are involved in the spontaneous cyclization reaction these include, tryptophan 2, 3-dioxygenase (Bna2), arylformamidase (Bna7), kynurenine 3-monooxygenase (Bna4), kynureninase (Bna5), and 3-hydroxyanthranilic acid dioxygenase (Bna1). Consequently, the phosphoribose moiety of PRPP transferred from Bna6 to QA catalysis NaMN

production where it serves as convergence point for the de novo synthesis and NA/NAM salvaging pathways. Notably, NA generated in the NA/NAM salvage pathway via the NAM deamidase reaction utilize pyrazineamidase and nicotinamidase (Pnc1); a vital enzyme in the salvage reactions. On hydrolyses of the amide group of NAM with Pcn1, the NA are produced (Hong and Huh 2021; Ghugari et al. 2020), thereby suggesting that at mutant of PNC1, NAM concentration could be tremendously increase while also inhibiting sirtuin functions (Mei and Brenner 2014; Jiang et al. 2016). At this convergence point, nicotinamide mononucleotide adenylyltransferase 1 & 2 (Nma1 and Nma2) participates in the conversion of NaMN to nicotinic acid adenine dinucleotide (NaAD) by adenosine monophosphate (AMP moiety) addition (Pinson et al. 2019), which undergoes amidation by glutamine (Q)-dependent NAD synthetase (Qns1) to yield NAD molecules (Kropotov et al. 2021; Chi and Sauve 2013). Aside from Qns1 which carries out amidation of NaAD to NAD, there exist also other de novo route which uses molecular oxygen as a substrate (Bna2, Bna4 and Bna1), thus suggesting utilization of anaerobic cellular growth conditions on the salvage pathways for NAD synthesis (Croft et al. 2020).

In specific, the de novo synthesis pathway does not appear essential for Sir2-dependent silencing or in lifespan extension by CR as biosynthesis of nicotinic acid 1 or *BNA6* deletion does not elicit an rDNA-silencing defect (Kato and Lin 2014). This condition however is repealed in the absence of NA in the growth medium, which together with QA, is often acquired from the extracellular sources (Fig. 2). This acquisition is done using a NA transporter called transporter of nicotinic acid 1 (Tna1) (Ohashi et al. 2013; Brickman et al. 2017). At abundant concentrations of NAD biosynthesis of nicotinic acid proteins are silenced by sirtuins and Hst1. Outright depletion activates the de novo pathway and/or salvage pathway during which sirtuin mediated protein deacetylation occur, thereby producing many NAD by the consumption of NAM.

#### NR-mediated NAD biosynthesis

Phosphorylation of NR to NMN using NR kinase is the determining step for NR-mediated NAD biosynthesis (Croft et al. 2020). NR get converted to NMN

by nicotinamide riboside kinase (NrK1) catalyzed phosphorylation, which in turn get adenylylated to NAD by Nma1 and Nma2 upon addition of ATP (Kato and Lin 2014). NR salvage holds advantage over NA/NAM salvage by being conferred with branch flexibility due to its enzymes and precursors compartmentalization. In addition to the vacuole storage of NAD intermediate such as NR and NMN (Lu and Lin 2011), it is also considered the most economical as it requires no PRPP. In *S. cerevisiae*, the NR salvage connects to the NA/NAM salvage pathway using the nucleosidases methylthioadenosine phosphorylase (Meu1), uridine hydrolase (Urh1) and purine nucleoside phosphorylase (Pnp1) where they convert NR to NAM, or nicotinic acid riboside (NaR) to NA (Tempel et al. 2007; Fletcher and Lavery 2018). NrK1 also converts NaR, a deamidated form of NR, to NaMN where it assists in the supply of needed metabolites for NAD biosynthesis (Belenky et al. 2007). Phosphorylation of NR by Pnp1 and Urh1 also triggers rising intracellular NAM levels and connects NR to the NA/NAM salvage pathway. Nrt11, a transporter for NR, allows for efficient conversion to NAD after phosphorylation of NR to NMN by nicotinamide riboside kinase NrK1 (Belenky et al. 2008; Fletcher et al. 2017). Constitutively, cytosolic nucleotidases IMP-specific 5'-nucleotidase (Isn1), suppressor of disruption of TFIIS (Sdt1), vacuolar acid phosphatase (Pho8) and equilibrative transporter (Fun26), have been shown to contribute to NR metabolism by converting NMN to NR (Bogan et al. 2009; Lu and Lin 2011; Kato and Lin 2014). In an alternative route, conversion of NAD intermediates to produce NAD may occur just as seen in recombinant Pnc1 which convert NAM to NA, and deamidate NMN and NR (Lu et al. 2009; Croft et al. 2018).

#### Bidirectional flux and transport of NAD precursors

Even though NR is absent in the yeast growth media, unlike NA, it catabolizes and ensures stability in the supply of intracellular NAD (Belenky et al. 2007). NA and NR are released extracellularly into culture medium; giving rise to a bidirectional flux (Fig. 2) between the intra and extracellular compartments to achieve optimum NAD pool (Belenky et al. 2011). The yeast cells appear to produce NAD during its log phase growth mainly by using the NA/NAM salvage pathway (Sporty et al. 2009). This is

as a result of the presence of niacin (NA and NAM) in its growth media; the uptake of NAM, NA and QA are aided by NA transporter protein Tna1 (Croft et al. 2018), while that of NR utilize Nrt1- an enzyme whose role in NAD biosynthesis remains to be fully elucidated (Ohashi et al. 2013). Conversely, because NR is an endogenous metabolite and also not found in the growth media (Lu et al. 2009), it negatively influences NAD synthesis in mutants defective de novo and NA/NAM salvaging pathways. The constant release and retrieval of NR to and from the growth culture medium create a form of clouding or traffic between the extracellular and intracellular compartments. Constitutively, the rate of NR relief in the growth medium is largely depending on genetic background and the yeast cell growth conditions. On average, the intracellular level of NR in the wild type cell ranges from ~2–10  $\mu\text{M}$  (Evans et al. 2010; Lu et al. 2009). As a complex driven process, understanding mechanisms cell uses to participate in networks such as nutrient sensing, transcriptional control, enzyme compartmentalization and feedback inhibition remain largely essential and also incompletely understood (Aman et al. 2018; Pinson et al. 2019; James Theoga Raj et al. 2019).

#### NAD and its analogues act as molecules for cellular aging and longevity

##### The replicative and chronological lifespan models

Replicative Lifespan (RLS) in the yeast *S. cerevisiae* measures the number of daughter cell (bud) single mother yeast produced before it dies (Steinkraus et al. 2008; Orlandi et al. 2020). For over half a century, RLS has been used in the study of lifespan in model organisms (Mortimer and Johnston 1959). It has also provided insight about aging progression in higher eukaryotes. In this study model, the mother cell produces daughters, which are physically smaller in size. According to Longo et al (2012), the concept is simple as it takes advantage of the asymmetric division in budding yeast. One of the physical characteristics of the aging mother is its appearance. Here, numerous scars on the mother yeast illustrate repeated budding and detachment/separation of the daughter cell. With the understanding that cells do not live forever, the concept

of RLS was designed to quantify the number of progeny a yeast mother generates before senescence. With advances in techniques and methodologies, recent decades have witnessed upsurge in RLS study owing to the understanding of the molecular mechanisms, identification of genes, and regulatory pathways. Traditionally, cells grown in a Yeast Peptone Dextrose (YPD) medium are physically separated using a manual micromanipulator equipped with a fiber-optic needle. These are monitored for 2–3 weeks and thereafter, the generated data set plotted against time to determine the survival percentage (Kaeberlein and Kennedy 2005; Steffen et al. 2009). More so, microfluidic techniques (detail in Sect. 2) and advanced high-throughput methods also provide protocol for the selective quantification of daughter cells.

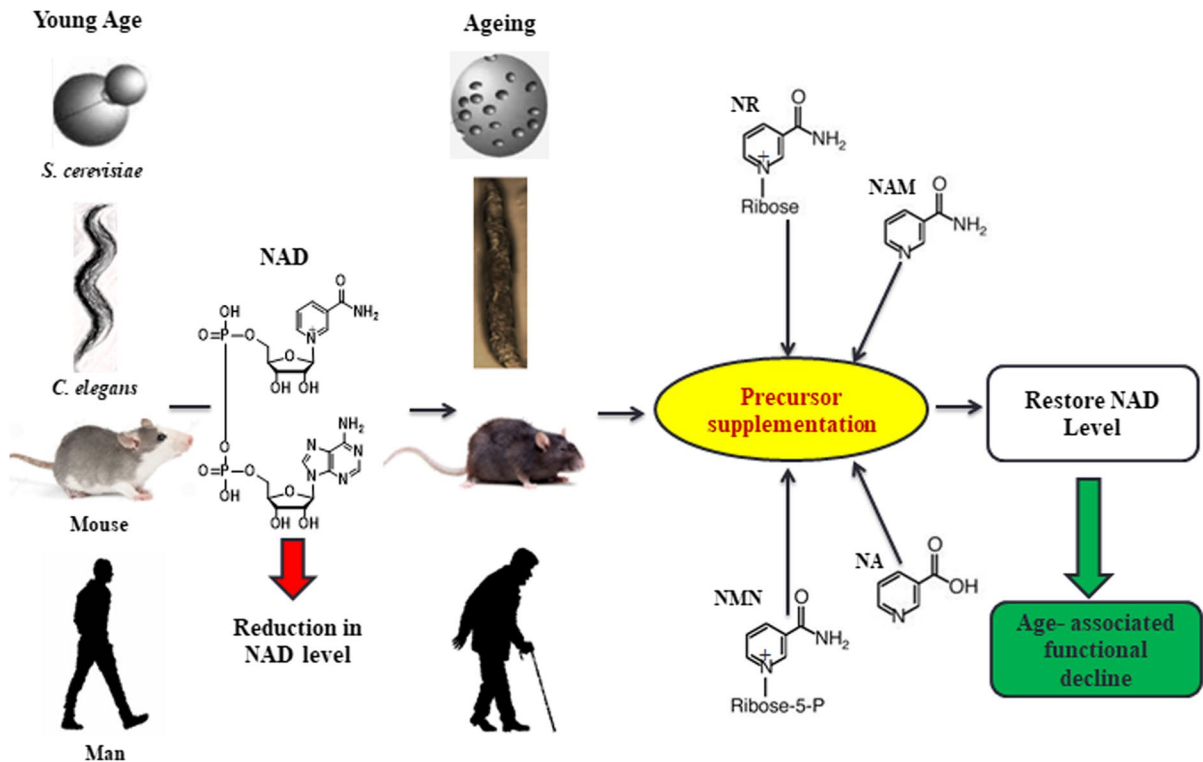
Chronological lifespan (CLS) is the length of time that a non-dividing yeast cell remains viable in a quiescent state. CLS estimates the amount of time a cell could possibly remain viable in a quiescent state i.e., cells that could regenerate upon return in a nutrient rich medium. Typically, cells are grown in a culture medium up to the post-diauxic state; a condition at which most cells exit the cell cycle. At this time, extracellular glucose has already been depleted, thereby triggering a switch to mitochondrial respiration and dramatically reducing cell propagation. Notably, the respiratory driven metabolism relies solely on the ethanol obtained during glucose fermentation (Werner-Washburne et al. 1996). Ocampo et al., (2012), mitochondrial respiration activation above an optimum level, sufficiently stores nutrients which induce stress resistance and results in the extension of CLS. In addition, CLS is characterized by lower metabolic rates and up-regulation of stress-resistance pathways. It is also an aging model to understudy aging progression in higher organisms or non-dividing cells (Longo et al. 2012; Fabrizio and Longo 2007; Longo 1997; Longo et al. 1996). Mechanistically, down-regulation of TOR pathway activity has proven to increase both CLS and RLS (Cao et al. 2016). Deletion of the *SCH9* kinase, a major TOR effector, also promotes CLS (Fabrizio et al. 2001). *TOR1* deletion increases mitochondrial respiration. Experimentally, it has been established that *tor1Δ* or *sch9Δ* cells produce mitochondrial ROS during growth. This ROS provides an adaptive

hormetic signal which activates the stress response dependent on the activity of Msn2/4 and Gis1. This results in a reduced ROS level in stationary phase, thus triggering elevated cell survival (Pan et al. 2011).

### Lifespan modulating principles

Compounding evidence of correlation between NAD depletion and aging abound. NAD decline triggers dysfunction of a cells physiological state and aging related conditions (Sedlackova and Korolchuk 2020; Aman et al. 2020; Fang et al. 2017). Even though the consequences of NAD decline is universally conserved (Fig. 3), it is unclear if its reduction is as a result of increased NAD consumption or reduced synthesis (Misiak et al. 2017). The progressive drop in NAD level is also likened to high turnover orchestrated by hyperactivation of PARP upon exposure to excessive DNA damaging agents (e.g., free radicals). An active approach has been to modulate NAD metabolism in cells through strategies that would involve (a) application of chemical inhibitors, (b) use of biologically active compound and (c) use of other NAD precursors (Depaix and Kowalska 2019; Okabe et al. 2019; Strømland et al. 2019). It is difficult to establish which of whether changing NAD metabolism and protocol is most important for NAD biology and which method that would ensure optimal state. It is imperative to presume a multiplier effect of the redox reaction, just as the rate-limiting NAD biosynthesis (Namp1), may differ in biological systems and according to their cellular state (Yaku et al. 2018a; Okabe et al. 2019). Strategic to this is the NAD-consuming enzymes (sirtuins); which mediate deacetylation and other processes involving NAD levels reduction. A constant supply of NAD replenishing precursors is proposed to increase organismal viability and attenuate deficiencies linked to aging process (Fig. 3) (Imai and Guarente 2016; Rajman et al. 2018; Lautrup et al. 2019). Furthermore, metabolism of the related molecule, NADPH, is essential for proper redox state within the cell. Genetic manipulation of the pentose phosphate pathway leads to NADPH imbalance, which disrupts the cell's redox state and decreases replicative potential without affecting the total cellular lifespan (Kwolek-Mirek et al. 2019).

The extension of lifespan in model organisms depends on the NAD utilizing enzyme, Sir2, a pioneering member of the Sirtuin family (Kaeberlein



**Fig. 3** NAD decline during aging is universally conserved. Across species; unicellular organisms (*S. cerevisiae*) and higher multicellular model animals (*C. elegans*, Mouse (*M. musculus*)) including Man, NAD level are reduced with increase in age. At young age, the cellular NAD concentration is presumably at an optimum state which guarantees viabil-

ity, vitality and fitness. During aging, this molecule steadily decreases leading to the onset of pathophysiological dysfunctions, disease progression and even death. The cell's dysfunction gets ameliorated upon supplementation with NAD precursors resulting in the restoration of NAD level with a corresponding decline in age-associated functions

et al. 1999). This NAD consuming enzyme is crucial for longevity as it suppresses rDNA recombination, silences telomere genes, mating-type loci (*HML* and *HMR*) and maintains a hypoacetylated chromatin state (Gartenberg and Smith 2016). As a determining component of lifespan in yeast, *SIR2* deletion increases frequency of rDNA recombination causing accelerated aging. In other words, it serves as a point of interest for NAD and its precursor's assessment in RLS. Increased recombination distorts genomic stability and negatively impacts the replicative lifespan (Lindstrom et al. 2011; Ganley and Kobayashi 2014). Enhanced NAD improves positive metabolic effects mediated by the induction of *SIR2*. This is evident where the redox precursors boost the level of NAD and promote longevity (Table 2) (Mouchiroud et al. 2013; Fang et al. 2017; Rajman et al. 2018).

Unlike in RLS, assessment of the effect of NAD precursors on yeast chronologically aging cells provide insight on the enzymes/protein mechanisms and pathways within which NAD are metabolized. Traditionally, CLS estimates the cell's relative survival i.e., percentage of cell that is capable of resuming growth upon return or activation in fresh rich medium. In this model, a shift from glucose-driven fermentation to ethanol/acetate-driven respiration (diauxic shift) at the 72 h usually triggers a global metabolic reconfiguration (Orlandi et al. 2020). The hallmark of this metabolic change is an increase mitochondrial respiration capacity and activation of gluconeogenesis; a key feature presumed to be fundamental for chronological lifespan extension and production of longevity supportive metabolites (e.g., trehalose) (Table 3) (Ocampo et al. 2012; Casatta et al. 2013; Baccolo et al. 2018). These metabolites and second

**Table 2** NAD analogues and their roles on yeast lifespan

NAD analogues	Mechanisms	Regulatory network/ pathways	Aging model	Responses	Organism/strain	References
NR	Promotes Sir2-dependent repression and improves gene silencing	Increases net NAD synthesis through Nrk1 pathway mediated by NR kinase and the Urh1/Php1/Meul1 pathway	RLS	Triggers extended RLS lifespan	Deletant yeast strains derivatives of BY4742 <i>S. cerevisiae</i>	Belenky et al. (2007)
NAM	Activate cellular response for up regulation of longevity gene	Regulates Sir2 activity by modulating NAM levels using Pnc1	RLS	Modulate yeast lifespan at a concentration dependent manner	<i>S. cerevisiae</i>	Anderson et al. (2003)
INAM (iso nicotinamide)	Stimulates Sir2 to promote intracellular NAD and rDNA locus silencing	Induce NAD level rise via the Pnc1 and Npt1 genes of the NAM salvage pathway	RLS	Extends lifespan in a <i>SIR2</i> -dependent manner with a corresponding rise in NAD level	<i>S. cerevisiae</i>	McClure et al. (2012)
NADPH	Provide electrons for thioredoxin (TRR) and glutathione reductase systems (GTR)	Catalyze via the Zwf1- and Gnd1-dependent reactions of the pentose phosphate pathway (PPP) and the Ald4-, Pos5-, Mae1-, and Idp1-driven reactions in the yeast mitochondria	CLS	TRR and GTR an NADPH-dependent reductase systems maintains the intracellular redox homeostasis	<i>S. cerevisiae</i>	Arlia-Ciommo et al. (2014), Leonov and Titorenko et al. (2013)
NAD-depleted environment	Allow for enzymatic function in the NAD-depleted environment by screening for mutant in Sir2 catalytic domain	Extend RLS by mutations in <i>SIR2</i> and increase enzymatic activity at low NAD	RLS	Sirtuin mutants permit function in NAD-limited environments and extend RLS	<i>S. cerevisiae</i> & mammalian cells	Ondracek et al. (2017)
NAM	Promotes Pck1 enzymatic activity, gluconeogenesis and phenotype <i>SIR2</i> inactivation	Regulate anabolic and respiratory activity and maintain low burden of superoxide anions	CLS	Extended the CLS of the wild type (WT) and affecting <i>Sir2</i> mutant even at high concentration	<i>S. cerevisiae</i> mutants and wild strains	Orlandi et al. (2017)
NAM	Induce inhibition of yeast silencing and increase rDNA recombination	Function as a physiologically relevant regulator of <i>Sir2</i> enzymes	RLS	Shortens RLS of <i>S. cerevisiae Sir2</i> mutant	<i>S. cerevisiae Sir2Δ</i>	Bitterman et al. (2003)

**Table 2** (continued)

NAD analogues	Mechanisms	Regulatory network/ pathways	Aging model	Responses	Organism/strain	References
Endogenous NR	Modulate classical salvage pathways and the NR salvage branch using NAD-dependent Sir2 deacetylase	Catalyze the classical salvage pathways and the NR salvage branch	RLS and CLS	Severity of growth defects in mutants correlate with the amount of Nmr release and assimilation and are essential for CR-induced lifespan	BY4742 <i>S. cerevisiae</i> strain	Lu et al. (2009)

messengers interplay and are a major determinant in regulating aging cells in CLS culture. Just like Sir2 in RLS, phosphoenolpyruvate carboxykinase (Pck1), increases CLS and enhances gluconeogenesis (Orlandi et al. 2017). Addition of NAD precursors in yeast CLS is envisioned to involve mechanisms that would unravel metabolic pathways affecting CLS and secondly, identifying biochemical strategies governing the decline of NAD levels in aged cells (Orlandi et al. 2018; Demarest et al. 2019).

### Precursors for NAD and their influence on RLS and CLS lifespan

#### Nicotinic acid (NA)

NA is transported into the yeast cell by high affinity nicotinic acid permease (Tna1). NA is non-essential even though it contributes to rising NAD levels in cooperation with availability of precursors in the salvage pathway when found in growth medium. It has been established that cells grow optimally in NA-free synthetic medium, but then display a reduced RLS and poor telomeric silencing (Orlandi et al. 2020). Evaluating intracellular NAD (1 mM) and amount of NA (2 mM) in a culture medium point to the fact that, NAD even at lower concentration (1 mM), allow for cells metabolic supplies and growth while inhibiting Sir2 activity (Belenky et al. 2007). The bioavailability of NA in the growth medium along with other precursors triggers the salvage pathway and increases NAD levels; it also further promotes a Sir2 function which consequently extends RLS. In view of this, mutant *npt1* (NA salvage enzyme) characterized by normal growth expresses decrease in NAD levels, loss of Sir2-mediated silencing and then shortens RLS (Hanasaki et al. 2020). Transcription of genes of the de novo pathway is under the control of Hst1, and it conceived that this NAD-dependent deacetylase also function as an NAD sensor where it regulate NAD levels and the degree of biosynthesis of nicotinic acid gene repression. Uneven responses presented by activity of *npt1* null mutants in the de novo pathway (upregulation of genes), and the salvage pathway (genes unaffected); indicate a defective NA/NAM salvage and the de novo pathway for NAD biosynthesis (Lee

**Table 3** Metabolites and their roles on *S. cerevisiae* CLS

Metabolites	Mechanism	Pathways	Regulatory proteins/ genes	Sites of synthesis	Response	References
NADPH	Provides electrons for TRR and GTR reductase systems	Exhibit response via PPP	Associated genes regulating NADH reaction include <i>Zwf1</i> - <i>Gnd1</i> , <i>Ald4</i> , <i>Pos5</i> , <i>Mae1</i> , and <i>Idp1</i>	Cytosol and mitochondria	Maintain intracellular redox homeostasis and decrease cellular oxidative damage	Brandes et al. (2013)
Spermine and spermidine	Inhibits histone acetyltransferases	Uses arginine and methionine for reactions in the mitochondria, cytosol, and peroxisomes	Activates transcription of the <i>ATG</i> genes, to promote cytoprotective process of autophagy	Mitochondria cytosol, and peroxisomes	Ensures quality control by asymmetric segregation of damaged materials	Krüger et al. (2013)
Acetic acid	Activate direct or indirect age-related apoptotic cell death and intracellular acidification	Exert effect through the nutrient signaling pathways	Stimulating cAMP/PKA signaling pathway and ROS generation	Cytosol and mitochondria	Acidification accelerates chronological aging with a pro-aging effect	Eisenberg et al. (2014), Mohammad et al. (2018)
Glycerol	Increase glucose fermentation and trigger the lowering of ethanol and acetic acid level	Modify transcriptional activator of some genes and other adaptive stress response pathways	Took part in glucose fermentation and ensuring susceptibility to long term stress	Cytosol	Induce rise in intracellular NAD/NADH with a pro longevity chronological phenotype	Wei et al. (2009)
Hydrogen peroxide	Exert longevity effect in response to CR or inactivation of catalases	Hormesis mediated pathways extend CLS by CR and mutational inactivation of growth signaling pathways	Activate anti-aging cellular pattern by stimulating transcription of <i>Gis1</i> , <i>Msn2</i> , <i>Msn4</i> , <i>SOD1</i> and <i>SOD2</i> genes	Mitochondria and peroxisomes	Elicit oxidative damage of proteins, lipids, and nucleic acids at high concentration	Ludovico & Burhans (2014)
Sphingolipids	Modulate serine palmitoyltransferase (SPT) activity	The sphingolipid backbone base phosphorylates sphingosine stimulates protein kinase activities of <i>Pkh1</i> and <i>Pkh2</i> pathways	The stimulated proteins are phosphorylated to activate the nutrient-sensing protein kinase <i>Sch9</i>	Endoplasmic reticulum (ER), cytosol, Golgi apparatus	A pro chronological aging metabolites	Ren & Hannun (2016), Roelants et al. (2011)
Trehalose	Regulate proteostasis activities	Cellular proteostasis sustain an anti-aging program, decrease misfolding and oxidative damage	Binds to newly synthesized proteins in cells to help decrease damaging effects	Cytosol	Ensures cell survival during starvation, extend CLS. Exhibits either an anti-aging or pro-aging effect	Leonov et al. (2017), Ocampo et al. (2012)



**Table 3** (continued)

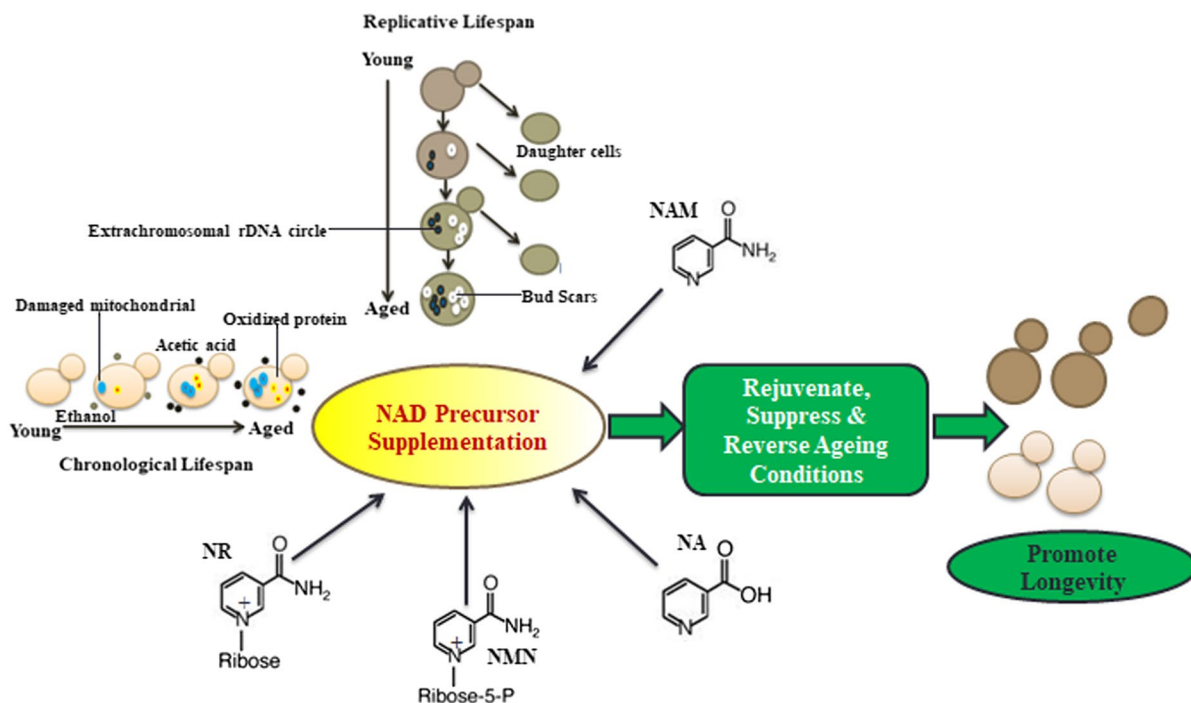
Metabolites	Mechanism	Pathways	Regulatory proteins/ genes	Sites of synthesis	Response	References
Amino acids	Regulates longevity-defining programs via downstream activation of Target of Rapamycin complex I (TORC1) and proteins (Sch9, Atg13, Tap42)	Nutrient-sensing protein kinase A (PKA), TORC1	Activation of protein kinase activity of the TORC1	Intermediates in the yeast cell mitochondria and in the TCA cycle	Pro-aging molecule which function by phosphorylating downstream protein targets (Sch9, Atg13, Tap42)	Swinnen et al. (2014)
Ethanol	Anaplerotic conversion of acetyl CoA to citrate and acetyl carnitine. Catalyze peroxisomal $\beta$ -oxidation of fatty acids to acetyl-CoA	Accelerate CLS by increasing age-related mode of regulated cell death (RCD) i.e., liponecrosis	Pro-aging of sirtuin deacetylase (Sir2) occur by inhibiting Adh2-driven conversion of ethanol to acetaldehyde	Mitochondrial and cytosol	Accelerates yeast chronological aging	Mohammad et al. (2018)
Free fatty acid (FFA) and diacylglycerol (DAG)	Accelerate the onset of age-related liponecrotic RCD	Ethanol-dependent suppression of peroxisomal $\beta$ -oxidation pathway	Accumulation of unoxidized FFA in peroxisomes elicits negative-feedback (buildup of FFA and DAG)	ER and lipid droplets	FFA and DAG serve as pro-aging metabolites shortened yeast CLS	Beach and Titorenko (2011)
Hydrogen sulfide ( $H_2S$ )	Endogenously synthesized in the transsulfuration (TSP) pathway from methionine to cysteine	Water and fat-soluble gas produce by assimilation of inorganic sulfate	Promote electron transport chain in mitochondria and activate transcription of stress-response genes	Mitochondrial electron transport chain	It release in culture triggers yeast CLS. It also inhibit yeast chronological aging by CR	Hine et al. (2015)

et al. 2013; Sauty et al. 2021). However, lack of Npt1 does not affect CLS differently as seen in RLS through the NA salvage pathway (Lu et al. 2009). Short-lived mutants *nrk1Δ*, *urh1Δ* and *pnp1Δ* upon supplementation of NA show no phenotype change on it CLS unlike the NR salvage pathway in which NR uptake might be required for cell survival in stationary phase (Orlandi et al. 2020; Belenky et al. 2007). NAD-dependent deacetylase is hypothesized to sense or regulate NAD levels by modulating the degree of biosynthesis of nicotinic acid gene repression. In principle, chronological aging is characterized by Pck1 enzymatic activity which regulate de/acetylation state; an increase in the acetylated (active) form that enhances gluconeogenesis and extends CLS (Orlandi et al. 2017; Casatta et al. 2013). At the negative control of Sir2, which is on the gluconeogenic activity of Pck1, negative effects on CLS are also reported. Activation of endogenous defense mechanisms is proven to contribute to prolong lifespan, especially some important metabolites which act as second messengers in CLS aging (Table 2). NAD precursors on chronological aging involve two key mechanisms; those that affect

physiological reduction of NAD levels observed in aging cells and those involved in metabolic pathways that affect CLS (Orlandi et al. 2018; Belenky et al. 2007).

#### Nicotinamide (NAM)

Studies on yeast *S. cerevisiae* lifespan extension have continuously pointed to Sirtuin activity, calorie response, and general cell redox balance (Nagarajan and Parthun 2020; Vatner, et al. 2020). This however excuses the continuous changing state of NAD which activates or inhibits Sir2, resulting in downstream changes on aging and lifespan related conditions (McCleary and Rine 2017). In addition to being activated by NAD, Sir2 is also inhibited by NAM. NAM is produced during deacetylation reactions, when Sir2 consumes a molecule of NAD (Hwang and Song 2020). This evolutionarily conserved NAD-dependent deacetylase is a critical regulator of metabolism and aging. The exogenous supplemented effects of NAM has been studied in both *S. cerevisiae* RLS and CLS models (Fig. 4). Accordingly, cells grown in NAM supplemented media phenocopy that of *SIR2*



**Fig. 4** Supplementation of NAD precursors promote RLS and CLS in yeast *S. cerevisiae*

deletion. They demonstrate similar shortened RLS and other features that mimics loss of *SIR2* functions, most importantly, a reduced silencing and increase in recombination at the rDNA locus, due to inhibition of Sir2 enzymatic activity (Sauve et al. 2005). NAM addition in yeast growth medium at initiation of chronological aging (diauxic shift) promotes CLS by inhibition of Sir2 activity. The metabolic reconfiguration during this shift confers in cells (*sir2Δ* cells and Sir2 activity) a metabolic change that ensures an extended CLS.

Elsewhere, supplemented yeast medium at NAM concentration of 5 mM led to the blockage of activity of Sir2 which phenocopies *SIR2* inactivation (Orlandi et al. 2017). This result confirms and corroborates the earlier prediction regarding the inhibition of Sir2 activity as previously reported (Anderson et al. 2003; Gallo et al. 2004). In addition, increase in the Pck1 enzymatic activity in NAM supplemented culture shows effects due to rising acetylated active form of the enzyme unlike in culture without NAM supplementation. This suggests therefore that NAM, in standard CLS experiment, inhibits Sir2-mediated deacetylation of Pck1 (Casatta et al. 2013; Lin et al. 2009). Hence, Pnc1 overexpression abrogates the inhibitory silencing effects of exogenously added NAM by converting the excess of NAM into NA and increasing RLS (Wierman and Smith 2014). The exogenously imported NAM creates a high local concentration of NAM; a phenomenon which, if not cleared by Pnc1 via the inhibition of Sir2, has a pro-replicative aging effect. Isonicotinamide (INAM), an isostere of NAM restores the intracellular NAD concentration to a level similar to those observed in the exogenous NA or NR (Belenky et al. 2007). This NAD rise requires Npt1 and Pnc1 and is consistent with an increased flux along the salvage pathway (McClure et al. 2012). It also activates Sir2 by relieving NAM inhibition and enhancing Sir2-mediated silencing (Sauve et al. 2005). INAM supplementation increases Sir2 activity and extends RLS by a combined effect of the relief of NAM inhibition and NAD buildup (McClure et al. 2012). In general, NAM-treated wide type (WT) cells mimic a *sir2Δ* strain by enhanced gluconeogenesis.

## Nicotinamide riboside (NR)

Early studies on NR date back to over half a century when it was reported as a precursor for NAD biosynthesis. NR-mediated NAD biosynthesis received much attention owing to the work of Brenner and coworkers, who reported NR phosphorylation to NMN by NR kinase in both yeast and humans (Bieganski and Brenner 2004). Following the discovery of Nicotinamide Riboside Kinase (NRK), an enzyme that efficiently bypasses NAMPT (which is rate limiting) in the salvage pathway to convert NR to NMN, there has been a broader understanding of NR mediated NAD metabolism (Belenky et al. 2011). This reaction is vital because it prevents the energetically costly PRPP, which is subject to feedback inhibition by NAD. Direct conversion of NR has seen the maintenance of appropriate cellular NAD levels in all compartments and energy homeostasis (Bogan and Brenner 2008; Dolle et al. 2013). In this pathway, NRK catalyses first the phosphorylation of NR to NMN (Fletcher and Lavery 2018), and subsequently converts the NMN to NAD using Nma1 and Nma2 (NMNAT) protein. Compartmentalization of enzymes and precursors in the yeast NR salvage pathways offers a whole lot of advantages unlike other pathways. In the vacuole, it enable the storage of NAD-intermediate (Lu and Lin 2011), cytosolic nucleotidases (Sdt1 and Isn1), conversion of NMN to NR (Bogan et al. 2009) and uptake of NA and QA using transporters enzyme (Tna1) (Ohashi et al. 2013). The equilibrative transporter of NR (Fun26) and Nrt11 also function in the uptake of NAD precursors (Belenky et al. 2008). Specifically, Fun26 ensures adequate transportation of the NR generated in the vacuole into the cytoplasm where it supports NAD synthesis. Using Pho8, NR is produced from NMN for cytosolic and vacuolar pools with efficient balancing of Fun26. NRK enzymes extend the lifespan (RLS) of yeast through the induction of Sir2 in an NAD-dependent manner, and shows to be an efficient NAD precursor for efficient NAD homeostasis (Pinson et al. 2019). Unlike in NA, whose NAD amount decreases over the course of the culture growth, NR supplementation in a culture medium remains stable (McClure et al. 2012; Orlandi et al. 2017). Since lack of Sir2 improves the pyridine nucleotides flow into NR branch enhance an increased release of NR (Lu and Lin 2011), it is

however presume that NR assimilation could be required for cell survival during CLS (Orlandi et al. 2020) (Fig. 4). In general, NR exhibit effects that are beneficial against aging and age-related diseases and have demonstrate to extend longevity and healthspan in multiples of model organisms (Poljsak and Milisav 2016; Zhang et al. 2016; Tsang et al. 2015) (Fig. 3). The *nrk1Δurh1Δpnp1Δ* mutants are highly impaired in NR utilization thus, displaying significant CLS reduction. Cells do not also utilize NR for NAD production when NRK1, PNP1 and URH1 genes are deleted.

#### Nicotinamide mononucleotide (NMN)

NMN is a stable natural compound and a relative of niacin (vitamin B3). Its role in ensuring normal cell functions, especially at late ages, has been well established (Mills et al. 2016). Mounting evidence supports that addition of NMN boosts NAD levels by improving mitochondrial functions and suppressing the biomarkers of aging (López-Otín et al. 2013). Some of the hallmarks of aging as extensively reviewed by López-Otín et al., (2013) which include: shortening of telomeres, cellular senescence, genetic mutations, accumulated DNA damage, diminished communication between cells, unregulated nutrient sensing, degradation of cellular proteins, and impaired mitochondrial functioning, limiting the decline upon treatment with NMN (Fig. 3,4). NMN promotes longevity by boosting NAD levels through a number steps (Tarantini et al. 2019), and mechanisms to prevent/reverse aging and rejuvenate organelle functions (Fang et al. 2016). In addition, NMN treatment reduces oxidative stress, DNA damage and also slows down cellular senescence (Mouchiroud et al. 2013; Amano et al. 2019; Kiss et al. 2020). Cells dual specificity of NaMN/NMN adenylyltransferase (Nmnats—Nma1 and Nma2), a molecule produce by NA/NAM salvage pathway or NaMN, are responsible for the conversion of NaMN to NaAD by the addition of the AMP moiety. Thereafter, amidation of NaAD is ensued by glutamine-dependent NAD synthetase Qns1. In addition, NR is converted to NMN by *Nrk1*, which helps to synthesize NAD using *Nma1*, *Nma2* and *Pof1*. In other words, NR is assimilated through the *Nrk1*-dependent and *Urh1/Pnp1*-mediated routes.

These routes of assimilation ensure the exogenous utilization of NR generated from NMN which is acted upon by the nucleotidase activities of *Isn1* and *Sdt1* in the cytosol (Bogan et al. 2009). Administration of NMN neutralizes aging-predisposing conditions, protects against age-associated functional decline, increases energy metabolism, mitochondrial oxidative metabolism and extended lifespan (Mills et al. 2016; Fang et al. 2017).

#### Biotechnological significance of yeast lifespan research

The growing shortage in the supply of plant-based renewable feedstock and natural products has positioned microbial cell factories as a viable platform for the sustenance of industrial production. In pharmaceutical industries, active ingredients required for drug production are often obtain from rare plants which in most cases are impacted by environmental conditions and socioeconomic upheaval such as Covid-19 pandemic which has affected global trade (Bolarin et al. 2021; Srinivasan and Smolke 2020). Fungal systems represent a promising avenue for drug discovery and production due to the many natural products and secondary metabolites they synthesize (Arnone 2020; Hagee et al. 2020). A good example is seen in the production of *scopolamine* and *atropine* – vital drugs used for the treatment of cardiac and Parkinson's diseases, and neuromuscular disorders (Srinivasan and Smolke 2020). Statistics have shown that active compounds used for medicine production account for over 50% of the totality of compounds used by industries. Limited supply of these molecules undermine progress and to a large extent result in marginal income (low production), and pose dire consequences during health emergencies. Hence softening this limitation and presenting a viable approach free of environmental and geopolitical dependencies is paramount. Therefore, reprogramming microbial cell factories (yeast cells) to increase biochemical production by modulating lifespan through genetic and rational metabolic engineering could serve as a sustainable avenue for efficient supply (Guo et al. 2020). Consequently, techniques that enables the development of protocol such as design-construction-evaluation-optimization (DCEO Biotechnology), and framework for flow manipulation

and directed carbon flux, along with approaches for the combination of different biochemical pathways for desired chemical synthesis, has been identified (Chen et al. 2018a; Kerfeld 2016). This method, though promising, is not without challenge, as metabolic channels modification for optimal biosynthesis of value added chemicals or products of interest is oftentimes a bottleneck. In overcoming this setback, genetic and metabolic competence, organismal/strain physiological state and maintaining suitable environmental conditions has proven vital for efficient synthesis in the microbial cell factory (Nielsen and Keasling 2016; Luo et al. 2016; Yu et al. 2018b).

Lifespan has been extensively studied using yeast *S. cerevisiae* (Mouton et al. 2020; Longo et al. 2012; Deprez, et al. 2018), fission yeast *S. pombe* (Ohtsuka et al. 2017) and *Escherichia coli* (Boehm et al. 2016; Lindner et al., 2008). As previously stated, each of these organisms passes through the classical aging models by measuring the number of daughter cells produced prior to senescence (RLS) or length of time the cells in stationary phase remain viable in quiescent state (CLS). In these models, manipulation of parameters such as generation time (Longo et al. 2012), cell size (Soifer et al. 2016), and stress tolerance (Dawes and Perrone 2020) influences lifespan and the cell's physiological state. In a recent study conducted, *E. coli* lifespan was engineered for the production of poly(lactate-co-3-hydroxybutyrate) (PLH) and butyrate using rational manipulative techniques (Guo et al. 2020). In their work, shortened RLS were recorded by the deletion of the carbon storage regulator and fine-tuned by two-output recombinase-based state machines. In the CLS extension, deletion of response regulator and modulation of multi-output recombinase-based state machine and overexpression of sigma-38 in *E. coli* cells were carried out. In general, *rssB*, *rpoS* and *csrA* genes were found to control lifespan and its overexpression and/or deletion could manipulate RLS and CLS of *E. coli*, thus, determining production of high value PLH and butyrate in a fermenter (Guo et al. 2020).

Fermentation of grape juice by *S. cerevisiae* during wine production halts cell division – a process that elongates the stationary phase – and exhibits a prolonged CLS (Orozco et al. 2012). During this process, a form of RLS is witnessed via asymmetric segregation of inclusion bodies by the modification of the phaM (poly (3- hydroxybutyrate) (PHB) and/

or polyhydroxyalkanoate (PHA) binding protein) expression, and the increase accumulation of inclusion bodies in the mother cell (Arlia-Ciommo et al. 2014; Wahl et al. 2012). To reprogram and regulate cell functions, genetic circuits with efficient platforms are designed (Farzadfard and Lu 2018; Roquet et al. 2016). Recombinase-based state machine (RSM); a site-specific DNA recombinases (SSRs) and an SsrA-tag mediated protein degradation system is found most efficient. These circuits are designed to perform different functions in the expression of DNA sequences and protein degradation (Karzai et al. 2000; Roquet et al. 2016).

Aside from the resemblance of RLS and CLS models in wine making and fermentation (Aranda et al. 2019), to the best of our knowledge, there has not been a full exploration of these lifespan models using *S. cerevisiae* that includes the characterization of added chemicals and compounds for industrial applications. This is evident in the dearth of literature on yeast lifespan reprogramming for chemical production. Presumably, this could be a result of the earliest line of research, which has been geared toward geroscience, coupled with *S. cerevisiae* evolutionary conserved mechanisms, and has provided clarity to the study of human aging and longevity. Therefore, we hypothesize that genetic manipulation of genes, and metabolic engineering of regulatory networks and pathways of yeast cell factories could potentially increase lifespan. It can also play a vital role in enhancing efficient production of value added chemical/compounds for pharmaceutical and industrial applications (Arnone 2020). This could be achieved either through genome or physiological state manipulation and application of some of the readily available RSM tools (Guo et al. 2020), as it will provide opportunity for sustainability in industrial supply.

## Future perspectives

NAD and its phosphorylated forms are consumed by a large proportion of oxidoreductases where they play role in the transport and storage of hydride, and later participate in myriad valuable biochemical reactions (Chen et al. 2018b; Zhang et al. 2019; You et al. 2017). One of the features of this molecule is its two-part structure. First the nicotinamide portion, which hosts its redox chemical functions and second,

the adenosine dinucleotide moiety, which confers the receptor-ligand recognition site (Knaus et al. 2016). The second adenosine dinucleotide moiety is amenable to engineering or replacement by other base dinucleotides, motifs, alkyl, or benzyl groups forming biomimetic nicotinamide analogues of interest (Wang et al. 2019; Paul and Hollmann 2016).

This feature has led to the creation of novel bio-orthologues – redox compounds termed non-natural cofactors. These cofactors are metabolically designed and are considered alternatives to the global redox cofactor (Liu et al. 2019; Depaix and Kowalska 2019). They are NAD analogues designed to target enzymes, functioning as an inhibitor of glycosidic bonds such as Sirtuins activity and ADP-ribosyltransferases' (Dai et al. 2018) and NAD-dependent metabolic pathways (e.g., the purine biosynthesis pathway) (Pankiewicz and Felczak 2015). Over the years, these compounds have evolved to become of great interest in industrial and biotechnological application, and are continuously being used to address confronting biological problems (Liu et al. 2018; Gan et al. 2018). Importantly, in designing these analogues, interaction between different sites within the constituent molecules usually occurs. This is illustrated by Inosine-50 -monophosphate dehydrogenase (IMPDH cofactor) that interacts with three different binding domains, namely (a) nicotinamide binding subsite (N-subsite), (b) adenosine binding subsite (A-subsite), and (c) pyrophosphate binding subsite (P-subsite) (Felczak and Pankiewicz 2011; Pankiewicz and Felczak 2015). Also, significant advances have been made in exploiting enzymes involved in natural product biosynthesis and new structure bearing non-natural substituents (Wang et al. 2017), as witness in the rising implementation and their application in chemical and synthetic biology (Rovira et al. 2017; Halle et al. 2018; Wang et al. 2021).

As a novel component for redox metabolism; non-natural nucleotides and non-natural amino acids have sufficiently enhanced our understanding of the mechanisms underlying bioenergetic and signaling pathways mediated by NAD, providing strategies for the effective regulation of redox reactions (Paul et al. 2013; Liu et al. 2019). This is significant owing to NAD complexes tight regulation and metabolic networks (Wang et al. 2019; Croft et al. 2018), which play fundamental roles in addressing biological challenges such as disease progression, aging, and

longevity. Even though sufficient knowledge on the synthesis of non-natural cofactors as alternatives to NAD exist, there are limited applications on cellular metabolism, biochemical functions and aging/lifespan biological processes. Some analogues possess dynamic properties making them unique in their functions and with excellent biocompatibility used in characterizing growth-dependent degradation of in vivo nicotinamide cofactors (Wang et al. 2019). A good example of these non-natural cofactor include nicotinamide hypoxanthine dinucleotide (NHD) which inhibits NAD synthesis in *E. coli* (Wang et al. 2019), and nicotinamide guanine dinucleotide (NGD) that together with NAD exhibit synergistic effects in ADH reactions.

In the earlier works, different NAD analogues and the adenosine moiety 1,2,3-triazole moiety were designed and tested for potential utilization as cofactors. The results with wild type malic enzyme (ME) and alcohol dehydrogenase showed appreciable activities, which suggests flexibility in the NAD-binding pockets of those enzymes (Hou et al. 2011, 2014; Ji et al 2013). Subsequently, on screening the analogues against ME mutants, a mutant (ME-L30K/L404S) characterized by over 1000-fold preference for a triazole derivative over NAD was characterized. This laid a strong foundation for the development of orthologue redox systems with the potential for metabolic pathway engineering and biology related applications. We envisage that these compounds may offer exciting and new opportunities in aging studies as molecular probes to regulate cofactor-associated processes (Wang et al. 2019).

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#### Declarations

**Conflict of interest** The authors declare no conflict of interest.

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