Boosting Cellular Longevity Through Intracellular ATP Modulation

Naci Oz[1,2], Hetian Su[3], Praveen Patnaik[4], Derek C. Prosser[1], Vyacheslav M. Labunskyy[4], Rohil Hameed[6], Vadim N. Gladyshev[5], Nan Hao[3], Alaattin Kaya[1,2,6#]

[1]Department of Biology, Virginia Commonwealth University, Richmond, VA, USA

[2]Integrative Life Sciences, Virginia Commonwealth University, Richmond, VA, USA

[3]Department of Molecular Biology, University of California San Diego, La Jolla, CA, USA

[4]Department of Dermatology, Boston University School of Medicine, Boston, USA

[5]Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

[6]Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, USA

#Corresponding author: Department of Biochemistry and Molecular Genetics, University of Virginia, 1340 Jefferson Park Ave. Charlottesville, VA, USA. E-mail: qcc6zm@virginia.edu

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Abstract

Aging results from the gradual accumulation of molecular damage as a result of cellular processes and is characterized by impaired functions, most notably an age-related decline in ATP production. However, the causal relationship between cellular ATP homeostasis and aging has not been established. In this study, we employed a nucleotide transporter from a eukaryotic intracellular parasite to directly alter ATP levels in budding yeast cells and exchange it with the extracellular milieu. We found that ATP depletion significantly shortens lifespan, whereas supplementation of the medium with ATP fully restores it. Analysis of gene expression showed inhibition of catabolic processes suggesting that increased ATP suppresses glucose metabolism. Our results also showed that ATP supplementation leads to lifespan extension. Overall, our study revealed the direct impact of cellular ATP homeostasis on the regulation of lifespan. This work offers new insights into the bioenergetic control of aging and positions energy metabolism as a promising target for longevity interventions.

Introduction

Aging, a complex and multifactorial process, is marked by the gradual decline in physiological function, culminating in increased susceptibility to diseases and mortality (1,2). At the heart of this process lies mitochondrial dysfunction, which emerges as a key player in the cellular and systemic manifestations of aging (3–5). Mitochondria are responsible not only for producing ATP through oxidative phosphorylation but also for regulating various cellular pathways such as calcium homeostasis, redox signaling, and apoptosis (6,7). Over the past decade, a growing body of research has established a strong connection between mitochondrial dysfunction and key characteristics of the aging process. Notably, alterations such as somatic mutations in mitochondrial DNA (mtDNA), impairments in the respiratory chain, and elevated production of mitochondrial reactive oxygen species (ROS) have been implicated. These changes lead to oxidative stress and the accumulation of molecular damage, ultimately disrupting cellular function and promoting senescence and age-related decline. Furthermore, mitochondrial dysfunction, resulting from structural and functional changes, is linked to age-related degenerative diseases (4,8,9). However, recent evidence suggests that mild mitochondrial stress, known as mitohormesis can promote adaptive responses, enhancing longevity through hormetic mechanisms (5,10–13).

Mitochondrial dynamics, encompassing fusion, fission, and mitophagy, play a crucial role in maintaining mitochondrial integrity and metabolic function throughout the lifespan (14–16). As organisms age, disruption in these dynamics can lead to the accumulation of dysfunctional mitochondria. Furthermore, mitochondrial quality control, mediated by mitophagy and fission/fusion processes, declines with age, exacerbating mitochondrial metabolic dysfunction there by resulting in an energy supply and demand imbalance (8,9,14–19). Caloric restriction (CR), a well-established intervention to extend lifespan, physical activity and pharmacological interventions targeting conserved longevity pathways improves mitochondrial biogenesis and reduces oxidative damage, indicating that metabolic interventions can mitigate age-related decline (20–23).

The concept of ATP homeostasis, essential for cellular metabolism and signaling, emerges as a pivotal factor in mitochondrial-mediated lifespan regulation (24–26). While several studies have explored how mitochondrial dysfunction impacts aging, few have directly examined the role of

intracellular ATP availability and energy supply and demand balance in modulating longevity (6,9,12,24). In *Caenorhabditis elegans*, for instance, a mild reduction in mitochondrial metabolic function during developmental stages confers increased lifespan, highlighting the nuanced relationship between energy production and aging (12). Similarly, research on mild mitochondrial uncoupling in mice demonstrates that metabolic shifts favoring increased energy expenditure can reduce ROS production and extend lifespan (11,27).

In this study, we investigated the impact of intracellular ATP homeostasis on cellular aging. We employed a novel synthetic biology approach, introducing a nucleotide transporter gene from an intracellular parasite (28,29) into budding yeast to enhance the uptake of extracellular ATP. This strategy allowed us to explore whether restoring ATP homeostasis could extend lifespan. Our findings revealed that ATP depletion significantly shortens lifespan, while ATP supplementation restores it. Intriguingly, we also observed that extracellular ATP alone could extend lifespan without cellular uptake, suggesting the existence of ATP-sensing pathways that modulate aging independently of conventional energy metabolism. RNA sequencing data further supported our hypothesis, indicating that increased ATP availability downregulates key catabolic processes, including glucose metabolism, thereby altering energy homeostasis. These findings underscore the intricate relationship between energy metabolism and cellular aging, with potential implications for developing energy-based interventions to promote healthy aging. Furthermore, our research suggests that mitochondrial bioenergetics, redox balance, and ATP homeostasis are tightly intertwined, with disruptions in these processes playing a central role in cellular lifespan. Overall, our study provides novel insights into the role of ATP homeostasis in lifespan regulation. Our findings highlight the potential of targeting ATP levels as a therapeutic strategy to delay aging and promote longevity. By elucidating the mechanisms underlying ATP-sensing pathways, we open new avenues for research aimed at developing interventions that optimize energy metabolism to enhance healthspan and mitigate the impact of age-associated mitochondrial functional decline.

Results

Overexpression of *NTT1* mediates ATP import into *S. cerevisiae* cells

To alter intracellular ATP levels through extracellular feeding, we created a yeast model expressing a nucleotide transporter (*NTT1*) from the intracellular fungal parasite *Encephalitozoon*

cuniculi. *E. cuniculi* naturally uses this exchanger to import ATP from the cytosol of eukaryotic host cells to sustain its survival (28,29). To examine Ntt1 localization in yeast, we modified its sequence by fusing the Pleckstrin homology (PH) domain of *SLM1* to its N-terminus—a well-established plasma membrane targeting signal. We generated expression cassettes with or without a C-terminal GFP tag and integrated them into the genome of wild-type (*Wt-BY4741*) yeast. *NTT1* expression was driven by the constitutive *TEF1* promoter (**Fig. 1A**). Fluorescence microscopy confirmed plasma membrane localization of the PH-Ntt1 fusion protein (Fig. 1B). This engineered system enables the exchange of extracellular ATP for intracellular ADP via *NTT1* (**Fig. 1C**).

Next, to test whether *NTT1* expression mediates ATP import from the extracellular environment, we created second yeast cell model by integrating the functional *NTT1*-cassette (without GFP) into the genome of a Wt yeast. We also genome-integrated a fluorescent ATP biosensor (Queen), to quantify intracellular ATP levels in single living yeast cells (30,31) (**Fig. 2A**). Based on this reporter strain, our analysis showed that *NTT1* expression in cells severely depleted intracellular ATP levels, compared to Wt control cells (**Fig. 2B**). Supplementing the medium with 5 mM ATP increased ATP significantly (p=7.5E-09) exceeding levels observed in Wt controls, indicating efficient ATP uptake via Ntt1 from the extracellular environment (**Supplementary File 1**). Interestingly, Wt cells lacking *NTT1* exhibited a significant increase in ATP levels (p = 2.22E-16) upon ATP supplementation, though to a lesser extent than NTT1-expressing cells (**Fig. 2B**). Since *S. cerevisiae* lacks known ATP transporters, this suggests that extracellular ATP may activate downstream signaling via unknown nucleotide-sensing receptors, promoting mitochondrial function and ATP synthesis. Alternatively, an unidentified transporter might facilitate ATP uptake.

To validate these findings, we designed an experiment to inhibit endogenous ATP synthesis by treating cells with 2-deoxyglucose (2DG), a glucose analog that competitively inhibits glycolysis. Once phosphorylated by hexokinase, 2DG becomes 2DG-6-phosphate, which accumulates and inhibits glycolysis, leading to rapid ATP depletion (32). As expected, 30 minutes of 2DG treatment sharply reduced intracellular ATP levels in both wild-type and NTT1-expressing cells (**Fig. 2C**). When NTT1-expressing cells were subsequently treated with 5 mM ATP, intracellular ATP levels increased, confirming ATP import via *NTT1*. In contrast, ATP treatment

did not restore ATP levels in wild-type cells, arguing against the presence of an unidentified ATP transporter (**Fig. 2C and Supplementary File 1**).

Altered ATP level targets cellular metabolic pathways

To investigate the effect of altered ATP abundance on gene expression in Wt and *NTT1*-expressing cells, we performed RNA-seq analysis (**Fig. 3A**). Our goal was to distinguish molecular signatures triggered by extracellular ATP-mediated signaling in wild-type cells from those driven by increased intracellular ATP via *NTT1*-mediated uptake in engineered cells. We analyzed differentially expressed genes (DEGs) under ATP-treated and untreated conditions for both strains and compared their expression profiles (**Fig. 3B and C**). In Wt cells, ATP treatment significantly altered the expression of 500 genes: 198 upregulated and 302 downregulated. In contrast, NTT1-expressing cells displayed a broader response: 1,034 genes were significantly changed, with 586 upregulated and 448 downregulated. Among these, 146 upregulated and 188 downregulated genes were shared between the two strains, suggesting that these changes result directly from ATP treatment and occur independently of *NTT1* expression (**Fig. 3B and C**).

To further dissect ATP-mediated responses in Wt cells, we performed Gene Ontology (GO) (**Fig. S1A, B**) and KEGG pathway enrichment analyses (**Fig. S1C, D**). ATP treatment led to significant upregulation of genes involved in ribosome biogenesis, ATP biosynthesis, MAP kinase (MAPK) signaling, and oxidative phosphorylation (**Fig. S1A, C**). Conversely, downregulated genes were enriched in carbohydrate metabolism and uptake, protein phosphorylation, autophagy, and meiosis pathways (**Fig. S1B, D**). These findings indicate that extracellular ATP triggers intracellular signaling cascades capable of modulating mitochondrial function, cytosolic translation, and protein phosphorylation (**Supplementary File S2**). Notably, genes in the MAPK cascade were significantly enriched (**Fig. S2**), suggesting this pathway might play a key role in mediating the ATP response.

We next applied the same enrichment analyses for the DEGs identified by comparing *NTT1*-expressing cells (treated vs non-treated). We found a complete rewiring of transcriptional regulation of metabolic genes in *NTT1*-expressing cells following ATP treatment. Similar to Wt cells, *NTT1*-expressing cells also showed upregulation of ribosome biogenesis and RNA

processing genes (**Fig. 3D and Fig. S3**). However, while ATP treatment upregulated genes associated with mitochondrial respiratory activity, oxidative phosphorylation, and MAPK signaling in Wt cells (**Fig. S1**), genes from these pathways were downregulated in *NTT1* cells **(Fig. 3D and Fig. S3)**.

Further, the downregulated gene set in NTT1-expressing cells was enriched in glucose uptake, energy generation from precursor metabolites, carbohydrate phosphorylation, general catabolic processes, and autophagy (**Fig. 3E and Fig. S3**). This repression mirrors SNF1-mediated metabolic rewiring. SNF1, the yeast homolog of mammalian AMP-activated protein kinase (AMPK), plays a pivotal role in energy homeostasis by modulating metabolic responses and glucose de-repression under varying nutrient and ATP levels (32). Low *SNF1* activity is expected under high ATP concentrations (33).

To confirm this, we examined the expression of *SNF1* downstream targets associated with nutrient stress. We observed significant downregulation of several genes, including *MIG1*, *TDA1*, *HXK1*, *HXK2*, and *HXT3,* which are involved in glucose transport and metabolism (**Fig. S4; Supplementary File S2**). Additionally, *RGT1*, a major transcription factor that represses glucose transporter (*HXT*) genes under high-glucose conditions, was significantly upregulated in *NTT1* cells (**Supplementary File S2**). Together, these results indicate strong repression of glucose uptake and metabolism, further supporting the presence of a global metabolic rewiring in response to ATP elevation in *NTT1*-expressing cells (34,35).

Collectively, our data reveal a striking biological phenomenon: NTT1-mediated ATP import leads to suppression of glucose uptake and catabolism, which in turn dampens mitochondrial energy production and reconfigures cellular metabolism. Given that catabolic pathways are a major source of intracellular damage through production of reactive by-products, this reduction in metabolic flux may also minimize damage accumulation. Consequently, autophagy, a key mechanism for clearing damaged macromolecules was downregulated in these cells.

These findings highlight two mechanistically distinct but complementary modes by which ATP influences cellular physiology and lifespan. In *NTT1*-expressing cells, ATP is directly

imported from the extracellular environment, leading to a robust and specific rewiring of cellular metabolism characterized by the suppression of glucose uptake, glycolysis, and mitochondrial respiration. This *NTT1*-dependent ATP influx mimics a high-energy state, repressing energy-generating catabolic pathways and reducing the need for damage-clearing processes such as autophagy, potentially preserving cellular integrity over time. In contrast, extracellular ATP also exerted effects in wild-type cells lacking *NTT1*, suggesting a separate, *NTT1*-independent mechanism possibly mediated through extracellular ATP sensing. This pathway upregulated mitochondrial oxidative phosphorylation and stress-responsive signaling cascades, including MAPK, indicating that cells can detect and respond to extracellular nucleotides without direct ATP uptake. Together, these data suggest that ATP can modulate aging-related pathways through both intracellular energy repletion and extracellular signaling, revealing an unexpected duality in how energy homeostasis interfaces with lifespan regulation. The unexpected divergence between *NTT1*-dependent and -independent effects underscores the complexity of cellular energy sensing and reveals ATP as both a metabolic substrate and a signaling molecule with profound implications for aging biology.

Altered intracellular ATP level modulates cellular lifespan

We further investigated the consequences of altered ATP abundance on cellular aging. Due to the unstable nature of ATP molecules in the culture medium, we employed a state-of-the-art microfluidic aging chamber (36–40), which allowed cells to be continuously exposed to a medium supplemented with fresh ATP. This system was also integrated with high-resolution fluorescence microscopy, enabling real-time measurement of ATP levels throughout the yeast lifespan at the single-cell level using the fluorescence intensity of a novel and reliable Queen-ATP reporter (30,31). Our analysis of ATP abundance throughout the yeast lifespan showed that yeast cells are born with low ATP levels, which gradually increase during their lifespan. Some cells completed their lifespan without any observable decrease in ATP abundance, while others showed a drastic decrease in ATP levels during late life (**Fig. 4A–D, Supplementary File S3**), consistent with previous observations supporting two modes of yeast lifespan, mediated by mitochondrial and/or *SIR2* function (36,40–43).

Consistent with our data presented in Figure 2, we also observed significantly lower ATP abundance in *NTT1*-expressing cells throughout their entire lifespan compared to Wt control cells (**Fig. 4A–C**). Furthermore, these cells displayed significantly reduced mean and maximum replicative lifespan (RLS), directly indicating that intracellular ATP depletion shortens lifespan (**Fig. 4D**). Next, we assessed RLS and age-associated ATP changes under ATP supplementation. We found that exposing *NTT1* cells to medium supplemented with 10 μM ATP restored intracellular ATP levels (**Fig. 4A–C**) and significantly ($p = 4.03E-18$) increased both mean and maximum RLS to levels comparable to WT cells (**Fig. 4D**).

In line with our previous observations, our analysis also showed a significant increase in ATP levels and RLS in Wt control cells upon ATP supplementation, although the observed changes in both ATP levels and RLS extension were not as pronounced as those in *NTT1*-expressing cells (**Fig. 4A–D**). These findings are consistent with our transcriptomic data, which suggests that extracellular ATP may activate downstream signaling through nucleotide-sensing receptors, thereby increasing mitochondrial function, ATP production, and other canonical pathways. This effect may, at least in part, explain the observed lifespan extension in Wt cells.

ATP homeostasis dictates aging pathway selection and longevity in yeast

To further understand how ATP availability influences aging dynamics, we examined the two known trajectories of yeast replicative aging mode-1 and mode-2 under both ATP-treated and untreated conditions (36). These modes are distinguished by their daughter cell morphology: mode-1 cells produce elongated daughters, whereas mode-2 cells give rise to small, round daughters. At the molecular level, mode-1 aging is characterized by rDNA instability and nucleolar enlargement and fragmentation, yet mitochondria remain functional. In contrast, mode-2 cells exhibit impaired mitochondrial function (36,40–43). Consistent with a previous report (36), we found that approximately 60% of Wt cells aged through mode-1, while 40% aged through mode-2 under control (no ATP treatment) condition. Cells, aged through mode-1 exhibited significantly longer lifespans than mode-2 cells (**Fig. 5A**).

We previously showed that mode-1 cells can maintain a relatively constant intracellular ATP level during aging, whereas mode-2 cells show a rapid depletion of ATP due to mitochondrial

dysfunction (42). Therefore, Ntt1-mediated ATP depletion may have a more dramatic effect on the lifespan of mode-1 cells than that of ATP-depleted mode-2 cells. As expected, under the condition of no ATP treatment, *NTT1* expression caused a significant reduction in the lifespan of mode-1 cells (Wt mode-1, median RLS = 19 vs. *NTT1* mode-1, median RLS = 14; p = 2.39E-4), but not in mode-2 cells (Wt mode-2, mean RLS = 14 vs. *NTT1* mode-2, mean RLS = 14, p = 0.74) (**Fig. 5A**). We then analyzed the effect of ATP treatment. In both Wt and *NTT1*-expressing cells, ATP treatment increased the lifespans of mode-1 and mode-2 cells (**Fig. 5B**). Furthermore, the treatment also enhanced the proportion of mode-1 cells (**Fig. 5C**), suggesting that ATP treatment can compensate for the age-induced ATP decline in mode-2 cells, biasing the fate decision toward mode-1 aging.

These results indicate that elevating intracellular ATP, whether through *NTT1*-mediated import or via extracellular sensing can extend cellular lifespan and influence the trajectory of aging. By restoring energy balance, ATP not only delays senescence but also shifts cells toward aging modes associated with greater mitochondrial integrity and extended replicative potential.

NTT1-driven ATP import alters lifespan through mitochondria-dependent mechanisms

To further understand the downstream effect of altered extracellular and intracellular ATP abundance and whether it interferes the mitochondrial function, we designed experiments with *rho*^{0} isolates, in which we eliminated mitochondrial DNA (mtDNA). These cells are unable to perform oxidative phosphorylation (OXPHOS) because they lack the necessary components of the electron transport chain encoded by the mitochondrial genome (44,45). RLS of Wt and *NTT1*-expressing cells as well as their *rho*^{0} isolates were assayed under the control and higher dose (10 mM) ATP supplemented medium conditions using microfluidic aging chamber. Interestingly, under the control condition (without ATP supplementation) eliminating mtDNA prevented the negative lifespan effect (**Fig. 4D and Fig. 6A, Supplementary File S3**) of *NTT1*-expression, consequently *NTT1 rho*^{0} cells displayed full lifespan to the level of Wt cells (**Fig. 6B**). It should be noted here that under the no ATP treatment condition, we did not observe any significant lifespan differences between Wt and Wt *rho*^{0} cells (**Fig. 6A, B**), further confirming that eliminating mtDNA was the primary driver of the increased lifespan, specific to the cells expressing *NTT1*. These data strongly suggest that the toxicity associated with *NTT1*-driven ATP import requires

functional mitochondria. In other words, high intracellular ATP levels become deleterious only when mitochondrial respiration is intact.

Next, 10 mM ATP treatment resulted in 39% mean RLS increase (p =4.52E-12) in WT cells (**Fig. 6A**), comparable to the altered Wt lifespan that we observed under 10 µM treatment condition (**Fig. 4D**). However, for the *NTT1*-expressing cells, in comparison to the 10 µM treatment condition, in which *NTT1* cells extended lifespan to the level of treated Wt cells (**Fig. 4D**), the positive effect of ATP treatment on lifespan was lost, indicating the increased ATP dosage causes toxicity thereby significantly (p= 1.25E-05) shortened the lifespan in comparison to the treated Wt cells (**Fig. 6B)**. However, we made and interesting observation that the toxicity effect of 10 mM ATP treatment on lifespan of *NTT1*-expressing cells was alleviated in *NTT1 rho0* isolates. The 10 mM ATP treatment significantly (p=3.07E-05) increased lifespan of *NTT1* rho^0 cells (**Fig. 6B)**, further supporting the conclusion that mitochondrial function mediates ATP toxicity.

Overall, these experiments allowed us to uncouple mitochondrial respiration from ATP-mediated lifespan effects. Strikingly, under baseline (no ATP treatment) conditions, the lifespan-shortening effect of *NTT1* expression was completely suppressed in rho^0 cells, suggesting that the toxicity of *NTT1*-driven ATP import requires functional mitochondria. In other words, high intracellular ATP becomes toxic only in the presence of intact mitochondrial function. Supporting this, at high extracellular ATP levels (10 mM), *NTT1*-expressing cells showed a shortened lifespan, whereas *NTT1* rho^0 isolates were protected and displayed significantly extended lifespan, again implicating mitochondria as mediators of ATP toxicity. Conversely, in wild-type cells lacking NTT1, extracellular ATP sensing (without import) robustly extended lifespan in both mtDNA-positive and rho^0 backgrounds, demonstrating that ATP sensing acts independently of mitochondrial function and does not confer toxicity. These combined observations point to a dual mechanism: (i) intracellular ATP import through *NTT1* affects lifespan in a mitochondria-dependent manner, with potential toxicity at high levels; and (ii) extracellular ATP sensing promotes lifespan extension independently of mitochondrial respiration. Thus, mitochondria serve as a key integrator of energy input via *NTT1*, while also distinguishing between beneficial signaling and detrimental metabolic overload. Additionally, the observation of that ATP treatment extended lifespan in both Wt and Wt rho^0 isolates (**Fig. 6B**), indicating the positive lifespan effect of ATP sensing and downstream signaling cascade is independent of mitochondrial function. In summary, these data further proved direct synergic interaction of *NTT1* function and altered ATP

abundance on mitochondrial function and differentiated effect of extracellular ATP treatment on Wt and *NTT1* cells.

Discussion

Catabolic systems (e.g., glycolysis, TCA cycle, respiration) are known to contribute significantly to cellular damage by generating by-products of cellular metabolism and reactive oxygen species (ROS) (46–48). A major purpose of catabolism is to generate ATP (47). However, downregulating subsets of these systems has been shown to extend lifespan in various organisms (49–52). Interestingly, as cells age, their ability to produce ATP often declines, leading to impaired cellular function and contributing to the aging process (53–55). The effect of altered ATP abundance in the context of aging remains largely unclear. This question has been difficult to address because ATP generation is essential for all life forms.

However, some eukaryotic intracellular parasites have evolved the ability to obtain ATP from their hosts. For example, *E. cuniculi* naturally uses nucleotide transporters to import ATP from the cytosol of eukaryotic host cells into its own cytosol as well as cellular compartments, such as mitochondria, to sustain its obligate intracellular lifestyle (28,29). We applied xenotopic-based synthetic biology strategy (56) to incorporate a nucleotide transporter from *E. cuniculi* into a budding yeast model and investigated the effects of altered ATP abundance on aging. Specifically, we expressed the *E. cuniculi* nucleotide transporter gene (*NTT1*) in yeast to enable ATP import from the extracellular environment, thereby aiming to downregulate much of the cellular catabolism while preserving energy supplies. This novel approach of directly providing energy to cells in the form of ATP alleviated organelle dysfunction, and revealed a complex picture of metabolic and phenotypic alteration resulting in lifespan extension.

We found that overexpression of *NTT1* led to severe depletion of intracellular ATP and reduced lifespan. Follow-up experiments in cells lacking mitochondrial respiratory function revealed that this effect was linked to mitochondrial stress, as rho^0 isolates (lacking mitochondrial DNA and OXPHOS) of *NTT1*-expressing cells restored lifespan to the level of WT controls. In addition, while low-dose ATP treatment significantly increased intracellular ATP levels and extended lifespan, high-dose ATP supplementation was detrimental to lifespan in *NTT1*-

expressing cells but not in WT cells. However, *NTT1- rho*^{0} isolates did not display decreased lifespan under toxic ATP concentrations.

These observations strongly indicate that *NTT1* overexpression and altered ATP abundance interfere with mitochondrial function. Lifespan mode analysis under both treated and non-treated conditions further confirmed that age-associated decline in mitochondrial function can be compensated for by ATP supplementation, thereby promoting a positive lifespan effect. Our data showed that the percentage of cells aging through mitochondrial dysfunction (mode-2) was significantly reduced when the medium was supplemented with ATP. The majority of cells then followed a mode-1 aging trajectory, which is associated with a loss of Sir2 function. Interestingly, mode-1 cells also showed increased lifespan following ATP treatment. This suggests that an elevated ATP levels are tightly connected in regulating cellular aging. In fact, previous studies that characterized these lifespan modes demonstrated that overexpression of *HAP4* (a transcription factor regulating mitochondrial respiratory function) (57) alone did not extend lifespan. However, combined overexpression of *SIR2* and *HAP4* had a synergistic lifespan-extending effect, where cells lived significantly longer than those overexpressing *SIR2* alone (36). This supports the idea that mitochondrial function and Sir2 activity work together to modulate lifespan.

Perhaps the most striking observation from our transcriptome data was that *NTT1*-expressing cells downregulated glucose uptake and catabolic pathways, including energy generation and mitochondrial respiration, when supplemented with ATP. This observation suggests that when cellular energy demand is supported through a direct import of ATP, cells can adapt by suppressing carbohydrate catabolic processes to balance energy homeostasis. Furthermore, the observed concurrence of decreased expression of autophagy pathway genes and lifespan extension can be attributed to the fact that catabolic processes are a major source of cellular damage through the generation of reactive by-products (e.g., oxidants and secondary metabolites). Reduced catabolic activity may therefore generate fewer damaging agents, leading to decreased activation of damage-clearing pathways such as autophagy.

Remarkably, extracellular ATP sensing independent of Ntt1 also exhibited life-extending effects following ATP treatment in Wt cells. Our transcriptomics data suggest the presence of a previously unknown nucleotide-sensing mechanism that links extracellular ATP to downstream

cellular processes in yeast. Considering the altered expression of genes enriched in MAPK and glucose homeostasis pathways in these cells, we hypothesize that the MAPK pathway may link extracellular ATP sensing to downstream pathways that promote mitochondrial respiration and increase ATP abundance. In fact, extracellular ADP treatment has also been shown to increase intracellular ATP levels in yeast (31). This suggests the existence of specific nucleotide-sensing mechanisms in yeast, similar to mammalian purinergic signaling, where purinergic receptors modulate downstream signaling cascades (58,59). A well-conserved *p38/HOG1* MAPK module in yeast is essential for cell survival under various stress conditions, such as osmotic, cell wall, nutrient, and oxidative stress (60–62). In addition, a wide variety of stimuli can elicit MAPK activation; however, it is still unclear how MAPKs regulate the distinct coupling of signals to downstream cellular responses (63,64). It is also known that MAPK is involved in cellular metabolic regulation and mitochondrial function and dynamics (65–67). Hence, our study suggests a previously uncharacterized MAPK–mitochondria signaling axis that regulates mitochondrial function in response to extracellular nucleotide sensing and provides new insights into the molecular and physiological adaptations of cells to ATP homeostasis. However, the components of the upstream sensing and downstream signaling cascades connecting ATP sensing to mitochondrial function require further investigation. It should also be highlighted that the increased mitochondrial respiration in these cells was not essential for the lifespan extension mediated by ATP supplementation, indicating modulation of other downstream canonical pathways. It is also possible that the presence or absence of mitochondrial OXPHOS function might mediate alternative pathways that differentially regulate cellular lifespan following ATP treatment in Wt cells. It is also possibility that the life-extending effect is simply through mild osmotic stress from extracellular ATP in yeast, which may have a hormetic effect.

In part, this positive lifespan effect may also be associated with increased mitochondrial function and ATP abundance in Wt cells. However, we observed that WT *rho*^{0} isolates also showed significantly increased lifespan following ATP treatment, indicating that mitochondrial function is not essential for ATP signaling-mediated lifespan extension. It should be noted that caloric restriction (CR) also induces stress response pathways increases respiration and intracellular ATP abundance in yeast (68,69). However, CR-mediated lifespan extension is also independent of mitochondrial function, since WT *rho*^{0} isolates extend lifespan under CR conditions (70). This

suggests that ATP-sensing mechanisms may regulate overlapping CR-mediated pathways to induce lifespan extension and may modulate alternative pathways mediated by mitochondrial functional status. However, the exact mechanism of ATP-mediated lifespan extension in yeast requires further exploration.

Overall, our study directly validates that maintaining energy metabolism modulates longevity. Decline in mitochondrial function is considered one of the key hallmarks of aging a finding supported by numerous previous studies. However, our study is novel in directly demonstrating the role of ATP homeostasis in modulating cellular metabolism and aging. This research establishes that cellular energy balance and mitochondrial function are interconnected regulators of aging, and targeting ATP metabolism provides a promising strategy for extending lifespan and improving cellular health.

Materials and Methods

Yeast Strains and Plasmids

The Pleckstrin Homology (PH) domain of the *SLM1* gene was amplified from genomic DNA of the wild-type *S. cerevisiae* (BY4741) strain. Primers *PH-F* and *PH-R* were designed to amplify the PH domain with overlapping sequences compatible with assembly (primer sequences are listed in **Supplementary File S4**). The *NTT1* gene was amplified from a plasmid (28) using primers NTT1-F and NTT1-R, which included overlaps with the PH domain at the 5' end and GFP at the 3' end. The yeast-enhanced green fluorescent protein (yeGFP) sequence was amplified from the pYM25 plasmid (71) using primers GFP-F and GFP-R, incorporating overlaps with the *NTT1* sequence. The purified PH domain, *NTT1*, and yeGFP PCR products were fused using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, NEB #E2621).

The assembled *PH_NTT1_GFP* (**Supplementary File S4**) fusion construct was used as a template for PCR amplification with Gateway-compatible attB primers (*attB1-F* and *attB2-R*), which were designed to add recombination sites for Gateway cloning (primer sequences are provided in **Supplementary File S4**). The purified attB-flanked *PH_NTT1_GFP* fragment was recombined into the *pAG413GPD-ccdB* plasmid (Addgene plasmid #14142) (Alberti et al., 2007). The confirmed plasmid was transformed into BY4741 cells using the lithium acetate method.

A fusion cassette (**Supplementary File S4**) containing the PH sequence and *NTT1* (with a SmaI restriction site between them) was amplified by PCR from the appropriate DNA template using primers *Attb_PH_F* and *Attb_NTT1_R*. The reverse primer included a stop codon at the 3′ end of *NTT1*. The resulting attB-PCR product was purified and transferred into pAG306-GPD-ccdB (Addgene plasmid #41894). The final plasmid construct was integrated into the yeast genome after NotI digestion (Hughes et al., 2012). The empty pAG306-GPD plasmid (Addgene plasmid #41895) was integrated into the yeast genome as a control strain.

Finally, the yeast strains containing the QUEEN ATP reporter gene were obtained from the National BioResource Project Yeast Genetic Resource Center. The *PH_NTT1* construct (with a stop codon) in pAG306-GPD-ccdB was integrated into the BY29034 strain. The empty pAG306-GPD plasmid was also integrated into the BY29034 genome as a control strain. All sequences were verified by Sanger sequencing after PCR amplification and cloning.

RNA Isolation and RNA-seq Analysis

Cells (three independent cultures for each strain) were cultivated in Complete Synthetic Medium (CSM) at 30°C with constant agitation (200–220 rpm) until they reached an optical density at 600 nm (OD600) of approximately 0.4–0.5. To examine the effect of extracellular ATP, a 50 mM ATP stock solution was prepared in CSM to maintain a consistent medium composition during treatment. Cells were treated with ATP at a final concentration of 5 mM for 1 hour at 30°C under continuous shaking. Untreated control cultures of the same strains were grown under identical conditions but without ATP supplementation.

After treatment, cells were harvested by centrifugation at 4°C. Total RNA was extracted using the Quick-RNA Fungal/Bacterial MiniPrep™ Kit (Zymo Research R2014) according to the manufacturer's instructions. The resulting RNA samples were used for downstream RNA-sequencing analysis.

To prepare RNA-seq libraries, the Illumina TruSeq RNA library preparation kit was used according to the user manual. RNA-seq libraries were loaded onto the Illumina NovaSeq 6000 platform to produce 150 bp paired-end sequences. After quality control and adapter removal, paired-end clean reads were aligned to the yeast reference genome (Ensembl, R64-1-1) using

Hisat2 v2.0.5. FeatureCounts v1.5.0-p3 was used to count the number of reads mapped to each gene. Fragments per kilobase of transcript per million mapped reads (FPKM) values were calculated based on the length of the gene and the read count mapped to that gene.

Differential expression analysis was performed using the DESeq2 R package (v1.20.0). The resulting p-values were adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate. Genes with an adjusted p-value ≤ 0.05 were classified as differentially expressed. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented using the clusterProfiler R package, which corrects for gene length bias. GO terms with corrected p-values less than 0.05 were considered significantly enriched among differentially expressed genes.

Microscopy for NTT1 Localization

The *S. cerevisiae* strain harboring *NTT1* was grown in yeast peptone dextrose (YPD) medium at 30°C with constant agitation (200–220 rpm) until mid-log phase. For visualization of *NTT1* localization, cells were harvested and immediately examined by confocal fluorescence microscopy using a GFP-compatible excitation/emission range (e.g., 488 nm excitation/505–530 nm emission). Images were acquired under identical settings for all samples to enable direct comparisons of fluorescence signals.

Relative ATP Measurement Using QUEEN ATP Reporter

The *S. cerevisiae* strains expressing the QUEEN ATP reporter (NTT1 and WT control) were cultivated in Complete Synthetic Medium (CSM) at 30°C with constant agitation (200–220 rpm) until reaching mid-log phase (OD600 ~ 0.4–0.6). The cells were retained in CSM for one set of experiments and either treated with 5 mM ATP for 30 minutes or left untreated as a control.

In a separate experiment, cells were transferred to CSM supplemented with 20 mM 2DG (Sigma #D-8375) for 30 minutes to modulate intracellular ATP levels. Following the indicated treatments, cells were harvested, washed once in fresh CSM, and resuspended in CSM for microscopy. Cell viability was assessed using propidium iodide (PI) staining.

Images were acquired using a Leica DMi8 inverted fluorescence microscope equipped with 405, 488 and 561 nm lasers, an LED3 fluorescence illumination system, a 100X, 1.47 numerical aperture Plan-Apochromat oil immersion lens, a Flash 4.0 v3 sCMOS camera (Hamamatsu), a W-View Gemini beam splitting device (Hamamatsu), and LAS X v3.7.6.25997 software. All strains within an experiment were imaged under identical exposure settings. A series of four images were captured for each field: one bright-field image and three fluorescence images corresponding to excitation/emission filters optimized for QUEEN (QWF-T ex405, QWF-T ex488), or for PI (QWF-T ex561).

Digital images were processed using Fiji software. First, the background signal was subtracted from the fluorescence channels after converting images to 32-bit floating-point format. Intracellular ATP was estimated by calculating a QUEEN ratio, defined as the pixel intensity of the ex405 image divided by the corresponding intensity of the ex488 image on a per-pixel basis. The mean QUEEN ratio for each cell was determined by averaging pixel ratios within a manually defined region of interest (ROI) encompassing the cell. Higher QUEEN ratios indicate elevated intracellular ATP levels. PI fluorescence (captured via ex561) was used to identify and exclude nonviable or compromised cells from the analysis. Based on the normality and homogeneity assumptions (Shapiro-Wilk and Levene's tests, respectively), a non-parametric Kruskal-Wallis test was chosen for comparison of groups. Post hoc pairwise comparisons using the Wilcoxon rank sum test with Bonferroni adjustment were used when comparing only two groups.

Setting up Microfluidics Experiments

The microfluidics device fabrication and experimental setup have been described previously (38,40,72).Yeast cells were inoculated into 2ml synthetic complete medium (SD, 2% glucose) and incubated on a 30^0C shaker overnight. From this culture, 2 µL of saturated culture was transferred to 20ml SD and incubated until its OD_{600} reached ~0.6 (early exponential growth phase). A microfluidics chip containing 4 microfluidics channels was placed under vacuum for at least 15min. In the meanwhile, 50ml SD with 0.04% Tween-20 (Sigma) was transferred to a 60ml syringe, to which plastic tubing (TYGON, ID 0.020 IN, OD0.060 IN, wall 0.020 IN) was connected. For samples that have additional ATP in the medium, ATP was added to this 50ml medium to reach the corresponding concentrations. The syringe was sealed with aluminum foil. 4 such syringes

were prepared, 1 for each microfluidics channel. After vacuum, the microfluidics chip was quickly connected to the syringes through plastic tubing from its inlet port to avoid air flowing into the microfluidics channels. The outlets were then connected to plastic tubing filled with ddH2O. The chip with inlets and outlets was then fixed to a microscope stage. For cell loading, cells were transferred into a 60ml syringe with plastic tubing connected. Connection of the microfluidics inlets to the media supply syringe was then replaced by connection to the cell loading syringe. The flow of medium in the device was maintained by gravity to drive cell loading into the traps. After loading, the media supply tubing was switched back to the inlet. Heights of all supply syringes were then adjusted to have a height difference between inlet and outlet of around 240cm. Tween-20 is a non-ionic surfactant that helps reduce friction during cell loading. We have validated previously that this low concentration of Tween-20 has no significant effect on cellular lifespan or physiology (73).

For assaying rho0 isolates, yeast cells were cultured overnight in synthetic complete (SC) media supplemented with 2% glucose. The following day, cultures were diluted 1:300 into 4 mL fresh SC media and incubated at 30°C for 3 hours. Prior to loading into the microfluidic device, 0.05% bovine serum albumin (BSA) was added to the culture to reduce nonspecific adhesion. Cells were then introduced into the microfluidic device, and all experiments were conducted using a Nikon Eclipse Ti2 inverted microscope equipped with a 60× 1.4 NA oil immersion objective (Nikon) and maintained at 30°C using a stage-top incubation system (Okolabs). Media was continuously delivered to the device via two 30 mL syringe pumps (total flow rate of 4 µL/min) to ensure nutrient availability and waste removal. Image acquisition was carried out every 5 minutes for up to 72 hours using NIS-Elements software, with the Nikon Perfect Focus System (PFS) engaged throughout all imaging sessions to preserve focal stability. Cell viability and death events were monitored in real time, and all downstream image segmentation, cell tracking, and data extraction were performed using MATLAB (MathWorks).

Time-lapse Microscopy

Time-lapse Microscopy experiments were conducted using a Nikon Ti-E inverted fluorescence microscope with an EMCCD camera (Andor iXon X3 DU897). The light source is a spectra X LED system. Images were taken using a CFI plan Apochromat Lambda DM 60X oil immersion

objective (NA 1.4 WD 0.13mm). In the yeast aging experiments, images were taken for each imaging channel every 15min for a total of 90 hours. The setting of each channel was set as the following and remains consistent across time-lapse imaging aging experiments: Phase, 60ms; GFP, 100ms at 10% lamp intensity with an EM gain of 100.

Quantification of Single-cell Aging Traces

The procedure of image quantification has been described previously (40). Image processing was done using ImageJ and custom Matlab codes. The background of fluorescence channel was subtracted by rolling ball background subtraction. Cells were masked and tracked by thresholding the GFP channel. The correctness of cell tracking was manually checked. The mean intensity of the top 40% pixels was quantified for each mother cell at each time point.

Cell aging mode was determined by daughter cell morphology (72). Cell replicative lifespan was manually quantified. Cells that showed obvious abnormal morphology upon loading were filtered out of RLS analysis.

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Author contributions

A.K. and V.N.G. designed research; N.O., H.S., P.P., R.H., D.C.P., performed research. N.O., H.S., P.P., R.H., V.M.L., N.H., and A.K. analyzed data; and N.O., H.S., P.P., R.H., V.M.L., D.C.P., V.N.G., N.H., and A.K, wrote the paper.

Competing interests

The authors declare no competing interest

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Figures:

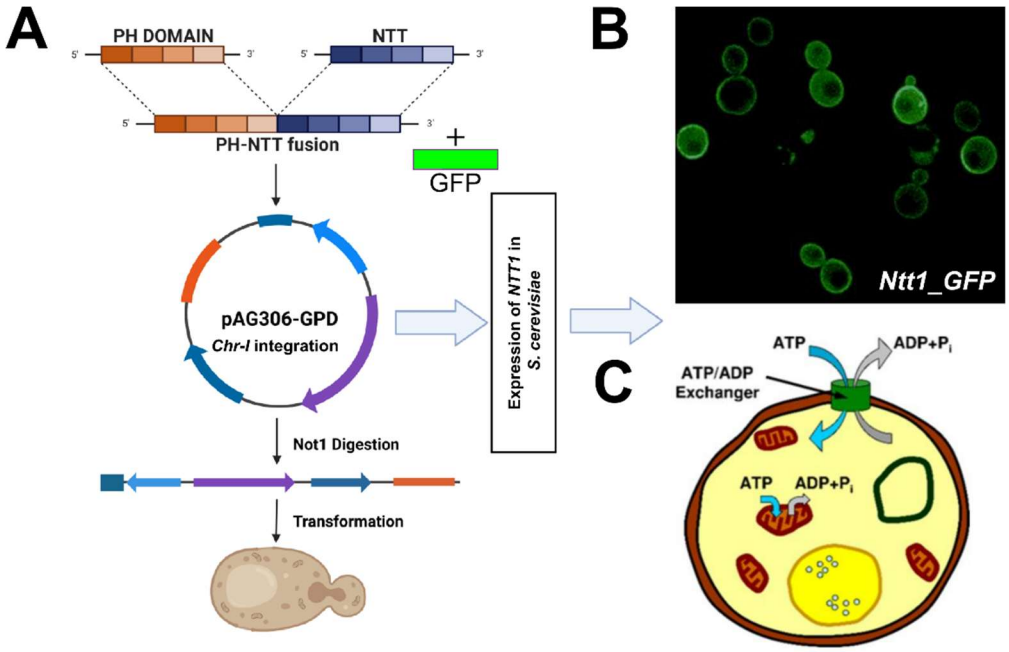

Figure 1: Strain design: (**A**) Pleckstrin homology (PH) domain of *SLM1* gene fused with *NTT1* and inserted in the vector (*pAG306GPD-ccdB-chr1*). The constructed (*PH-NTT1*) plasmid, digested with *NotI*, was integrated into the yeast genome. (**B**) In a separate construct, *yeGFP* sequence was fused to the 3' end of *PH-NTT1* sequence and expressed in yeast and visualized by confocal microscopy to assess cellular localization. (**C**) Overall yeast strain design with *NTT1* overexpression mediating ATP import from extracellular environment.

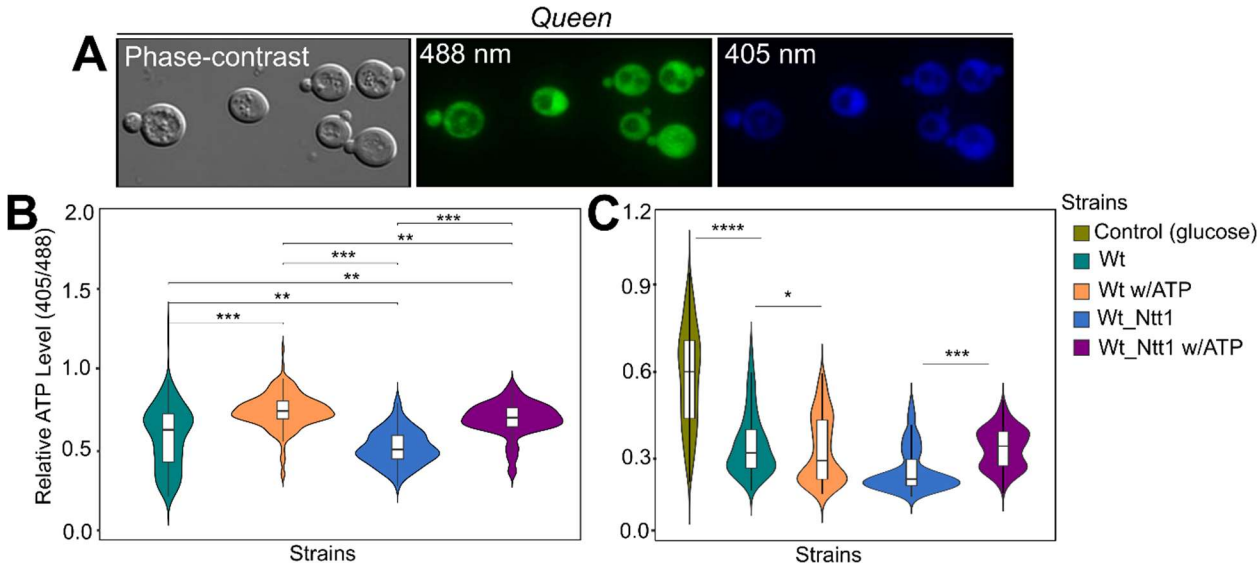

Figure 2: Measurement of relative ATP levels (**A**) Yeast cells expressing the fluorescent QUEEN ATP reporter were imaged using fluorescence microscopy. Relative ATP levels were measured in cells grown in media containing (**B**) 2% glucose or (**C**) 20 mM 2DG, and are presented as violin plots. Sample sizes for each strain are between 265 and 315 cells. Statistical significance between groups and raw QUEEN ratio data are available in **Supplementary File 1.**

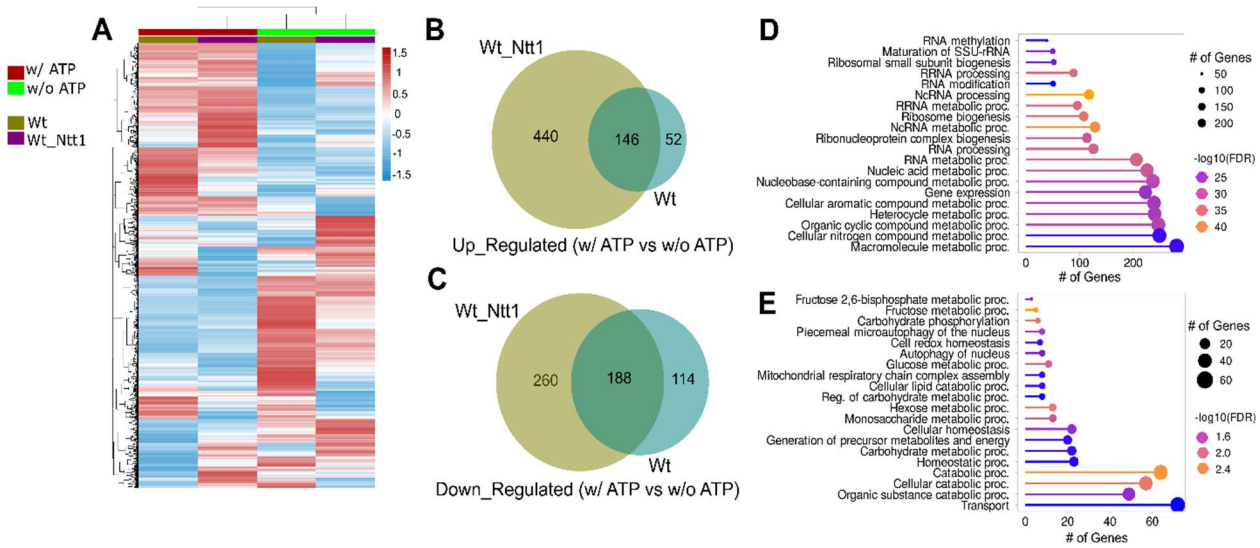

Figure 3: Transcriptomic analysis of Wt and Wt_Ntt1 cells with and without ATP treatment. (**A**) Heatmap showing hierarchical clustering of gene expression profiles from Wt and Wt_Ntt1 cells cultured with ATP (red) and without ATP (green). Expression values are Z-score normalized, with red indicating higher expression and blue indicating lower expression. Three independent cultures for each group were used to isolate total RNA and perform RNA-seq analysis. (**B, C**) Venn diagrams displaying the number of genes uniquely or commonly (**B**) upregulated or (**C**) downregulated in Wt and Wt_Ntt1 cells treated with ATP compared to untreated controls. (**D, E**) Enrichment analysis showing significantly enriched gene ontology (GO) terms associated with genes (**D**) upregulated and (**E**) downregulated in Wt_Ntt1 cells compared to their corresponding untreated controls. Circle size reflects the number of genes in each GO term, and color indicates significance level as represented by -log10(FDR). The normalized counts and the complete list of DEGs with *p* values can be found in **Supplementary File S2**.

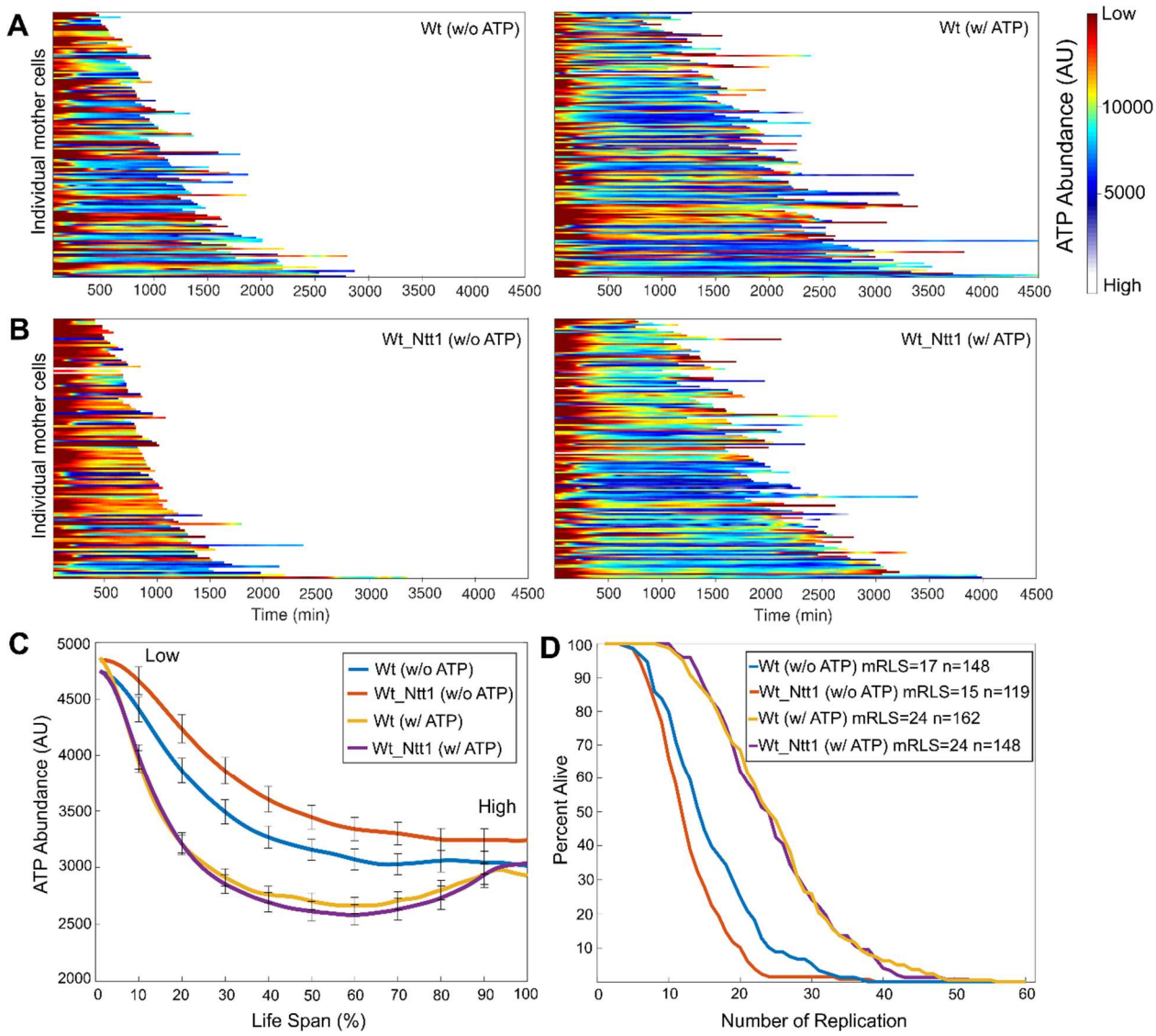

Figure 4: Quantifications of ATP levels and replicative lifespans of the Wt strain and NTT1 strain under ATP treatment condition. (**A**) Heatmaps show the fluorescent signal of the QUEEN intracellular ATP reporter throughout cells whole lifespan. Each line is a color-coded (Red=low ATP and Blue = high ATP) time trace of a single yeast cell. Wt control without addition of external ATP showed rapid increase of cellular ATP during the aging process (left), while Wt control with ATP showed much higher level (p = 1.15E-143) and similar trend of cellular ATP (right). (**B**) NTT1 strain without ATP show lower level of cellular ATP compared to WT without ATP throughout the aging process whereas addition of ATP restored the cellular ATP level to that similar to Wt control with addition of ATP (right). (**C**) Average of single-cell time traces of the QUEEN reporter signal aligned by percent lifespan. Error bars are standard errors. (**D**) Addition of ATP significantly increased the replicative lifespan of the Wt strain (p = 1.28E-12) and the NTT1 strain (p = 4.03E-

18). Without ATP, the NTT1 strain showed significantly shorter lifespan than the Wt strain ($p = 0.005$), while with ATP, the 2 strains showed similar lifespans ($p = 0.669$). With addition of ATP, the NTT1 strain showed a significantly enhanced replicative lifespan as compared to Wt without ATP ($p = 1.03E-18$). P-values were calculated with Mann-Whitney U test. The AU units and lifespan data can be found in **Supplementary File 3.**

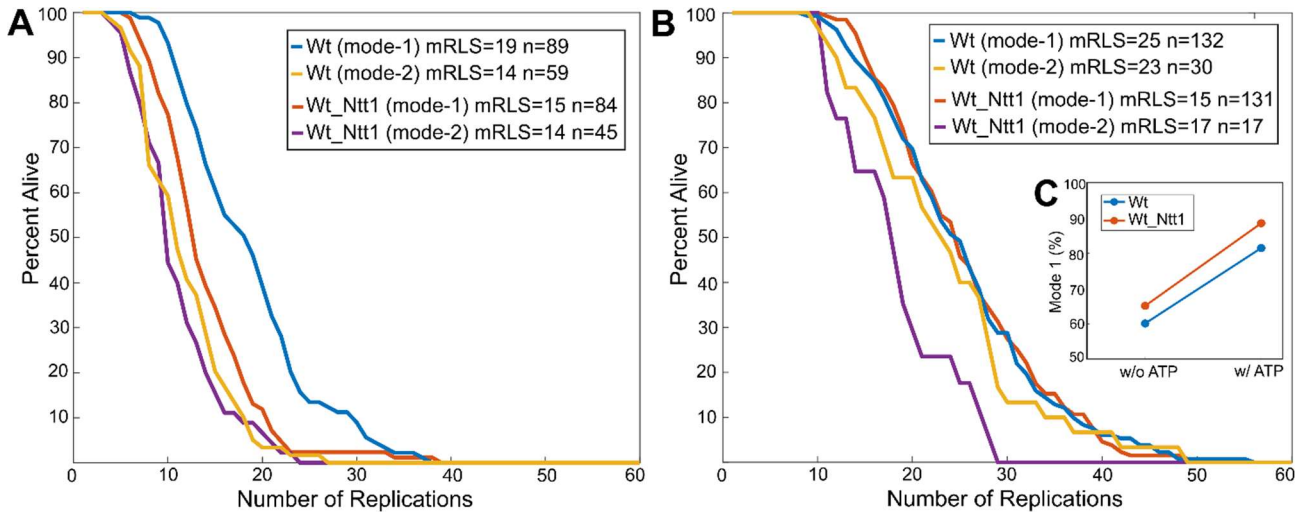

Figure 5: Replicative lifespan of different aging modes and mode distribution under ATP treatment condition. (**A**) Without ATP, the NTT1 strain showed similar lifespans between the two modes of yeast cell aging (p = 0.74), while Wt strain showed different lifespans (p = 2.18E-4) between 2 modes of aging. While the mode-2 lifespans between the 2 strains showed no significant difference (p = 0.826), NTT1 strain showed significantly shorter mode-1 lifespan than that of the Wt strain (p = 2.39E-4). (**B**) With ATP, the NTT1 strain showed significantly increased mode-1 lifespan (p = 1.24E-16), but not mode-2 lifespan (p = 0.15), whereas the Wt strain showed significantly increased mode 1 lifespan (p = 3.17E-7) and also mode-2 lifespan (p = 5.98E-6), which was similar to mode 1 lifespan (p = 0.4). The mode 1 lifespans between the 2 strains were similar (p = 0.849). (**C**) Both strains show increased percentage of mode-1 cells under the effects of ATP. The p-values were calculated using Mann-Whitney U test. The lifespan data can be found in **Supplementary File 3.**

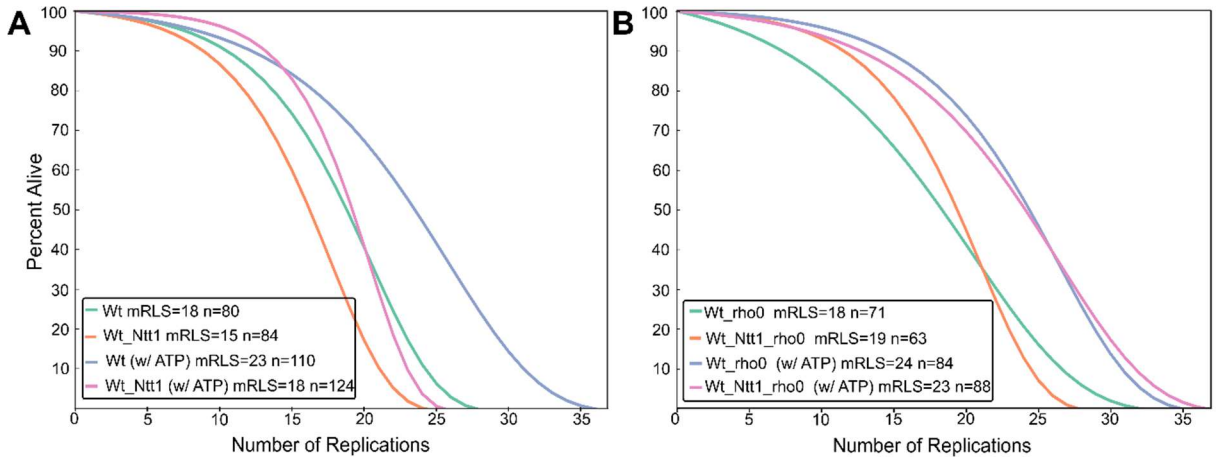

Figure 6: Replicative Lifespan analyses of *rho^0* Isolates. Replicative lifespan (RLS) phenotypes of (**A**) parental controls and (**B**) their *rho^0* isolates were measured using a microfluidic chip under untreated and ATP-treated (w/ATP) conditions. Elimination of mtDNA in rho0 isolates alleviate the toxic effect of ATP treatment on Wt_Ntt1 cells. Raw lifespan data and statistical significance are provided in **Supplementary File 3**.

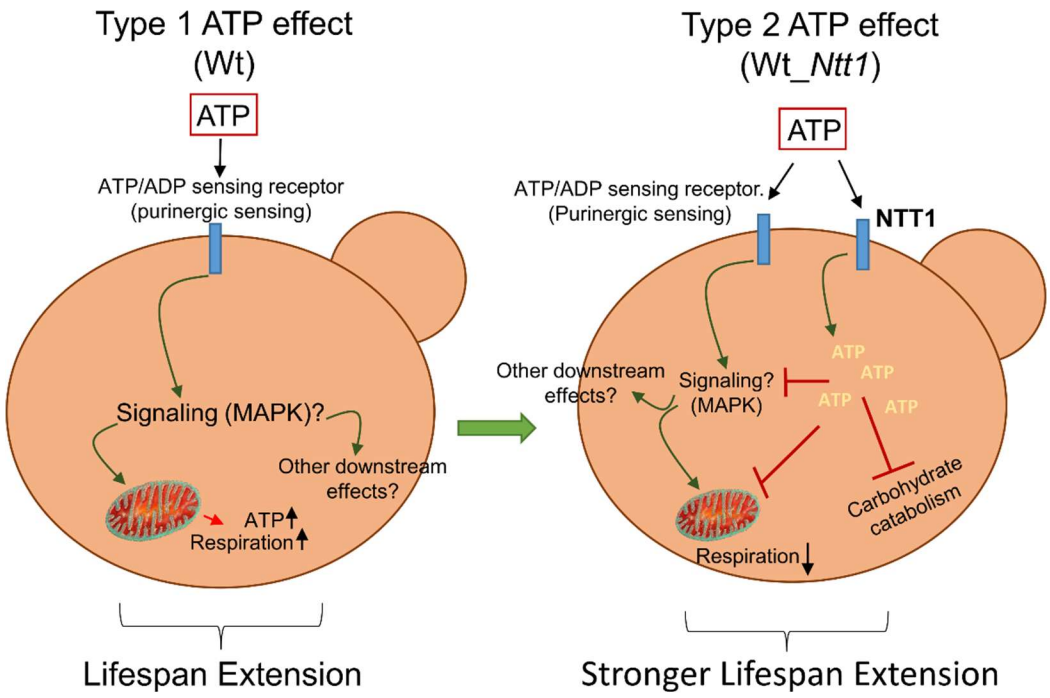

Figure 7: Proposed Models for ATP Treatment Effects in Wt and Wt_Ntt1 Cells. The type-1 effect involves ATP sensing, analogous to mammalian purinergic signaling, which activates a kinase cascade (e.g., MAPK) to modulate downstream pathways and mitochondrial function (**left panel**). In contrast, the type-2 effect is specific to Wt_Ntt1 cells and is regulated through increased ATP abundance imported from the medium via Ntt1. In this model, elevated ATP levels inhibit mitochondrial respiration and catabolic processes, potentially modulating some pathways associated with the type-1 effect (**right panel**). Both type-1 and type-2 effects increase replicative lifespan (RLS), with the type-2 effect producing a stronger lifespan extension.

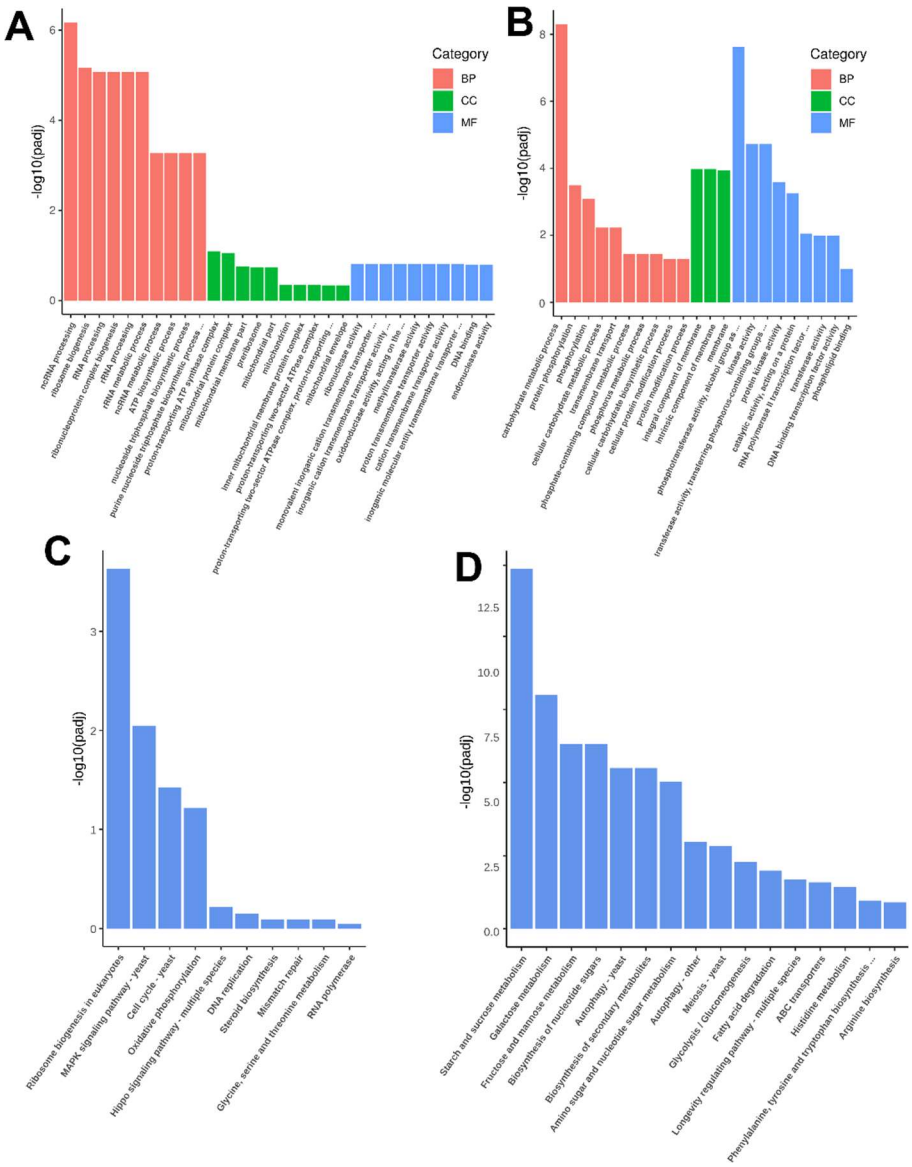

Figure S1: Gene Ontology and KEGG Pathway Analyses for Wt Cells. Gene ontology (GO) enrichment analysis for (**A**) upregulated and (**B**) downregulated genes, and KEGG pathway analysis for (**C**) upregulated and (**D**) downregulated genes in Wt cells after ATP treatment. GO categories include BP (Biological Process), CC (Cellular Component), and MF (Molecular Function).

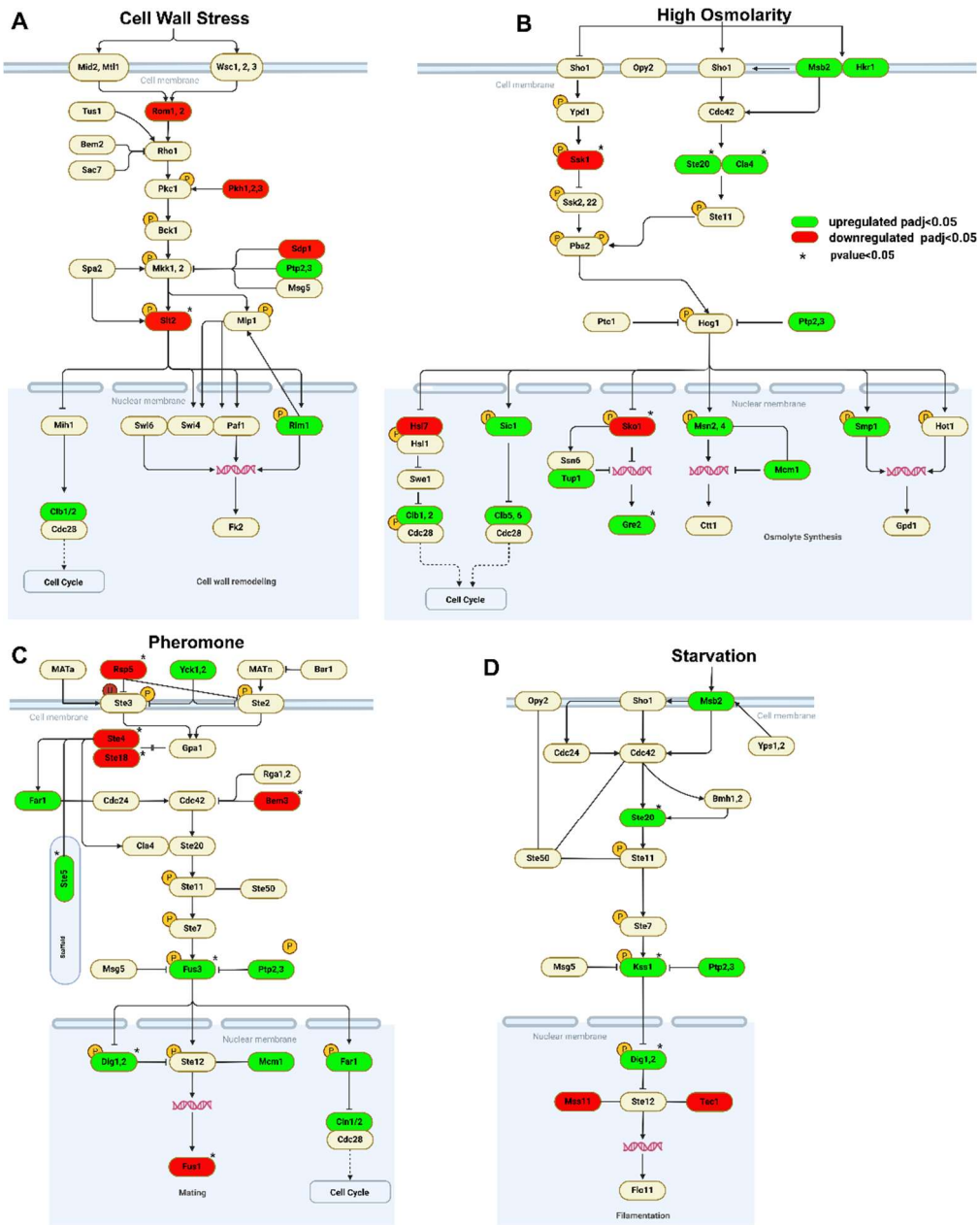

Figure S2: Alteration of MAP Kinase Pathway in Wt Cells. Genes uniquely upregulated (green) or downregulated (red) are shown for each component of the MAPK pathway in Wt cells under ATP treatment: (A) Cell Wall Stress, (B) High Osmolarity, (C) Pheromone, and (D) Starvation.

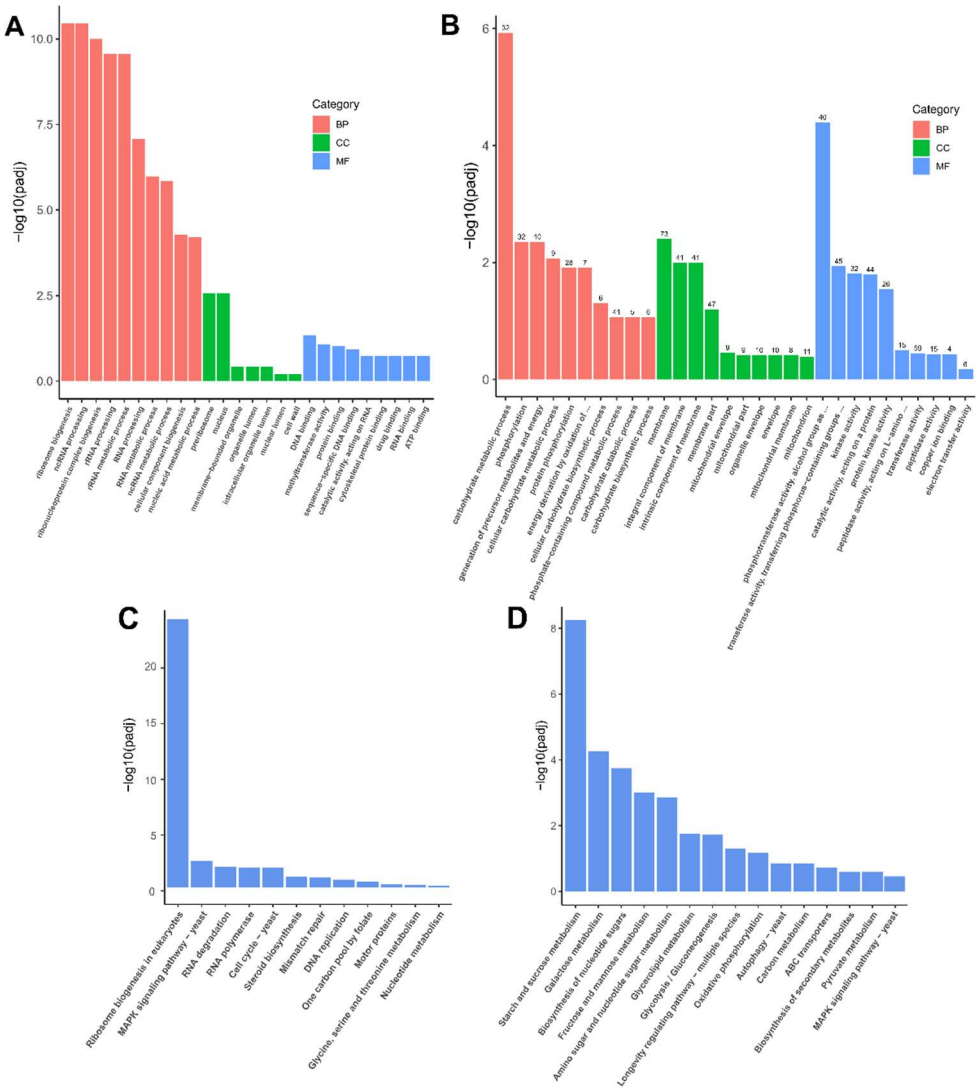

Figure S3: Gene Ontology and KEGG Pathway Analyses for Wt_Ntt1 Cells. Gene ontology (GO) enrichment analysis for (**A**) upregulated and (**B**) downregulated genes, and KEGG pathway analysis for (**C**) upregulated and (**D**) downregulated genes in Wt_Ntt1 cells after ATP treatment compared to untreated controls. GO categories include BP (Biological Process), CC (Cellular Component), and MF (Molecular Function).

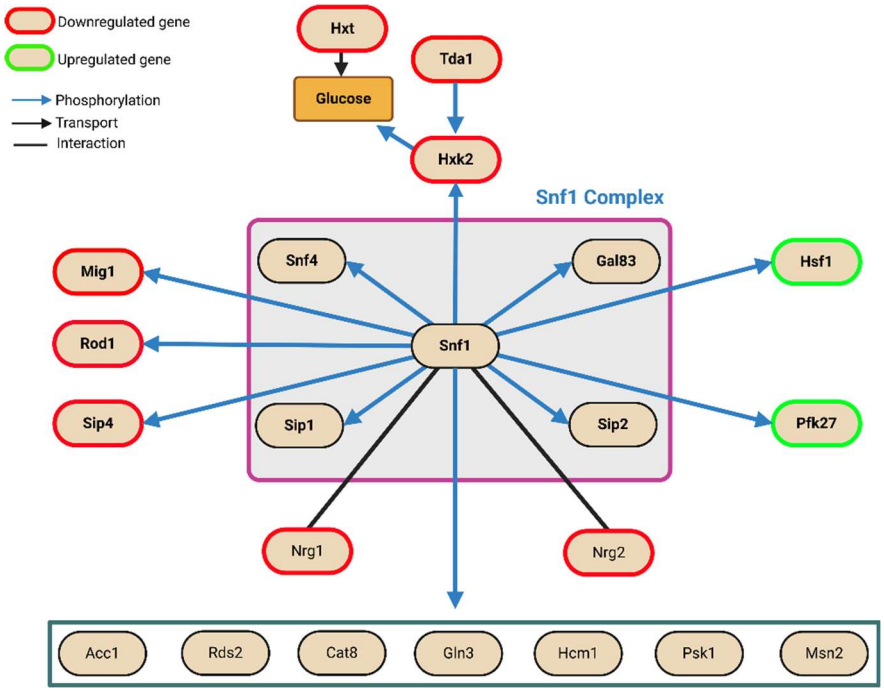

Figure S4: SNF1-Mediated Regulatory Gene Network in Wt_Ntt1 Cells. Genes regulated by SNF1 in Wt_Ntt1 cells under ATP treatment are depicted in red (downregulated), green (upregulated), and black (no expression changes). The types of interactions are represented by arrows and lines.