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[MASTER'S THESIS]

[EFFECT OF ANTIDIABETIC DRUG METFORMIN on GENE
EXPRESSION PROFILE in *Schizosaccharomyces pombe*]

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CONTENTS

Page No

ACKNOWLEDGEMENTS	iv
CONTENTS	v
TABLE OF FIGURES	viii
TABLE OF TABLES	x
TABLE OF ABBREVIATIONS	xi
ÖZET	xvi
SUMMARY	xvii
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1. AGING	4
2.2. AGING PATHWAYS	5
2.2.2. AMPK Signaling and Aging	6
2.2.3. Sirtuin Signaling	8
2.2.4. MTOR Signaling	9
2.3. METFORMIN	12
2.4. METFORMIN AS A DRUG FOR DIABETES AND THE PATHWAYS IT FOLLOWS	13
2.4.1. Sirtuin Signaling and Metformin	13
2.4.2. Metformin and AMPK Signaling	13
2.4.3. Pharmacokinetics Pathway	15
2.4.4. Pharmacodynamics Pathway	17
2.5. ANTIAGING PROPERTIES OF METFORMIN ACROSS DIFFERENT MODEL ORGANISMS THEN FOCUSING DOWN TO <i>S.POMBE</i>	19
2.5.1. Different Model Organisms	19
2.5.2. Fission Yeast - <i>Schizosaccharomyces pombe</i>	20
3. MATERIALS AND METHODS	24
3.1. GROWTH MEDIA	24
3.1.1. SD media	24
3.1.2. YEA media	24
3.2. CONDITIONS FOR CELL GROWTH	25

3.3. CELL COUNTING.....	25
3.4. INOCULATION.....	25
3.5. METFORMIN	26
3.6. DETERMINATION OF MID-LOG PHASE AND OPTIMIZATION	26
3.7. TOTAL RNA EXTRACTION	26
3.8. RNA SEQUENCING.....	27
3.8.1. Experimental Methods and Workflow	27
3.11. NANO-DROP™ 2000/2000C SPECTROPHOTOMETER	29
4. RESULTS	30
4.1. MID-LOG PHASE	30
4.2. RNA EXTRACTION.....	31
4.3. EXPRESSION PROFILE – UPREGULATED AND DOWNREGULATED GENES	32
4.3.1. 5%M / 3%M Comparison	34
<i>Molecular Function</i>	34
<i>Biological Process</i>	35
<i>Cellular Component</i>	37
<i>Protein Class</i>	37
<i>Panther Pathway</i>	39
4.3.2. 3%M / 5%M Comparison	41
<i>Molecular Function</i>	41
<i>Biological Process</i>	42
<i>Cellular Component</i>	43
<i>Protein Class</i>	44
<i>Panther Pathway</i>	46
4.3.3. 3%M / 3%N Comparison.....	47
<i>Molecular Function</i>	47
<i>Biological Process</i>	49
<i>Cellular Component</i>	50
<i>Protein Class</i>	51
<i>Panther Pathway</i>	52
4.3.4. 5%M / 5%N Comparison.....	54
<i>Molecular Function</i>	54
<i>Biological Process</i>	56
<i>Cellular Component</i>	57

<i>Protein Class</i>	58
<i>Panther Pathway</i>	60
5. DISCUSSION	62
5.1.1. UP-REGULATED GENES.....	63
5.1.2. DOWN-REGULATED GENES.....	65
5.4. CONCLUSION	74
KAYNAKLAR	76
ÖZGEÇMİŞ	87

]



TABLE OF FIGURES

	Page No
Figure 2.1: Schematic diagram of the structure of AMPK heterotrimers, with the different subunits colour coded (α , yellow; β , lilac; γ blue) (Vara-Ciruelos et al., 2019).....	6
Figure 2.2: Constituents of mTORC and mTOR signaling in the cell. (Yu et al., 2021)	10
Figure 2.3: Illustration of the roles of the AMPK, SIRT1 and mTOR signaling pathways in aging. The blue arrows indicate a positive effect, and the orange arrows indicate a negative effect. (Yu et al., 2021).....	12
Figure 2.4: Figure: Pharmacokinetics pathway of metformin. Stylized cells depicting genes involved in the transport and clearance of metformin. (Gong et al., 2012).	17
Figure 2.5: Pharmacodynamics pathway of metformin. Stylized cells depicting the mechanism of action of metformin. (Gong et al., 2012).....	19
Figure 4.1: The effect of 25 ml metformin on growth curve in <i>S. pombe</i> using 3% and 5% SD growth media.	31
Figure 4.2: RNA Sequencing Agarose Gel Electrophoresis	31
Figure 4.3: Molecular Function of 5%M/3%M upregulated and downregulated genes using Panther.....	355
Figure 4.4: Biological Process of 5%M/3%M upregulated and downregulated genes using Panther.....	366
Figure 4.5: Cellular Component of 5%M/3%M upregulated and downregulated genes using Panther.....	37
Figure 4.6: Protein Class of 5%M/3%M upregulated and downregulated genes using Panther	39
Figure 4.7: The Panther Pathway of 5%M/3%M upregulated and downregulated genes	40
Figure 4.8: Molecular Function of 3%M/5%M upregulated and downregulated genes using Panther.....	422
Figure 4.9: Biological Process of 3%M/5%M upregulated and downregulated genes using Panther.....	433
Figure 4.10: Cellular Component of 3%M/5%M upregulated and downregulated genes using Panther.....	444
Figure 4.11: Protein Class of 3%M/5%M upregulated and downregulated genes using Panther	455

Figure 4.12: The Panther Pathway of 3%M/5%M upregulated and downregulated genes	477
Figure 4.13: Molecular Function of 3%M / 3%N upregulated and downregulated genes using Panther.....	488
Figure 4.14: Biological Process of 3%M / 3%N upregulated and downregulated genes using Panther.....	50
Figure 4.15: Cellular Component of 3%M / 3%N upregulated and downregulated genes using Panther.....	51
Figure 4.16: Protein Class of 3%M / 3%N upregulated and downregulated genes using Panther	522
Figure 4.17: The Panther Pathway of 3%M / 3%N upregulated and downregulated genes	544
Figure 4.18: Molecular Function of 5%M / 5%N upregulated and downregulated genes using Panther.....	554
Figure 4.19: Biological Process of 5%M / 5%N upregulated and downregulated genes using Panther.....	577
Figure 4.20: Cellular Component of 5%M / 5%N upregulated and downregulated genes using Panther.....	588
Figure 4.21: Protein Class of 5%M / 5%N upregulated and downregulated genes using Panther	59
Figure 4.22: The Panther Pathway of 5%M / 5%N upregulated and downregulated genes	61
Figure 5.1: The putative interaction map of proteins that encoded by all genes whose expression were significantly changed according to glucose availability. Network was created through STRING website (ver. 11.0). Interaction sources included experiments, databases, co-expression, neighbourhood, gene fusion and co-occurrence. Minimum required interaction score was set as 0.400. Detailed explanation is given in the text.	75

TABLE OF TABLES

	Page No
Table 3.1: SD Media Recipe.....	24
Table 3.2: YEA Media Recipe.....	25
Table 4.1: Mid-Log Phase Optimisation	30
Table 4.2: Pooling of repeats together for RNA sequencing.....	32
Table 4.3: Raw data stats.....	33
table 4.4: Trimming data stats.....	33
Table 4.5: Mapped data stats.....	34

TABLE OF ABBREVIATIONS

Symbol	Abbreviation
2-HE2	: 2-hydroxy estradiol
ACC1	: Acetyl Coenzyme A Carboxylase 1
ADP	: Adenosine diphosphate
AKT	: AK strain transforming
AMP	: Adenosine monophosphate
AMPD	: Adenosine deaminase
AMPK	: AMP-activated protein kinase
AMPK	: AMP-activated protein kinase
ATP	: Adenosine triphosphate
BDNF	: Brain-derived neurotrophic factor
C. elegans	: Caenorhabditis elegans
CBM	: Carbohydrate-binding molecule
cGMP	: Cyclic guanosine monophosphate
CLS	: Chronological lifespan
CRM	: Calorie restriction mimetic
DEGs	: Differentially expressed genes
DEPTOR	: DEP-space containing mTOR-connecting protein
DN	: Double negative

DNA	: Deoxyribonucleic Acid
DNMT1	: DNA methyltransferase 1
DPP-4	: Dipeptidyl peptidase-4
E2	: Estradiol
eHSP70	: Heat shock protein 70
eNOS	: Endothelial nitric oxide synthase
ERK1/2	: Extracellular signal-regulated kinase 1/2
ERs	: Estrogenic receptors
ETC	: Electron transport chain
Fabp4	: Fatty acid-binding protein 4
FPKM	: Fragments Per Kilobase of transcript per Million Mapped reads
G6pc	: Glucose 6-phosphatase
Ght7	: AMP-activated protein kinase complex
GLP-1	: Glucagon-like peptide-1
GLUT1	: Glucose transporter protein type 1
GLUT1/GLUT4	: Glucose transporter protein type
H3K9	: Histone H3 Lysine 9
HGPS	: Hutchinson-Gilford progeria syndrome
HMG-CoA	: Hydroxymethylglutaryl-coenzyme A
Hsp16	: Heat shock protein 16
HuR	: Hu-Antigen Receptor

IGF-1	: Insulin-Like Growth Factor-1
IGF1R	: Insulin-like growth factor 1 receptor
IRS2	: Insulin receptor substrate 2
ISCs	: Intestinal stem cells
LKB1	: Liver kinase B1
MATE1	: Multidrug and toxin extrusion 1
MBNL1	: Muscleblind like splicing regulator 1
Mde7	: RNA-binding protein
met14	: Adenylylsulfate kinase
mfm3	: Myofibrillar myopathy-3
MLCK	: Myosin light chain kinase
mRNA	: Messenger ribonucleic acid
mSIN1	: Mammalian stress-activated protein
mTOR	: Mammalian target of rapamycin
NAD⁺	: Nicotinamide adenine dinucleotide
NADs	: Nicotinamide adenine dinucleotide
NDGA	: Nordihydroguaiaretic
NIH	: National Institutes of Health
pAMPK	: Phosphorylated AMP-activated protein kinase
PC12	: Pheochromocytoma Cell Line 12

PCOS	: Polycystic ovarian syndrome
PH3	: Hydrogen phosphide
PI3Ks	: Phosphoinositide 3-kinases
PMAT	: Plasma membrane monoamine transporter
p-MLC	: Phosphorylated myosin light chain
PP2Cα	: Protein phosphatase 2C dephosphorylates
PPG	: Postprandial plasma glucose
PQ	: Plastoquinone
PRAS40	: Proline-rich Akt substrate 40 kDa
Rag	: Ras-related GTP-binding protein
RBP	: RNA binding protein
Rictor	: Rapamycin-insensitive mTOR companion
RNA	: Ribonucleic Acid
ROCK1	: Rho-kinase 1
ROS	: Reactive oxygen species
RPKM	: Reads Per Kilobase of transcript per Million mapped reads
RPM	: Revolutions per minute
S6K	: S6 kinase
SD	: Synthetic Dextrose
SIRT1	: Sirtuin (silent mating type information regulation 2 homolog) 1
SREBP1c	: Sterol regulatory element-binding protein 1c

TAK1	: Transforming growth factor- β -activated kinase 1
TGF-1	: Transforming growth factor-1
TOR	: Target of Rapamycin
TPM	: Transcripts Per Kilobase Million
TSC	: Tuberous sclerosis complex
TSC1/2	: Tuberous sclerosis
ULK1/2	: Unc-51 like autophagy activating kinase
UPR	: Unfolded protein response
YEA	: Yeast Extracted Solid
YNB	: Yeast Nitrogen Base
γ-TuRCs	: γ -tubulin ring complexes

]

ÖZET

[EFFECT OF ANTIDIABETIC DRUG ETFORMIN ON GENE EXPRESSION PROFILE IN *Schizosaccharomyces pombe*

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[Even though calorie constraint is the most frequently utilized experimental intrusion to lengthen longevity in many model organisms, mounting data points to the possibility that excessive glucose shortens the life span in a variety of organisms. It is still crucial to identify genetic relationships, molecular responses and gene expression patterns dependent on glucose accessibility to completely comprehend the molecular basis of the pro-aging impact of glucose. Here, we used the RNA sequencing technique to evaluate the gene expression profiles in *Schizosaccharomyces pombe* mid-log-phase cells cultured on three distinct Synthetic Dextrose mediums with 3%, and 5% glucose. Our findings elucidate the association between excessive glucose, various cellular functions, and aging. The most affected genes are catalytic activity, ATP-dependent activity, and growth..]

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SUMMARY

[EFFECT OF ANTIDIABETIC DRUG ETFORMIN ON GENE EXPRESSION PROFILE IN *Schizosaccharomyces pombe*

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[Even though calorie constraint is the most frequently utilized experimental intrusion to lengthen longevity in many model organisms, mounting data points to the possibility that excessive glucose shortens the life span in a variety of organisms. It is still crucial to identify genetic relationships, molecular responses and gene expression patterns dependent on glucose accessibility to completely comprehend the molecular basis of the pro-aging impact of glucose. Here, we used the RNA sequencing technique to evaluate the gene expression profiles in *Schizosaccharomyces pombe* mid-log-phase cells cultured on three distinct Synthetic Dextrose mediums with 3%, and 5% glucose. Our findings elucidate the association between excessive glucose, various cellular functions, and aging. The most affected genes are catalytic activity, ATP-dependent activity, and growth

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1. INTRODUCTION

[Aging is defined as the aggregation and build-up of changes in our body affecting the functions of our organs at the cellular levels, increasing our susceptibility to diseases and eventually leading to death. These changes at the cellular level are linked to defects in our genes, environmental influence, infectious diseases, and the general course of development of the body over time (Harman, 2001). To put it another way, aging is a process that results in changes in our bodies' regular processes and the accumulation of damage over time, that fundamentally every living organism goes through.

It has been forecasted that the 21st century will be an unparalleled time for positive aging, where people try to keep a positive outlook and attitude toward aging and make the most out of old age. This idea is being upheld by the media too as it portrays positive characteristics of aging such as movement, independence, decision-making, freedom, and good health, all of which go against the conventional negative views of aging that suggest decline, weakness, and reliance on others (Katz, 2001).

The obsession of the human race with anti-aging and the public burden of the current aging population has compelled scientists to focus on anti-aging research work. Considering that inducing pathological harm and physiological damage at the molecular and cellular level is what leads to aging, scientists have worked hard to find a mechanism in organisms that causes aging. However, it doesn't seem possible to link aging to any single mechanism (Karan M.A & Tufan F, 2010).

Although, aging itself isn't seen as a disease itself but it isn't refutable to say that it has a direct connection to life and is a leader in all diseases related to old age. One cannot deny that aging and diseases associated with it are among the top causes of death. Research carried out in recent years has revealed various compounds and molecules that help in the healthy extension of the lifespan of living organisms. One of these compounds has been identified as metformin (N, N-dimethyl biguanide). The drug Metformin has been administered for a long time in treating the type-2 diabetes for its effect of reducing blood glucose levels. Other than its uses in antidiabetic treatment it has also been found useful in neurodegenerative disease, anti-aging, cancer treatment, and a remedy to improve lipid patterns and reduce weight in

patients with coronary heart disease (Carlsen et al., 1996; Podhorecka et al., 2017; Soukas et al., 2019).

By decreasing IRS2 and IGF1R in neurons and activating the pAMPK, eNOS / cGMP, and pMLCK actin remodelling pathways, metformin has been shown to prevent aging, reducing the accumulation of AGEs, regulating ROS levels in mitochondria, inhibiting oxidative stress and interleukin (IL)-1 β -induced osteoarthritis, regulating protein homeostasis, reducing Insulin-Like Growth Factor-1 (IGF-1) signaling and activation of autophagy via inhibition of the mTOR signaling pathway (Hu et al., 2021). It has also exhibited reduction and suppression of reactive oxygen species (Kulkarni et al., 2020). It is still unknown, nevertheless, whether metformin's numerous effects are caused by a single mechanism that leads to a series of events, or if it affects numerous routes simultaneously (Barzilai et al., 2016). Understanding which genes and cellular functions metformin affects in the anti-aging process is critical.

The research during the previous two decades on anti-aging has sped up a lot, thanks to different model organisms. As a result of these studies, it has been realized that researching non-human organisms is very crucial to better understanding various diseases and subsequently, aging. Some organisms such as flies, worms, yeast, and mice have been found to share common aspects of aging. Replicative aging studies in yeast cells and chronological aging studies in nematodes and fruit flies have been discovered to be very similar to that of human cell lines (Bitto et al., 2015; Paradis & Ruvkun, 1998).

To examine many cellular and molecular processes, such as eukaryotic mRNA modification, cell cycle control mechanisms, and nucleosome organizations, conserved in eukaryotic organisms, yeasts have emerged as important model organisms (Botstein & Fink, 2011; Kevin Struhl & Victoria Lundblad, 2008). In the recent years, studies on aging have centred on more and more on yeasts as they have emerged to be a very promising and powerful research area. Considering the chronological and replicative lifetimes of yeast cells as model organisms, they have helped researchers to accomplish very effective and ideal results in the field of aging (Breitenbach et al., 2012).

One such yeast is *Schizosaccharomyces pombe*. It has been deducted that *S. pombe* is among the yeasts that have many similar characteristics as higher eukaryotes, which are very

different than *S. cerevisiae* (MacKenzie & Lacefield, 2020). Over time, it has been accepted as a very useful organism to study the molecular biology of higher eukaryotes (Moreno et al., 1991). *S. pombe* has also been found to be among the best models to study signal transduction for sexual differentiation, cell-cycle control, and chromosomal structure (Giga-Hama & Kumagai, 1999). *S. pombe* has displayed antioxidant and anti-aging activities by regulating stress tolerance response, increase in *ctt1* gene expression, and inducing stress mitochondrial activity (Astuti et al., 2021).

In the light of this thesis, our objective is to comprehend and identify the impacts of metformin, a drug that has acted as a mule to impact various pathways and mechanisms in eukaryotic cells, on longevity and aging and to understand through which pathways these changes take place by using the wild strain of the yeast. We also hope to understand the genes that get upregulated and downregulated in wild strains of *S. pombe* when exposed to metformin. This research work will help in understanding the aging mechanism in *S. pombe*, which in return would give us a better understanding of aging in higher eukaryotes.

2. LITERATURE REVIEW

2.1. AGING

Aging, as a process known to humans, is as old as Earth itself; 3.5 billion years. Aging works by causing negative alterations not only in organs but in tissues and cells which eventually leads to death. It can be attributed to environmental effects, inborn defects, genetic diseases, and viral or bacterial diseases (Harman, 2001). The body's degradation as we age is the primary risk factor for a variety of human illnesses, including as heart disease, diabetes, neurological conditions, and cancer (López-Otín et al., 2013).

Even though, aging is generally defined as a process affecting an organism; the detection of it can be brought down to cellular levels. Contrary to previous studies, aging has been found to affect single-cell organisms such as bacteria too, which were previously believed to thrive and divide for as long as it lasts provided suitable conditions. A study focusing on the model organism *Escherichia coli* that divides without a juvenile phase used automated timelapse microscopy to observe repeated cycles of reproduction and found out that when a cell inherits the older pole, its growth rate decreases, its production of off-springs reduces, and the death rate increases (Stewart et al., 2005).

Lately, aging studies have emerged to be an area of interest for research. The fundamental question of aging being a suitable subject of study wasn't out of debate until very recently. In today's age where we consider aging as a very suitable topic for research, only thirty years ago it was a highly contested debate whether exploring this topic is befitting for scientific research or not (Zainabadi, 2018). The work in this field has advanced so quickly with different simpler studies on various model organisms such as yeasts and nematodes, the results of which are being tested on mice, being a relatively advanced mammalian, using small-molecule drugs. The remarkable extension of this work in recent times can be observed in the identification of certain evolutionary preserved biochemical processes which control the rate of aging (López-Otín et al., 2013).

2.2. AGING PATHWAYS

Aging, a basic biological phenomena, is accompanied by a general loss in tissue function. Age-related problems, such as memory or Alzheimer's, will in fact become an increasing public health problem as people live longer. Maturation is a crucial risk factor for various age-related mishaps. People today desire a longer lifespan and greater health. Understanding the fundamental molecular and cellular processes that control aging is essential for altering the process of aging for optimal longevity and curing age-related illnesses (Yu et al., 2021).

Aging pathways reflect the many biological actions and mechanisms which trigger living things, including people, to age. These routes entail intricate interactions between several genetic, epigenetic, cellular, and environmental elements that affect how quickly organism ages (Sen et al., 2016).

2.2.1 AMPK Signaling

The protein kinase AMP-activated (AMPK) detects cellular energy. It helps to restore energy balance by triggering catabolic pathways and inhibiting cell growth and expansion that had previously been influenced by increases in cell AMP: ATP ratios. Age and longevity have recently been associated with AMPK signalling. Cellular homeostasis, stress tolerance, cell survival and proliferation, cell death, and autophagy can all be regulated by AMPK signalling. Because of specific AMPK activation, *Caenorhabditis elegans* (*C. elegans*) and rodents lived longer (Vara-Ciruelos et al., 2019).

In mammalian cells, glucose deprivation leads to the activation of AMPK. The AMPK is well recognized for its ability to measure the energy state of both cells and the entire body. When AMP and/or ADP replace ATP anchored at a critical position on its regulatory subunit, AMPK is activated. Additionally encouraged by upstream kinases are allosteric activation and net phosphorylation of the catalytic subunit. ADP increases and ATP decreases during times of cell energy stress, pushing the process that adenylate kinases catalyse ($2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$) rightwards, ensuring that AMP ascends to a much greater degree than ADP and subsequently activating AMPK sensitively. Additionally, non-canonical, AMP/ADP-independent mechanisms that activate AMPK include DNA damage, glucose deprivation, and elevations in intracellular Ca^{2+} . AMPK not only encourages ATP generation to sustain cell viability but also to restore energy equilibrium but also prevents the development and division of cells by

phosphorylating targets downstream, which activate catabolic pathways while deactivating anabolic pathways and other ATP-consuming activities, such as the progression through the cell cycle (Vara-Ciruelos et al., 2019).

The AMPK can be found in mammalian cells in three different isoforms: two for the alpha and beta subunits, respectively; a sum of 12 particular alpha-beta-gamma ($\alpha\beta\gamma$) isoforms. Because it has a C-terminal regulatory domain and an activation sequence threonine 172 (T172) in its N-terminal protein kinase domain, the (α) subunit is in charge of AMPK's catalytic activity. The beta (β) subunit consists of a carbohydrate-binding molecule (CBM). The C-terminal domain of the CBM links the alpha (α) and gamma (γ) subunits. The AMP/ATP ratio is monitored and controlled by the four cystathionine-(β)-synthase (CBS) domains on the (γ) subunit, which refers to adenine nucleotide binding. The gamma ($\gamma 1$, $\gamma 2$, $\gamma 3$), and beta ($\beta 1$, and $\beta 2$) subunits control phosphorylation and activation of AMPK (Morgunova & Klebanov, 2019).

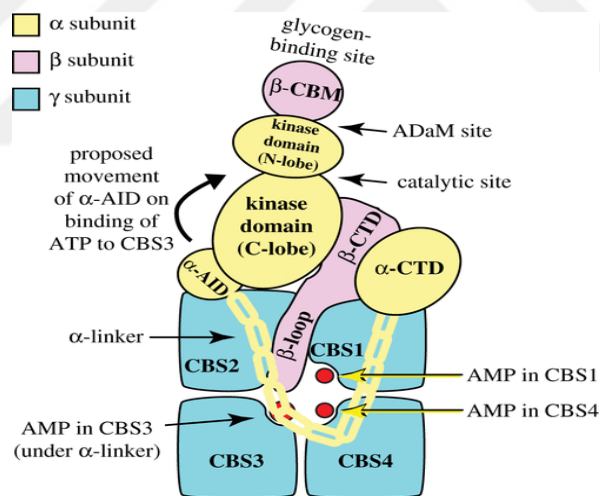


Figure 2.1: Schematic diagram of the structure of AMPK heterotrimers, with the different subunits colour coded (α , yellow; β , lilac; γ blue) (Vara-Ciruelos et al., 2019).

2.2.2. AMPK Signaling and Aging

LKB1, CaMKK β , TAK1, and PP2C α are upstream of AMPK, and ULK1/2, SIRT1, TSC1/2, SREBP1c, ACC1, HuR, p53, and GLUT1/GLUT4 are downstream pathways under its control.

At the cellular level:

- The AMPK upstream regulator LKB1 is essential for cell growth, migration, and death. This is achieved by inhibiting the induction of phosphorylated myosin light chain (p-MLC) and myosin light chain kinase (MLCK) by the potent antioxidant flavonoid quercetin (Kim et al., 2017).
- Through the LKB1/AMPK signalling pathway, 100 mM testosterone increased GLUT4-dependent the absorption of glucose in 3T3-L1 adipocytes (Mitsubishi et al., 2015).
- Estradiol (E2) is a crucial sex hormone that guards against aging in women, especially in the case of cardiovascular illnesses (D'Eon et al., 2008).
- E2 interacts with estrogenic receptors (ERs) through the metabolite 2-hydroxy estradiol (2-HE2) in C2C12 myotubes to activate AMPK (D'Eon et al., 2008).
- According to a different study, E2 and its mimic nordihydroguaiaretic (NDGA) increased the expression of ER, AMPK, and LKB1, which prevented angiotensin II-induced VSMC proliferation (S. Y. Kim et al., 2016).

At organism level

- Protein Kinase B2 (*Akt2*) and AMPK were both knocked down twice, which led to an increase in cardiac aging but not a reduction in longevity. This shows that as we age, AMPK may provide protection against faulty autophagy and mitophagy (Wang et al., 2019).
- Through the AMPK route, strawberries greatly increased the anti-oxidant activity, mitochondrial plant matter, and activity of aged rats. (Giampieri et al., 2017).
- In the brain and kidneys of mice lacking the growth hormone receptor gene, males expressed AMPK, PGC1- and SIRT1 at higher levels than females, highlighting the importance of these proteins in mitochondrial biogenesis.
- By boosting blood fibroblast growth factor 21 (FGF-21) levels to enhance the generation of mitochondrial enzymes and further raise AMP/ATP levels, which in turn activate AMPK, a methionine-restricted diet might restore a youthful metabolic phenotype in adult mice (Zawada et al., 2015).
- In older mice, β -adrenergic decreased AMPK activation and increased β -arrestin 1, which accelerated myocardial fibrosis (J. Wang et al., 2016).

- Additionally, hippocampus neurogenesis declines with aging and has been linked to cognitive impairment through AMPK signaling (B. Wang et al., 2019).

Together, these findings imply that the AMPK pathway is linked to age-related changes. The question of whether AMPK activity changes with age, however, is still a contentious one. While some studies imply that AMPK activity increases with aging, other evidence suggests that it decreases.

2.2.3. Sirtuin Signaling

Sirtuin is an enzyme that belongs to the class of NADs (nicotinamide adenine dinucleotide) and is thought to be a viable target for the treatment of human disorders because it is involved in many cellular pathways. Mammals have SIRT1 through SIRT7 sirtuins. Sirtuin is a crucial element that postpones cell senescence and lengthens the life expectancy of biological entities by regulating a number of cell activities. By preventing age-related telomere attrition, preserving genomic integrity, and supporting DNA damage repair, sirtuin essentially prevents cellular senescence. A few flagging mechanisms that regulate life expectancy, including as the insulin/IGF-1 flagging route, AMP-enacted protein kinase, and forkhead box O, are also influenced by Sirtuin, which affects how long an organism survives. Despite being debatable it is believed that Sirtuin's ability to prolong life depends on its expression level and tissue type. Researchers have focused on Sirtuin activators to create treatments for age-related disorders because Sirtuin is thought to also mediate the longevity impact of calorie restriction. Numerous studies have been conducted to determine whether the phytochemical resveratrol, which is prevalent in the outermost layer of red grapes and wine, might boost sirtuin activity and halt the aging process (Lee et al., 2019).

The two main ways that Sirtuin promotes DNA damage repair and prevents telomere attrition are how it suppresses cellular senescence. Sirtuins are essential for preserving the integrity of the genome because they help to keep chromatin in its normal state of condensation and respond to and repair DNA damage. SIRT1, SIRT6, and SIRT7 function as regulators of transcription to inhibit high-quality articulation by controlling the chromatin structure (Toiber et al., 2011). SIRT1 deacetylates more than 50 non-histone proteins, notably DNMT1, transcription variables, and DNA-repairing proteins. The SIRT1 enzyme also deacetylates histones H3, H4, and H1. Like human Sirtuins, *Drosophila* dSir2 participates in the epigenetic transfer of quiet chromatin configurations. Mutations in dSir2 have been shown to reduce the

position effect variegation, a heterochromatin-mediated silencing phenomenon. Telomere reverse transcriptase is necessary for telomere lengthening and maintaining the integrity of the telomeric nucleus, and it is known that SIRT1 and SIRT6 control how much of it is produced. Additionally, they deacetylate H3K9, H3K56, and histone 3 lysine 9. Additionally, it has been demonstrated that SIRT1 and SIRT6 are drawn to the sites of damaged DNA and aid in DNA repair by deacetylating repair proteins like Werner (WRN) helicase, Ku70, NBS, and poly (ADP-ribose) polymerase (PARP)-1. SIRT4 controls the mitochondrial glutamine metabolism, contributing to DNA damage (Jeong et al., 2013).

2.2.4. MTOR Signaling

The serine/threonine kinase known as mammalian target of rapamycin (mTOR) is a member of the kinase family connected to phosphoinositide 3-kinase (PI3K). In addition to serving as an intracellular energy sensor, mTOR plays a crucial role in controlling a variety of activities, including growth, proliferation, metabolism, aging, and others. The mTOR gene is composed of two distinct protein complexes known as mTOR complex 1 (mTORC1) and 2 (mTORC2) (Albert & Hall, 2015). The five components that make up mTORC1 are the mTORC1 administrative related protein (Raptor), the DEP-space containing mTOR-connecting protein (DEPTOR), the mammalian deadly with *Sec13* protein 8, and the proline-rich *Akt* substrate 40 kDa (PRAS40). DEPTOR, mLST8, mSIN1, rapamycin-insensitive mTOR companion (Rictor), mammalian stress-activated protein (mSIN1), and protein associated with Rictor-1 (Protor-1) are the six components that make up mTORC2. Both mTORC1 and mTORC2 act adversely to control DEPTOR. Activating ribosomal protein S6 kinase (S6K), *Akt*, and SGK1 in response to DEPTOR inhibition will promote cell growth and proliferation (Peterson et al., 2009).

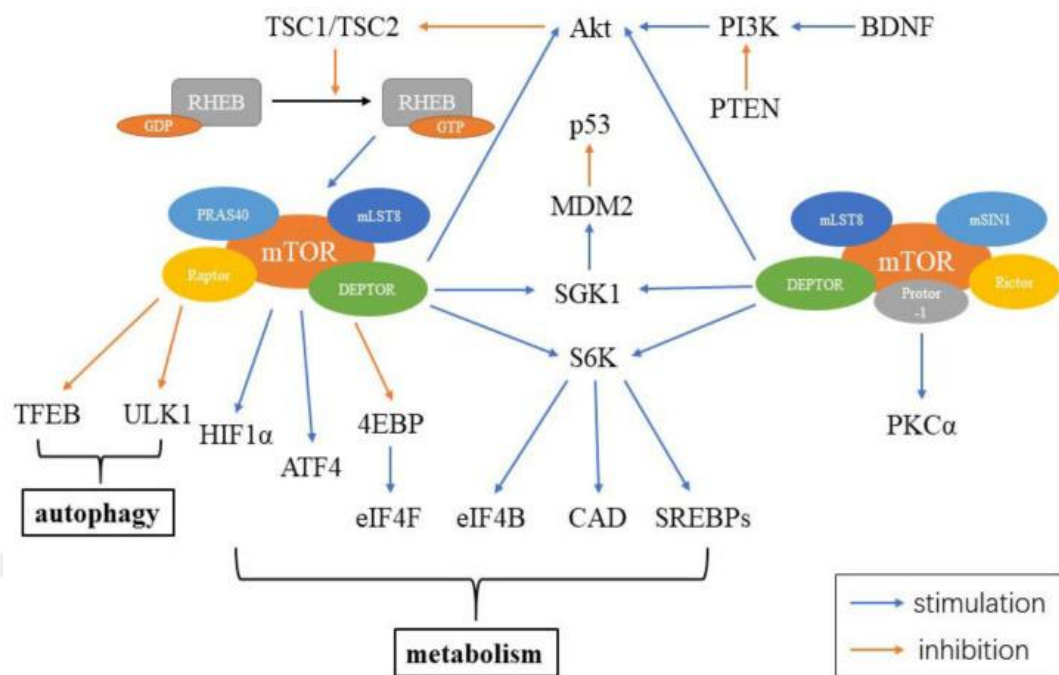


Figure 2.2: Constituents of mTORC and mTOR signaling in the cell. (Yu et al., 2021).

From worms to warm-blooded vertebrates, it has been demonstrated that there is a link between mTOR flagging and developing, nevertheless, the fundamental processes are still not well comprehended (Yu et al., 2021). The Ras-related GTP-binding protein (Rag) and the Tuberous Sclerosis Complex (TSC)-Rheb pathway can both regulate mTORC1 which controls amino acid signalling. According to several studies, mTORC1 controls a wide range of cellular functions, including protein translation, autophagy, and cell development. Environmental factors including oxygen, amino acids, glucose, and growth affect how sensitive it is (Widlund et al., 2013).

However, numerous kinases must be activated for mTORC2 to function as an effector of the insulin/insulin-like growth factor-1 (IGF-1) pathway (IIS) downstream of PI3K. For instance, Akt/PKB, a crucial regulator of cell survival, is phosphorylated and activated by mTORC2. Given that mTOR has negative effects on aging, it seems reasonable that as people age, the activity of mTOR will rise. However, as people aged, mTOR activity as well as BDNF/PI3K/Akt signaling, which is upstream of mTOR, reduced. Inhibition of mTORC2 results in immunosuppression, decreased activity level, glucose intolerance, and diabetes. As

a result, mTORC2 inhibition impairs health and lifespan. While mTORC1 suppression slows aging and lengthens life. mTOR activity and cognitive performance have an inverted U-shaped dose-effect curve, although chronic mTOR inhibition diminishes the effects of aging on spatial learning and memory (Chellappa et al., 2019).

Aging may have intricate systems and be an irreversible process. Therefore, rather than rejuvenation, the actual aim of antiaging may be to promote good aging, which means reducing physiological decline and dysfunction with age. Specific signalling pathways, such as the AMPK, SIRT1, and mTOR pathways, are important regulators of aging. They have the power to slow down aging either individually or collectively. More particular, metformin, resveratrol, and exercise activate AMPK whereas AMPK inhibits autophagy. Exercise, NAD⁺, CR, and resveratrol all raise SIRT1 expression. Exercise, CR, resveratrol, and rapamycin disrupt mTOR and affect autophagy. Additionally, SIRT1 and mTOR can activate SIRT1, AMPK, and SIRT1 can activate mTOR, and SIRT1 and mTOR can inhibit mTOR. The identification of these aging signalling pathways opens up new opportunities to develop effective strategies for healthy aging by enhancing longevity pathways or inhibiting aging pathways (Yu et al., 2021).

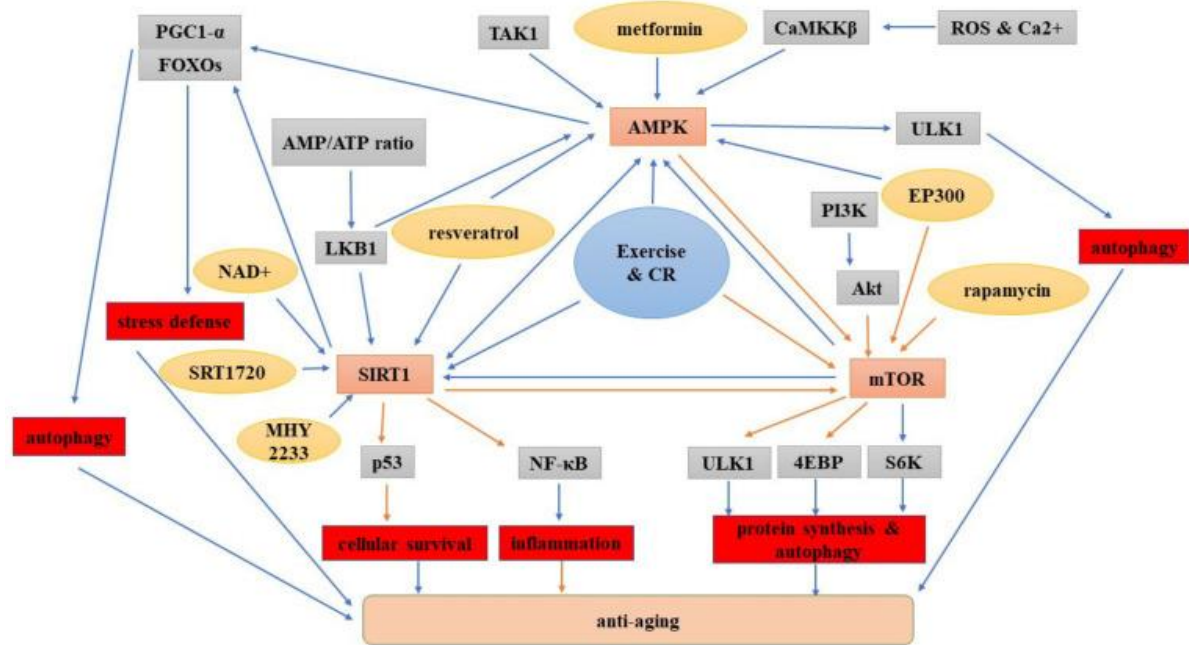


Figure 2.3: Illustration of the roles of the AMPK, SIRT1 and mTOR signaling pathways in aging. The blue arrows indicate a positive effect, and the orange arrows indicate a negative effect. (Yu et al., 2021).

2.3. METFORMIN

The main first-line therapy for type 2 diabetes, especially in obese people, is metformin, which is marketed under the brand names Glucophage and others. It is also used in the management of polycystic ovarian syndrome. It is used orally and has no adverse effects on weight. The biguanide family of medications includes metformin. You can manage your blood sugar, or glucose, with metformin. Both the quantity of glucose your liver generates and the amount you take in from meals are reduced. Metformin also improves the way the body reacts to insulin, a hormone that normally controls blood sugar levels. Diabetes type 1 is not managed with metformin (Metformin: MedlinePlus Drug Information, 2023.).

Metformin has antiaging properties and can be used to stop aging-related diseases by interfering with nutrition sensing, DNA damage, ROS accumulation, autophagy, telomere attrition, inflammation, cellular senescence, and stem cell depletion. Over time, metformin has been found to have positive pleiotropic impacts on human health. Medication containing metformin has the ability to treat hyperglycemia and may also lower the risk of a wide range

of other aging-related disorders, including as cancer, cardiovascular disease, and neurodegeneration (Soukas et al., 2019).

2.4. METFORMIN AS A DRUG FOR DIABETES AND THE PATHWAYS IT FOLLOWS

Metformin decreases ambient and postprandial plasma glucose (PPG) since it is a biguanide. It can be taken by itself or in combination with insulin, thiazolidinediones, DPP-4 inhibitors, sulfonylureas, and GLP-1 agonists, which are all used to treat diabetes. Metformin acts by improving intestinal glucose absorption and utilisation while reducing hepatic glucose production. Metformin not only lowers blood sugar levels but may also help with weight reduction, lower plasma lipid levels, and avoid certain vascular issues. The usage of metformin is rising along with the prevalence of obesity in the United States. Metformin is frequently prescribed for conditions including polycystic ovarian syndrome (PCOS), among others. Most people have good tolerance for metformin. However, there is a wide range in the glucose tolerance to metformin. Although some people react quite well, others do not (Gong et al., 2012).

2.4.1. Sirtuin Signaling and Metformin

Metformin is believed to mimic the majority of metabolic responses to calorie restriction by acting as an agonist for SIRT1, a nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylase. Enzyme experiments revealed that metformin and other biguanides, including phenformin, increased SIRT1's catalytic effectiveness when it was operating in vitro with low NAD⁺ levels. (Cuyàs et al., 2018).

2.4.2. Metformin and AMPK Signaling

The FDA has approved metformin, a well-known medication for type 2 diabetes. The primary generic blood glucose-lowering medicine prescribed for the management of type 2 diabetes is metformin, an AMPK activator. In the 17th century, it was first separated from the French lilac, which was a conventional medicinal plant. Research from the National Institutes of Health (NIH) in the early 2000s showed that metformin might improve mice's health and longevity while lowering their chance of acquiring cancer and other age-related illnesses.

Consistently, metformin increased *C. elegans*' youthful mobility, increased health span, and median lifespan.

There are now 16 clinical trials looking into metformin with longevity and aging. The current classification of metformin places it in the benign family of drugs known as calorie restriction mimetic (CRM), which can reduce protein acetylation levels and induce autophagy (Pietrocola et al., 2018). Metformin can either directly or indirectly activate AMPK by lowering the ADP/ATP and AMP/ATP ratios and partly inhibiting Complex I of the mitochondrial electron transport chain (ETC) (Hardie et al., 2012).

By activating AMPK in response to cellular stress, metformin can treat Hutchinson-Gilford progeria syndrome (HGPS) accelerated aging deficiencies (Finley, 2018). Using metformin locally helped the AMPK pathway to improve vascularization, which helped wound healing and cutaneous integrity in older skin (Zhao et al., 2017). By blocking both upstream and downstream Rho-kinase 1 (ROCK1) and TGF-1/Smad3 signalling, metformin additionally stimulates AMPK signalling to decrease the resistance to insulin in obesity, transform the extracellular structure in adipose tissue, and decrease related to age hepatic lipid accumulation. This has been demonstrated to speed up obesity-induced steatosis in mice (Luo et al., 2016).

By increasing ATP, cyclic guanosine monophosphate (cGMP), and the activity of mitochondria through the AMPK and endothelial nitric oxide (NO) pathways, metformin lessens age-related alterations in liver sinusoidal endothelial cells (Hunt et al., 2019). The unfolded protein response (UPR), which is connected to age-related hearing loss, cell death, and neurodegeneration, is also inhibited by metformin in d-galactose-induced aging mice through the AMPK/extracellular signal-regulated kinase 1/2 (ERK1/2) signalling pathways (Cai et al., 2020). The PC12 cells and hippocampus neurons were protected from oxidative damage by metformin at clinically relevant dosages by activating AMPK (X. Zhao et al., 2019). Astrocyte activity was decreased by metformin in both Parkinson's disease (PD) and healthy aging by affecting AMPK and brain-derived neurotrophic factor (BDNF) signalling (Ryu et al., 2020). By activating AMPK signalling, metformin guards against mitochondrial malfunction, oxidative stress, autophagy control, neurodegeneration, and neuroinflammation in addition to preventing the phosphorylation and accumulation of α -synuclein (Paudel et al., 2020).

Despite metformin has various anti-aging effects through AMPK signaling, its use is still limited. For instance, its lactic acidosis side effect is crucial to be aware of even if there is presently no evidence to support these effects in healthy humans and animals.

2.4.3. Pharmacokinetics Pathway

The half-life of metformin is approximately 5 hours, and it is eliminated unaltered in urine. The general population's average renal clearance (CL_r) ranges from 510 to 120 ml/min. Active secretion from tubes in the kidney is the primary mechanism by which metformin is eliminated from the body. The drug is widely transferred into bodily tissues like the liver, kidney, and intestine through organic cation transporters. The range of 54 to 4133 ng/ml for the trough steady-state metformin plasma levels suggests that there is substantial interindividual variation in the metformin pharmacokinetics (Christensen et al., 2011).

Metformin absorption through the gut may be significantly influenced by the plasma membrane monoamine transporter (PMAT, which is expressed on the luminal side of enterocytes and is produced by the gene SLC29A4). The pointed border of enterocytes may express the gene OCT3 (SLC22A3), which may enhance metformin absorption. Additionally, the SLC22A1 characteristic known as OCT1 may allow metformin to pass through the interstitial fluid more easily in the cytoplasm and basolateral layer of the enterocytes (Graham et al., 2011).

OCT1 (SLC22A1) and potentially OCT3 (SLC22A3) play a major role in the hepatic absorption of metformin. On the basolateral membrane of hepatocytes, both transporters are expressed. OCT1 is crucial for metformin absorption in the liver, as evidenced by the fact that the concentration of metformin in the liver was significantly lower in Oct1-deficient animals than in control mice. In the Oct1-deficient rats, the metformin's glucose-lowering benefits were likewise completely reversed. MATE1 (human multidrug and toxin extrusion 1) and MATE2-K (gene SLC47A2) are also effective metformin substrates. Metformin is excreted from the kidney and liver via the MATE1 (SLC47A1) pathway, which is often expressed in skeletal muscle, the liver, and the kidney. However, because metformin's biliary excretion seems to be insufficient in people, MATE1's role in hepatic release has been called into doubt (Tsuda et al., 2009). Data from a Mate1 knockout mouse research show that rodents at least excrete metformin in their bile (Ito et al., 2010).

Metformin is retained in renal epithelial cells from the blood via the property OCT2 (also known as SLC22A2), which is mostly communicated at the basolateral film in the renal tubules. The kidney's elimination of metformin from the tubular cell to the stream is mediated by MATE1 (SLC47A1) and MATE2-K (SLC47A2). Metformin excretion in healthy people has been demonstrated to include MATE1 and MATE2-K. They are expressed by the renal tubule proximal cells' apical membrane. Both the apical and subapical subdomain sides of the proximal and distal renal tubules appear to express OCT1, suggesting that it is necessary for metformin reabsorption in those tubules (Kusuhara et al., 2011). The expression of PMAT (gene SLC29A4) on the apical membrane of kidney epithelial cells may help the kidneys absorb metformin. However, there is no evidence to support this involvement in vivo at this time. In addition, the genes P-gp (gene ABCB1) and BCRP (gene ABCG2) facilitate the efflux of metformin across placental apical membranes (Hemauer et al., 2010).

Drug interactions brought on by the blocking of metformin transporters (OCTs and MATEs) are clinically important since metformin is not metabolised in the liver. Mutations in these transporter genes may also directly affect the pharmacokinetics of metformin and the diversity of medication responses (see the Pharmacogenomics section). According to current studies on drug-drug interactions, metformin is not absorbed in vitro when proton-pump inhibitors block OCT1, OCT2, and OCT3. The oral diabetes medications repaglinide and rosiglitazone also impeded OCT1-mediated in vitro metformin transport (Nies et al., 2011). The H2 blocker cimetidine is linked to decreased renal cylindrical discharge and increased fundamental receptivity to metformin when the two prescriptions are taken at the same time. The most plausible reason for pharmaceutical associations with cimetidine in renal function is the blocking of MATEs, but not OCT2. According to a recent study, metformin and several tyrosine kinase inhibitors (such as imatinib, nilotinib, gefitinib, and erlotinib) may interact with transporters, which might have an impact on the drug's distribution, potency, and side effects when used in clinical settings (Minematsu & Giacomini, 2011).

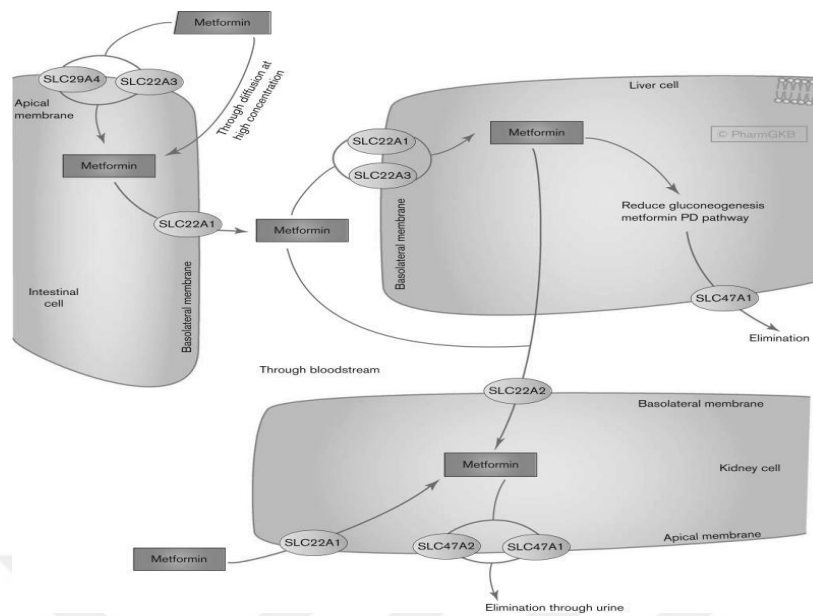


Figure 2.4: Pharmacokinetics pathway of metformin. Stylized cells depicting genes involved in the transport and clearance of metformin. (Gong et al., 2012).

2.4.4. Pharmacodynamics Pathway

Metformin lowers basal and PPG levels. Reduced gluconeogenesis essentially chokes down excessive hepatic glucose union. In addition to these effects, metformin may also improve insulin signalling, reduce the production of fatty acids and triglycerides, and promote the oxidation of -fatty acids. While reducing food intake and intestinal glucose absorption, metformin may also boost the utilisation of glucose in peripheral organs. Metformin does not promote endogenous insulin emission, therefore the subsequent negative effects of other anti-diabetic medications, such as hypoglycemia and hyperinsulinemia, are not desired (Gong et al., 2012). The liver's AMP-activated protein kinase (AMPK) is activated and phosphorylated when metformin is administered. As a result, among other pharmaceutical adverse effects, the production of fat and glucose may be inhibited. It has been demonstrated that the subatomic elements LKB1/STK11 and ATM contribute to the phosphorylation of AMPK when metformin is present. However, metformin does not directly target ATM, LKB1, or AMPK. Metformin's ability to reduce hepatic glucose production has recently been demonstrated in a study utilizing mouse with liver-specific AMPK knockouts. This finding suggests that metformin may suppress hepatic gluconeogenesis in a way that is both LKB1- and AMPK-independent (Foretz et al., 2010).

In a separate study, metformin activated AMPK and reduced gluconeogenesis in mice lacking the gene for Oct-1 (Shu et al., 2007). According to a different study, metformin reduces hepatic gluconeogenesis by regulating SHP in an AMPK-dependent manner. So, a decrease in gluconeogenesis could happen both ways, AMPK-dependent and AMPK-independent. Even though the exact goal is not well understood, metformin specifically impairs complex I of the mitochondrial respiratory chain. It is possible that this inhibition may result in an increase in cellular AMP, which will activate AMPK: ATP to a ratio (Shackelford & Shaw, 2009). AMPK is a crucial cell regulator of the breakdown of lipids and glucose. HMG-CoA reductase, MTOR (target of rapamycin), ACC-2, ACC, glycerol-3-phosphate acyltransferase, and carbohydrate response element-binding protein are all proteins that are phosphorylated and rendered inactive by activated AMPK. Metformin's activation of AMPK also inhibits the production of SREBP-1, a crucial lipogenic transcription factor that is encoded by the gene SREBF1. Phosphorylated AMPK increases the nucleus production of Pgc-1a, which is encoded by the gene PPARGC1A, and later promotes mitochondrial biogenesis. SiRT1 is also activated by phosphorylated AMPK. Metformin interferes with the coactivation of PXR and SRC1, which lowers the declaration of the CYP3A4 quality (Krausova et al., 2011).

The SLC2A4 gene, which codes for the GLUT4 translocation, is rendered more active by an active AMPK, which in turn facilitates glucose absorption by skeletal muscle. AMPK activation in the liver enhances fatty acid oxidation while limiting the synthesis of cholesterol and triglycerides as part of its general pharmacological activity. Peripheral effects include a rise in insulin sensitivity as well as a speeding up of skeletal muscle glucose and fatty acid oxidation (Shackelford & Shaw, 2009).

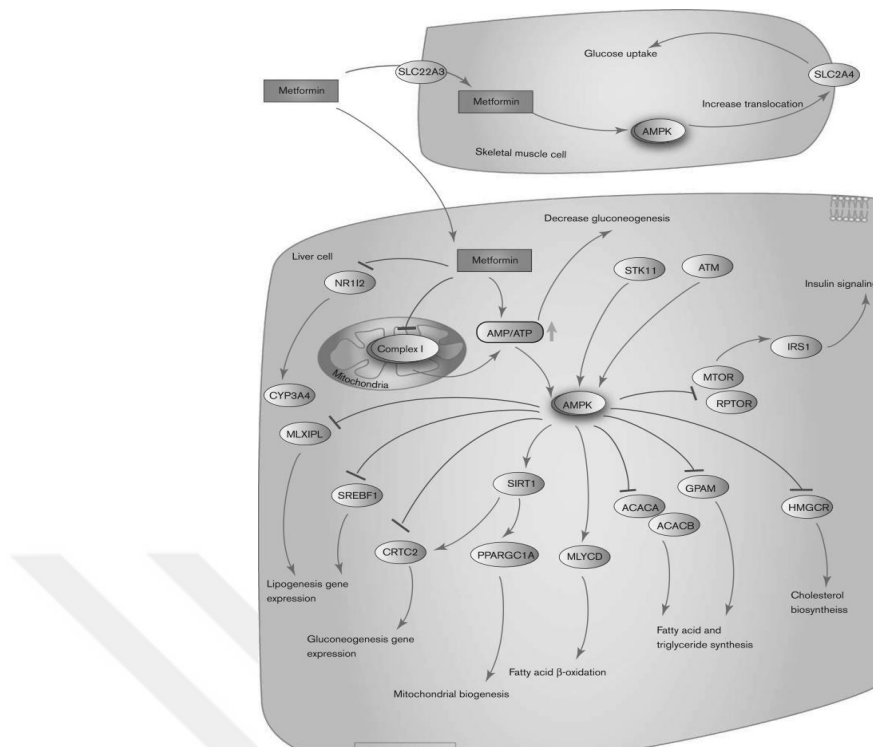


Figure 2.5: Pharmacodynamics pathway of metformin. Stylized cells depicting the mechanism of action of metformin. (Gong et al., 2012).

2.5. ANTIAGING PROPERTIES OF METFORMIN ACROSS DIFFERENT MODEL ORGANISMS THEN FOCUSING DOWN TO *S.pombe*

2.5.1. Different Model Organisms

Metformin is gaining popularity among non-diabetics as a way to extend life and delay the onset of age-related disorders. Numerous model species have been used in studies on the impact of metformin on lifespan and aging, with various degrees of success. After receiving metformin, healthy *Caenorhabditis elegans* worms and *Mus musculus* mice were tested in a lab to see how long they survived. In separate meta-analyses, the lifespan outcomes for mice and nematodes were examined, and there was a large level of variation within experiments within each species (Parish & Swindell, 2022).

Despite the fact that experiments are often conducted under well controlled laboratory circumstances, the impact of an anti-aging drug on model animal longevity might vary significantly between experiments. For instance, there is no doubt that strain influences the effects of food restriction in mice, with cross-breed mice displaying more potent and

beneficial effects than inbred animals and, for the most part, less beneficial effects in wild-determined mice (Swindell, 2016).

The research was conducted where the *Caenorhabditis elegans* (*C. elegans*) nematode and the laboratory mouse *Mus musculus* were used to investigate the effect of metformin on lifespan. The researchers conducted a comprehensive review and meta-analysis of published survival data from controlled experiments in which metformin was administered to laboratory mice or *C. elegans*. To assess overall effect sizes, evaluate whether there was significant heterogeneity among experiments, and uncover experimental characteristics related to such heterogeneity, the researchers attempted to comprehensively integrate investigations (Swindell, 2016).

The results discovered that neither mice nor nematodes' total lifespans were appreciably extended by metformin. In contrast, when fed live OP50 *Escherichia coli* in studies, nematodes lived longer; this effect was more real when metformin was given prior to throughout daily life. Our research emphasises the value of drug testing in a variety of model animals. Investigation of metformin's effects on development in both young and elderly partners across all species, including humans, is also extremely likely to be predicted (Parish & Swindell, 2022).

2.5.2. Fission Yeast - *Schizosaccharomyces pombe*

According to a study, metformin considerably increases the chronological lifespan (CLS) of the model organism *S. pombe* by imitating mechanisms in mammalian cells and other cells. In addition to increasing ATP production and carbohydrate consumption, metformin also decreased the generation of reactive oxygen species and improved signs of oxidative damage such lipid peroxidation and carbonylated proteins. The effect of metformin on extending lifespan was examined to see if it changed with the timing of its addition to the medium. The effect was discovered to be negatively associated with the amount of glucose present in the medium, and it did not prolong the life of the organism when glucose was supplied after the media had entirely run out of glucose. On the other hand, metformin-treated cells that were injected into a medium devoid of glucose likewise exhibited an extension in lifetime. This implies that mechanisms other than those that depend only on the presence of glucose may contribute to the extension of a cell's life span. These findings imply that metformin extends lifespan, particularly by influencing energy metabolism and stress tolerance, and that fission

yeast can be successfully employed to study metformin's antiaging processes (Şeylan & Tarhan, 2023).

Metformin is crucial for addressing aging mechanisms, according to research on a variety of model species and human cell lines. In addition to inhibiting the mTOR pathway (Pérez-Revuelta et al., 2014), activating AMPK signaling (Cho et al., 2015), and inhibiting mitochondrial complex 1 in the electron transport chain (Bridges et al., 2014), metformin also lowers insulin levels, IGF-1 signaling (Liu et al., 2011), and the formation of reactive oxygen species (ROS) (Bridges et al., 2014), as well as reducing DNA damage (Algire et al., 2012). Inflammation (Saisho, 2015), autophagy (Song et al., 2015), and cellular senescence (Jadhav et al., 2013) are among the metabolic and cellular aging processes that metformin favorably influences. Metformin has a wide range of impacts on the cell, however it is unclear if these effects are brought on by one mechanism connected to aging and sequential biological processes, or if the medication may have simultaneous effects on different targets in numerous pathways (Barzilai et al., 2016). Therefore, it is crucial to clarify how genes and cellular pathways impacted by metformin affect the aging process (Şeylan & Tarhan, 2023).

On the contrary, not all of the research using various model species found that metformin had the same impact on lifespan and health span. According to Novelle et al. (2016), fruit flies do not appear to have evolved with the same preservation of metformin's advantages across nematode longevity. For instance, metformin therapy in *Drosophila melanogaster* significantly increased AMPK activity and reduced lipid storage but did not affect longevity (Slack et al. 2012). The bulk of research looking at the long-term effects of metformin on animals were centred on young animals. Only a few research using older animals have been unable to find metformin-induced lengthening of life or have shown the toxicity of high dosages of the drug. Middle-aged worms display considerable toxicity when exposed to 50 mM metformin, prolonging the lives of young nematodes. Therefore, careful consideration of several factors is necessary when determining the dosage and length of metformin administration (Şeylan & Tarhan, 2023).

The growing popularity of yeasts in aging research has revealed that the processes that regulate longevity in other eukaryotes, including animals, are also conserved in yeasts (Kaeberlein & Kennedy, 2005). For instance, *Saccharomyces cerevisiae*'s replicative (RLS) and chronological longevity (CLS) are both increased by calorie restriction, the most popular

experimental strategy for extending lifespan in many model organisms (Fabrizio et al., 2005). *S. pombe*, a famous model organism in aging studies in recent years, has also been intensively studied for CLS. According to this research, CLS may be involved in cellular functions such as nutrition signaling, mitochondrial activity, ROS generation, stress tolerance, proteasome activity, and autophagy (Şeylan & Tarhan, 2023).

On the other hand, metformin has only been the subject of a small number of investigations in yeasts, and as far as we are aware, no such research has been conducted in *S. pombe*. The goal was to ascertain how metformin affected *S. pombe*'s longevity and a few cellular functions as ATP synthesis, intracellular oxidation, and stress tolerance. The longevity of *S. pombe* is increased by about 50% when 25 mM metformin is used, but the effects varied depending on when the drug was added to the medium (Borklu-Yucel et al., 2015). The effects of metformin on ATP production, intracellular ROS, and carbonylation varied from measurement day to measurement day, but it considerably boosted stress resistance and carbohydrate consumption. These findings may clarify the anti-aging effects of metformin, show that *S. pombe* is a suitable model organism for metformin research, and deepen our understanding of *S. pombe* aging (Avelar-Rivas et al., 2020).

This research provides early findings that advance knowledge of metformin's antiaging effects in *S. pombe*. All things considered, our findings imply that metformin extends *S. pombe*'s lifetime in part through regulating energy and promoting stress tolerance, two processes also shown in other organisms. The findings also suggest that *S. pombe* can be a useful model organism for elucidating the antiaging effects of metformin. Further research on the genes involved in the fundamental metformin-affected pathways can be conducted using mutant *S. pombe* cells, and genomic studies like transcriptome, proteome, and epigenetics analysis can help shed light on the drug's effects on the aging process from a wider perspective (Şeylan & Tarhan, 2023).

In genetic research, *S. pombe* is utilized as a yeast in model organisms. However, little is known about how anti-diabetic medications like metformin affect the gene expression profile of this organism. Metformin is known to promote the AMP-activated protein kinase (AMPK)

pathway, which regulates cellular energy balance by altering gene expression (De Haes et al., 2014).

Metformin's impact on *S. pombe* has been the subject of several investigations. For instance, a 2015 study discovered that metformin increased the longevity of *S. pombe* cells by triggering the AMPK pathway and promoting autophagy, which is the process by which cells degrade and recycle damaged or extraneous components. The research also discovered that metformin changed the expression of genes related to energy metabolism and oxidative stress response (Borklu-Yucel et al., 2015).

Metformin has been seen to decrease *S. pombe* cell growth and cause cell cycle arrest. It has also been discovered that metformin stimulated autophagy and the AMPK pathway (Saini & Yang, 2018).

Collectively, these findings demonstrate that metformin affects *S. pombe* in a number of ways, including by inducing autophagy and activating the AMPK system, changing the expression of genes involved in stress response and energy metabolism, reducing cell growth, and halting the cell cycle. These findings have consequences for how metformin is used in various healthcare settings and provide helpful details about the mechanisms by which the medication works (Borklu-Yucel et al., 2015).

To fully comprehend how metformin affects gene expression in this organism, more study is necessary. Such research may shed light on the mechanisms by which metformin exerts its anti-diabetic effects and may have repercussions for how metformin is applied in other medical settings.

3. MATERIALS AND METHODS

3.1. GROWTH MEDIA

The media used as a basis for the production and storage of *S. pombe* cells within the scope of the study are given in Table 3.1 and 3.2 with their contents.

3.1.1. SD media

Initially for 3% SD media, 300ml of distilled water was poured in a flask and 0.85g of yeast nitrogen base, 2.5g of ammonium sulphate, and 15g of glucose were added and the dissolved by stirring. Then, the total volume was made upto 500ml by adding distilled water. Same process was followed for 5% SD media with 25g of glucose. All media used were sterilized by autoclaving at 121 °C, 1.2 atm pressure, for 15 min.

Table 3.1: SD Media Recipe.

Medium	Contents
3% Synthetic Dextrose (SD) Broth (500 ml)	Yeast Nitrogen Base (YNB) – 0.85 g Ammonium Sulphate – 2.5 g Glucose – 15 g Distilled Water (dH ₂ O) – 500 ml
5% Synthetic Dextrose (SD) Broth (500 ml)	Yeast Nitrogen Base (YNB) – 0.85 g Ammonium Sulphate – 2.5 g Glucose – 25 g Distilled Water (dH ₂ O) – 500 ml

3.1.2. YEA media

YEA media was made by pouring 700ml of distilled water in a flask. Then, 5g of yeast extract, and 30g of glucose were added. These contents were mixed by stirring before adding 20g agar. The total volume was then made upto 1000ml by adding distilled water. The media was autoclaved at 121 °C, 1.2 atm pressure, for 15 min. It was then cooled down to approximately 50 °C and poured into petri dishes before solidifying.

Table 3.2: YEA Media Recipe.

Medium	Contents
Yeast Extracted Solid (YEA) Medium (1000 ml)	Yeast Extract – 5 g Glucose – 30 g Agar – 20 g Distilled Water (dH ₂ O) – 1000 ml

3.2. CONDITIONS FOR CELL GROWTH

Synthetic Dextrose (SD) medium containing 3% glucose and 5% glucose was used to establish suspended cultures of the wild strain *S. pombe*. SD medium was prepared by dissolving all components in 500 ml of distilled water. Yeast Extract Agar (YEA) medium was used for pre-experiment resuscitation of wild breed stocks at +4 °C, colony counting in aging experiments, and storage of cells at +4 °C.

3.3. CELL COUNTING

To count the number of cells, present in the media, the serial dilutions were prepared. Three Eppendorf's were taken and filled with 900 µl of autoclaved distilled water. A healthy streak of yeast was taken from the plate and was submerged into the first Eppendorf. The first Eppendorf was vortexed, then the 100 µl was taken from it and it was mixed into the sec Eppendorf for the 10x dilution. The sec Eppendorf was vortexed and these steps were repeated for 100x dilution. Then from the third Eppendorf (100x diluted), 40 – 50 µl was extracted and poured onto the Marenfield Molassez cell counting slide. The cells present in the 5 squares diagonally were counted 5 times under the microscope. The average was calculated by adding all the total numbers and then dividing them by 5 for the average number of the cells. The number was then multiplied by 10^5 and multiply dilution factor 10^2 .

3.4. INOCULATION

For the inoculation 4 flasks were taken and 25 ml of media was added into each flask. We started with 5×10^4 cells to get 0.5 million cells. The flasks were then placed on the orbital shaker at 180 rpm at 30 – 32 °C. Later, the cells were extracted at the mid-log phase and the RNA extraction was initiated.

Note: In our case, we use 3% SD media (3%N), 3% SD media with metformin (3%M), 5% SD media (5%N), and 5% SD media with metformin (5%M). We also use control for both 3% and 5%.

3.5. METFORMIN

To examine the effect of metformin on lifespan and aging-related processes in *S. pombe* throughout the study, cells were grown in media with and without metformin (control). The stock metformin solution to be added to the medium was prepared by dissolving 0.664 g metformin1,1 Dimethylbiguanide hydrochloride 97% (Sigma-Aldrich) in 4 ml dH₂O with a final concentration of 1 M in a falcon tube. The stock solution was sterilized using a filter (Millipore, Millex-GS, pore opening: 0.22 µm) and stored at + 4°C. As a result of the calculation made after the cell count, *S. pombe* cells were seeded in 25 ml (625 µl of metformin solution was utilised) of 3% SD broth with and without metformin (control) at 5×10^4 cells per milliliter. Suspended cultures were allowed to grow in a shaking incubator at 180 rpm at 30°C.

3.6. DETERMINATION OF MID-LOG PHASE AND OPTIMIZATION

Once the cells were inoculated and were on the shaker incubator, their OD after intervals was measured. The cells typically showed change in OD / noticeable growth after 24 hours. So, after 24 hours, the OD was measured every 2 hours. This was done by taking 200 µl of culture from the flasks in a 96 well plate. Then the Geu 5 software was used to measure the OD. Then the graph was plotted and mid-log phase was spotted. The experiment was repeated and RNA extraction was started at the mid-log phase.

3.7. TOTAL RNA EXTRACTION

For the RNA extraction, Hibrigen General RNA Isolation kit (MG-RNA-01-50, Turkey) was used. Guanidium isothiocyanate, a chaotropic salt that shields RNA from endogenous RNases, is used to digest and homogenise samples. The material was given an ethanol addition following homogenization. Samples were then transferred into the filtered tubes to which RNA could have been attached. Impurities were effectively removed by washing. The pure RNA was then collected with DEPC-treated water and was ready for the use for different application. The protocol for the RNA extraction is as follows. The cell suspension of the

yeast cells was poured into the centrifugal tube and was centrifuged at 7000 rpm for 10 min to form a pellet. After ten min, the tube was taken out and supernatant was discarded. Then 1 ml HI tampon was added. This was vortexed until the cells and liquid reached the homogeneity (it was approximately from 30 sec to 1 min). The half of the Eppendorf was loaded with glass beads and the remaining half was loaded with the sample solution. The Eppendorf was then vortexed for 5 – 6 min in 30 sec sections (30 sec vortexed and 30 sec ice incubation to protect the RNA quality) or the Sartorius Mikro-Dismembrator S could also be used at 3000 – 4000 rpm. An injection needle was heated to puncture the Eppendorf on the bottom to transfer the supernatant into the new Eppendorf by short spins in the centrifuge. In order to get more pure RNA, the Eppendorf was centrifuged at 4°C, 12000 RPM for 5 min and the supernatant was transferred to a new Eppendorf. Then 200 µl of chloroform was added and then it was vortexed for 15 sec to achieve homogeneity and was then incubated in ice for 10 min. It was then centrifuged at 4°C, 12000 rpm for 15 min. After this, two layers were achieved. The top layer was colourless and contained RNA, and the bottom layer contained phenol chloroform waste. The top layer of supernatant was transferred to the maximum into a new Eppendorf tube. Then, into this half (of the total amount of supernatant) EtOH (100% ethanol) was added. It was made sure that this ethanol was from the freezer (already cold) and vortexed for homogeneity. The solution was then transferred into a filter tube and was centrifuged at 4°C, 12000 RPM for 30 sec. The accumulated liquid at the bottom was thrown. Then 500 µl of RY1 solution was added in the filter and was centrifuged at 4°C, 12000 RPM for 30 sec. Then 500 µl of RY2 was added in the filter and left for 1 min at room temperature, and was then centrifuged at 4°C, 12000 RPM for 30 sec. This step was then repeated. Then to dry the filter, it was centrifuged at 12000 RPM for 2 min. Then 30 – 100 µl DEPC was added in the filter, and was incubated at room temperature for 2 min. The filter was then placed in a new Eppendorf and centrifuged at 12000 RPM for 2 min. The RNA tube was then stored at -80°C.

3.8. RNA SEQUENCING

3.8.1. Experimental Methods and Workflow

The total RNA from Sample of interest (Cells) was extracted. The DNA contamination was eliminated using DNase. Depending on the kinds of RNA, the proper kit was selected for the library preparation process. An mRNA cleaning kit was utilized for mRNA with a poly-A tail, and a ribo-zero RNA elimination kit was used to filter non-coding RNAs like lincRNA. The

quality check to ensure integrity on raw sequences was done by using FastQC v0.11.7 program. In order to verify the integrity of files, md5sum is used. If the values of md5sum are the same, there is no forgery, modification or omission. The purified RNA was then randomly fragmented for short read decoding using Trimmomatic 0.38 program. Then, cDNA was produced from fragmented RNA using HISAT2 version 2.1.0 which is known to handle spliced read mapping through Bowtie2 aligner. The cDNA segments have ligate adapters attached to both ends. Following PCR amplification, fragments with insertion lengths between 200 and 400 bp were chosen. In Illumina-specific paired-end sequencing, the length of the read was used to sequence both ends of the cDNA.

3.8.2. Analysis Methods and Workflow

The sequenced raw reads' quality control was examined. The total bases, total reads, GC (%), and fundamental statistics were all computed. Artefacts such low-quality reads, adaptor sequences, contaminated DNA, or PCR duplicates were eliminated in order to lessen biases in analysis. With the aid of the splice-aware aligner HISAT2, the trimmed reads have been assigned to the reference genome. Aligned readings were used by StringTie to build the transcript. Based on transcript length and depth of coverage, read count and normalisation values were used to describe the expression profiles. FPKM (Fragments Per Kilobase of Transcript per Million Mapped Reads), RPKM (Reads Per Kilobase of Transcript per Million Mapped Reads), and TPM (Transcripts Per Kilobase Million) were used to produce standardisation values. In our study, we used TPM to compare the change in gene expression across cells grown in different media and metformin concentration.

3.9. 50X TAE BUFFER (100 ml)

Distilled water was used to dissolve 24.2 g of tris base. Then, 10.7 ml of 0.5 M isoanyl alcohol buffer and 5.7 ml of glacial acetic acid were added. After being vortexed, the samples were centrifuged at 13000 rpm for a period of ten min. After being transferred to a fresh Eppendorf, the supernatant was mixed with cooled isopropanol (0.6*volume of supernatant). After being vortexed, the samples underwent incubation at -20°C for an hour. The specimens were centrifuged for 5 min at a speed of 13000 rpm, and the resulting liquid was eliminated. The particle went through a centrifuge at 13000 rpm for a minute after being rinsed with 200 l of 70% ethanol. The sample was removed from the ethanol and let to air dry until it turned

translucent. In 15–35 l of nuclease-free water, the pellet was reconstituted. On a 0.8% agarose gel that had been produced with 1 x TAE buffer, the DNA's purity was examined.

3.10. GEL ELECTROPHORESIS

Agarose was heated until it was dissolved after being combined with 100 cc of 1 X TAE. 10 l of 1 mg/ml ethidium bromide were added once it had cooled down. The gel was then poured into a caster with the combs, and it was left to solidify.

3.11. NANO-DROP™ 2000/2000C SPECTROPHOTOMETER

Firstly, the software NanoDrop 2000/2000c was opened to which the NanoDrop spectrophotometer was connected. The desired RNA was selected. The hinged arm which was hanging horizontally on the instrument was gently lifted. The small dark spot, also known as the pedestal is where the sample was to be placed. Therefore, the pedestal was gently cleaned and buffed with a lint-free wipe. Then 1 - 2 µl of the blank solution was added. The arm was gently lowered. The measure blank option was selected. The arm was lifted up and cleaned with the lint-free wipe. These steps were repeated after replacing the blanking solution with the sample.

4. RESULTS

4.1. MID-LOG PHASE

The results did not show any significant increase in all media; 3%N, 3%M, 5%N, and 5%M. The OD in all the media was almost equivalent, and not much noticeable increase was observed. The RNA extraction was done after 50 hours in 3%N, 47 hours in 3%M, 49 hours in 5%N, and 51 hours in 5%M.

Table 4.1: Mid-Log Phase Optimisation.

Sample	Hours
3% N	50
3% M	47
5% N	49
5% M	51

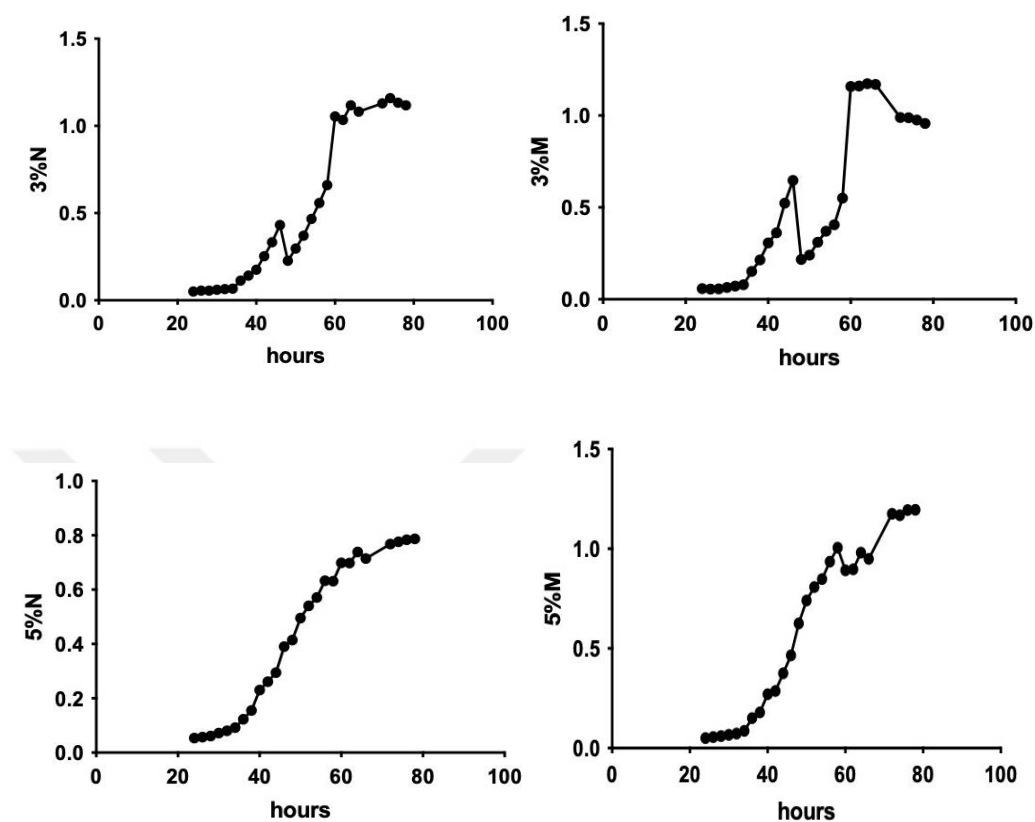


Figure 4.1: The effect of 25 ml metformin on growth curve in *S. pombe* using 3% and 5% SD growth media.

4.2. RNA EXTRACTION

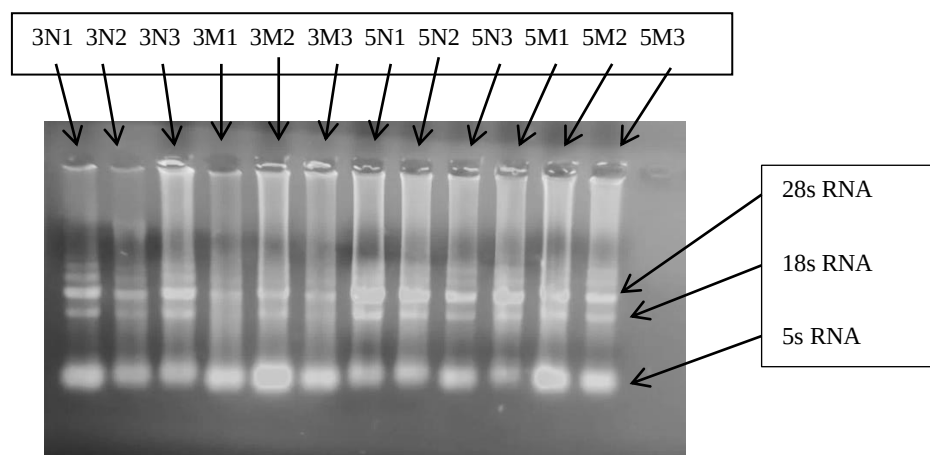


Figure 4.2: RNA extraction Agarose Gel Electrophoresis.

Figure 4.2 shows the bands representing 5s, 18s and 28s RNA after performing gel electrophoresis. The samples labeled are 3 extractions of each 3%N, 3%M, 5%N and 5%M which were then pooled in together for RNA sequencing as shown in the table below. Because of them being pooled together and not sequenced separately, there is no statistical data available.

3N1	3%N
3N2	
3N3	
3M1	3%M
3M2	
3M3	
5N1	5%N
5N2	
5N3	
5M1	5%M
5M2	
5M3	

Table 4.2: Pooling of repeats together for RNA sequencing

4.3. EXPRESSION PROFILE – UPREGULATED AND DOWNREGULATED GENES

The total number of bases, reads, GC (%), Q20 (%), Q30 (%) are calculated for 4 samples. For example, in 3%M, 109,744,636 reads are produced, and total read bases are 11.1Gbp. The GC content (%) is 43.14% and Q30 is 94.26%. Table 4.3 shows raw data stats, Table 4.4 shows trimming data stats and Table 4.5 shows mapped data stats.

Sample id	Total read bases	Total reads	GC (%)	Q20 (%)	Q30 (%)
3%M	11,084,208,236	109,744,636	43.14	97.87	94.26
3%N	13,025,156,344	128,961,944	41.13	98.06	94.41
5%N	12,776,672,104	126,501,704	40.53	98.20	94.67
5%M	9,413,494,314	93,202,914	40.62	97.82	93.93

Table: 4.3: Raw data stats

Sample id	Total read bases	Total reads	GC (%)	Q20 (%)	Q30 (%)
3%M	10,725,666,497	107,041,694	43.14	98.69	95.34
3%N	12,692,307,958	126,567,132	41.11	98.68	95.25
5%N	12,472,568,603	124,355,496	40.54	98.76	95.42
5%M	9,131,478,850	91,082,410	40.62	98.58	94.92

Table 4.4: Trimming data stats

Sample id	No of processed reads	No of mapped reads (%)	No of unmapped reads (%)
3%M	107,041,694	94,473,105 (88.26%)	12,568,589 (11.74%)
3%N	126,567,132	119,396,290 (94.33%)	7,170,842 (5.67%)
5%N	124,355,496	118,735,444	5,620,052

		(95.48%)	(4.52%)
5%M	91,082,410	86,941,815 (95.45%)	4,140,595 (4.55%)

Table 4.5: Mapped data stats

The differentially expressed genes (DEGs) classification is done using PANTHER Up-regulated genes (A) and down-regulated genes (B) are categorised by molecular operation, cellular element, physiological process, peptide class, and panther route. in 5%M / 3%M, 3%M / 5%M, 3%N% / 3%M, and 5%N / 5%M genes.

4.3.1. 5%M / 3%M Comparison

Molecular Function

Both dysregulated gene sets showed genes that were primarily involved in ATP-dependent activity (GO:0140657), molecular adaptor activity (GO:0060090), cytoskeletal motor activity (GO:0003774), catalytic activity (GO:0005488), binding (GO:0005488), and no Panther category is assigned (unclassified). In contrast to down-regulated genes, up-regulated genes had increased catalytic activity, no specific category assignment (unclassified), ATP-dependent activity, molecular adaptor activity, bind, and cytoskeletal motor activity.

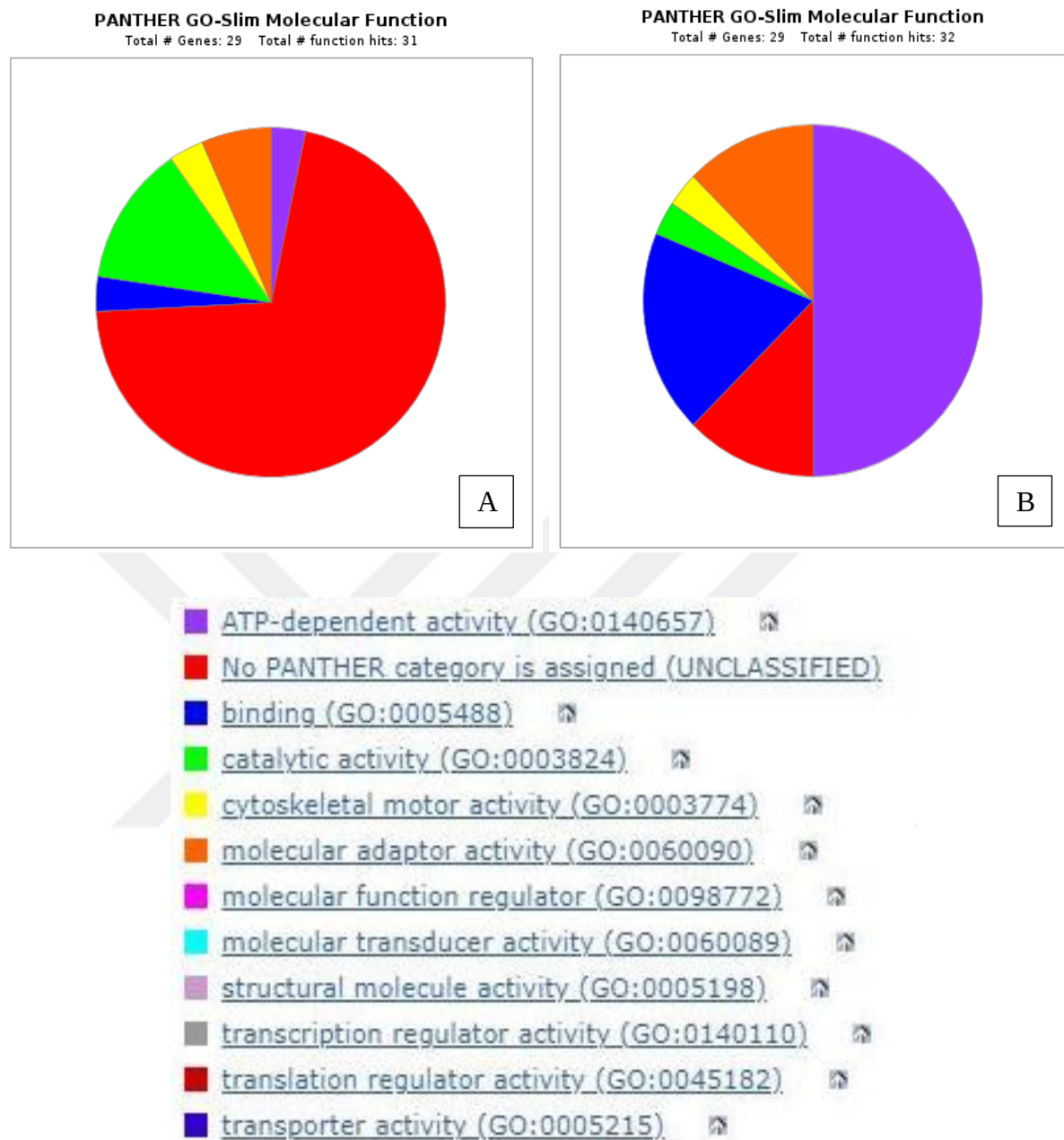


Figure 4.3: Molecular Function of 5%M/3%M upregulated and downregulated genes using Panther.

Biological Process

Biological phase (GO:0044848), biological regulation (GO:0065007), cellular process (GO:0009987), developmental process (GO:0032502), response growth (GO:0040007), and localization (GO:0051179) were the biological processes that both dysregulated groupings of genes were most heavily engaged in and no Panther category is assigned (unclassified). No panther category (unclassified), biological regulation, growth, and localisation is higher in

upregulated genes and biological phase, cellular process, and developmental process is higher in down-regulated genes.

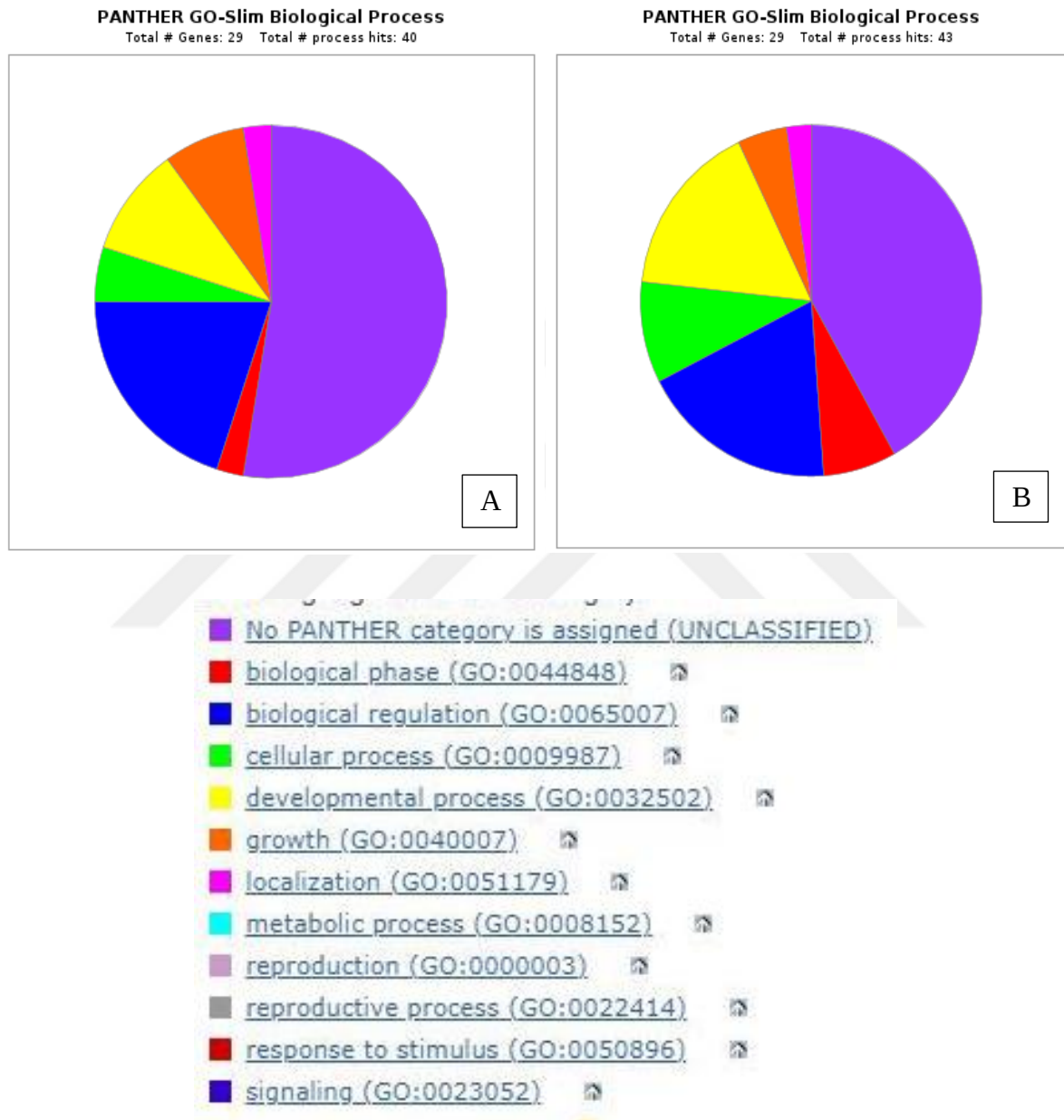


Figure 4.4: Biological Process of 5% M/3% M upregulated and downregulated genes using Panther.

Cellular Component

In terms of cellular component, Genes mostly engaged in cellular anatomical entities (GO:0110165) and protein-containing complexes (GO:0032991) were reported in both dysregulated gene groupings, and no Panther category was assigned (unclassified). The no panther category (unclassified) and protein-containing complex is higher in up-regulated genes, and cellular anatomical entity is higher in down-regulated genes.

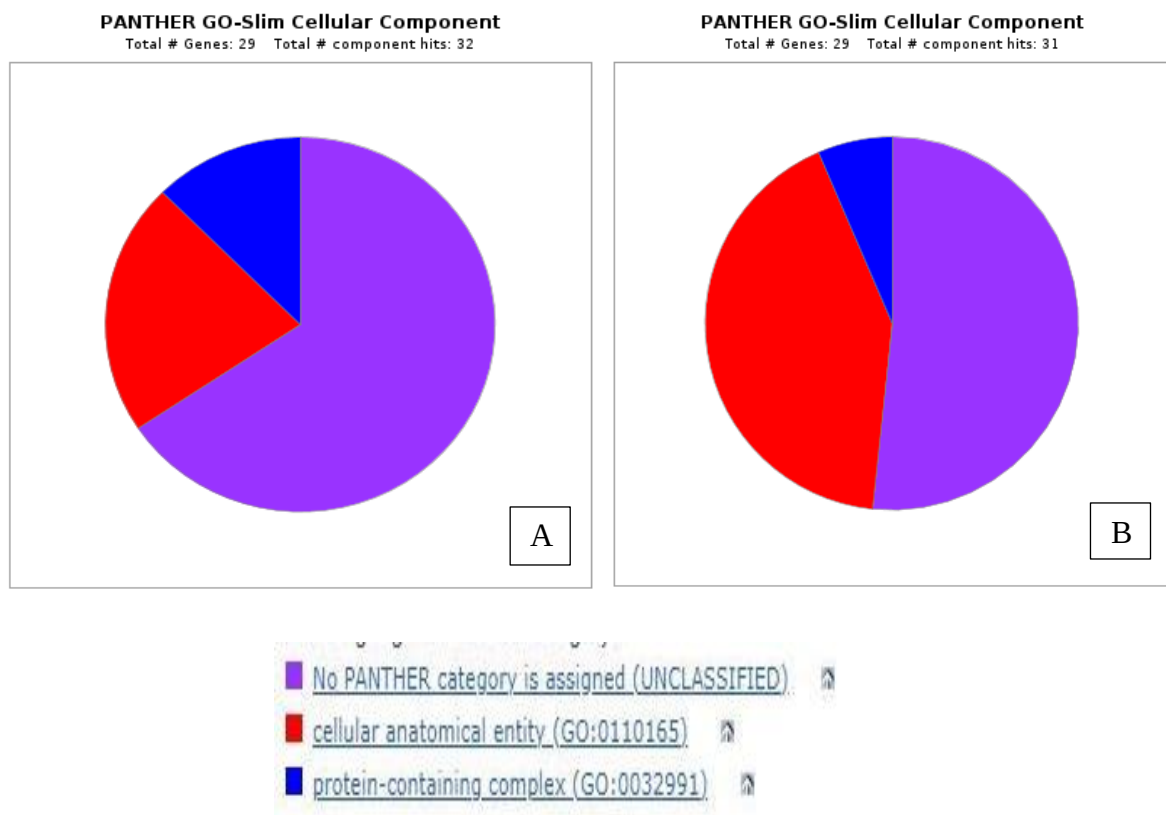


Figure 4.5: Cellular Component of 5%M/3%M upregulated and downregulated genes using Panther.

Protein Class

In terms of protein class, Genes mostly involved in DNA metabolism protein (PC000009), cytoskeletal protein (PC00085), chromatin / chromatin-binding or regulatory protein (PC00077), chaperone (PC00072), and calcium-binding protein (PC00060) were found in both dysregulated sets of genes, RNA metabolism protein (PC00031), and no Panther

category is assigned (unclassified). The no panther category (unclassified) is more in up-regulated genes and all the rest of the proteins are more in the down-regulated genes.

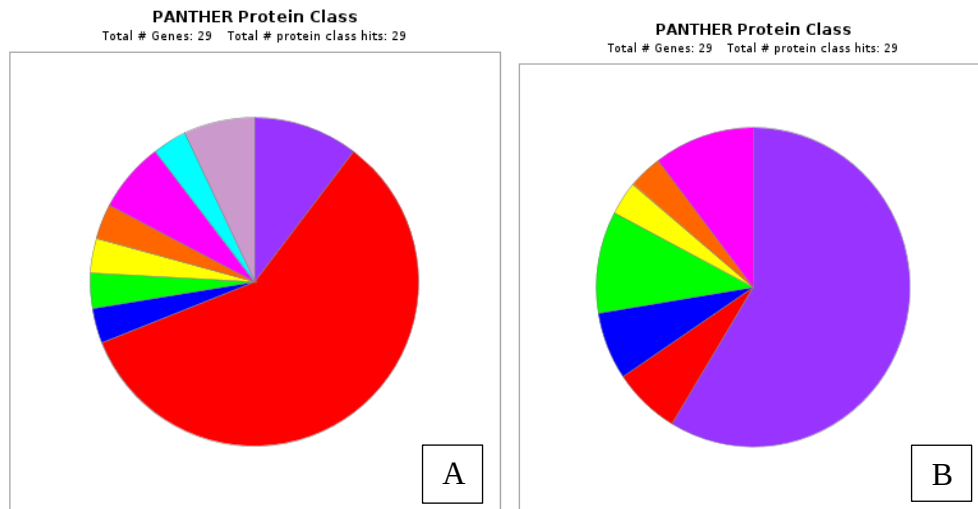




Figure 4.6: Protein Class of 5%M/3%M upregulated and downregulated genes using Panther.

The extracellular matrix protein (PC00102) and gene-specific developmental regulator (PC00264) are also components of the up-regulatory genes.

Panther Pathway

In terms of the panther pathway, the majority of the genes in both dysregulated gene groups were those involved in the breakdown of 5-hydroxytryptamine (P04372). Up-regulated genes contains 100% 5-Hydroxytrptamine degradation whereas downregulated genes consist of 95%.

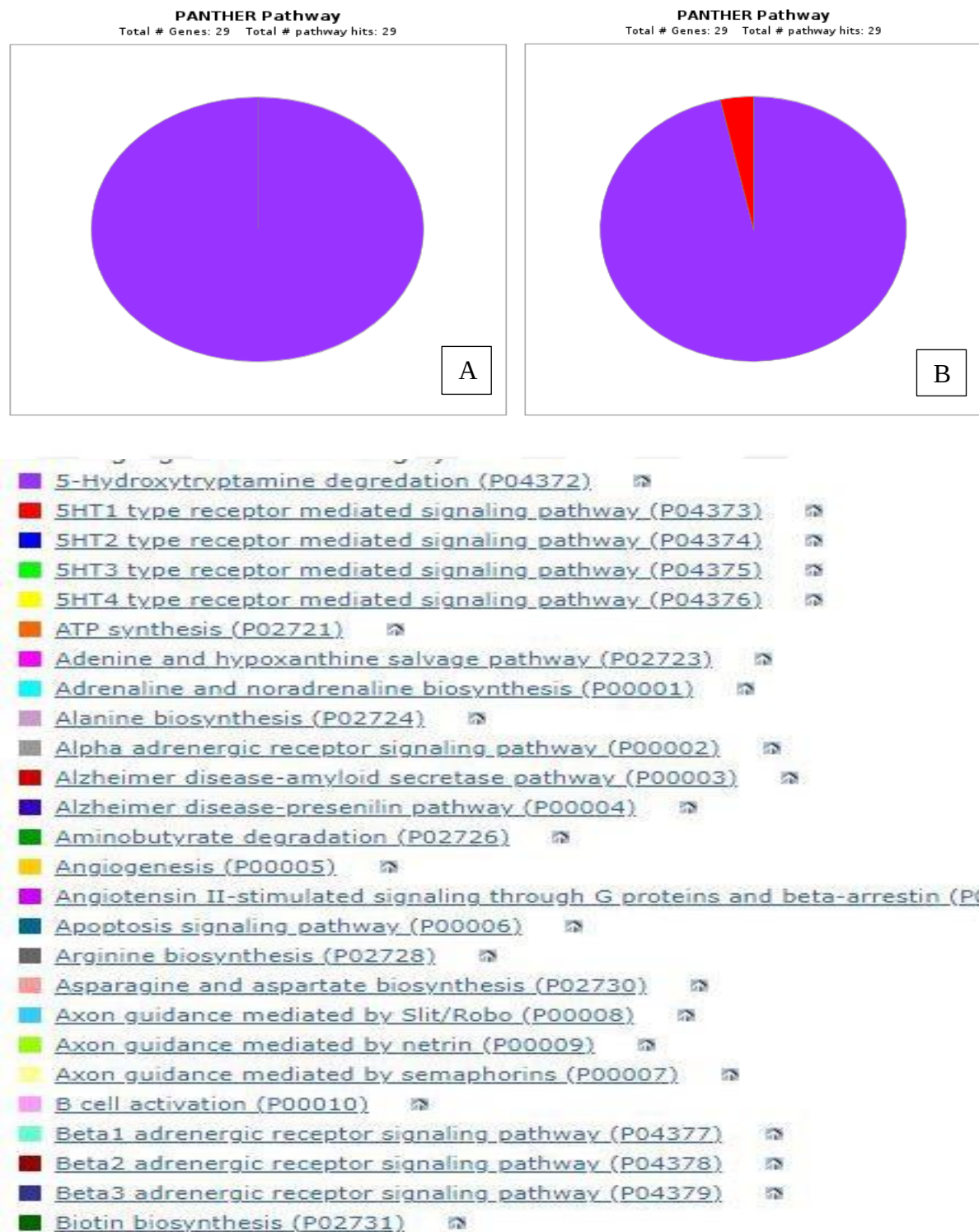


Figure 4.7: The Panther Pathway of 5%M/3%M upregulated and downregulated genes.

Additionally, the 5HT1 type receptor-mediated signalling pathway (P04373) is included in the down-regulatory genes.

4.3.2. 3%M / 5%M Comparison

Molecular Function

Both dysregulated gene groupings displayed genes that were mostly associated with ATP-dependent activity in terms of cellular function (GO:0140657), catalytic activity (GO:0005488), binding (GO:0005488), and no Panther category is assigned (unclassified). The ATP-dependent activity is higher in upregulated genes; and catalytic activity, binding and no panther category (unclassified) is higher in down-regulated genes.

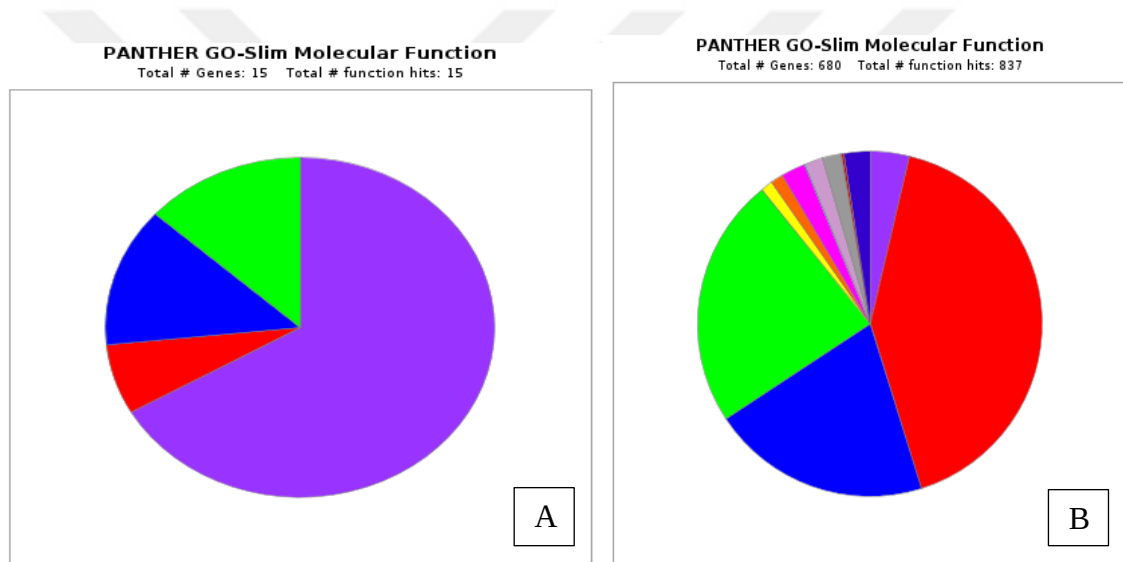




Figure 4.8: Molecular Function of 3%M/5%M upregulated and downregulated genes using Panther.

Along with transporter activity (GO:0005215), translation regulator activity (GO:0045182), transcription regulator activity (GO:0140110), structural molecular activity (GO:0005198), molecular function regulator activity (GO:0098772), molecular adaptor activity (GO:0060090), and cytoskeleton motor activity (GO:0003774), the down-regulatory genes also include genes that regulate these processes.

Biological Process

Both dysregulated gene sets displayed genes that were mostly engaged in biological regulation (GO:0065007) in terms of biological processes, growth (GO:0040007), cellular process (GO:0009987), and no Panther category is assigned (unclassified). The no panther category (unclassified) and biological regulation is higher in up-regulated genes; and growth and cellular process is higher in down-regulated genes.

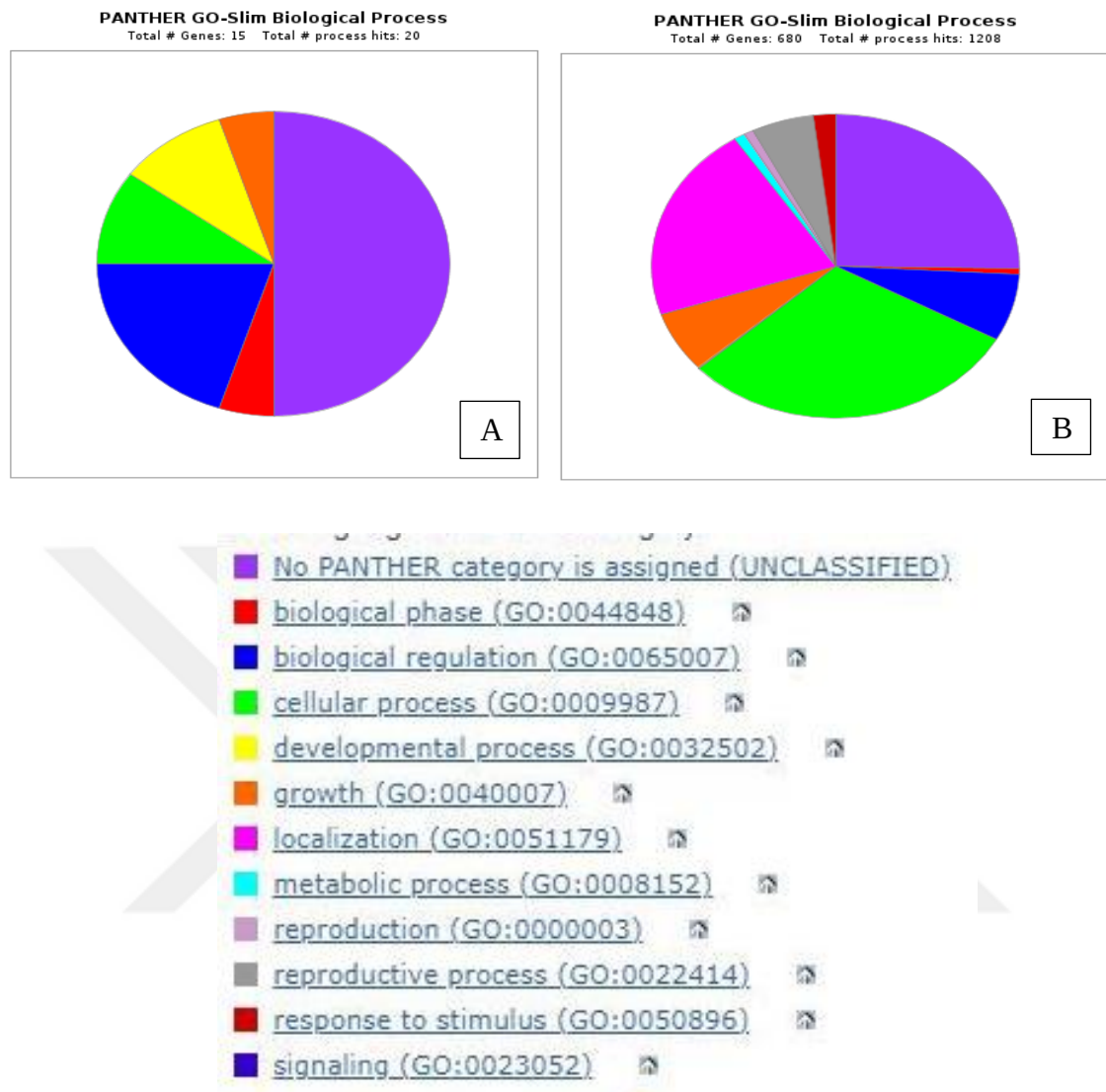


Figure 4.9: Biological Process of 3%M/5%M upregulated and downregulated genes using Panther.

The up-regulatory genes also consist of developmental process (GO:0032502).

Response to stimuli (GO:0050896), localisation (GO:0051179), metabolic process (GO:0009152), reproduction (GO:0000003), and reproductive process (GO:0022414) are other genes that are down-regulated.

Cellular Component

Both dysregulated gene sets showed genes that were mostly engaged in cellular anatomical entities in terms of cellular component (GO:0110165) and no Panther category is assigned

(unclassified). The cellular anatomical entity is higher in up-regulated genes as compared to the down-regulated genes.

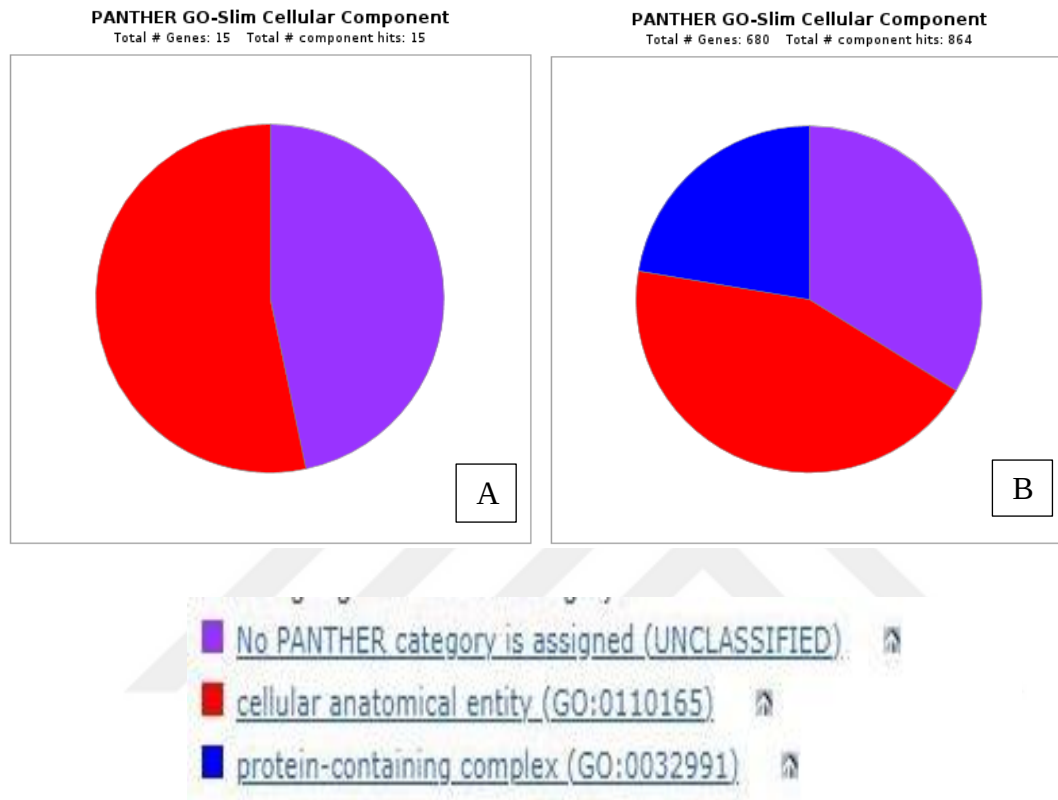


Figure 4.10: Cellular Component of 3% M/5% M upregulated and downregulated genes using Panther.

The down-regulatory gene also consist of protein-containing complex (GO:0032991).

Protein Class

Both dysregulated gene sets displayed proteins (PC000009) that are primarily involved in DNA metabolism, RNA metabolism protein (PC00031), and no Panther category is assigned (unclassified). The DNA metabolism protein is higher in the up-regulated genes; and RNA metabolism protein and no panther category (unclassified) is higher in the down-regulated genes.

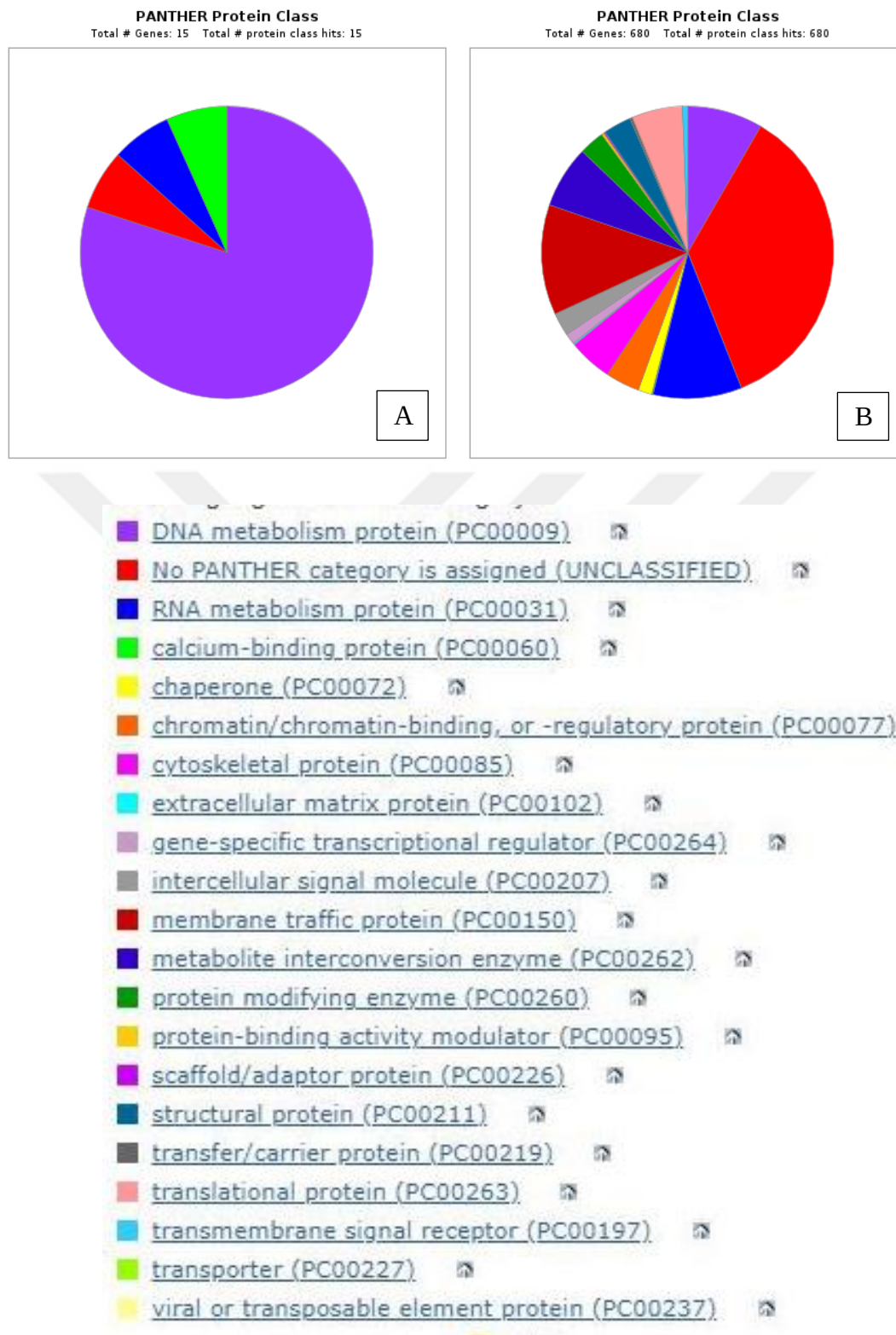


Figure 4.11: Protein Class of 3%M/5%M upregulated and downregulated genes using Panther.

The up-regulatory genes also consist of cytoskeletal protein (PC00085).

The down-regulatory genes also consist of extracellular matrix protein (PC00102), transcriptional protein (PC00263), structural protein (PC00211), membrane traffic protein (PC00260), intercellular signal molecule (PC00207), metabolite interconversion enzyme (PC00262), protein-binding activity modulator (PC00095), protein modifying enzyme (PC00260), protein modifying enzyme (PC00260), protein modifying enzyme (PC00260), gene-specific transcriptional regulator (PC00264), scaffold / adaptor protein (PC00226), chromat and protein binding activity modulator (PC00095).

Panther Pathway

In terms of the panther pathway, the majority of the genes in both dysregulated gene groups were those involved in the breakdown of 5-hydroxytryptamine (P04372). The up-regulated genes consist of 100% 5-Hydroxytrptamine degradation whereas the down-regulated genes consist of 80%.

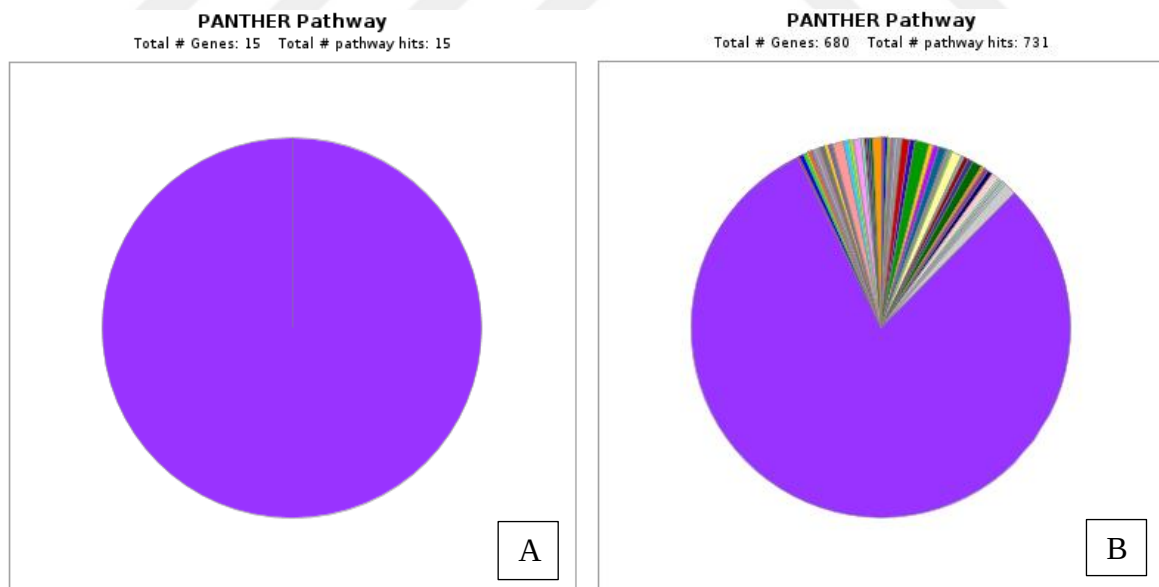




Figure 4.12: The Panther Pathway of 3%M/5%M upregulated and downregulated genes.

The down-regulatory genes also consist of almost all the genes from the list above.

4.3.3. 3%M / 3%N Comparison

Molecular Function

Both dysregulated gene sets showed genes that were primarily engaged in ATP-dependent activity (GO:0140657), catalytic activity (GO:0005488), cytoskeletal motor activity (GO:0003774), molecular function regulator (GO:0060090), and molecular transducer activity (GO:0060089), among other molecular functions, binding (GO:0005488), and no Panther category is assigned (unclassified). Catalytic activity, cytoskeletal motor activity,

molecular function regulator, and binding are greater in down-regulated genes whereas ATP-dependent activity, molecular transducer activity, and no defined category (unclassified) are higher in up-regulated genes.

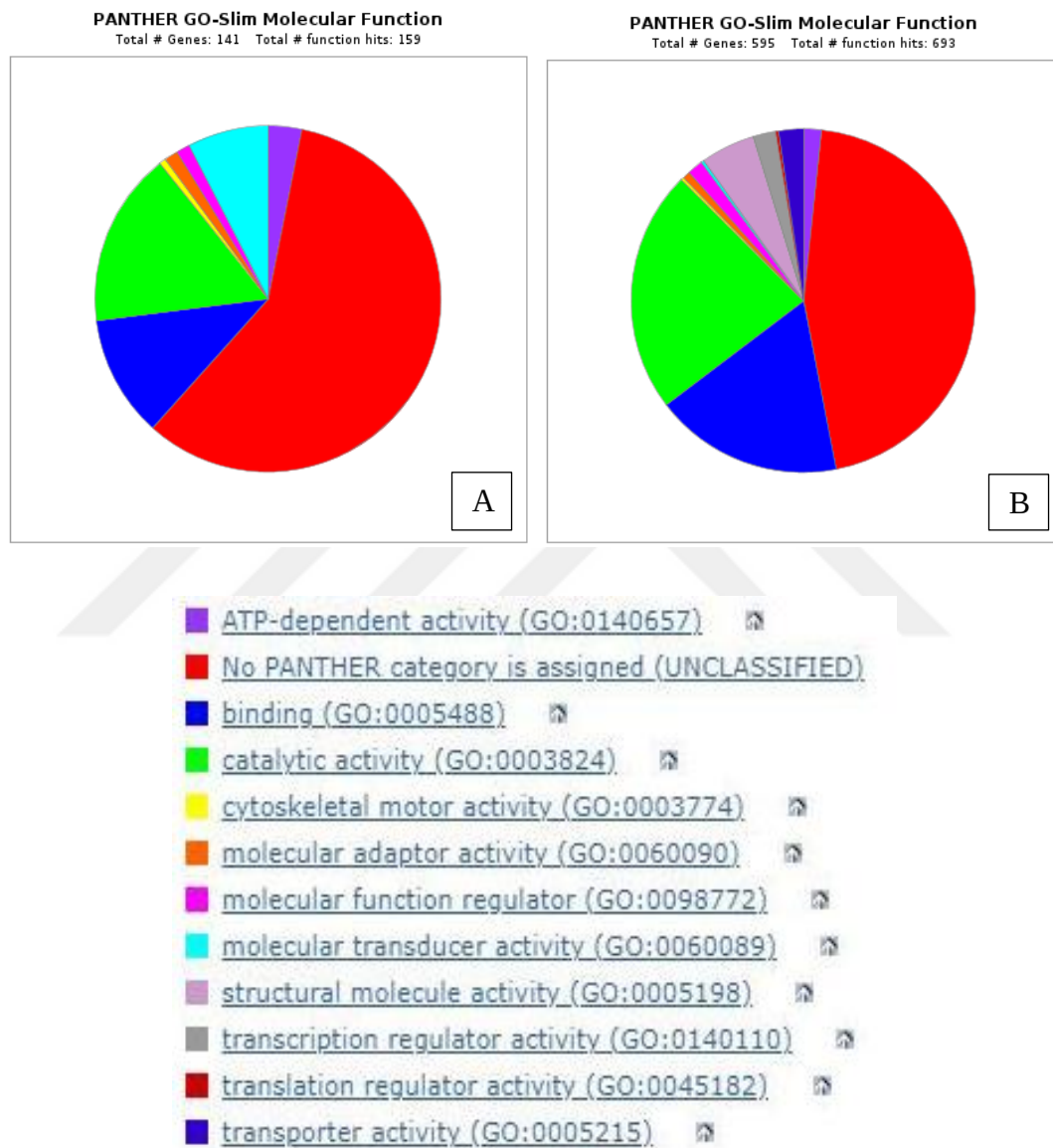
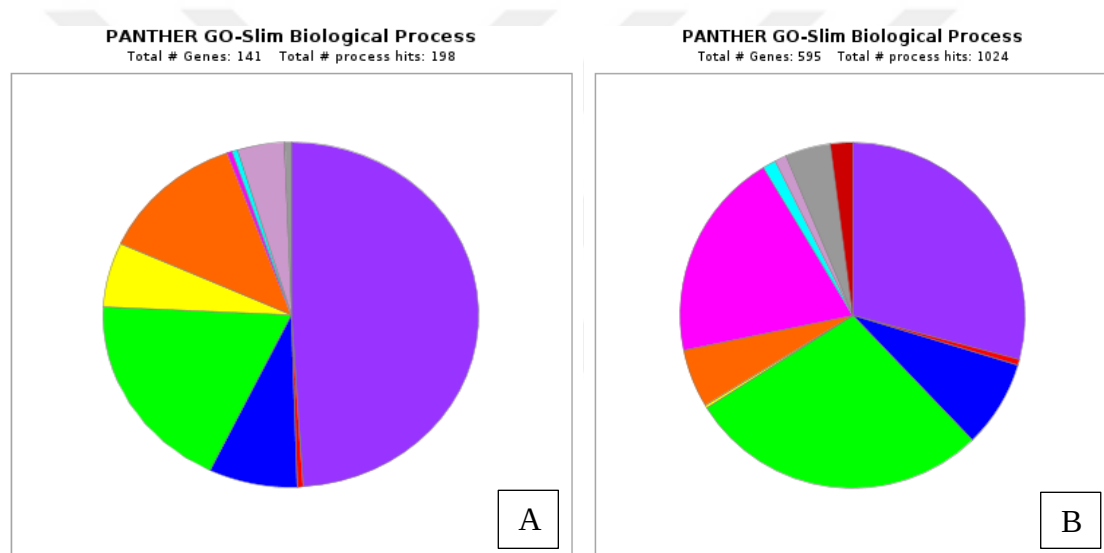


Figure 4.13: Molecular Function of 3%M / 3%N upregulated and downregulated genes using Panther.

Transporter activity (GO:0005215), translation regulator activity (GO:0045182), transcription regulator activity (GO:0140110), and structural molecular activity (GO:0005198) are additional features of the down-regulatory genes.

Biological Process

Biological phase (GO:0044848), biological regulation (GO:0065007), growth (GO:0040007), cellular process (GO:0009987), localization (GO:0051179), reproduction (GO:0000003), metabolic process (GO:0009152), and reproductive process (GO:0022414) were the primary biological processes that both dysregulated groups of genes were involved in and no Panther category is assigned (unclassified). No panther category (unclassified), reproduction and growth is high in up-regulated genes; the biological regulation is equal in up-regulated and down regulated genes; and biological phase, cellular process, localization, metabolic process, and reproductive process is higher in down-regulated genes.



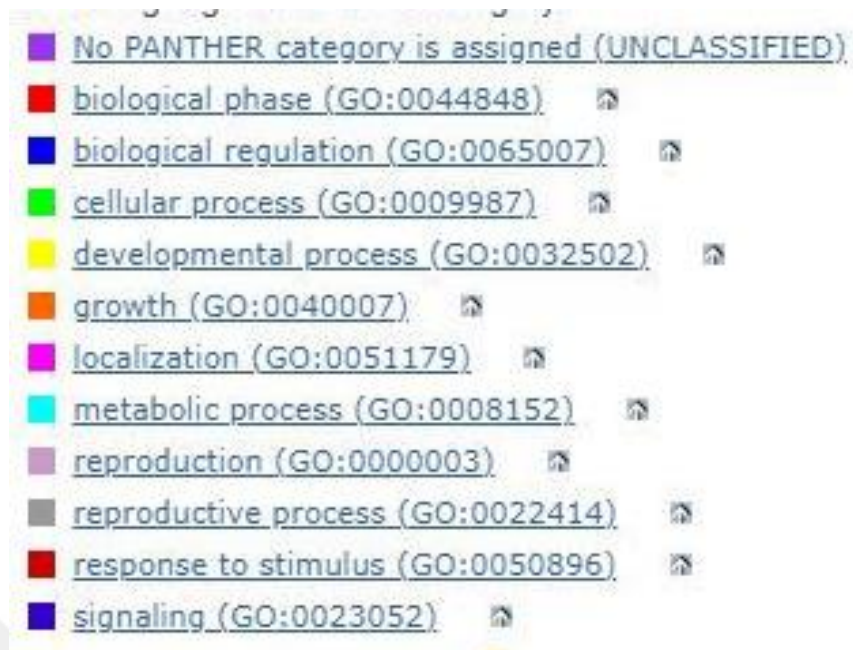
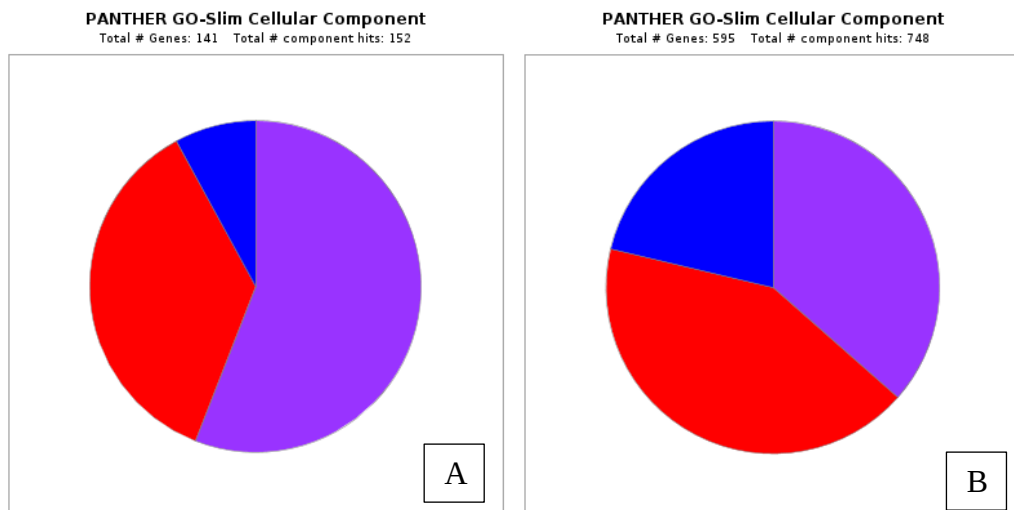


Figure 4.14: Biological Process of 3%M / 3%N upregulated and downregulated genes using Panther.

The down-regulatory genes also consist of response to stimulus (GO:0050896).

Cellular Component

Both dysregulated gene sets showed genes that were mostly engaged in biological processes involving cellular anatomical entities (GO:0110165) and protein-containing complexes (GO:0032991) and no Panther category is assigned (unclassified). No panther category (unclassified) is high in up-regulated genes; and protein-containing complex and cellular anatomical entity is higher in down-regulated genes.



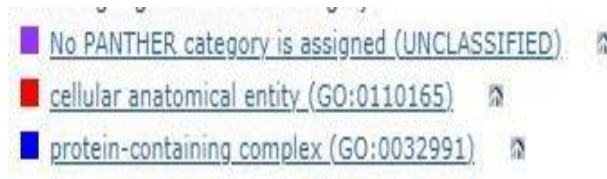


Figure 4.15: Cellular Component of 3%M / 3%N upregulated and downregulated genes using Panther.

Protein Class

In terms of protein class, DNA metabolism protein (PC000009), RNA metabolism protein (PC00031), cytoskeletal protein (PC00085), scaffold/adaptor protein (PC00226), membrane traffic protein (PC00150), intercellular signal molecule (PC00207), extracellular matrix protein (PC00102), chaperone (PC00072), chromatin / chromatin-binding or regulatory protein (PC00077), and chaperone (PC00072) were the main genes involved in both dysregulated groups of genes and no Panther category is assigned (unclassified). No panther category (unclassified), extracellular matrix protein and cytoskeletal protein is high in up-regulated genes, the membrane traffic protein, chromatin/ chromatin-binding or regulatory protein and chaperone is equal in up-regulated and down-regulated genes; and DNA metabolism protein, RNA metabolism protein, cytoskeletal protein, scaffold/adaptor protein and intercellular signal molecule is high in down-regulated genes.

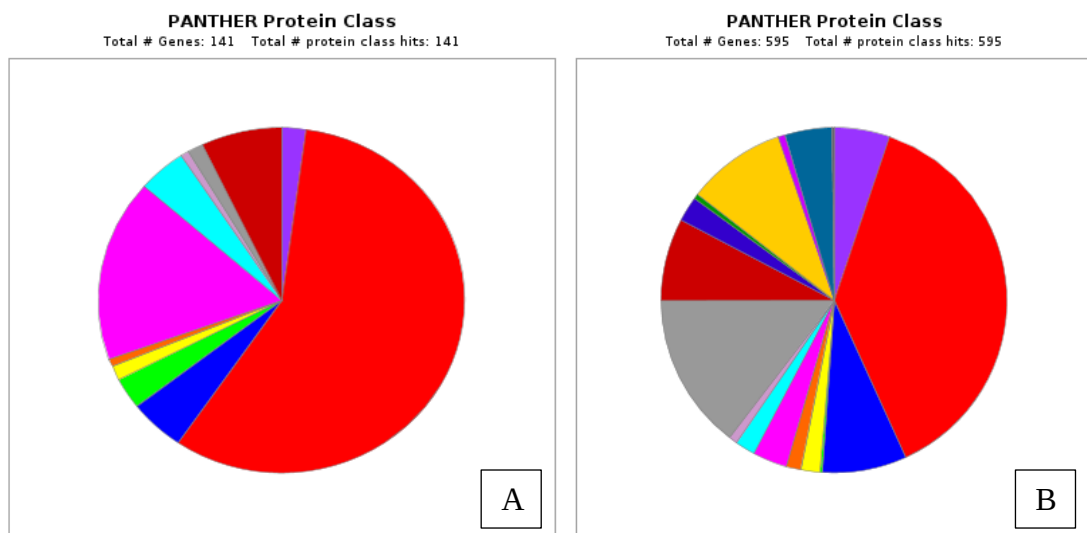




Figure 4.16: Protein Class of 3%M / 3%N upregulated and downregulated genes using Panther.

The up-regulatory genes also consist of calcium binding protein (PC00060).

Aside from metabolite interconversion protein enzyme (PC00262), protein-binding activity modulator (PC00095), structural (PC00211), and protein modifying enzyme (PC00260), the down-regulatory genes also include these.

Panther Pathway

Both dysregulated gene sets in the panther pathway included genes mostly involved in the breakdown of 5-hydroxytryptamine (P04372). Alpha adrenergic receptor signalling pathway (P00002), Alzheimer disease-amyloid secretase pathway (P00003), ATP synthesis (P02721), 5HT1 type receptor mediated signalling pathway (P04373), 5HT2 type receptor mediated signalling pathway (P04374), 5HT3 type receptor mediated signalling pathway (P04375), and

5HT4 type receptor mediated signalling pathway (P05911) are examples of Angiotens Up-regulated genes produce the most adrenaline and noradrenaline, whereas down-regulated genes produce the most ATP.

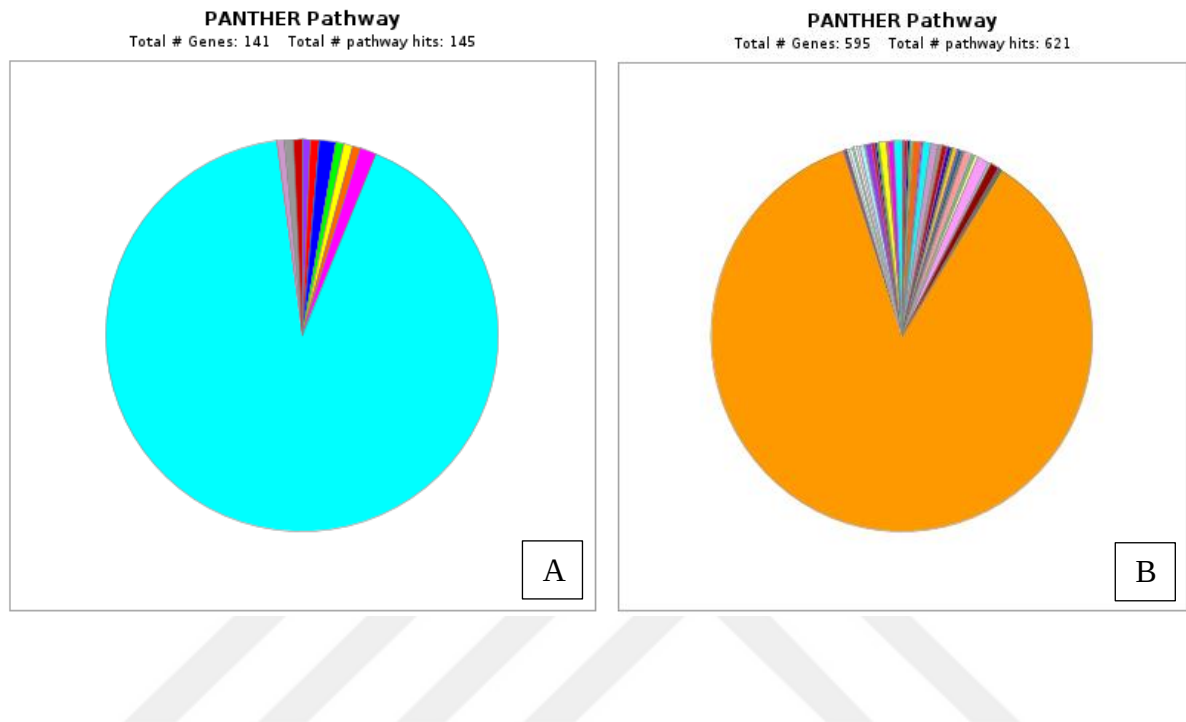




Figure 4.17: The Panther Pathway of 3%M / 3%N upregulated and downregulated genes.

4.3.4. 5%M / 5%N Comparison

Molecular Function

Both dysregulated gene sets showed genes primarily involved in ATP-dependent activity (GO:0140657), molecular transducer activity (GO:0060089), molecular adaptor activity (GO:0060090), catalytic activity (GO:0005488), cytoskeletal motor activity (GO:0003774), binding (GO:0005488), and other molecular functions and no Panther category is assigned

(unclassified). No panther category (unclassified), ATP-dependent activity and cytoskeletal activity is higher in up-regulated genes; and molecular transducer activity, molecular adaptor activity, catalytic activity, and binding is higher in down-regulated genes.

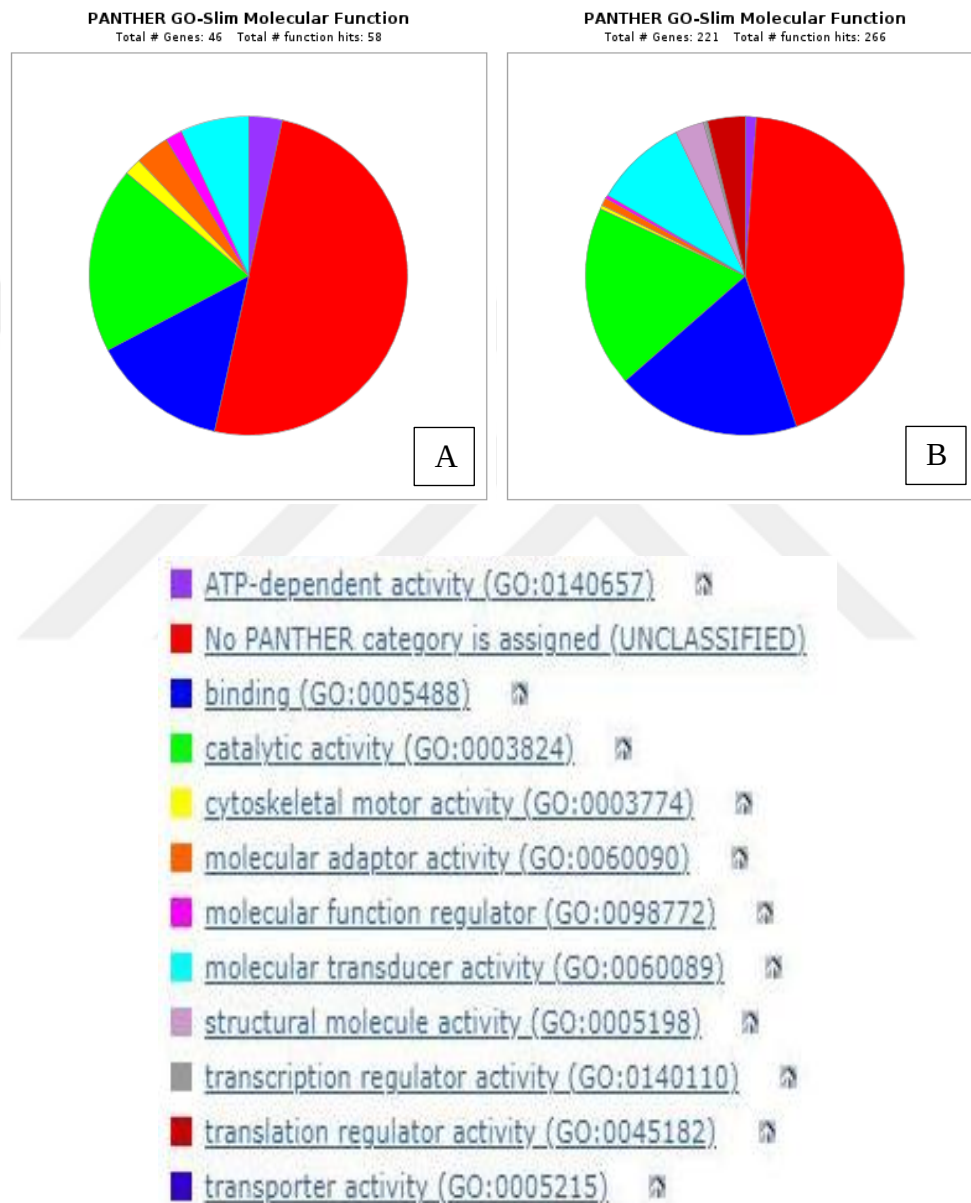
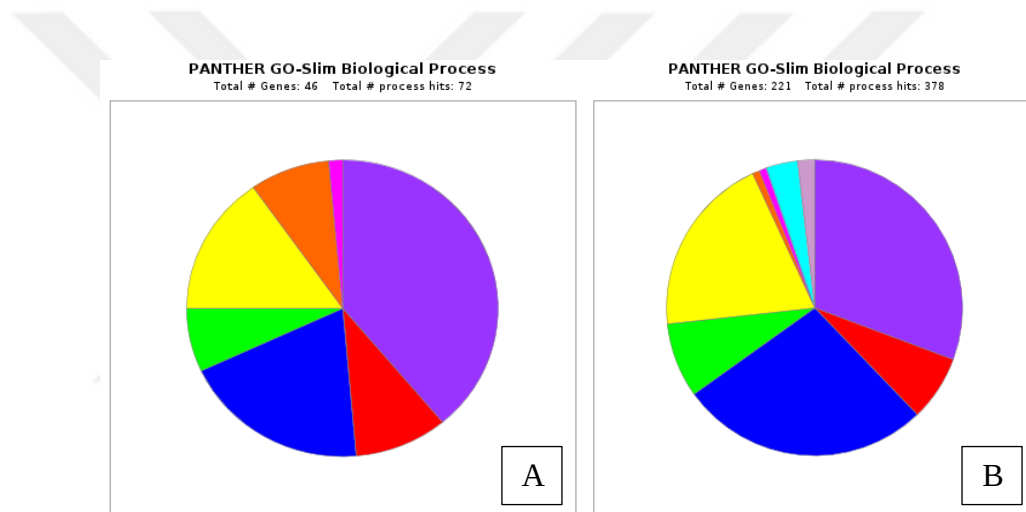


Figure 4.18: Molecular Function of 5%M / 5%N upregulated and downregulated genes using Panther.

Transporter activity is also present in the up-regulatory genes (GO:0005215). Transcription regulator activity (GO:0140110) and translation regulator activity (GO:0045182) are also components of the down-regulatory genes.

Biological Process

In terms of biological process, both dysregulated groups of genes presented genes mainly involved in biological phase (GO:0044848), biological regulation (GO:0065007), cellular process (GO:0009987), developmental process (GO:0032502), growth (GO:0040007), localization (GO:0051179), and no Panther category is assigned (unclassified). No panther category (unclassified), biological phase, and growth is high in up-regulated genes; the cellular process is equal in up-regulated and down-regulated genes; and biological regulation, developmental process and localization is high in down-regulated genes.



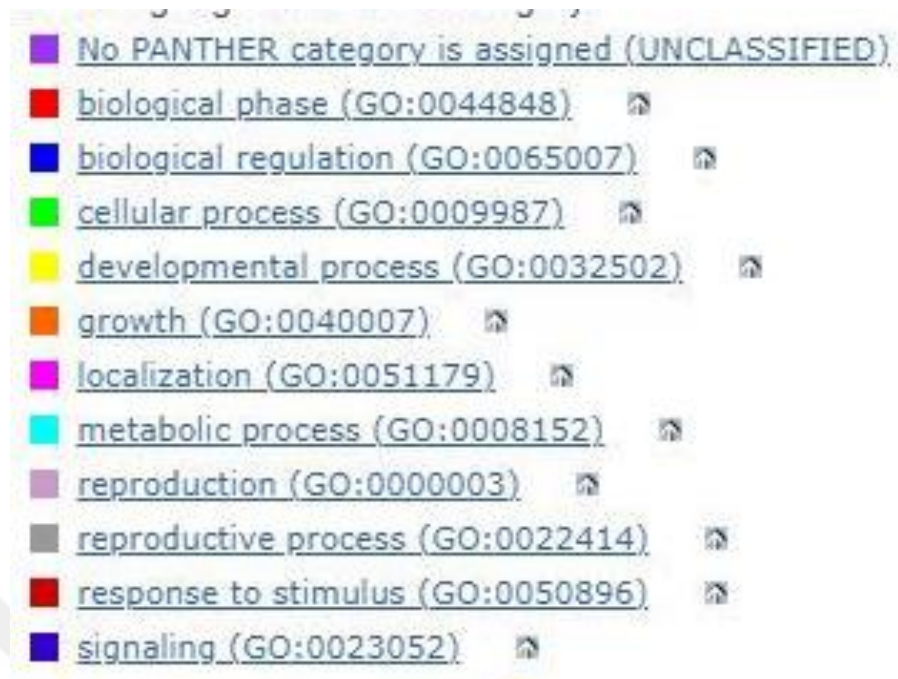


Figure 4.19: Biological Process of 5% M / 5%N upregulated and downregulated genes using Panther.

The down-regulatory genes also consist of reproduction (GO:0000003) and metabolic process (GO:0009152).

Cellular Component

Both dysregulated gene sets showed genes that were mostly engaged in biological processes involving cellular anatomical entities (GO:0110165) and protein-containing complexes (GO:0032991) and no Panther category is assigned (unclassified). Unclassified No Panther category has the largest level of up-regulated genes, while the highest level of down-regulated genes is found in cellular anatomical entities and protein-containing complexes.

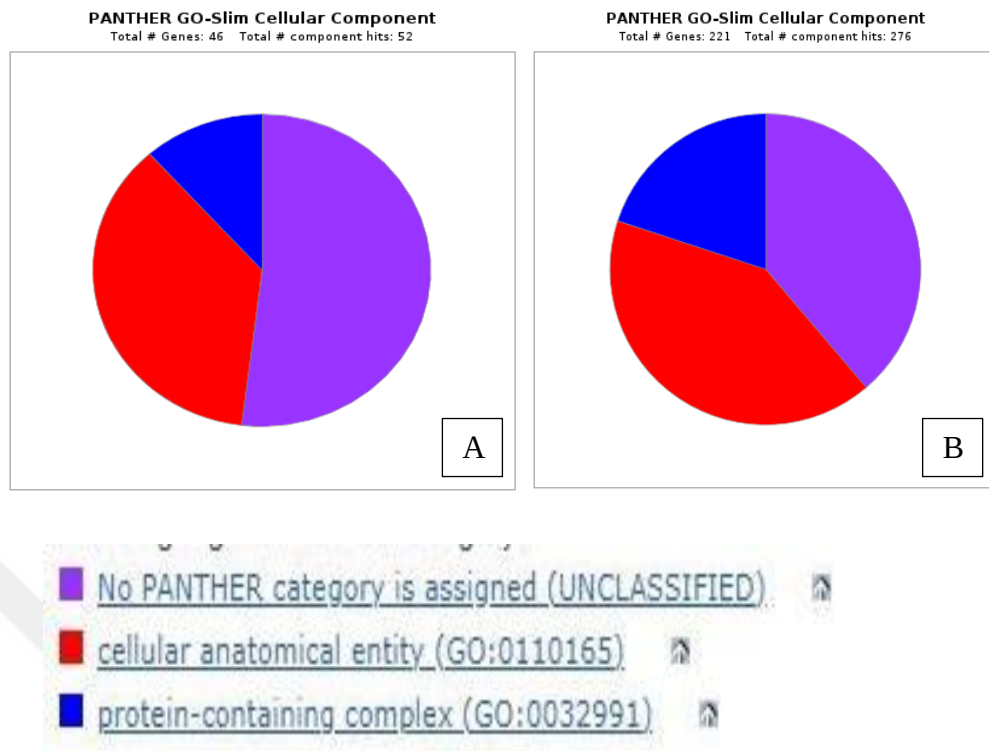


Figure 4.20: Cellular Component of 5%M / 5%N upregulated and downregulated genes using Panther.

Protein Class

Both dysregulated groups of genes were mainly involved in the following protein classes: chaperone (PC00072), calcium binding protein (PC00060), chromatin / chromatin-binding or regulatory protein (PC00077), cytoskeletal protein (PC00085), extracellular matrix protein (PC00102), gene-specific transcriptional regulator (PC00264), intercellular signal molecule (PC00207), and membrane traffic protein (P000009) and no Panther category is assigned (unclassified). No panther category (unclassified), DNA metabolism protein, membrane traffic protein, extra-cellular matrix protein, and cytoskeletal protein is high in up-regulated genes; and RNA metabolism protein, Down-regulated genes have high levels of the chaperone, chromatin/chromatin-binding or regulatory protein, gene-specific transcriptional regulator, and intercellular signal molecule.

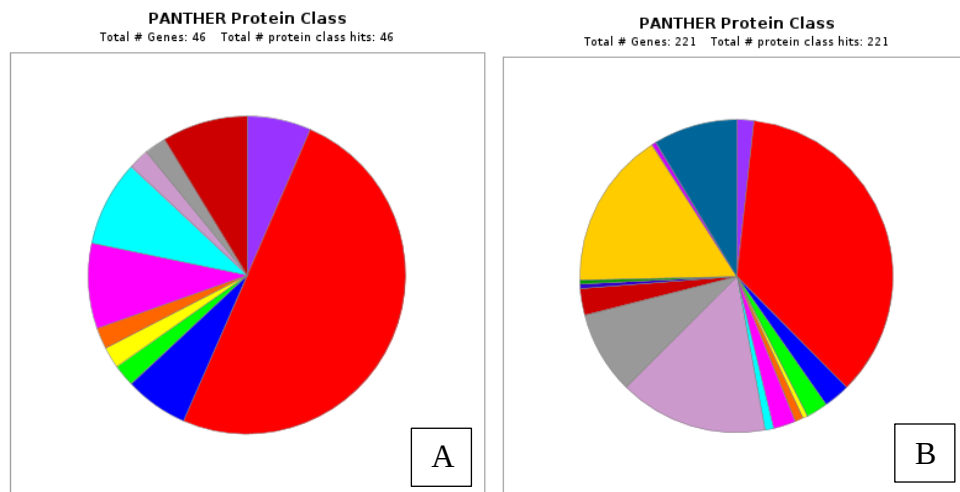


Figure 4.21: Protein Class of 5%M / 5%N upregulated and downregulated genes using Panther.

The scaffold/adaptor protein (PC00226), the protein modifying enzyme (PC00260), the metabolite interconversion enzyme (PC00262), and the structural protein (PC00211) are also included in the list of down-regulatory genes.

Panther Pathway

Specifically speaking about the panther route, both dysregulated gene sets showed genes mostly related in the apoptotic signalling pathway (P00006), fructose galactose metabolism (P02744), and interleukin signaling pathway (P00036), Parkinson disease (P00049), and no panther category is assigned (unclassified).

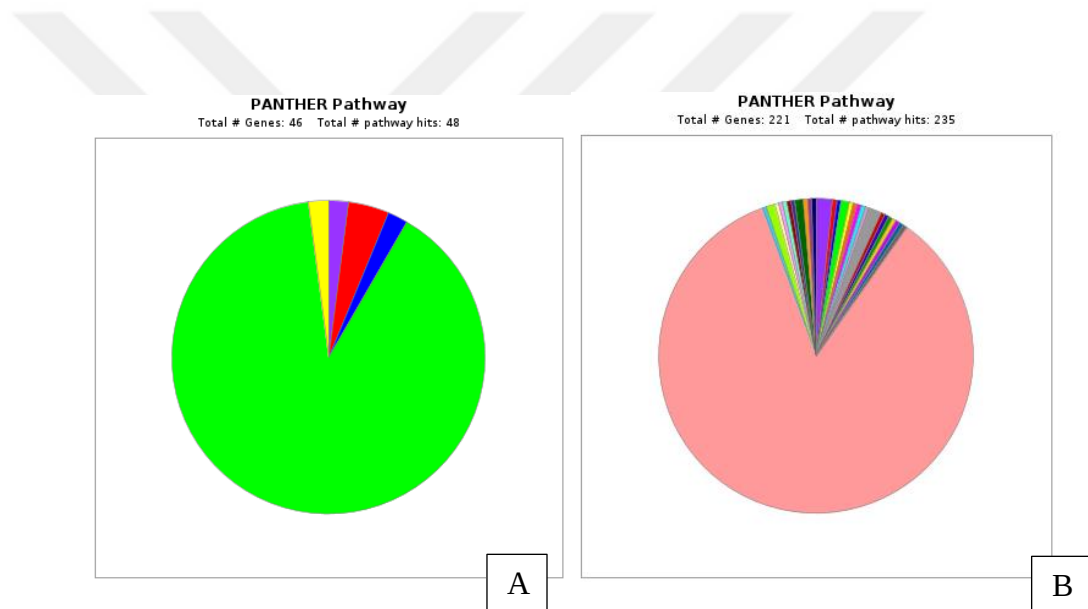




Figure 4.22: The Panther Pathway of 5%M / 5%N upregulated and downregulated genes.

5. DISCUSSION

[Aging is characterized by a gradual decline in function and a higher likelihood of death. Over time, the accumulation of cellular damage, such as DNA mutations, protein misfolding, and lesions, impairs the function of cells and organelles. These cellular changes lead to the buildup of dysfunctional cells and disrupt homeostasis, limiting the body's regenerative abilities. This process triggers mild inflammation, alters cell-to-cell communication, and hampers overall tissue function. Epigenetic modifications, gene expression dysregulation, and the accumulation of molecular damage resulting from changes in endocrine signaling contribute to the aging process and are considered the primary risk factors for age-related diseases and multiple health issues (Moosavi & Ardekani, 2016). There is growing interest in identifying drugs that can treat and prevent age-related diseases in humans. One important objective of studying model organisms, such as yeast and mice, is to gain a better understanding of the underlying mechanisms, therapeutic effects, and potential side effects of drugs, with the aim of applying this knowledge to humans (Kennedy & Pennypacker, 2014).

In the field of aging research, it is crucial to have an ideal model system that can be used to explore potential anti-aging agents and evaluate their effects on health. The preferred model system should closely resemble human aging processes, including the presence of highly conserved genes and signaling pathways (Folch et al., 2017). It is increasingly recognized that pathways associated with human aging are also highly conserved in yeast species like *Saccharomyces cerevisiae* and others. Yeast, being unicellular eukaryotes, have gained prominence as a valuable tool for investigating and testing potential anti-aging interventions, as well as for understanding the signaling pathways and molecular players involved in the aging process (Zimmermann et al., 2018).

In addition to *S. cerevisiae*, other yeast species, particularly the fission yeast *S. pombe*, are utilized as model organisms for studying aging. *S. pombe* offers greater similarity to the cellular properties of more complex organisms, along with advantages such as ease of manipulation and short life cycles, making it an excellent model for molecular studies, especially those related to aging (Şeylan & Tarhan, 2023).

Metformin, also known as N,N-dimethylbiguanide, is a commonly prescribed medication for type-2 diabetes and has recently garnered attention in anti-aging research (Bailey, 2017). Metformin exhibits versatile effects due to its proven ability to reduce blood glucose levels, as well as its protective properties for the heart, anti-inflammatory characteristics, and potential in combating cancer. Moreover, metformin impacts various metabolic and cellular processes associated with age-related diseases, including inflammation, oxidative damage, protein glycation, cellular senescence, apoptosis, and the growth of specific types of cancer. An additional advantage of metformin for further investigation of its anti-aging potential is its extensive use in humans over many years, which has established a well-defined safety profile and known potential side effects. These characteristics make metformin a more practical option for administering as an anti-aging treatment compared to non-approved clinical drugs (Şeylan & Tarhan, 2023).

This study aimed to investigate the change in gene expression in *S. pombe*. The cells were cultured in media as such that we had 3% and 5% SD media with and without metformin namely; 3%M, 3%N, 5%M and 5%N. The media contained 25mM concentration of metformin. Subsequently, then the effects of the drug on gene expression was examined by the process of RNA sequencing. It was observed that *S. pombe* cells grown in the presence of metformin had various upregulated and downregulated genes. In this discussion section we try to compare our data with other researches and infer what we have found.

5.1. GROUPING GENES THAT HAVE SIMILAR FUNCTIONS

By examining and grouping both upregulated and downregulated genes with common function, we can obtain a comprehensive view of the molecular changes induced by metformin treatment. This information allows us to identify the affected biological processes, potential mechanisms of action, and potential implications for cellular function. It also helps to uncover any imbalances or disruptions in gene expression that may contribute to the observed effects of metformin.

5.1.1. Up-regulated genes

- Molecular function:

The catalytic activity (GO:0005488) suggests that metformin treatment may stimulate enzymatic reactions or metabolic processes in *S. pombe*. This finding is consistent with the

known effects of metformin on cellular metabolism. The presence of unclassified genes among the upregulated genes indicates that these genes may have unique or less characterized functions. Further investigation of these genes can provide novel insights into the specific mechanisms affected by metformin.

- Biological process:

The biological regulation (GO:0065007) suggests that metformin treatment may impact regulatory processes within the cell, potentially influencing gene expression and cellular responses. The upregulation of genes associated with growth (GO:0040007) indicates that metformin may have an impact on cellular growth and proliferation in *S. pombe*. The localization (GO:0051179) that metformin treatment may influence the movement or distribution of cellular components within *S. pombe* cells. The reproduction (GO:0000003) suggests that metformin treatment may affect the reproductive processes in *S. pombe*, such as cell division or the formation of spores.

- Cellular component:

The presence of unclassified genes among the upregulated genes indicates that these genes may have unique or less characterized cellular locations. Further investigation of these genes may provide more data about their specific cellular roles influenced by metformin.

- Protein class:

Unclassified: The enrichment of unclassified proteins suggests that there may be novel protein classes affected by metformin in *S. pombe* that have not been extensively characterized. The DNA metabolism protein (PC00009) indicates that metformin may influence processes related to DNA replication, repair, or recombination in *S. pombe*.

Extracellular matrix protein (PC00102): The presence of upregulated genes associated with the extracellular matrix suggests that metformin may impact the cell's interaction with its external environment. The Cytoskeletal protein (PC00207) implies that metformin treatment may affect the organization and dynamics of the cytoskeleton in *S. pombe*. The membrane traffic protein (PC00150) indicates that metformin may influence processes involved in the transport of molecules within the cell.

- Panther pathway:

5-Hydroxytryptamine degradation and adrenaline and nonadrenaline; the upregulation of genes associated with these pathways suggests that metformin treatment may impact the metabolism or signalling of these compounds in *S. pombe*.

Overall, the enrichment of specific gene categories in different functional classifications provides insights into the potential cellular processes affected by metformin treatment. It suggests that metformin may influence metabolic pathways, cellular regulation, growth, localization, and reproductive processes in *S. pombe*. Furthermore, it indicates potential effects on DNA metabolism, cytoskeletal organization, and membrane dynamics. However, further experimental validation is needed to confirm and understand the precise mechanisms underlying these observations.

5.1.2. Down-regulated genes

- Molecular function:

The down-regulation of genes associated with ATP-dependent activity suggests that metformin treatment may suppress ATP-dependent processes in *S. pombe*. These processes could include ATP-dependent enzyme activities or ATP-dependent molecular transport. The molecular adaptor activity indicates that metformin treatment may impact the ability of proteins to mediate interactions between different molecules or cellular components. The binding suggests that metformin treatment may influence the ability of molecules to bind to specific targets or substrates. The catalytic activity indicates a potential decrease in the expression of enzymes involved in various metabolic pathways or cellular processes. The cytoskeletal motor activity suggests that metformin treatment may affect the movement and dynamics of the cytoskeleton in *S. pombe* cells. The molecular function regulators indicate that metformin treatment may modulate the activity or expression of regulatory molecules that control specific molecular functions within the cell.

- Biological process:

The down-regulation of genes associated with cellular processes suggests that metformin treatment may affect various fundamental cellular activities, including cell cycle progression, cell division, and intracellular signaling. The biological regulation indicates that metformin treatment may impact the regulation of gene expression, signaling pathways, or cellular responses to environmental cues. The growth suggests that metformin treatment may influence cellular growth and proliferation in *S. pombe*. The localization implies that metformin treatment may impact the movement or targeting of cellular components within *S. pombe* cells. The reproduction indicates that metformin treatment may affect reproductive processes in *S. pombe*, such as cell division or spore formation. The down-regulation of

unclassified genes suggests that there may be genes with unknown functions or roles that are affected by metformin treatment.

- Cellular component:

The down-regulation of genes associated with down-regulatory gene and protein-containing complex suggests that metformin treatment may impact the expression or activity of genes and proteins involved in the regulation of cellular processes or the formation of protein complexes.

- Protein class:

The down-regulation of unclassified proteins indicates that there may be genes with uncharacterized functions or roles that are affected by metformin treatment.

RNA metabolism protein: The down-regulation of genes involved in RNA metabolism suggests that metformin treatment may influence processes related to RNA processing, stability, or translation. The genes associated with DNA metabolism indicates that metformin treatment may impact processes related to DNA replication, repair, or recombination.

Cytoskeletal protein: The down-regulation of genes involved in cytoskeletal proteins suggests that metformin treatment may affect the organization and dynamics of the cytoskeleton in *S. pombe*. The scaffold/adaptor proteins implies that metformin treatment may modulate the ability of proteins to mediate specific

In summary, the downregulated genes identified in various functional categories provide insights into the specific molecular functions, biological processes, cellular components, and protein classes that are influenced by metformin treatment in *S. pombe*. The downregulation of genes associated with ATP-dependent activity, molecular adaptor activity, binding, catalytic activity, and cytoskeletal motor activity indicates the suppression or reduction of these molecular functions in response to metformin. Similarly, the downregulation of genes related to cellular processes, biological regulation, growth, localization, and reproduction suggest a dampening of these processes under metformin treatment. The downregulation of genes involved in protein-containing complexes indicates potential alterations in the composition or activity of these complexes upon metformin exposure.

Furthermore, the downregulation of genes in specific protein classes such as RNA metabolism, DNA metabolism, cytoskeletal proteins, scaffold/adaptor proteins, intercellular signal molecules, extracellular matrix proteins, calcium-binding proteins, and membrane

traffic proteins highlights the impact of metformin on these specific protein classes and their associated functions.

Overall, these findings suggest that metformin treatment in *S. pombe* leads to the downregulation of genes associated with various molecular functions, biological processes, cellular components, and protein classes. Understanding the specific genes and pathways affected by metformin provides insights into the mechanisms through which metformin influences cellular processes, and further investigation into these downregulated genes can shed light on the functional consequences of metformin treatment in *S. pombe*.

These upregulated and downregulated genes represent the molecular changes in gene expression profile in response to metformin treatment in *S. pombe*. The upregulated genes indicate an increase in their expression levels, potentially leading to enhanced metabolic activities, regulation of cellular processes, and alterations in cellular components and protein classes. On the other hand, the downregulated genes suggest a suppression or reduction in their expression levels, impacting ATP-dependent activities, molecular interactions, binding processes, catalytic activities, cellular processes, and protein classes involved in various biological functions. These gene expression changes provide insights into the molecular mechanisms and pathways affected by metformin in *S. pombe*.

The effects of metformin on gene expression in *S. pombe* were examined in the article Effect of Antidiabetic Drug Metformin on Gene Expression Profile in *S. pombe*. Genes that were elevated and downregulated in several functional areas were used to display the results. Based on the observed changes in gene expression, the investigation concentrated on the possible effects of metformin on cellular functions, pathways, and protein subclasses.

Specifically exploring the impact of metformin on the gene expression of phosphoenolpyruvate carboxykinase (PEPCK), the Effect of metformin on gene expression of phosphoenolpyruvate carboxykinase research was given. The findings demonstrated that insulin and metformin together further suppressed PEPCK gene expression that was induced by cAMP/dexamethasone. Metformin alone dramatically lowered baseline PEPCK mRNA levels. The study also found that, in contrast to the MAPK pathway, the PI3K pathway mediates the inhibitory effects of insulin on PEPCK gene expression.

When comparing the two researches, it can be seen that they explore various studies and the effects of metformin on gene expression in various situations. This study gives a more comprehensive overview of the gene expression alterations and potential cellular processes

that metformin may have an impact on in *S. pombe*, whereas the study Effect of metformin on gene expression of phosphoenolpyruvate carboxykinase focuses specifically on the effects of metformin on PEPCCK gene expression and the involvement of insulin signaling pathways (Yuan et al., 2002).

Both findings emphasize how metformin affects gene expression and may have an impact on cellular functions. Although the precise genes and pathways covered in each talk vary, they all help us understand how metformin affects cellular function and modify gene expression.

The changes in longitudinal whole-blood transcriptome profiles of healthy persons following a one-week metformin intervention were examined in the article Metformin substantially impacts transcriptome of peripheral blood cells in healthy individuals. The results showed that metformin-induced differential expression of genes involved in IgA synthesis and cytokine-cytokine receptor interaction pathways in the intestinal immune network. Additionally, the study discovered subject-specific differential gene expression clusters linked to cholesterol homeostasis and insulin production. The findings indicated both a significant inter-individual variance in the metformin effect on transcriptional regulation as well as a robust link between the drug and the gut immune system (Ustinova et al., 2019).

The Metformin Resistance Is Associated with Expression of Inflammatory and Invasive Genes in A549 Lung Cancer Cells study examined changes in gene expression, cellular behavior, and mitochondrial dynamics linked to metformin resistance and focused on the establishment of metformin-resistant lung cancer cells (Seo et al., 2023). Both studies offer insights into the effects of metformin on cellular processes and highlight potential implications for metabolic pathways, DNA repair, cellular responses, and the invasiveness of cancer cells. The two studies differ in their model systems (yeast vs. lung cancer cells) and experimental approaches.

5.2.ANALYSING INDIVIDUAL GENES

5.2.1. Upregulated Genes

Amongst the upregulated genes, *mde7* had the high gene fold of 202.8166759 in the 3%M / 3%N comparison. This is the RNA binding specific protein gene and although its function is not well documented in *S.pombe*, *S.cerevisiae* ortholog WHI13 is known to modulate mRNA stability. It does this by regulating genes that play a role in cell cycle, sister chromatid

cohesion, and stress response. In a study, the RNA binding proteins (RBPs) have been discovered to be essential in recent years as much effort has been made into deciphering the posttranscriptional gene regulation network of diabetes and diabetic complications. RBPs control the expression of genes. Results indicate that metformin promotes the expression of RBP MBNL1; it reduces the senescence of renal tubular epithelial cells in DN (Chen et al., 2022). This brings us to the inference that if metformin leads to life longevity, it maybe because of proteins like this playing their role in stress response and cell cycle. This would need further research to confirm our results. The function of this thesis is to find candidate genes for further studies to understand the life-prolonging effect of metformin. Therefore, *mde7* is now one of these candidates.

The *mzt1* gene had gene fold of 37.71254819 in the 3%M / 3%N comparison. This is the protein coding gene for Mitotic Spindle Organizing Protein 1. *Mzt1* function is to make sure that the chromosomes are distributed evenly among the dividing cells (Ward et al., 2014). In a study, it was found that *mzt1*, expressed in testes of *Drosophila*, binds with core γ -TuRC components to facilitate the interaction between γ -TuRC and the proteins that attach γ -TuRC to MTOCs. The mutants of *mzt1*, though viable, are defective in basal body positioning and γ -TuRC recruitment to centriole adjuncts and effect the sperm formation. This in return has shown age-related decrease in male fertility and sperm mortality. (Tovey et al., 2018). The high fold change in upregulation when treated with metformin thus shows us that *mzt1* slows down the age related decrease in reproductive health of male drosophila. In another study, the results demonstrated that to evaluate the increase in centrosome amplification in stem cells in relation with age, *Drosophila* ISCs are an appropriate model. Metformin suppresses age-related centrosome amplification in *Drosophila* midgut stem cells through the AKT/TOR pathway. Additionally, the well-known anti-cancer medication metformin prevents age- and oxidative stress-related centrosome proliferation in ISC. ISCs are solely mitotic cells, and after PQ treatment in wild type flies pretreated with metformin, the proportion of PH3-positive cells and mitotic ISCs with extra centrosomes reduced (Na et al., 2015).

The *ght7* gene had the gene fold of 17.67884276 in the 3%M / 3%N comparison. This is the protein coding gene for plasma membrane hexose transmembrane transporter. It enables the passage of a structurally similar substance to hexose via the blood-brain barrier (Gupta et al., 2019). Hexose transport system regulation by metformin in vascular endothelial and smooth

muscle cells was studied. Western blot examination revealed a correlation between the increased abundance of GLUT1 protein in the plasma membrane and the metformin-induced increase in hexose transport in BSMC treated for 24 h with the medication. These results suggest that metformin impacts the hexose transport mechanism in vascular cells in addition to its recognised effects on hexose metabolism in insulin responsive tissues. This could enhance its ability to reduce blood glucose levels in those with Type 2 diabetes who are not insulin-dependent (Lee, 2014).

The *hsp16* gene had the gene fold of 4.770588155 in the 3%M / 3%N comparison. This is a protein coding gene for heat shock protein 16. For cells that are subjected to stressful situations, these genes serve as the first line of defence. By maintaining effective signalling pathways that are essential for cell survival and regular cell activity, they help to protect the integrity of cells (Dubrez et al., 2020). Extracellular heat shock protein 70 and insulin resistance in type 2 diabetes were explored in a study. The findings suggested that the results may have been skewed by metformin medication. Since medication was taken into account in each model, it's interesting to note that the correlation between eHSP70 and insulin resistance remained strong (Alemi et al., 2018).

5.2.2. Downregulated Genes

The *mfm3* gene had the gene fold of 0.024 in the 3%M / 3%N comparison. This is a protein coding gene in mice for myopathy myofibrillar 3. This gene primarily affects skeletal muscles, which are muscles that the body uses for movement. Effects of a long-term treatment with metformin in dystrophic *mdx* mice were investigated in a study. The analysis of the metformin effect on AMPK activation evaluated by western blot and the expression of mechanical-metabolic response genes revealed no discernible protective effects on dystrophic muscle metabolism. The reason of absence of metabolic impacts could be due to the inability of metformin to increase the levels of l-arginine, l-citrulline and taurine (Mantuano et al., 2018). Considering that in this study metformin had no protective effect on dystrophic muscle metabolism, it is tough to conclude the downregulation of *mfm3* in our study. Further study on different model organisms might provide more insight on metformin's effect on this gene.

The *met14* gene had the gene fold downregulation of 0.137 in the 3%M / 3%N comparison. This is a protein coding gene for adenylyl-sulfate kinase. In a research it was discovered that

pure AMP deaminase (AMPD) activity was reduced by metformin in isolated skeletal muscle L6 cells. AMPD is an enzyme that facilitates the conversion of adenosine monophosphate to inosine monophosphate and release an ammonia molecule as a by-product. In addition, fatty acid oxidation and glucose absorption were enhanced by a known inhibitor of AMPD. Both metformin and the AMPD inhibitor prevented the cells from accumulating ammonia. The metformin-induced enhancement of glucose transport was prevented by AMPD knockdown. We come to the conclusion that the mechanism of action of metformin is AMPD inhibition (Ouyang et al., 2011).

In our gene expression results *spc1* was found to be downregulated by 0.499 folds in 3%M / 3%N comparison. This gene is closely related to *hog1* gene in *S. cerevisiae* and to the *p38* kinases in mammals. The activation of *spc1* gene is essential for the cell to survive in various stress conditions and it gets activated by different stressors such as oxidative stress and high temperature. The encoding of stress-related proteins such as the trehalose-6-phosphate synthase (*tps1+*) and glycerol-3-phosphate dehydrogenase (*gpd1+*) are regulated by *spc1* (Degols et al., 1996). By understanding this we infer that the downregulation of *spc1* in presence of metformin decreases the cell viability under oxidative and temperature related stress conditions. However, because this role is biologically inferred and experimentally not proven, there is a possibility that the same protein has different function in other cellular processes. So, this requires more investigation and further research to be fully understood.

The *rpb11* gene which was observed to be downregulated by 0.499 folds in our gene expression study in 3%M / 3%N comparison encodes for the *rpb11* protein which is the subunit of RNA polymerase II. This protein plays an important role in the assembly and stability of RNAPII complex and facilitates in making of the functional core enzyme. It is known to be involved in various processes which in one way or another are related to transcription (Woychik et al., 2018). Even though no study has been done to understand the effect of metformin on *rpb11* gene, we infer that the downregulation of this gene would interfere with the normal function of RNA polymerase II thus effecting mRNA synthesis. The downregulation of this gene inhibits the production of certain proteins and somehow the lifespan of the cell is still prolonged. Which brings us to an inference that these proteins were somehow limiting the lifespan of the cell. This inference is supported by previous studies which mention that cell saves the energy by not producing certain proteins and using that

saved energy for more important cellular processes such as DNA repair and other cellular organelles (Ledford, 2008).



5.3. COMPARISON OF GENE UPREGULATION AND DOWNREGULATION IN 3%M / 3%N AND 5%M / 5%N

By comparing the fold change in expression of various genes between 3%M / 3%N and 5%M / 5%N we understand the effect of metformin on gene expression in relation to the available amount of glucose in the media. It was observed that the downregulated genes showed a high change fold in 3%M / 3%N as compared to 5%M / 5%N . For instance, *jmj4* showed a fold change of 0.032 in 3%M / 3%N as compared to 0.113 in 5%M / 5%N . Similarly, *mde5* had a fold change of 0.0476 and 0.2528 in 3%M / 3%N and 5%M / 5%N respectively.

Somewhat similar pattern of fold change was observed in upregulated genes. *Mde7* showed a fold change of 202.81 in 3%M / 3%N and 112.61 in 5%M / 5%N . *Ght7* showed a fold change of 17.68 in 3%M / 3%N and 9.01 in 5%M / 5%N .

This implies that the effect of metformin is demonstrating independence from glucose. The relationship between glucose and metformin appears to be weak. The fold change in the genes of cells grown in the 3% solution is higher compared to the 5% solution overall. This suggests that even when glucose concentration is low, metformin is still able to regulate the function of the gene.

5.4. CONCLUSION

In addition to serving as a universal energy source, cells use glucose as a signal molecule. As a consequence, it influences a variety of biological functions such cell division, proliferation, and aging. In order to better understand how the presence of glucose impacts the regulation of all genes across the genome, we performed a genome-wide analysis. Our findings showed that, as predicted, too much glucose down-regulated the expression of numerous genes involved in metabolism. Additionally, in the qualitative gene expression tab of pombase it is mentioned that many of our upregulated genes' protein level increased in presence of pyruvate in absence of glucose. From this we infer that cells when treated with metformin behave as if there are calorie restriction conditions and as if there is no glucose present. However, in mid log phase there is excess glucose in both the 3% and 5% SD media. Knowing that calorie restriction leads to longer life, this supports the argument that metformin alters gene expression regardless the presence of glucose, increasing the antiaging effects.

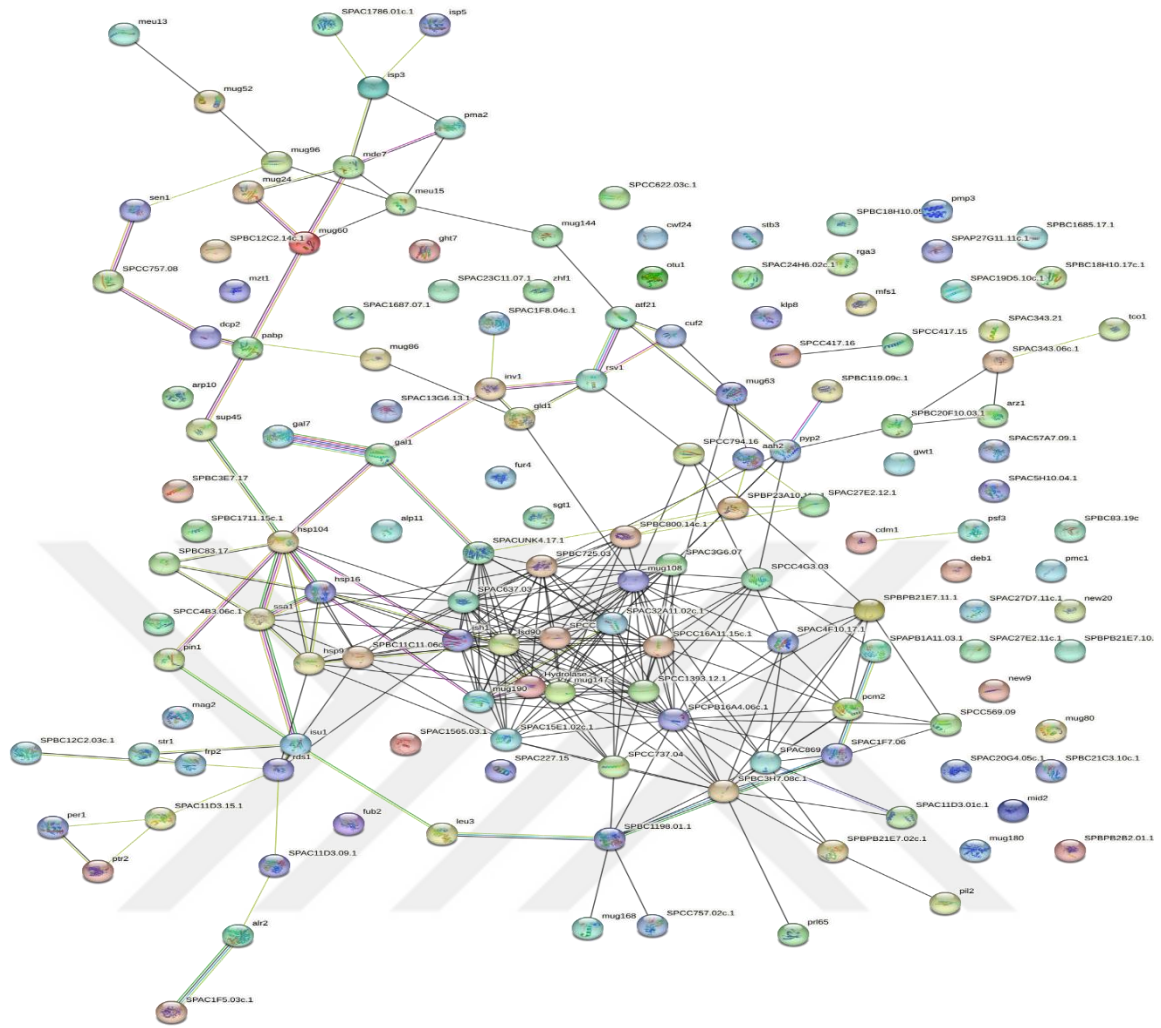


Figure 5.1: The putative interaction map of proteins that encoded by all genes whose expression were significantly changed according to glucose availability. Network was created through STRING website (ver. 11.0). Interaction sources included experiments, databases, co-expression, neighbourhood, gene fusion and co-occurrence. Minimum required interaction score was set as 0.400. Detailed explanation is given in the text.

In order to understand the potential anti-aging effects of excess glucose, we propose some complimentary roles and potential interactions of these gene expression variations. These concepts might add to the information discovered recently about the interaction between various cellular systems and glucose availability. Additionally, we noticed notable alterations in the expression of a few genes that have not yet been thoroughly studied in *S. pombe*. Future research might therefore focus on their functions in glucose metabolism and glucose-dependent cellular response. We anticipate that the extensive analysis we conducted will serve as the foundation for further research on the connection between glucose availability and aging.]

KAYNAKLAR

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ÖZGEÇMİŞ

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Doğum Yeri	
Doğum Tarihi	
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Lisans	
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Mezuniyet Yılı	Tarih girmek için tıklayın veya dokununuz.

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Enstitü Adı	Fen Bilimleri Enstitüsü
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Makale ve Bildiriler	