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RESEARCH ARTICLE

Functional Annotation of Uncharacterised Proteins Whose Expression Patterns Affect the Lifespan under Metformin Treatment in Fission Yeast

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ABSTRACT

Objective: Metformin, a well-known anti-diabetic drug and a caloric restriction mimetic, seems to attenuate aging through myriad cellular processes, wherein most of its mode of action is still elusive. Thus, bioinformatic analyses that might direct experimental studies are crucial. Moreover, uncharacterised proteins with unknown molecular functions might withhold information regarding metformin's mode of action. Here, we aimed to elucidate genes encoding uncharacterised proteins that are somehow involved in metformin metabolism and elaborate their involvement through functional annotation to reveal novel cellular processes in which metformin interferes.

Materials and Methods: Total RNA isolation was conducted from *Schizosaccharomyces pombe* wild-type cells that were grown in standard and overnutrition conditions. Following the gene expression analysis of the uncharacterised proteins, the bioinformatics analysis of the up- and down-regulated uncharacterised proteins upon metformin treatment in both was conducted using the functional annotator called PANNZER2.

Results: Genes that might be related to cellular processes such as meiosis, protein folding, calcium homeostasis, and heme production are up- and down-regulated upon metformin treatment. Moreover, the up-regulation of apoptosis and antioxidation-related genes and the down-regulation of mitosis, DNA damage, apoptosis, mitochondria, and telomere-capping-related genes were also determined.

Conclusion: We effectively identified associations between metformin and a wide range of cellular processes and genetic mechanisms through the comprehensive annotation of uncharacterised genes. Our findings are consistent with the literature, and many of these uncharacterised proteins could be used as targets for research into aging in the future.

Keywords: Metformin, Aging, Uncharacterized Proteins, *Schizosaccharomyces pombe*

INTRODUCTION

Aging is defined as a gradual deterioration of physiological integrity that diminishes the function at molecular, cellular, tissue, and systemic levels and increases the tendency of mortality. A majority of serious human pathologies, such as cancer, diabetes, cardiovascular problems, and neurological diseases are at high risk owing to this degradation.[1] Although aging is not considered a disease in itself, it is undeniable that it is the main cause of many age-related diseases.

To date, many chemicals and compounds that contribute to the healthy prolongation of the life span of various organisms have been identified. Since they contain unique properties that affect nine hallmarks of aging, one of these compounds, metformin (N,N-dimethylbiguanide), has been identified as extremely important. Thus, it became the first drug to be tested for its anti-aging effects in the large clinical trial-TAME (Targeting Aging by Metformin) study (visit https://www.afar.org/tame-trial). Since it lowers blood glucose levels, metformin has been used for nearly 65 years to treat type-2 diabetes. In addition to its anti-diabetic properties, it has also been found to be effective in the treatment of cancer, neurological diseases, and biological aging. It is also helpful for treating coronary heart disease by inducing weight loss and improving cholesterol levels.[2–4]

Metformin delays aging by regulating adenosine monophosphate-activated protein kinase (AMPK), endothelial nitric oxide synthase (eNOS)/cyclic guanosine monophosphate (cGMP) and phospho-myosin light chain kinase (p-MLCK) actin remodelling pathways, decreasing

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insulin receptor substrate 2 (IRS2) and insulin-like growth factor 1 receptor (IGF1R) in neurons, reducing the buildup of the advanced glycation end products (AGEs), reducing reactive oxygen species (ROS) levels in mitochondria, inhibiting oxidative stress, balancing protein homeostasis, and enhancing autophagy by inhibiting the target of rapamycin (mTOR) signaling pathway.[5] The anti-aging effects of metformin can be used to treat age-related diseases through regulating nutrition sensing. It is effective in treating certain hallmarks of aging, such as DNA damage, the production of ROS, telomere attrition, inflammation, cellular senescence, stem cell depletion, and autophagy.[4] In various model organisms such as mice and *Caenorhabditis elegans*, it has been demonstrated that metformin extends lifespan through interacting with myriad metabolic pathways.[6–8] In a recent study, the autophagy-inducing effect of metformin was shown to delay muscle aging in *Drosophila melanogaster* adults.[9] Şeylan and Tarhan[10] report that metformin significantly extends *Schizosaccharomyces pombe (S. pombe)*'s chronological lifespan (CLS) by mechanisms resembling those identified in mammalian cells and other model organisms. It was demonstrated that metformin increases the production of ROS, glucose uptake, and adenosine triphosphate (ATP) synthesis while decreasing oxidative stress markers such as lipid peroxidation and carbonylated proteins.[10]

Proteins must have accurate functional annotations for biological research to be successful. Unfortunately, functional characterisation or empirically confirmed annotations are absent from the great majority of protein sequences.[11] If a protein's role and relevance in cellular processes are not completely comprehended or annotated, it is said to be uncharacterised.[12] The most accurate technique to characterise proteins with unknown activity is by experimental determination of protein function, however with so many potential uses for a protein, it can be challenging to decide which functional research to prioritise. Several computer methods for protein function prediction have been developed to assist experimentalists.[13] A significant fraction of these proteins lack human analogues and may serve as a valuable source for new antibacterial drug targets.[14]

Sequence homology is a common method for predicting protein function since it assumes that proteins with similar amino acid sequences should have comparable functions. To search a database of known amino acid sequences and their functions, early methods used sequence search tools like BLAST or DIAMOND.[15,16] The main drawback of these approaches is that they are constrained by the databases they use; annotation errors may occur, and it is sometimes challenging to establish a suitable threshold for transferring protein function, leading to low specificity and sensitivity.[17] Researchers have been able to investigate machine learning algorithms that are data-driven because of the improved data availability. In the early days of function prediction, supervised machine learning models like neural networks (NNs), support vector machines (SVMs), or k-nearest neighbour (KNN) methods were employed to extract characteristics from the sequence of interest.[11,18] Multiple Gene Ontology (GO) predictors are implemented within the Protein ANNotation with Z-scoRE (PANNZER2), and they all are based on enrichment statistics of the sequence neighbourhood that the authors of the publication referred to as scoring functions.[19] Although score calculation differs from one scoring function to another, they all accept the same filtered sequence neighbourhood as an input. The authors state that the PANNZER2 uses the ARGOT scoring function by default, as it performs best. Likewise, the same filtered sequence neighbourhood is clustered according to the description similarity based on word frequencies using hierarchical clustering with average linkage for free text description prediction. The authors use a regression model to select the best cluster, and the output is the most representative description, i.e., the most frequent one within the best cluster.

The discovery of previously unidentified proteins that might be implicated in the aging process is made possible by uncharacterised protein prediction. The creation of thorough networks and pathways involved in aging is made possible by combining prediction algorithms with other high-throughput approaches, including transcriptomics and proteomics. Researchers can find proteins that might act as markers of aging or disease development by identifying uncharacterised proteins and evaluating their expression patterns during aging. These proteins may be used to identify healthy aging biomarkers. Additionally, the identification of targets for therapeutic interventions targeted at slowing down the aging process and age-related disorders can be aided by the prediction of protein function.

In the present study, we focused on uncharacterised proteins that are differentially expressed under metformin treatment in *S. pombe* and estimated their functions using the PANNZER2. In these types of studies, researchers generally focus on the expression pattern of the genes/proteins that have already been characterised, whereas this study focused on genes that have not yet been annotated and we aimed to identify new target genes that may be involved in the life-prolonging effect of metformin. These proteins play roles in many cellular processes such as meiosis, mitosis, DNA damage, protein folding, apoptosis, autophagy, antioxidative effect, mitochondrial changes, heme production, and telomere capping. These results are in line with previous studies. Moreover, most of these non-annotated proteins might serve as targets for further aging studies.

MATERIALS AND METHODS

Organism and Media

S. pombe wild-type strain 972- and Synthetic Dextrose (SD) medium was used in the study. Chen and Runge[20] report that this medium is suitable for chronological lifespan experiments as it recapitulates the evolutionarily conserved response of lifes-

pan shortening due to overnutrition for cells grown in SD with excess glucose. SD medium with 3% glucose (standard condition) and 5% glucose (overnutrition condition) were used in this study. To understand how metformin affects gene expression in these two different conditions, gene expression in cells grown in a 3% glucose medium with metformin was compared with the gene expression profile of cells grown in a 3% glucose medium without metformin (control medium). The same comparison was made for a medium containing 5% glucose. Cells from a single colony with a 5x104 cell/ml density were inoculated in 25 ml SD medium with and without 25 mM Metformin hydrochloride (SIGMA) in a 100 ml flask and orbitally shaken at 180 rpm and at a temperature of 30 °C until respective mid-log phase. Determination of the dose of metformin and its application method were given in our previous study.[10]

Total RNA Isolation

The Hibrigen Total RNA Isolation Kit was used following the manufacturer's instructions for the total RNA isolation from mid-log cells. Briefly, samples were digested and homogenized while exposed to guanidium isothiocyanate, a chaotropic salt protecting RNAs from endogenous RNases. Subsequently, we conducted ethanol precipitation to isolate nucleic acids and transferred the samples into filtered tubes that could selectively withhold RNAs. The attached RNAs were then eluted with DEPC-treated water, and total RNA purity and quantity were assessed using a Nanodrop 2000 spectrophotometer (Nanodrop Technologies, USA). For each sample, three biological replicates were used. Finally, the replicates were pooled for sequencing according to their concentrations.

Library Preparation and RNA Sequencing

Library preparation, fragmentation, adapter binding, RNA sequencing, and bioinformatic analysis of sequence data were performed by Macrogen, Inc. (Seoul, South Korea). Briefly, the contaminating DNAs were eliminated using DNase. TruSeq Stranded Total RNA LT Sample Prep Kit (Gold) was used for the library preparation. The purified RNAs were then randomly fragmented for short-read sequencing, and these fragmented RNAs were reverse transcribed into cDNA. Adapters were ligated onto both ends of the fragments, and those with insert sizes between 200 and 400 base pairs were selected after amplification with PCR. Both ends were sequenced by the read length for paired-end sequencing using the Illumina platform.

Bioinformatic Analysis of Sequence Data

Quality control of the raw sequencing data was conducted using FastQC (ver.0.11.7). Afterward, adapter sequences and bases with a base quality lower than three were removed from the ends using Trimmomatic (ver.0.38). Additionally, bases of reads that do not qualify for window size four and mean quality 15 were trimmed using the sliding window method. Finally, reads shorter than 36 base pairs were dropped to yield trimmed data. The quality of the trimmed data was checked again using FastQC. Subsequently, the trimmed reads were mapped onto the reference genome using HISAT2 (ver.2.1.0), which handles mapping through Bowtie2 (ver.2.3.4.1) aligner. Lastly, transcripts were assembled using StringTie (ver.2.1.3b). After the assembly, the gene/transcript abundance was calculated by using the FPKM (fragments per kilobase per million reads) and TPM (transcripts per kilobase million) for each sample.

Gene Expression Analysis

For the gene expression analysis, TPM values for different conditions (metformin-treated versus control) were rationed. We assumed that changes in gene expression are significant if the ratio is at least twice as high or lower than 1.5-fold for one condition versus the other, a common assumption for such analyses. Among these significantly differentially expressed genes, the uncharacterised ones that lack functional annotation in the literature were filtered, and further research was conducted.

PANNZER2 and the Analysis of the Results

PANNZER2 was used to functionally annotate uncharacterised proteins that the gene expression analysis yielded.[11] PANNZER2 accomplishes functional annotation of uncharacterised proteins by predicting GO classes and free text description lines required for new sequence submission into databases based on enrichment statistics and sequence similarity, respectively. The tool comprises three servers—a web server containing the user interface, the SANSparallel server for homology search, and the DictServer for handling metadata associated with the uncharacterised proteins. First, a sequence similarity search against the UniProtKB database (https://www.uniprot.org/) using SANSparallel is conducted. The output is a subset of sequences called a sequence neighbourhood. Next, the sequence neighbourhood is filtered following several criteria. Finally, the remaining sequences' GO annotations and free text descriptions are gathered using the DictServer.

The sequences of uncharacterised proteins were submitted in FASTA format from the web server and the batch queue option was used to download the results later. For all other parameters, default options were selected. The output is a summary table containing the sequence identifier, description predictions, and GO predictions for biological processes, molecular functions, and cellular components.Color-coded probabilities from green to red that correspond to high-confidence to low-confidence predictions are also provided. After the results were generated, we filtered uncharacterised proteins with at least a GO class or free text description line prediction. Subsequently, we exam-

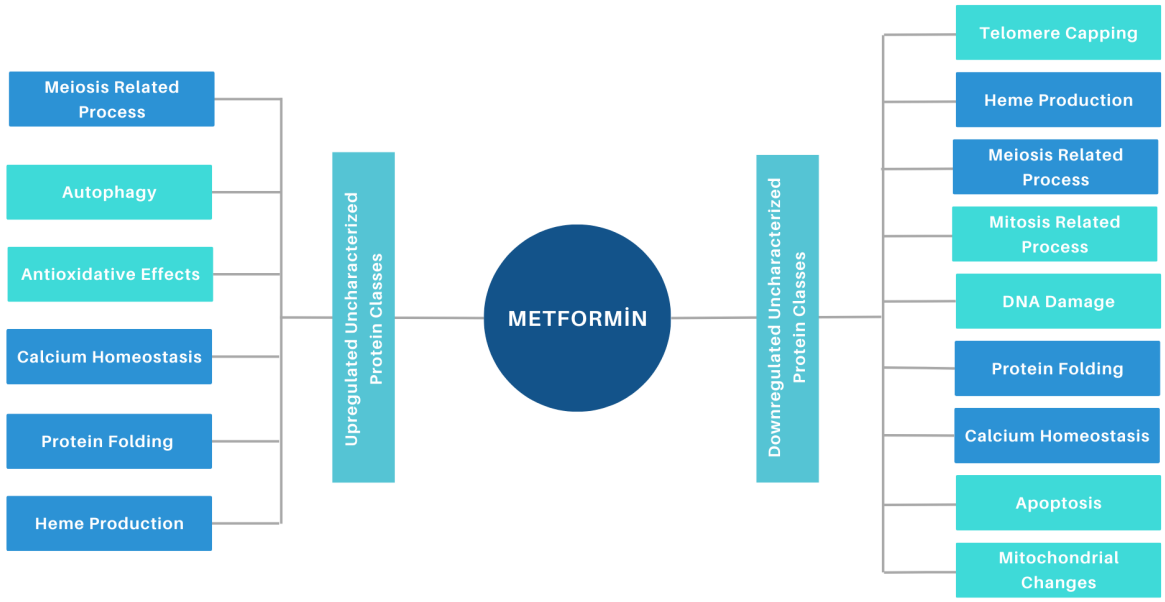

Figure 1. Metformin affects many cellular processes. Boxes with dark blue colours are common uncharacterised genes belonging to both up-regulated and down-regulated classes. Boxes with turquoise colors are uncharacterised genes that belong to only one class. Common cellular processes include meiosis, protein folding, calcium homeostasis, and heme production.

ined the filtered predictions to designate joint GO classes or descriptions, aiming to reveal crucial cellular processes involved in metformin metabolism and to have a more comprehensive perspective.

RESULTS

Based on observations, it appears that metformin treatment can impact the expression of certain genes in various ways. Our gene expression analysis yielded 561 uncharacterised proteins and PANNZER2 predicted at least a GO class or free text description line for 250 of these proteins (44%). Tables 1 and 2 outline the up-regulated and down-regulated proteins, respectively, that were predicted by PANNZER2 and provide details on their molecular and biological functions, as well as their fold changes.

It was found that there are shared up-regulated and uncharacterised genes in standard and overnutrition conditions. These genes play a crucial role in responding to iron ion starvation, as well as in regulating the meiotic cell cycle, and in metabolic processes related to lipids. They also play a role in macroautophagy, in transporting proteins, and in responding to oxidative stress. Proteins named conidiation-specific protein 6 (NP_592798.1), meiotically up-regulated gene 52 protein (NP_593587.1), and meiotically up-regulated gene 144 protein (NP_593215.2) were identified as being commonly expressed in both media. Specifically, the expression of meiotically up-regulated gene 52 protein was found to increase significantly in standard conditions, with a quantitative increase of 636 times.

PANNZER2 predicted myriad biological processes and molecular functions that the uncharacterised down-regulated proteins are involved in and possess, and some of these annotations are crucial to cell viability. We classified these proteins according to their predictions to reveal the cellular processes in which metformin interacts. The classes include meiosis, mitosis, DNA damage, protein folding, apoptosis, mitochondrial changes, heme production, and telomere capping (Figure 1).

DISCUSSION

The primary objective of our study was to illuminate the cellular and molecular repercussions of metformin by establishing associations between the expression profiles of previously uncharacterised proteins. Through the systematic classification of uncharacterised genes predicated on their altered expression patterns, we successfully delineated correlations between metformin and a myriad of cellular processes and genetic mechanisms (Figure 1). Significantly, genes that showed changes in their expression levels held particular importance due to their involvement in pivotal processes, including meiosis, protein folding, and calcium homeostasis.

While we observed alterations in the expression levels of numerous proteins during meiosis, striking ones were meiotically up-regulated genes. One of these genes, meiotically up-regulated gene 52 protein (636.37 fold change in standard condition and 5.1 fold change in overnutrition condition) is up-regulated in both conditions along with conidiation-specific protein 6 (5.46 fold change in standard condition and 5.51 fold change in overnutrition condition). Expressions of meioti-

Table 1. Up-regulated uncharacterised proteins upon metformin treatment both in (3% glucose) standard and (5% glucose) overnutrition conditions.

Query	Description	Biological Process	Molecular Function	Fold Change (3M/3C)	Fold Change (5M/5C)
NP_593587.1 SPAC17H9.18c	Meiotically up-regulated gene 52 protein	No prediction	● GO:0051321 meiotic cell cycle	636.37	5.1
NP_592798.1 SPAC11D3.01c	Conidiation-specific protein 6	No prediction	No prediction	5.46	5.51
NP_593215.2 SPAC30D11.02c	Meiotically up-regulated gene 144 protein	No prediction	● GO:0051321 meiotic cell cycle	3.61	
NP_594740.1 SPAC20G4.05c	Protein adenylyltransferase SelO, mitochondrial	0.64 GO:0016779 nucleotidyltransferase activity 0.56 GO:0005524 ATP binding 0.54 GO:0046872 metal ion binding 0.39 GO:0016209 antioxidant activity 0.36 GO:0016491 oxidoreductase activity	● GO:0018117 protein adenylylation ● GO:0045454 cell redox homeostasis ● GO:0098869 cellular oxidant detoxification ● GO:0034599 cellular response to oxidative stress	2	
NP_592791.1 SPAC1F8.02c	High affinity heme transporter	0.91 GO:0140488 heme receptor activity 0.64 GO:0020037 heme binding	● GO:1904334 heme import across plasma membrane ● GO:0010106 cellular response to iron ion starvation ● GO:0006897 endocytosis		5.2
NP_592777.1 SPAC977.05c	Velum formation-protein	0.36 GO:0008270 zinc ion binding	No prediction		4.01
NP_595084.1 SPBC660.05	WW domain-containing protein C660.05	No prediction	No prediction		3.57
NP_593689.1 SPAC4G9.07	Meiotically up-regulated gene 133 protein	No prediction	● GO:0051321 meiotic cell cycle		3.38
NP_592814.1 SPAC5H10.01	Hydro-lyase	0.62 GO:0016829 lyase activity 0.39 GO:0009975 cyclase activity	● GO:0006536 glutamate metabolic process		2.93
NP_592939.1 SPAC24H6.13	Phosphate metabolism protein 7	0.81 GO:0005227 calcium activated cation channel activity	● GO:0098655 monoatomic cation transmembrane transport		2.11
NP_587988.1 SPCC16A11.01	Plasma membrane protein	No prediction	● GO:0048017 inositol lipid-mediated signalling ● GO:0006629 lipid metabolic process		2.06
NP_594405.1 SPAC27E2.04c	Meiotically up-regulated gene 155 protein	No prediction	● GO:0051321 meiotic cell cycle		2.05
NP_596308.1 SPBC405.05	Autophagy protein 16	0.71 GO:0019776 Atg8 ligase activity	● GO:0016236 macroautophagy ● GO:0051321 meiotic cell cycle ● GO:0015031 protein transport		2.01
NP_595648.1 SPBC83.16c	Inclusion body clearance protein IML2	No prediction	● GO:0071218 cellular response to misfolded protein ● GO:1990748 cellular detoxification		2.01
NP_593616.2 SPAC25A8.03c	Protein arginine methyltransferase NDUFAF7 homolog, mitochondrial	0.85 GO:0035243 protein-arginine omega-N symmetric methyltransferase activity	● GO:0032259 methylation		1.99

Table 2. Down-regulated uncharacterised proteins upon metformin treatment both in (3% glucose) standard and (5% glucose) overnutrition conditions.

Query	Description	Biological Process	Molecular Function	Fold Change (3M/3C)	Fold Change (5M/5C)
NP_596062.1 uncharacterized protein SPBC2G5.01 [Schizosaccharomyces pombe]	0.37 UPF0674 endoplasmic reticulum membrane protein C2G5.01	0.84 GO:0032469 endoplasmic reticulum calcium ion homeostasis 0.62 GO:0045048 protein insertion into ER membrane 0.53 GO:0006457 protein folding	0.69 GO:0005509 calcium ion binding 0.56 GO:0044183 protein folding chaperone	1.484839734	1.244282751
NP_595355.1 uncharacterized protein SPBC216.03 [Schizosaccharomyces pombe]	0.34 NAD(P)-binding domain-containing protein	0.74 GO:0042167 heme catabolic process	0.41 GO:0016829 lyase activity	1.358003583	1.478227884
NP_595087.2 uncharacterized protein SPBC660.08 [Schizosaccharomyces pombe]	0.40 Meiotically up-regulated gene 167 protein	0.79 GO:0016236 macroautophagy 0.67 GO:0051321 meiotic cell cycle	0.56 GO:0005515 protein binding	1.355431673	1.430388762
NP_593856.1 uncharacterized protein SPAC7D4.03c [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.85 GO:0051403 stress-activated MAPK cascade 0.80 GO:0043410 positive regulation of MAPK cascade	0.85 GO:0005078 MAP-kinase scaffold activity	1.339964572	1.316245774
NP_587772.3 uncharacterized protein SPCC553.01c [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.85 GO:0000712 resolution of meiotic recombination intermediates 0.74 GO:0006302 double-strand break repair		1.310073372	0.900329932
NP_593156.1 uncharacterized protein SPAC821.03c [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.88 GO:1903360 protein localization to lateral cortical node 0.88 GO:1903359 lateral cortical node assembly		1.281782062	1.027685654
NP_595544.1 uncharacterized protein SPBC27B12.14 [Schizosaccharomyces pombe]	0.63 Assembly factor cbp4	0.84 GO:0017062 respiratory chain complex III assembly 0.79 GO:0033108 mitochondrial respiratory chain complex assembly		1.16944588	1.416262734
NP_587971.1 uncharacterized protein SPCC1393.13 [Schizosaccharomyces pombe]	0.65 Sugar phosphate phosphatase	0.43 GO:0006974 DNA damage response 0.43 GO:1990748 cellular detoxification	0.85 GO:0103026 fructose-1-phosphatase activity 0.84 GO:0097023 fructose 6-phosphate aldolase activity 0.54 GO:0046872 metal ion binding 0.51 GO:0004427 inorganic diphosphate phosphatase activity	1.155098296	1.409568489
NP_588026.2 uncharacterized protein SPCPB16A4.02c [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.88 GO:0031566 actomyosin contractile ring maintenance 0.85 GO:1902410 mitotic cytokinetic process 0.72 GO:1902635 1-phosphatidyl-1D-myo-inositol 4,5-bisphosphate biosynthetic process	0.83 GO:0005546 phosphatidylinositol-4,5-bisphosphate binding 0.79 GO:0140550 phosphatidylinositol-4,5-bisphosphate sensor activity 0.52 GO:0005515 protein binding	1.109540038	1.480591308

Table 2. Continued

NP_593199.1 uncharacterized protein SPAC1A6.07 [Schizosaccharomyces pombe]	0.48 Eisosome protein sle1	0.80 GO:0007009 plasma membrane organization		1.029201593	0.811563261
NP_594986.3 uncharacterized protein SPAC29B12.08 [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.86 GO:0030466 silent mating-type cassette heterochromatin formation		1.017425083	0.927726427
NP_587691.1 uncharacterized protein SPCC613.03 [Schizosaccharomyces pombe]	0.18 EF-hand domain-containing protein		0.59 GO:0005509 calcium ion binding	0.985505116	1.090550772
NP_594939.1 uncharacterized protein SPAC11E3.14 [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.77 GO:0071218 cellular response to misfolded protein 0.63 GO:1990748 cellular detoxification		0.961099066	1.10032874
NP_588137.1 uncharacterized protein SPCC1322.09 [Schizosaccharomyces pombe]	0.34 Maintenance of telomere capping protein 1			0.953135185	1.161232568
NP_588130.1 uncharacterized protein SPCC1322.02 [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.88 GO:0007534 gene conversion at mating-type locus 0.86 GO:0000729 DNA double-strand break processing 0.85 GO:0045002 double-strand break repair via single-strand annealing	0.90 GO:0140656 endodeoxyribonuclease activator activity 0.63 GO:0005515 protein binding	0.933931645	0.926896946
NP_001342866.1 uncharacterized protein SPAC1420.01c [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.87 GO:0031930 mitochondria-nucleus signaling pathway 0.78 GO:0000122 negative regulation of transcription by RNA polymerase II 0.77 GO:0006808 regulation of nitrogen utilization		0.914501233	1.073219792
NP_596723.1 uncharacterized protein SPBC1861.06c [Schizosaccharomyces pombe]	0.40 Meiotically up-regulated gene 131 protein	0.77 GO:0051321 meiotic cell cycle		0.893999889	1.15681355
NP_594513.1 uncharacterized protein SPAC2C4.10c [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.88 GO:0061509 asymmetric protein localization to old mitotic spindle pole body 0.87 GO:0031030 negative regulation of septation initiation signaling		0.889591019	0.915033714

Table 2. Continued

NP_588087.1 uncharacterized protein SPCC4B3.03c [Schizosaccharomyces pombe]	0.80 Related to MAM3 Protein required for normal mitochondrial morphology	0.83 GO:0010960 magnesium ion homeostasis 0.49 GO:0030026 intracellular manganese ion homeostasis 0.40 GO:0007005 mitochondrion organization 0.37 GO:0006914 autophagy	0.36 GO:0019787 ubiquitin-like protein transferase activity	0.88878451	1.244341597
NP_594906.2 uncharacterized protein SPAC14C4.01c [Schizosaccharomyces pombe]	0.40 Mitophagy receptor atg43	0.85 GO:0000423 mitophagy	0.86 GO:0140580 mitochondrion autophagosome adaptor activity 0.63 GO:0005515 protein binding	0.865426311	1.009510393
NP_594896.1 uncharacterized protein SPAPJ691.03 [Schizosaccharomyces pombe]	0.55 MICOS complex subunit MIC10	0.85 GO:0042407 cristae formation		0.857530553	0.8515606
NP_596357.1 uncharacterized protein SPBC25H2.09 [Schizosaccharomyces pombe]	0.36 MICOS complex subunit mic19	0.85 GO:0042407 cristae formation		0.844529172	0.456016818
NP_596816.1 uncharacterized protein SPBC1539.02 [Schizosaccharomyces pombe]	0.20 Eukaryotic nuclear protein implicated in meiotic chromosome segregation	0.71 GO:0051321 meiotic cell cycle		0.838771377	0.931903577
NP_588164.1 uncharacterized protein SPCC338.02 [Schizosaccharomyces pombe]	0.40 Meiotically up-regulated gene 112 protein	0.77 GO:0051321 meiotic cell cycle		0.82624801	1.121878485
NP_594883.1 uncharacterized protein SPAC26F1.12c [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.70 GO:0061077 chaperone-mediated protein folding	0.78 GO:0061770 translation elongation factor binding 0.46 GO:0044183 protein folding chaperone 0.45 GO:0003677 DNA binding	0.818094711	0.839081199
NP_593192.1 uncharacterized protein SPAC1A6.01c [Schizosaccharomyces pombe]	0.96 Thyroid receptor interacting protein	0.60 GO:0045893 positive regulation of DNA-templated transcription 0.54 GO:0006357 regulation of transcription by RNA polymerase II	0.65 GO:0003713 transcription coactivator activity 0.63 GO:0008270 zinc ion binding	0.810588765	0.982837431
NP_593840.1 uncharacterized protein SPAC823.13c [Schizosaccharomyces pombe]	0.61 Sensitive to high expression protein 9, mitochondrial	0.55 GO:0007007 inner mitochondrial membrane organization		0.80229592	0.870844396

Table 2. Continued

NP_596268.1 **uncharacterized protein SPBC30D10.17c [Schizosaccharomyces pombe]**	0.97 Cell wall biosynthesis/cell cycle regulator	0.68 GO:0070880 fungal-type cell wall beta-glucan biosynthetic process 0.63 GO:0140278 mitotic division septum assembly 0.49 GO:0071555 cell wall organization	0.69 GO:0030674 protein-macromolecule adaptor activity 0.43 GO:0003677 DNA binding	0.800795426	0.702029951
NP_595536.1 **uncharacterized protein SPBC27B12.04c [Schizosaccharomyces pombe]**	0.94 Eukaryotic protein implicated in cell cycle regulation	0.88 GO:0061509 asymmetric protein localization to old mitotic spindle pole body 0.87 GO:0031030 negative regulation of septation initiation signaling 0.58 GO:0007010 cytoskeleton organization 0.55 GO:0007049 cell cycle		0.769817529	0.810148092
NP_595853.1 **uncharacterized protein SPBC18E5.07 [Schizosaccharomyces pombe]**	0.0 Uncharacterized protein	0.70 GO:0007015 actin filament organization		0.753490375	0.998961254
NP_588319.1 **uncharacterized protein SPCC1442.05c [Schizosaccharomyces pombe]**	0.39 MICOS complex subunit	0.85 GO:0042407 cristae formation		0.75240903	0.414149709
NP_596443.1 **uncharacterized protein SPBC2G2.14 [Schizosaccharomyces pombe]**	0.0 Uncharacterized protein	0.90 GO:0072766 centromere clustering at the mitotic interphase nuclear envelope 0.80 GO:0000070 mitotic sister chromatid segregation	0.63 GO:0005515 protein binding	0.751819481	1.303609909
NP_593089.2 **uncharacterized protein SPAC2G11.09 [Schizosaccharomyces pombe]**	0.11 Calcium permeable stress-gated cation channel 1	0.61 GO:0098655 monoatomic cation transmembrane transport 0.59 GO:0006816 calcium ion transport 0.51 GO:0098662 inorganic cation transmembrane transport	0.81 GO:0005227 calcium activated cation channel activity 0.60 GO:0015085 calcium ion transmembrane transporter activity 0.52 GO:0003676 nucleic acid binding	0.746571084	1.159343596
NP_593280.1 **uncharacterized protein SPAC1565.01 [Schizosaccharomyces pombe]**	0.37 Respiratory supercomplex factor 2 homolog C1565.01	0.65 GO:0033617 mitochondrial cytochrome c oxidase assembly		0.723308517	0.745748355

Table 2. Continued

NP_001342840.1 uncharacterized protein SPAC11E3.12 [Schizosaccharomyces pombe]	0.11 NADH-ubiquinone oxidoreductase 24 kDa subunit homolog C11E3.12, mitochondrial	0.60 GO:0017004 cytochrome complex assembly	0.65 GO:0051537 2 iron, 2 sulfur cluster binding 0.54 GO:0046872 metal ion binding 0.52 GO:0016491 oxidoreductase activity	0.715916213	0.850030937
NP_595539.1 uncharacterized protein SPBC27B12.07 [Schizosaccharomyces pombe]	0.0 Uncharacterized protein	0.58 GO:0006560 proline metabolic process		0.704886509	0.868992993
NP_594721.1 uncharacterized protein SPAC11H11.03c [Schizosaccharomyces pombe]	0.49 Smr domain-containing protein	0.71 GO:0070481 nuclear-transcribed mRNA catabolic process, non-stop decay 0.53 GO:0006281 DNA repair	0.69 GO:0046404 ATP-dependent polydeoxyribonucleotide 5'-hydroxyl-kinase activity 0.54 GO:0004519 endonuclease activity	0.698691652	1.228533546
NP_594721.1 uncharacterized protein SPAC11H11.03c [Schizosaccharomyces pombe]	0.49 Smr domain-containing protein	0.71 GO:0070481 nuclear-transcribed mRNA catabolic process, non-stop decay 0.53 GO:0006281 DNA repair	0.69 GO:0046404 ATP-dependent polydeoxyribonucleotide 5'-hydroxyl-kinase activity 0.54 GO:0004519 endonuclease activity	0.698691652	1.228533546
NP_596045.1 uncharacterized protein SPBC365.16 [Schizosaccharomyces pombe]	0.37 Mitochondrial fission process protein 1			0.60030676	0.771552988
NP_593522.2 uncharacterized protein SPAPB1A10.08 [Schizosaccharomyces pombe]	0.67 Meiotically up-regulated protein PB1A10.08			0.550616579	0.784518803
NP_594400.1 uncharacterized protein SPAC11G7.06c [Schizosaccharomyces pombe]	0.40 Meiotically up-regulated gene 132 protein	0.77 GO:0051321 meiotic cell cycle		0.55013418	0.790413974
NP_588079.1 uncharacterized protein SPCC4B3.11c [Schizosaccharomyces pombe]	0.75 BolA-like protein 3	0.79 GO:0106035 protein maturation by [4Fe-4S] cluster transfer		0.540823457	0.643180973
NP_001343089.1 uncharacterized protein SPCC757.15 [Schizosaccharomyces pombe]	0.43 Cytochrome c oxidase assembly protein	0.64 GO:0033617 mitochondrial cytochrome c oxidase assembly		0.508254753	0.612148701

Table 2. Continued

NP_001343011.1 **uncharacterized protein SPAC8C9.19 [Schizosaccharomyces pombe]**	0.90 ERMES regulator 1	0.84 GO:0007008 outer mitochondrial membrane organization 0.61 GO:0120010 intermembrane phospholipid transfer	0.42 GO:0005515 protein binding	0.338334348	0.444875858
NP_595535.2 **uncharacterized protein SPBC27B12.02 [Schizosaccharomyces pombe]**	0.40 CENP-A recruiting complex protein mis19	0.85 GO:0051315 attachment of mitotic spindle microtubules to kinetochore 0.85 GO:0071459 protein localization to chromosome, centromeric region 0.66 GO:0051301 cell division	0.63 GO:0005515 protein binding	0.328300005	0.938710618
NP_594342.3 **uncharacterized protein SPAC4H3.06 [Schizosaccharomyces pombe]**	0.48 Meiotic recombination protein			0.314154011	0.888067929
NP_594483.1 **uncharacterized protein SPAC694.03 [Schizosaccharomyces pombe]**	0.43 nicotinamide-nucleotide adenylyltransferase	0.75 GO:0034356 NAD biosynthesis via nicotinamide riboside salvage pathway 0.61 GO:0007124 pseudohyphal growth 0.61 GO:0001403 invasive growth in response to glucose limitation 0.55 GO:0030433 ubiquitin-dependent ERAD pathway	0.83 GO:0000309 nicotinamide-nucleotide adenylyltransferase activity 0.53 GO:0016887 ATP hydrolysis activity 0.43 GO:0005524 ATP binding	0.301149026	0.719119844
NP_001018224.1 **uncharacterized protein SPAPB17E12.09 [Schizosaccharomyces pombe]**	0.67 Meiotically up-regulated protein PB17E12.09	0.77 GO:0051321 meiotic cell cycle		0	0.240788533

cally up-regulated gene 133 protein, 155 protein, and 144 (3.61 fold change in standard condition) proteins also increased in the presence of metformin. PANNZER2 predicted five down-regulated proteins among jointly down-regulated proteins in both conditions: the meiotically up-regulated genes 43, 112, 131, 132, and 167 (1.35 fold change in standard condition and 1.43 in overnutrition condition) are involved in the meiotic cell cycle. Indeed, all five have been reported to be involved in meiosis, although their exact function remains elusive.[21] Meiotic recombination protein, early meiotic induction protein 1, and meiotically up-regulated proteins PB1A10.08 and PB17E12.09 are among other proteins predicted by PANNZER2 to be involved in the meiotic cell cycle. Indeed, a study revealed that the latter two belong to a class of late genes that are stimulated during meiotic divisions and whose expression is high until the end of sporulation.[22]

Nutrition depletion, particularly nitrogen, triggers a switch from a haploid state to a diploid and initiates meiosis in fission yeast.[23] Metformin is a caloric restriction mimetic that recapitulates the beneficial effects of caloric restriction without dietary limitations.[24] Therefore, the drug might induce nutrition depletion conditions, which leads to meiosis initiation. In accordance with this, PANNZER2 predicted that another down-regulated protein (NP_001342866.1 /Mks1) is involved in nitrogen utilisation regulation. The protein shares a high sequence similarity with the Mks1 of *S. cerevisiae*, which inactivates the nitrogen uptake systems upon its under-expression, a possible mechanism for how metformin induces nutrition depletion conditions.[25] Taken together, both the up-regulation and down-regulation of meiosis-related genes suggest metformin's significant role as a calorie restriction mimetic in meiosis.

One of the down-regulated proteins among jointly down-

regulated proteins is UPF0674 endoplasmic reticulum membrane protein (1.48 fold change in standard condition and 1.24 fold change in overnutrition condition), and it is predicted to be involved in ER calcium ion homeostasis, protein insertion into ER membrane, and protein folding. It also has a calcium ion binding and protein folding chaperone activity. The protein shares a high sequence and structure similarity with the PAT complex subunit CCDC47 of *Homo sapiens*.[26] It functions as an intramembrane chaperone that maintains cellular protein. CCDC47 is also reported to regulate calcium ion homeostasis in the ER and is required for the misfolded protein degradation ER-associated degradation (ERAD) pathway.[27,28] Additionally, PANNZER2 predictions for two proteins suggest a role in calcium ion homeostasis. Indeed, the latter shares a high sequence similarity with the calcium permeable stress-gated cation channel 1 of *Homo sapiens*.[29]

ER stress triggers the unfolded protein response (UPR) which reduces unfolded proteins to maintain cell viability and functionality.[30] Conza et al.[31] demonstrated that metformin affects UPR upon ER stress in endometrial cancer cells. One of the thirteen proteins up-regulated in overnutrition conditions, inclusion body clearance protein IML2, is predicted to be involved in the cellular response to misfolded protein and cellular detoxification by PANNZER2. This protein has a significant similarity in sequence with the IML2/YJL082W protein found in *S. cerevisiae*. The latter is essential for removing inclusion bodies, and this protein is known to localise to inclusion bodies that form due to protein misfolding stress.

PANNZER2 predicted that one of the down-regulated proteins (NP_594883.1/Hgh1) is involved in the chaperone-mediated protein folding and has a translation elongation factor binding and protein folding chaperone activity. The protein shares a high sequence similarity with the Hgh1 of *S. cerevisiae,* which is a chaperone involved in the Eukaryotic elongation factor 2 (eEF2) folding.[32] Another down-regulated protein among jointly down-regulated proteins is EF-hand domain-containing protein which is predicted to be involved in the cellular response to misfolded protein and cellular detoxification. The protein shares a high sequence similarity with the inclusion body clearance protein IML2 of *S. cerevisiae*, and this is necessary for inclusion body clearance upon protein folding stress.[33] Thus, both the up-regulation and down-regulation of protein misfolding and calcium homeostasis-related genes indicate metformin's central role in such cellular processes.

Autophagy protein 16 (atg16), is predicted to be involved in the meiotic cell cycle, macroautophagy, and protein transport. Indeed, Gregan et al.[34] report that this protein is required for chromosome segregation during meiosis. PANNZER2 predicted atg16 to be a component of the phagophore (belonging to the autophagy process) assembly site. The autophagosome outer membrane fuses with the vacuole and forms the autophagic body where vacuolar hydrolases degrade cellular material and permeases release the resulting materials to be recycled in the cytosol.[35] Atg16 interacts with the atg5-atg12 conjugate through atg5, and the atg5-atg12/atg15 complex is required for the atg8 conjugation to phosphatidylethanolamine that leads to the expansion of the phagophore, and atg8 localization to the pre-autophagosomal structure.[36] Autophagy is induced through the AMPK-MTOR-ULK1-mediated signaling or SIRT1-FOXO pathway.[37,38] Metformin is known to activate both AMPK and SIRT1 and, therefore, can induce autophagy.[39]

Protein adenylyltransferase SelO (mitochondrial) is predicted to be involved in protein adenylation, cell redox homeostasis, and cellular response to oxidative stress. The probable protein transfers adenosine 5'-monophosphate (AMP) to Ser, Thr, and Tyr residues of its protein substrates involved in redox homeostasis and, therefore, regulates the cellular response to oxidative stress.[40] Metformin decreases intracellular ROS production, lipid peroxidation, and protein carbonylation in fission yeast.[10] Thus, the up-regulation of Protein adenylyltransferase SelO (mitochondrial) in fission yeast upon metformin treatment suggests that metformin's antioxidative effect might be dependent on this enzyme.

There are at least three types of cortical nodes for distinct cellular processes to take place on the nongrowing middle part of the fission yeast plasma membrane.[41] One type includes the mitotic inhibitor Skb1, a PRMT5-like methyltransferase, which interacts with Slf1 to form the node. Moreover, Skb1 nodes ensure correct cell cycle progression by sequestering Skb1. PANNZER2 predicted that Slf1 is involved in protein localisation to the lateral cortical node assembly. Consequently, Slf1 down-regulation upon metformin treatment might have reduced the cortical node number, which ultimately leads to suppressed mitosis through the freed Skb1. PANNZER2 predicted that another down-regulated protein among jointly down-regulated proteins (NP_596443.1/csi1) is involved in centromere clustering at the mitotic interphase nuclear envelope and mitotic sister chromatid segregation. Indeed, it is reported that csi1 regulates chromosome segregation by positioning the centromeres at the spindle pole body during the interphase and organising the bipolar spindle.[42,43] Another type of cortical node is eisosomes, which regulate phosphatidylinositol (4,5)-bisphosphate levels.[41] PANNZER2 predicted that another protein (NP_588026.2/Opy1) among jointly down-regulated proteins is involved in actomyosin contractile ring maintenance, mitotic cytokinetic process, and 1-phosphatidyl-1D-myo-inositol 4,5-bisphosphate biosynthetic process, and it has a role in phosphatidylinositol metabolism. The precursor of phosphatidylinositol 3,4,5-trisphosphate and actin polymerization regulator phosphatidylinositol 4,5-bisphosphate has a vital role in insulin-stimulated glucose transport.[44] Metformin increases glucose uptake in peripheral tissues, possibly by directly binding to the lipid phosphatase Src homology 2 domain-containing inositol-5-phosphatase 2 (SHIP2).[45] SHIP2 is up-regulated in diabetic rodent models, which leads to insulin resis-

tance and diminished glucose uptake. The down-regulation of Opy1 might be another possible mechanism for how metformin increases glucose uptake as Opy1 binds phosphatidylinositol 4,5-bisphosphate, which leads to reduced cellular amounts of phosphatidylinositol 4,5-bisphosphate.

PANNZER2 predicted that one of the down-regulated proteins (NP_587772.3/Dbl2) is involved in the resolution of meiotic recombination intermediates and double-strand break repair. Indeed, dbl2 gene deletion leads to the failure of homolog chromosome segregation to opposite poles due to DNA double-strand break repair intermediates during meiosis in fission yeast as it is required for Fbh1 DNA helicase foci formation at the DNA double-strand break repair sites that process these intermediates.[46] Another down-regulated protein described as sugar phosphate phosphatase is predicted to be involved in the DNA damage response (DDR) and cellular detoxification. The protein shares a high sequence similarity with the damage-control phosphatase YMR027W of *S.cerevisiae*, according to the UniProt database. Damage-control phosphatase YMR027W is a metal-dependent phosphatase, and its substrates are fructose-1-phosphate and fructose-6-phosphate.[47] The enzyme favors fructose-1-phosphate, which is a strong glycating agent that causes DNA damage, indicating a protective function against such phospho-metabolites in hexose phosphate metabolism.

One of the down-regulated proteins (NP_593856.1, 1.34 fold change in standard condition and 1.32 fold change in overnutrition condition) is involved in the stress-activated mitogen-activated protein kinase (MAPK) cascade. The protein shares a high sequence similarity with the AHK1 of *S. cerevisiae*, according to the PomBase database. Osmotic stress triggers the Hog1 MAPK, which regulates myriad adaptive responses to such stimuli.[48] Moreover, Hkr1 is a putative osmotic sensor of one of the Hog1 upstream pathways called HKR1. Ahk1 binds to the cytoplasmic regulatory domain of Hkr1 (an osmotic sensor), and AHK1 gene deletion partially inhibits osmotic stress-induced Hog1 activation, suggesting that it serves as a scaffold protein. MAPKs can act as apoptosis activators or inhibitors, depending on the cell type and stimulus.[49] Proline dehydrogenase/proline oxidase (PRODH/POX) is a mitochondrial enzyme that degrades proline, producing ROS that induce apoptosis.[50] Metformin increases the expressions of PRODH/POX and AMPK, which also activates PRODH/POX leading to apoptosis. PANNZER2 predicted that another down-regulated protein (NP_595539.1) is involved in the proline metabolic process. The protein shares a high sequence similarity with the PUT7 of *S. cerevisiae*, which acts as a negative regulator of mitochondrial proline uptake.[51] Therefore, its down-regulation upon metformin treatment may lead to increased proline concentration in the mitochondria and subsequent ROS production through PRODH/POX activity and apoptosis.

Down-regulated in overnutrition and normal conditions, cytochrome c oxidase assembly protein COX is predicted to be involved in mitochondrial cytochrome c oxidase assembly by PANNZER2. Since COX is a protein that is entrenched in the mitochondrial membrane, its down-regulation may either be an early apoptotic signaling event or a late effect of apoptotic signaling.[52] HeLa cells were initially exposed to various respiratory chain complex inhibitors for 24 hours before being exposed to hydrogen peroxide for the same amount of time. Here, respiratory complex IV (COX) inactivation significantly increased the susceptibility of cells to treatment with hydrogen peroxide. The same study conclusively demonstrates that COX inhibition accelerates mitochondrial apoptotic response to oxidative stress.[53] This appears to be one of the countless theories explaining how metformin's impacts on energy metabolism prolong life.

Another down-regulated protein, assembly factor cbp4 (1.17 fold change in standard condition and 1.42 fold change in overnutrition condition) is involved in the respiratory chain complex III assembly and mitochondrial respiratory chain complex assembly. The protein shares a high sequence similarity with the assembly factor CBP4 of *S. cerevisiae*, which is essential for the assembly of ubiquinol-cytochrome c reductase with a direct effect on its subunits'.[54] One of the mechanisms by which metformin exerts its anti-aging effects is by selectively inhibiting respiratory chain complex I, consequently causing oxidative phosphorylation. This leads to AMP/ATP and NAD^+/NADH ratio increment that activates AMPK and upregulates SIRT1.[55] The down-regulation of Cbp4 suggests that the drug interferes with oxidative phosphorylation in different stages of the process.

Metformin disrupts the cristae and inner mitochondrial membrane and induces mitochondrial swelling by causing ER stress and subsequently increased calcium influx into the mitochondria.[56] The three down-regulated proteins, MICOS complex subunit MIC10, mic19, and Mic23/26/27 are involved in cristae formation, suggesting a possible mechanism for metformin to disrupt cristae and induce mitochondrial dysfunction. The down-regulated and sensitive to high expression protein 9 (mitochondrial), is also involved in inner mitochondrial membrane organization. The protein shares a high sequence similarity with the sensitive to high expression protein 9 (mitochondrial) (Mdm33) of *S. cerevisiae*. Its overexpression leads to growth arrest, mitochondria aggregation, and unusual inner membrane structure generation, including loss of inner membrane cristae.[57] Related to the MAM3 Protein required for normal mitochondrial morphology this protein was also down-regulated under metformin treatment. The down-regulated ERMES regulator 1 is predicted to be involved in outer mitochondrial membrane organisation and intermembrane phospholipid transfer. In yeasts, the ER-mitochondria encounter structure (ERMES) complex plays an important role in mediating the formation of ER-mitochondria contact sites.[58,59] In addition to lipid transport, the ERMES complex regulates mitochondrial

fission, mtDNA inheritance, and mitophagy.[60,61] A study reports that the absence of Emr1 leads to abnormal mitochondrial morphology and that Emr1 regulates the number of ERMES foci.[62] The down-regulation of these proteins suggests that metformin interferes with energy metabolism not only through oxidative phosphorylation but also by disrupting mitochondrial structure.

PANNZER2 predicted that one down-regulated protein, mitophagy receptor atg43, is involved in mitophagy and has a mitochondrion autophagosome adaptor and protein binding activity. Indeed, atg43, a mitochondrial outer membrane protein, acts as a mitophagy receptor for selective mitochondria degradation by tethering Atg8 to mitochondria via an Atg8-family-interacting motif.[63] However, it is known that mitophagy contributes to mitochondrial function maintenance, and metformin induces mitophagy.[63,64] Another down-regulated protein Fis1 (mitochondrial fission process protein 1), may influence mitochondrial dynamics by inducing mitochondrial fission through interactions with the enzyme Drp1 or by preventing mitochondrial fusion through inhibition of Mfn2/Opa1. By bringing TBC1D15/17 and Syntaxin17 to the mitochondria, Fis1 takes part in mitophagy. Fascinatingly, Fis1 overexpression may play pathogenic roles in Parkinson's disease and diabetes mellitus, most likely through up-regulating mitochondrial fission and mitophagy. In light of this information, it is quite logical that metformin which is used in Diabetes Mellitus treatment down-regulates the Fis1 gene.[65,66]

Another down-regulated protein among jointly down-regulated proteins is the maintenance of telomere capping protein 1. Telomere attrition is one of the nine hallmarks of aging, and severe telomere uncapping can result from shelterin component deficiencies.[1] Shelterin is a specialized nucleoprotein complex that attracts DNA repair machinery to damaged telomeres through its formation. Metformin treatment prevented telomere attrition in male offspring of mothers with gestational diabetes, suggesting its beneficial effect against telomere attrition.[67]

CONCLUSION

This research concentrated on unannotated genes, aiming to pinpoint novel target genes potentially associated with the life-extending properties of metformin. These proteins are implicated in various cellular processes, including meiosis, mitosis, DNA damage response, protein folding, apoptosis, autophagy, antioxidative effects, mitochondrial changes, heme production, and telomere capping. Many of these unannotated proteins could serve as promising targets for future investigations into aging.

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