



Proteomic Analysis of Red Ginseng on Prolonging the Life Span of Male *Drosophila melanogaster*

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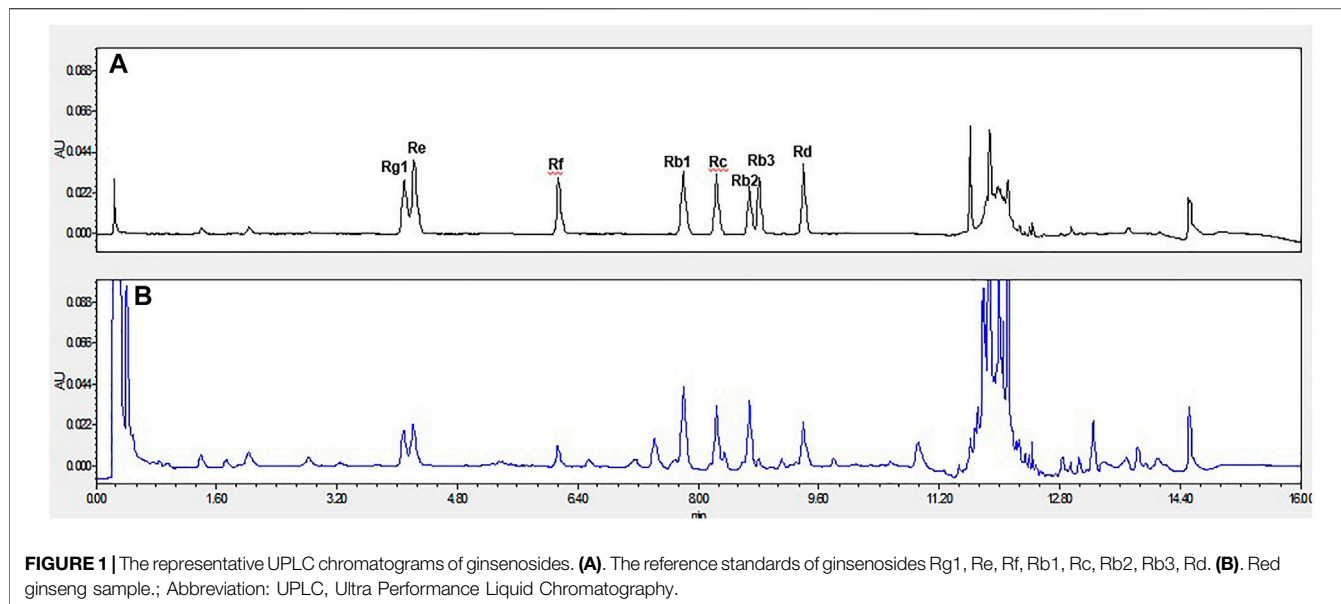
Ginseng (*Panax ginseng* C. A. Mey.) is a traditional medicine that has been utilized for over 2000 years in Asia and shows varied pharmacological effects. Red ginseng (RG) is steamed and dried ginseng root and is considered to be more effective. Heating inactivates its catabolic enzymes and increases the activities of RG, which can improve the immune system, alleviate fatigue, and has anti-inflammatory effects and antioxidant activity. In addition, RG has a good anti-aging effect, but its mechanism is unclear. Senescence, a side-effect of normal developmental and metabolic processes, is a gradual decline in physiological integrity and function of the body. Senescence is usually associated with a variety of diseases, including neurodegenerative diseases and diabetes. Research on anti-aging and the prolongation of life span has always been a focus topic. In this study, we investigated the molecular mechanism of RG that results in prolonged the life span for male *Drosophila melanogaster*. Isobaric tag for relative and absolute quantitation (iTRAQ) was used to identify protein changes in an old male *D. melanogaster* treated with RG. The differential proteins were verified by qRT-PCR and western blotting. The results showed that 12.5 mg/ml RG prolonged its life span significantly. iTRAQ results showed that, compared to the control group, 32 upregulated proteins and 62 downregulated proteins displayed significantly differential expression in the RG group. In this study, we explored the pathways that RG may participate in that extend the life span of *D. melanogaster*, and the results showed that the PI3K/AKT/FoxO pathway was involved. In addition, 4E-BP increased and participated in the regulation of life span.

Keywords: red ginseng, anti-Aging, isobaric tag for relative and absolute quantitation, proteome, drosophila

INTRODUCTION

Senescence is a gradual decline in the physiological integrity and function of the body, including molecules, cells, tissue structure, and function, as well as homeostasis (He and Jasper, 2014). Senescence is usually associated with various diseases, such as neurodegenerative diseases, diabetes, cardiovascular diseases, and cerebrovascular diseases. Research on anti-aging and the prolongation of life span has always been a point of focus.

Ginseng (*Panax ginseng* C. A. Mey.) is a traditional medicine that has been utilized for over 2000 years in Asia and has varied pharmacological effects (Wisniewski et al., 2009; Wu et al., 2017). The ancient Chinese Meteria Medica *ShenNong BenCao Jing* recorded that ginseng could be taken for



a long time and prolong life. Modern pharmacological studies have shown that ginseng extract enhances the activity of superoxide dismutase in aged rats (Ramesh et al., 2012). In addition, ginseng has been shown to be cardioprotective because of its antioxidative, anti-arrhythmic, and calcium channel-antagonistic activities (Yuan 2015). Red ginseng (RG) is steamed and dried ginseng root, which is considered more effective. Heating inactivates its catabolic enzymes and increases the activities of RG, which can improve the immune system and alleviate fatigue and has anti-inflammatory effects and antioxidant activity; it allows effective coping with the metabolic dysfunction of senescent cells and functional decline (Wu et al., 2017; Ham et al., 2019). Furthermore, RG can protect the brain and spinal cord from neurodegeneration and extend the life span of *Drosophila melanogaster* (Wisniewski et al., 2009; Kim 2013; Liu et al., 2018).

D. melanogaster is an excellent model for research on senescence due to its short life span and easy maintenance (Allocca et al., 2018). In our previous experiments, we fed *D. melanogaster* RG, and the results showed that RG extended the life span of *D. melanogaster* in both males and females (Liu et al., 2018; Hou et al., 2020). However, the mechanism by which RG extends the life span of male *D. melanogaster* has not been elucidated. In this study, an isobaric tag for relative and absolute quantitation (iTRAQ) was used to identify protein changes in senile male *D. melanogaster* treated with RG and is the first study to reveal the changes in signaling pathways.

MATERIALS AND METHODS

Materials

Red ginseng (6 years) was purchased from Changchun City (Jilin Province, China) and pulverized into a powder. The panaxoside content was determined by high-performance liquid

chromatography method (Zhang et al., 2018), and the contents were (all in mg/g) Re 1.470, Rg1 1.836, Rf 1.01, Rb1 5.21, Rc 4.447, Rb2 3.211, Rb3 0.317, and Rd 4.453 (Figure 1).

Red Ginseng Prolongs the Life Span of Male *D. melanogaster*

D. melanogaster (wild type) were a gift from Jilin Agricultural University (Changchun, China). All male *D. melanogaster* were housed in an artificial climate incubator at 25°C and 60% humidity with 12 h alternating dark and light phases. New eclosion *D. melanogaster* were separated by sex and males were subsequently divided into six groups ($n = 60$ each). *D. melanogaster* of the control group were fed a basic diet of water, agar, corn extract, sucrose, extra yeast powder, and propionic acid, and *D. melanogaster* of the RG group were fed basic diet supplemented with RG at a final concentration of 10.0, 12.5, 15.0, 17.5, or 20.0 mg/ml. The food was changed every 2–3 days. Survival was recorded at 8:00 AM for both the control and RG groups. *D. melanogaster* that died unnaturally were eliminated (accidental death), and the *D. melanogaster* that died naturally were removed from the cage. The test was repeated three times. All animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China, and the study was approved by The Animal Administration and Ethics Committee of Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences.

Climbing Ability Test

The male *D. melanogaster* of the control and RG groups were tested at the age of 36 days. The test was repeated three times. The locomotor activity of *D. melanogaster* was measured by climbing ability. *D. melanogaster* were placed into culture tubes (Diameter, 2.5 cm; Height, 12 cm). *D. melanogaster* was tapped down to the

bottom of tube and then the maximum height that the *D. melanogaster* climbed up the tube was measured over 15 s (Iliadi and Boulianne 2010; Minois et al., 2001).

Protein Preparation

The male *D. melanogaster* of the control and RG group were anesthetized at the age of 36 days. Liquid nitrogen was added to the samples (The male *D. melanogaster* body) and ground into a fine powder. Then, the powder was dissolved in SDT buffer (4% sodium dodecyl sulfate, 0.1 M; dithiothreitol, 100 mM; Tris-HCl, pH 7.6). The protein content was tested using a BCA protein assay (Thermo Fisher Scientific, United States). Protein digestion was performed according to the filter-aided proteome preparation procedure (Wisniewski et al., 2009). The peptides were desalted on MILI-SPE Extraction disk cartridge (C18-SD), lyophilized, and 40 μ L dissolution buffer was added.

Isobaric Tag for Relative and Absolute Quantitation Labeling

Each peptide mixture (100 μ g) was labeled with the iTRAQ reagent-8 plex Multiplex Kit (AB SCIEX United Kingdom, Limited, United Kingdom). The peptide mixture of control-male-1 was labeled as a 113 tag. Control-male-2 was labeled as a 114 tag, and control-male-3 was labeled as a 115 tag. RG-male-1 was labeled as a 116 tag, RG-male-2 as a 117 tag, and RG-male-3 as a 118 tag.

LC-MS/MS Analysis

Each peptide mixture was analyzed by nano LC-MS/MS coupled to an EASY nLC (Thermo Fisher Scientific). The sample was loaded into the column (Thermo Scientific Acclaim PepMap100, 100 μ m \times 2 cm, nanoViper C18) by an automatic sampler and connected to an analytical column (Thermo Scientific EASY Column, 10 cm, ID75 μ m, 3 μ m, C18-A2) in buffer A (0.1% formic acid) and buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific). The mass spectrometer was detected in positive ion mode, and the precursor ions scanning range was 300–1,800 m/z. The automatic gain control (AGC) target was set to 1e6 and maximum inject time (IT) to 50 ms. Survey scans were acquired at a resolution of 70,000 at m/z 200 and the resolution for HCD spectra was set to 17,500 at m/z 200, and an isolation width of 2 m/z. Normalized collision energy was 30 eV, and the underfill ratio was defined as 0.1% (Liu et al., 2017; Yu et al., 2017). Raw mass spectrometry data were submitted to China National Genomics Data Center (CNCB-NGDC, <https://bigd.big.ac.cn/omix/>) BioProject accession number under PRJCA004725.

Proteomic Analysis

The mass spectrometry data were noted and quantified using the MASCOT engine (version 2.2; Matrix Science, London, United Kingdom) and Proteome Discoverer 1.4 (Thermo Fisher Scientific). The selected database was UniProt *D. melanogaster* 42524 20180327. fasta. The following options were used to identify proteins: peptide mass tolerance = \pm 20 ppm;

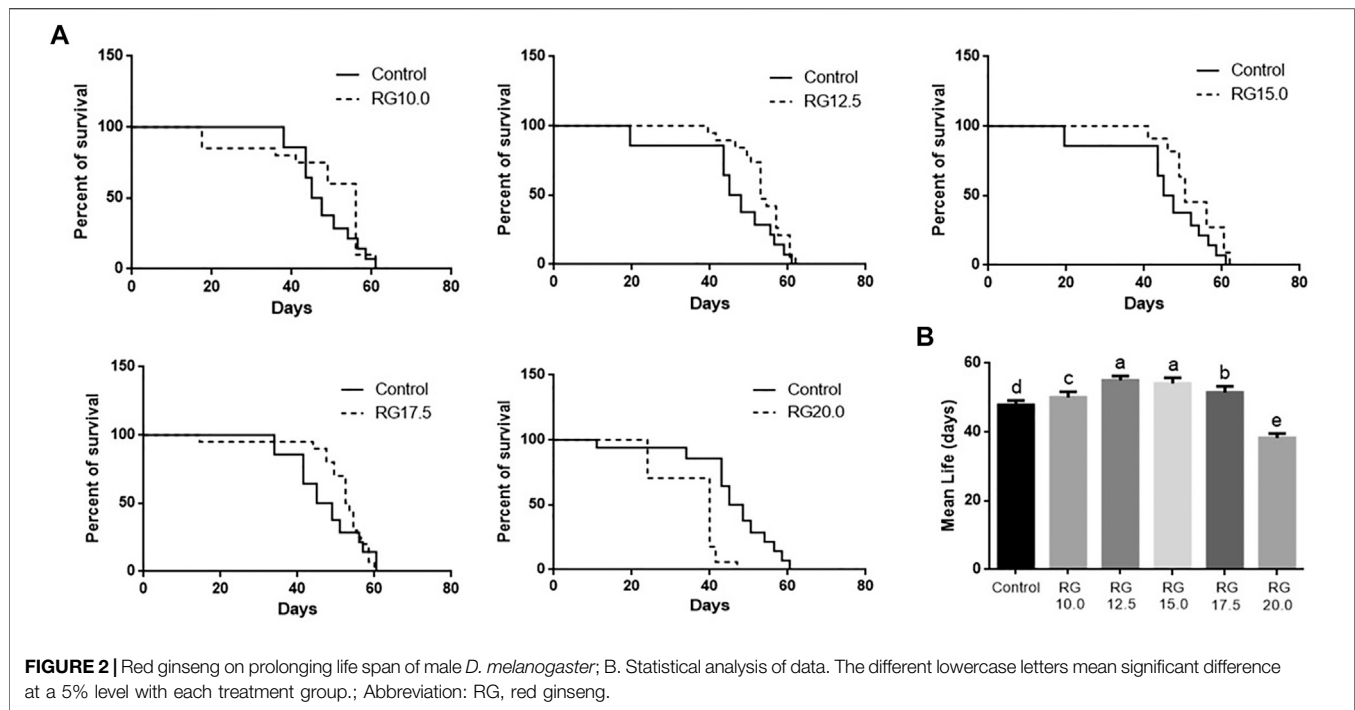
TABLE 1 | Primer sequences.

| <Gene | Primer sequences |
|---------------|---|
| GAPDH | F 5' CCTATGACGAAATCAAGGCTAA 3' R 5' GCTGAAGAAGTCCGGTGGAGA 3' |
| CG9286 | F 5' TGCCAGGGAATCTCTCAA 3' R 5' GCACAGAGGTGATTCCAAAGAT 3' |
| Vir-1 | F 5' CGACTGGCGACTCTGATGAT 3' R 5' AATGGTGGAAAGTGGTGTGG 3' |
| CG10472 | F 5' TTCGGTCAAGAACCTGAACATC 3' R 5' TTGGGCTCGGCAATCTGT 3' |
| Rheb | F 5' AAAACTGCTCGACGTAATGGG 3' R 5' CCCACGGACTCGTTCTGTTT 3' |
| Ent2 | F 5' GAGTTACGCGACCCATTTC A 3' R 5' AGGTCGCGCCAAAGTT 3' |
| ND-MLRQ | F 5' TCACTTGGAAATCGCACATCA 3' R 5' TAGTTTGGAAATAATCCCTCACAG 3' |
| Rab4 | F 5' GATCGTACTATCGCGGAGCA 3' R 5' GCATCATTACGCCAGTTTGTG 3' |
| Path | F 5' ACAATCCGCATCCAACAACC 3' R 5' GCAATTCGCGAAAGGTCAT 3' |
| Muskelin | F 5' GCCAGCAGGGCTATTTGAGT 3' R 5' TTGCCAGGCTTTATGTGCG 3' |
| FK506-bp2 | F 5' AAAAGGTCACGGTCCACTACAC 3' R 5' AACTGGGCAACTCCCTCATC 3' |
| Akh | F 5' GAATCCCAAGAGCGAAGTCC 3' R 5' CCCTGCTGTGTCTCGAAAAA 3' |
| Hgo | F 5' TTCGATGATCGGGATGTAAG 3' R 5' GGACAAGTGAGCACGGTAAAA 3' |
| CASK | F 5' GGACATCGGTGCGAATGAGTA 3' R 5' GCTGCCGTGTAATCTGCTAT 3' |
| CG31674 | F 5' GGTGGTGGGACATACTCAA 3' R 5' GTAAGATCGGCTACCGCAAC 3' |
| Lpin | F 5' TGGAGCGTCGCAACCTAA 3' R 5' GGCTTCTCTCGCCCTGA 3' |
| BcDNA:RH44935 | F 5' GTGGCTACAAGTGCCTGAAT 3' R 5' GTACTTTGGCCATTTCCACCTC 3' |
| Trp | F 5' AGGGCACGGACAAGTTCAA 3' R 5' TATGCTCCAGCAGGATCAC 3' |
| Hppy | F 5' CACAGTCACCACAATGCCAATA 3' R 5' GGTTCCCGAGCCAAATCTTT 3' |
| Chchd2 | F 5' TCATGGATTGACCTCGTGT 3' R 5' TGGAGATGGTGGCTCTGCTT 3' |
| DmelCG5510 | F 5' CTGTTCCGCGACGGATTT 3' R 5' GCTGAGATATGGATGTTGGTGG 3' |

fragment mass tolerance = 0.1 Da; enzyme = trypsin; max missed cleavages = 2; fixed modification: carbamidomethyl (C), iTRAQ4/8plex (N-term), iTRAQ4/8plex (K); and variable modification: oxidation (M), iTRAQ4/8plex (Y); database pattern = decoy. The selection criteria for differential proteins were set as fold-change with a comparison >1.2 or <0.83 , combined with an unadjusted $p < 0.05$. The Blast2GO program (<https://www.blast2go.com/>) was used to annotate the functions of the differentially expressed proteins. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to analyze the pathway enrichment of significant proteins (Liu et al., 2017; Yu et al., 2017).

qRT-PCR

The identified differentially expressed proteins were examined at the transcriptional level by qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Proteintech,



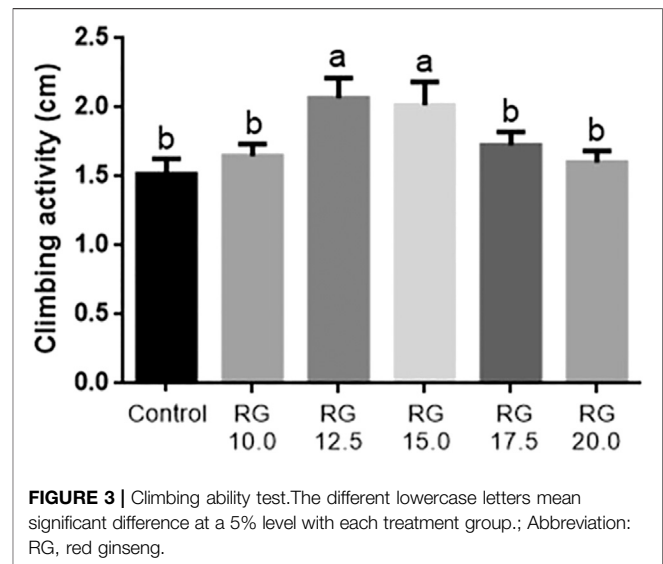
United States) was used as an internal reference. *D. melanogaster* from the control and RG group were euthanized at the age of 36 days. The test was repeated three times. Total RNA was extracted using the Total RNA Extraction Kit (UNIQ-10 Trizol, SK1321, Shanghai, China), and cDNA was synthesized using the Thermo Fisher Scientific cDNA Synthesis kit (EP0733). The $2^{-\Delta\Delta CT}$ method was used to analyze RNA expression. Primer sequences for qRT-PCR are shown in **Table 1**.

Western Blotting

The significant proteins were validated using western blotting. Male *D. melanogaster* from the control and RG group were euthanized at the age of 36 days. The test was repeated three times. Total protein was extracted from *D. melanogaster* with lysis buffer (Beyotime, Haimen, China) and Bullet Blender (NY, United States). Protein concentration was tested using the BCA protein assay reagent. GAPDH was used as the loading control. Samples were separated by 12% SDS-PAGE (Bio-Rad, Hercules, CA, United States) and transferred to polyvinylidene difluoride membranes (Millipore, United States). Membranes were blocked with 5% w/v nonfat dry milk (BD Biosciences, United States) and incubated with primary antibodies at 4°C overnight. The membranes were washed with Tris-buffered saline-Tween and incubated with horseradish peroxidase-labeled secondary antibodies (Proteintech, United States) for 1.5 h at 25°C. Finally, the enhanced chemiluminescence kit (GE Healthcare, United States) was used to visualize the immunobands. The protein bands were scanned using an imaging densitometer.

Statistical Analyses

SAS software (version 9.2) was used for statistical analysis. Fisher's LSD (least significant difference) was used for the



analysis of life span and climbing ability test data. The data of qRT-PCR and Western blotting were analyzed using *t*-test. A *p* value of less than 0.05 was considered statistically significant.

RESULT

Red Ginseng on Prolongs the Life Span of Male *D. melanogaster*

The male *D. melanogaster* in the RG group were fed RG at concentrations of 10.0, 12.5, 15.0, 17.5, or 20.0 mg/ml. The life

TABLE 2 | Identification of differential proteins in male *D. melanogaster*.

| Entry | Protein ID | Gene name | RG/Control | p | Change |
|-------|------------|--------------------|------------|-------------|--------|
| 1 | Q6IJ59 | HDC15811 | 1.735026 | 0.031516984 | Up |
| 2 | Q2XYE0 | CG8343 | 1.480231 | 5.98782E-05 | Up |
| 3 | Q4V509 | CG10445 | 1.406139 | 0.049010132 | Up |
| 4 | Q27279 | Su(fu) | 1.383591 | 0.010438172 | Up |
| 5 | Q9VFR0 | CG9286 | 1.359872 | 0.003587151 | Up |
| 6 | Q7JYV3 | CG12374 | 1.355848 | 0.020600274 | Up |
| 7 | Q960S8 | Unr | 1.34977 | 0.021517929 | Up |
| 8 | Q9VNI8 | Hpr1 | 1.316567 | 0.025014078 | Up |
| 9 | Q4V5T1 | CG14661 | 1.300725 | 0.032815710 | Up |
| 10 | Q8MRN5 | CG5839 | 1.296939 | 0.005629237 | Up |
| 11 | Q8SZ36 | Vir-1 | 1.293305 | 0.026735257 | Up |
| 12 | Q86NQ5 | CG8773 | 1.289529 | 0.013560964 | Up |
| 13 | Q8MR67 | CG10472 | 1.278358 | 0.028262384 | Up |
| 14 | A0A0B4KFH1 | Muskelin | 1.276112 | 0.046799449 | Up |
| 15 | Q95TG5 | Svil | 1.264427 | 0.016375094 | Up |
| 16 | Q9GQR6 | Stai | 1.245608 | 0.039443523 | Up |
| 17 | Q9VND8 | Rheb | 1.245072 | 0.014024349 | Up |
| 18 | Q7JZN0 | Sec61beta | 1.243214 | 0.025590847 | Up |
| 19 | A1Z6I7 | BubR1 | 1.242469 | 0.020906239 | Up |
| 20 | Q9VEH4 | Dmel\CG14325 | 1.238543 | 0.010173205 | Up |
| 21 | A8JV30 | Dmel\CG34327 | 1.233242 | 0.005286876 | Up |
| 22 | Q9VMB6 | Ent2 | 1.226665 | 0.01309915 | Up |
| 23 | Q8SYJ2 | ND-MLRQ | 1.223182 | 0.025171248 | Up |
| 24 | Q9VYY3 | Uba5 | 1.222387 | 0.001308629 | Up |
| 25 | Q9VIU8 | Dmel\CG10132 | 1.217911 | 0.016758497 | Up |
| 26 | Q7KY04 | Rab4 | 1.212045 | 0.029160556 | Up |
| 27 | Q8SYU2 | RpL7-like | 1.211114 | 0.031963084 | Up |
| 28 | M9WDW1 | Lds-RA | 1.210729 | 0.007067795 | Up |
| 29 | Q9VT04 | Path | 1.207497 | 0.012074239 | Up |
| 30 | Q9VNX8 | CG7414 | 1.205446 | 0.00520136 | Up |
| 31 | P40301 | Prosalpha2 | 1.201562 | 0.026267051 | Up |
| 32 | Q8MS69 | Dmel\CG9596 | 1.201365 | 0.043806106 | Up |
| 33 | A8YPP4 | CG30296 | 0.832942 | 0.015104015 | Down |
| 34 | Q9VS47 | Anon-WO0118547.349 | 0.83233 | 0.037428812 | Down |
| 35 | Q4V5Y8 | CG13309 | 0.83139 | 0.00628201 | Down |
| 36 | Q9VSN0 | Zasp66 | 0.829811 | 0.011645056 | Down |
| 37 | Q29QY4 | | 0.829644 | 0.023284602 | Down |
| 38 | Q9VLP2 | Dmel\CG7781 | 0.823622 | 0.023643643 | Down |
| 39 | P48375 | FK506-bp2 | 0.823203 | 0.013030252 | Down |
| 40 | Q9VND4 | Dmel\CG14671 | 0.813542 | 0.032412725 | Down |
| 41 | P61855 | Akh | 0.813533 | 0.000702961 | Down |
| 42 | B3DNM3 | | 0.813445 | 0.023169705 | Down |
| 43 | Q8MR80 | EG:BACR42117.2 | 0.811568 | 0.033391311 | Down |
| 44 | A0A0B4JCT6 | Kank | 0.810175 | 0.039877023 | Down |
| 45 | G2J5Y4 | CG8446-RE | 0.810138 | 0.000136072 | Down |
| 46 | Q9VKJ0 | Hgo | 0.8088 | 0.002827784 | Down |
| 47 | Q9U5V9 | Su(p) | 0.804879 | 0.040446674 | Down |
| 48 | Q24407 | ATPsynCf6 | 0.804015 | 0.007844585 | Down |
| 49 | O61345 | Peng | 0.803825 | 0.020630303 | Down |
| 50 | Q9VZ34 | Dmel\CG2076 | 0.803381 | 0.048727204 | Down |
| 51 | O76877 | BcDNA:LD03613 | 0.803176 | 0.032438469 | Down |
| 52 | Q24210 | CASK | 0.80112 | 0.041871672 | Down |
| 53 | Q6AWS3 | CG31674 | 0.798807 | 0.026233971 | Down |
| 54 | Q709R6 | Bocks | 0.798735 | 0.02187602 | Down |
| 55 | Q7JRA7 | CG15096 | 0.793707 | 0.039789077 | Down |
| 56 | Q9W078 | Cpr62Bb | 0.790797 | 0.033351008 | Down |
| 57 | Q8SZM0 | Cpr92F | 0.789962 | 0.000668051 | Down |
| 58 | K7ZE33 | PUG | 0.781302 | 0.002310275 | Down |
| 59 | Q9VSN3 | Cpr66D | 0.781155 | 0.000250163 | Down |
| 60 | Q7K5J8 | Cpr57A | 0.778778 | 0.031569703 | Down |
| 61 | Q8SZA8 | Fdx2 | 0.772951 | 0.000578374 | Down |
| 62 | Q9VFX3 | BcDNA:RE53127 | 0.772918 | 0.041415202 | Down |
| 63 | E5DK16 | Lpin | 0.764213 | 0.026655127 | Down |
| 64 | A0A0B4K6X5 | Dmel\CG43093 | 0.763455 | 0.007419164 | Down |

(Continued on following page)

TABLE 2 | (Continued) Identification of differential proteins in male *D. melanogaster*.

| Entry | Protein ID | Gene name | RG/Control | p | Change |
|-------|------------|---------------|------------|-------------|--------|
| 65 | Q9VZ01 | BcDNA:RH44935 | 0.76059 | 0.004836408 | Down |
| 66 | Q7JZZ3 | CG13321 | 0.751225 | 0.046743486 | Down |
| 67 | M9NDL7 | Reps | 0.749973 | 0.004348601 | Down |
| 68 | P19334 | trp | 0.747794 | 0.03200816 | Down |
| 69 | Q9W4D2 | Rnp4F | 0.747663 | 0.011693647 | Down |
| 70 | Q9XZS3 | CG13364 | 0.742911 | 0.023862935 | Down |
| 71 | Q9VJ22 | BEST:GH09876 | 0.741874 | 0.011097374 | Down |
| 72 | E1UIM5 | CG13675 | 0.741544 | 0.042732988 | Down |
| 73 | Q9VS21 | Dmel\CG15829 | 0.739049 | 0.044600699 | Down |
| 74 | Q9VKU5 | Dmel\CG6144 | 0.736171 | 0.014459095 | Down |
| 75 | A0A0B4LFQ3 | Hppy | 0.730729 | 0.002866597 | Down |
| 76 | M9PE69 | Dmel\CG43740 | 0.725764 | 0.016526142 | Down |
| 77 | Q8MYR7 | PI4KIIIalpha | 0.713236 | 0.006458911 | Down |
| 78 | Q4V5K3 | CG15653 | 0.683802 | 0.002240457 | Down |
| 79 | M9PH94 | Caz | 0.678528 | 9.17871E-05 | Down |
| 80 | Q9VCQ9 | Dmel\CG6733 | 0.672357 | 0.019442633 | Down |
| 81 | Q8T043 | CG3409-FA | 0.661867 | 0.036690684 | Down |
| 82 | Q9VAD3 | Vps13B | 0.657683 | 0.008481404 | Down |
| 83 | Q9VQF9 | Snapin | 0.649571 | 0.035362079 | Down |
| 84 | Q6IJB6 | HDC15303 | 0.637737 | 0.032958939 | Down |
| 85 | Q8SXL8 | Cln3 | 0.63377 | 0.005102953 | Down |
| 86 | A0A0B4K891 | CG8963 | 0.625873 | 0.014196561 | Down |
| 87 | Q8T412 | CG10749 | 0.60393 | 0.017466337 | Down |
| 88 | A9UN96 | CG18259 | 0.569743 | 0.047419921 | Down |
| 89 | Q8MZ62 | CG5103 | 0.559821 | 0.044463531 | Down |
| 90 | Q9V8I2 | CG5189 | 0.554082 | 0.025951457 | Down |
| 91 | Q6NP21 | CG12124 | 0.551103 | 0.038401808 | Down |
| 92 | Q9VX77 | Chchd2 | 0.533666 | 0.000874477 | Down |
| 93 | C1C5B1 | cact-RB | 0.461301 | 0.034408467 | Down |
| 94 | Q9VCC2 | Dmel\CG5510 | 0.402157 | 0.028519357 | Down |

p value <0.05 was considered statistically significant.

spans of male *D. melanogaster* treated with 12.5 mg/ml RG and 15.0 mg/ml RG were significantly extended compared with the control group, while the life spans of male *D. melanogaster* treated with 20.0 mg/ml RG decreased (Figure 2). These results indicated that RG could prolong the life span of male *D. melanogaster* within the appropriate dose range, while an overdose of RG had a negative impact on the life span.

Climbing Ability Test

The male *D. melanogaster* of the control and RG groups were tested at the age of 36 days. Climbing activity had already been successfully used to evaluate the rate of aging in *D. melanogaster*. The male *D. melanogaster* treated with 12.5 mg/ml and 15.0 mg/ml RG had better climbing activity compared with the control group (Figure 3). These results indicated that RG could slow down the rate of aging.

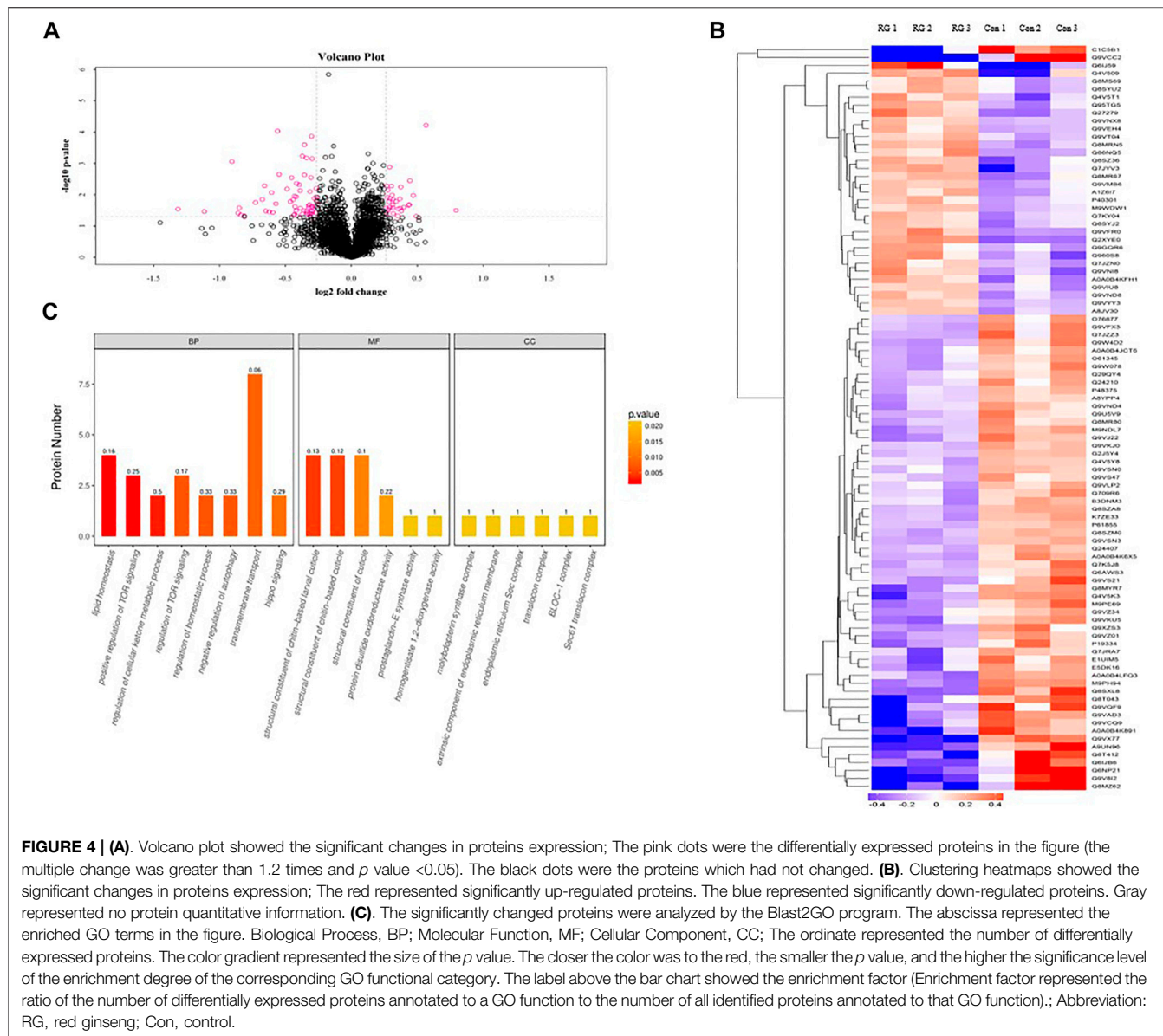
Identification of Significantly Changed Proteins

To investigate the effects of RG on male *D. melanogaster* life span, we treated male *D. melanogaster* with 12.5 mg/ml RG and used iTRAQ to identify protein changes. Using a threshold of >1.2 or <0.83 ($p < 0.05$), a total of 94 proteins were found to be differentially expressed between the RG and control groups.

Of these, the expression of 32 proteins increased and the expression of 62 proteins decreased in the experimental group (Table 2). Clustering heatmaps and volcano plots showed significant changes in protein expression (Figures 4A,B).

Bioinformatics Analysis of Significantly Changed Proteins

The significantly changed proteins were analyzed by the Blast2GO program and Fisher's exact test ($p < 0.05$). The enriched GO terms were from the following three categories: cellular component, molecular function, and biological process. The biological process category was significantly enriched in GO for proteins involved in lipid homeostasis, positive regulation of TOR signaling, and regulation of cellular ketone metabolic process. The molecular function category was significantly enriched in GO for structural constituents of the chitin-based larval cuticle, protein disulfide oxidoreductase activity, and prostaglandin-E synthase activity. The cellular component category was significantly enriched in GO for the extrinsic component of the endoplasmic reticulum membrane, molybdopterin synthase complex, and translocation complex (Figure 4C). Significantly changed proteins were analyzed by KEGG. The identified pathways were protein processing in the endoplasmic reticulum, oxidative phosphorylation, and the mTOR signaling pathway.



Validation of Significantly Changed Proteins by qRT-PCR

The mRNA levels of these differentially expressed proteins were tested by qRT-PCR. The mRNA level changes of CG9286, CG10472, FK506-bp2, Akh, hgo, CG31674, Lpin, BcDNA: RH44935, hppy, and Chchd2 were consistent with the changes analyzed by iTRAQ. No significant changes were evident in the levels of trp and Dmel\CG5510 between the control and RG groups (Figure 5).

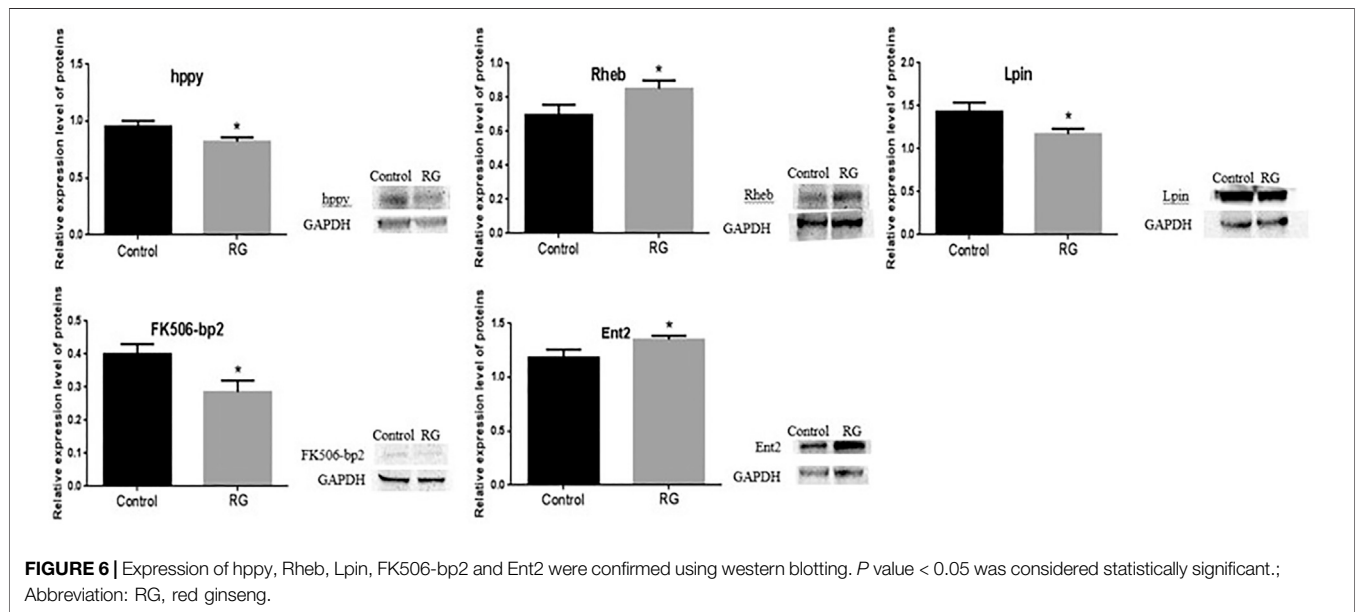
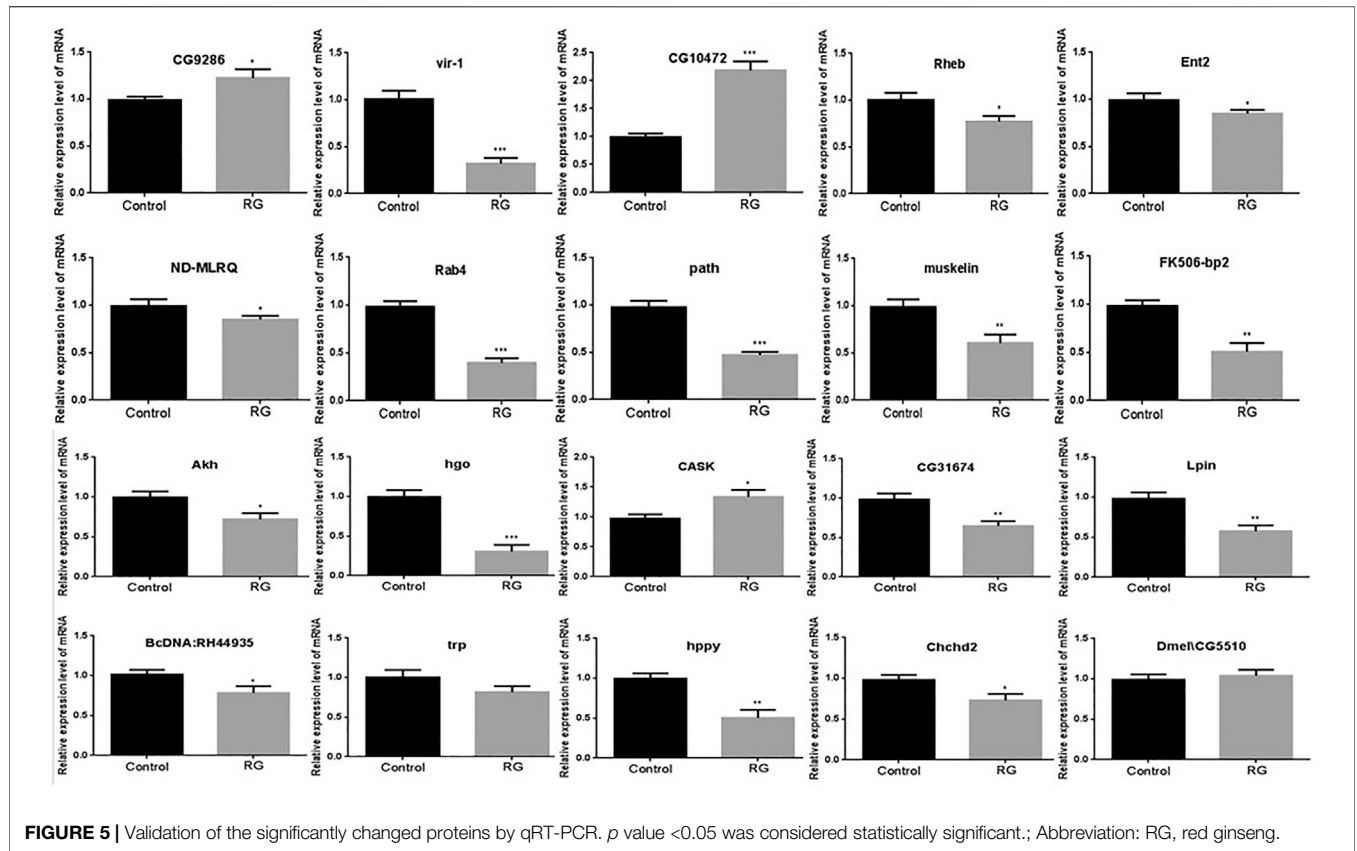
Validation of Proteins by Western Blotting

Expression of hppy, Lpin, Ent2, Rheb, and FK506-bp2 was tested by western blotting. The altered expressions of hppy, Lpin, Ent2, Rheb, and FK506-bp2 between the control and RG groups were consistent with the changes analyzed by iTRAQ. The expression

of hppy, Lpin, and FK506-bp2 was downregulated and the expression of Ent2 and Rheb was upregulated (Figure 6).

Effect of Hppy, Rheb, and Lpin on the PI3K/AKT/FoxO Pathway

The differentially expressed proteins hppy, Rheb, and Lpin participated in the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT)/forkhead box O (FoxO) signaling pathway and regulated the life span of *D. melanogaster*. We tested the changes of PI3K, AKT, p-AKT, mTOR, p-mTOR, S6K, 4E-BP, and FoxO proteins using western blotting. The expression of PI3K, AKT, and p-AKT was decreased in the RG group ($p < 0.05$), and the expression of mTOR and p-mTOR did not change significantly. The expression of 4E-BP and FoxO increased significantly ($p < 0.05$) and S6K did not change (Figures 7, 8).



DISCUSSION

Red ginseng prolongs the life span of *D. melanogaster*, but the mechanism has not been elucidated. In this study, iTRAQ was used to identify protein changes in senile (36 days old) male *D.*

melanogaster. The iTRAQ examination revealed that a total of 94 proteins were differentially expressed in the RG group. Of these, the expression of 32 proteins increased and the expression of 62 proteins decreased. qRT-PCR revealed variations in the mRNA levels of CG9286, CG10472, FK506-bp2, Akh, hgo, CG31674,

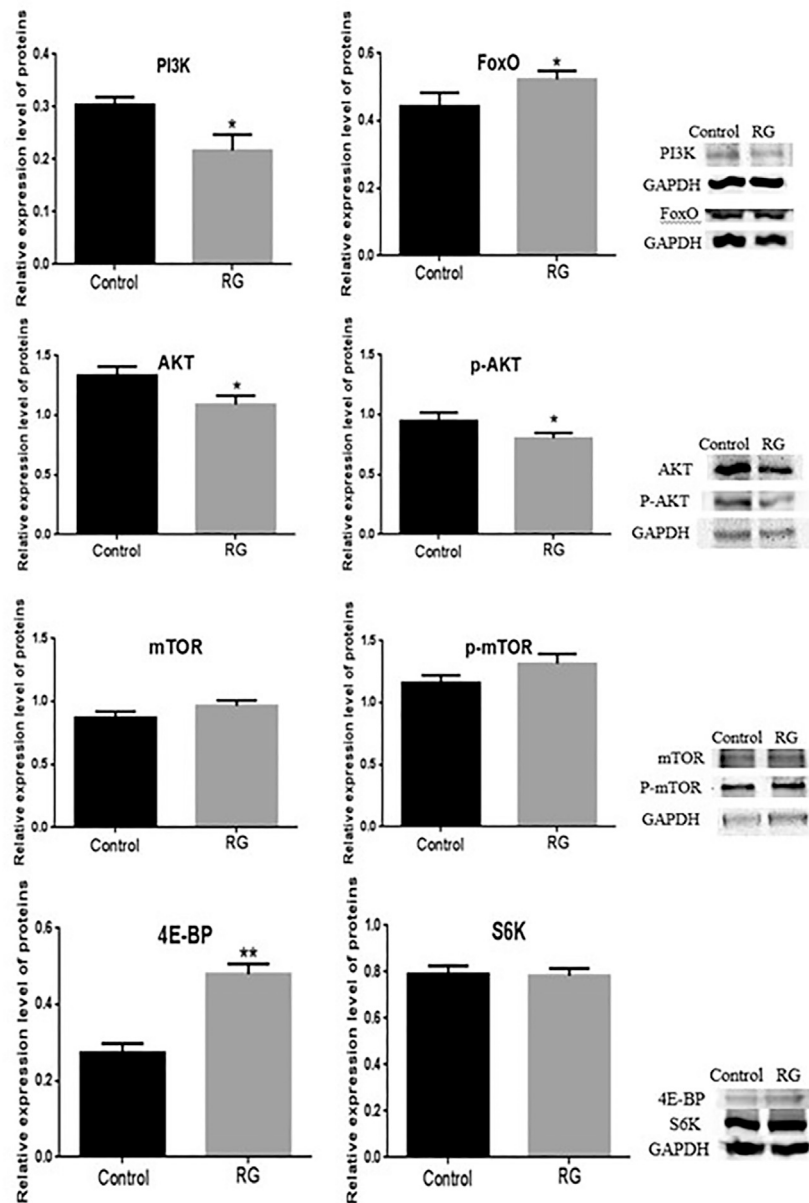
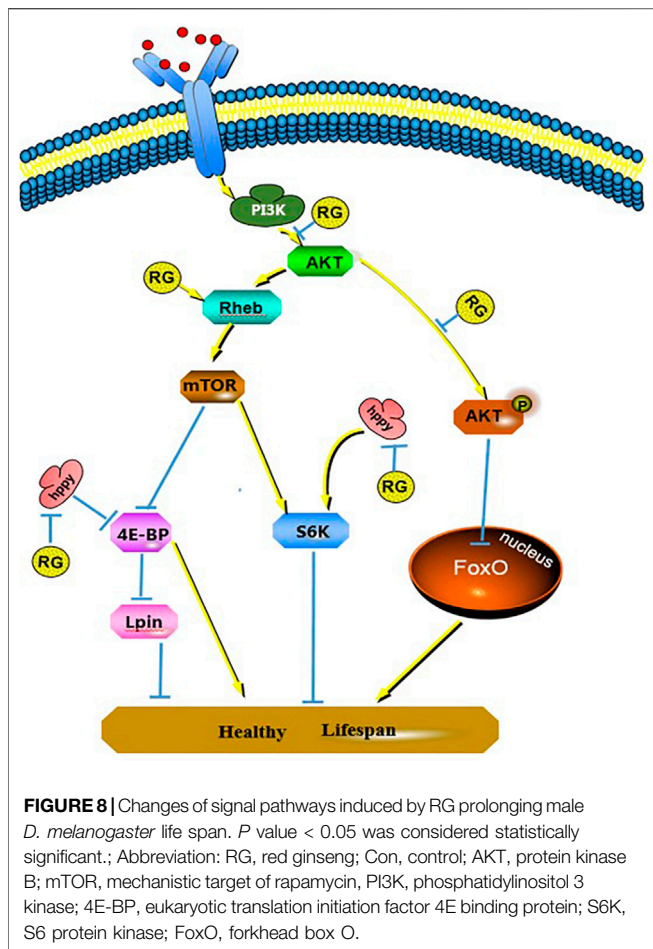


FIGURE 7 | Expression of PI3K, AKT, p-AKT, mTOR, p-mTOR, 4E-BP, S6K and FoxO were confirmed using western blotting. *P* value < 0.05 was considered statistically significant.; Abbreviation: RG, red ginseng; Con, control; AKT, protein kinase B; mTOR, mechanistic target of rapamycin, PI3K, phosphatidylinositol 3 kinase; 4E-BP, eukaryotic translation initiation factor 4E binding protein; S6K, S6 protein kinase; FoxO, forkhead box O.

Lpin, BcDNA: RH44935, hppy, and Chchd2, which were consistent with the iTRAQ results. No significant changes were seen in the levels of trp and Dmel\CG5510 between the control and RG groups. The mRNA expression of vir-1, Rheb, Ent2, ND-MLRQ, Rab4, path, muskelin, and CASK after qRT-PCR detection were not consistent with iTRAQ results (Figure 4). This inconsistency in changes may be because mRNA is regulated by many regulatory factors during translation. The altered expression of hppy, Lpin, Ent2, Rheb, and FK506-bp2 found in western blotting were consistent with the results of iTRAQ.

The differentially expressed protein hppy, which is homologous to human MAP4K3, regulates various signaling pathways, including the mTOR pathway (Lam et al., 2010). The mTOR pathway plays an important role in the process of *Drosophila* senescence. A decrease in MAP4K3 level can reduce the activity of mTOR (Bryk et al., 2010). S6 protein kinase (S6K) and translation inhibitor (4E-BP) are targets of mTOR activity, and MAP4K3 also regulates the activity of S6K and 4E-BP (Weichhart 2018). The effect of 4E-BP on extending life span has been shown in *Drosophila* before (Hay 2011). In our study, western blotting results showed that the expression of hppy in



male *D. melanogaster* was decreased after RG treatment. The expression of mTOR, p-mTOR, and S6K did not change. The expression of 4E-BP increased significantly ($p < 0.05$). The findings indicated that the decreased expression of hppy upregulated the expression of 4E-BP, directly inhibited protein synthesis, and participated in the regulation of life extension. In addition, MAP4K3 can also activate the JNK pathway and induce cell apoptosis. Based on these results, the decreased expression of hppy may inhibit the JNK pathway (Lam et al., 2010).

The differentially expressed protein Rheb, a homolog of Ras GTPase, participates in the PI3K/AKT/mTOR signaling pathway and upregulates Rheb activated mTOR activity (Karassek et al., 2010). Inhibiting mTOR activity and reducing protein synthesis can prolong the life span of organisms, including yeast, *D. melanogaster*, nematodes, and mice (Alic and Partridge 2011). However, long-term inhibition of mTOR activity can lead to inhibition of wound healing, anemia, proteinuria, pneumonia, and hypercholesterolemia (Lamming et al., 2012). The balance of the mTOR pathway is essential for cell health. In this study, the results showed that the expression of Rheb in male *D. melanogaster* was increased after RG treatment, and the expression of PI3K, AKT, and p-AKT was decreased in the RG group ($p < 0.05$). The expression of p-mTOR increased slightly in the RG group, but the difference was not significant ($p > 0.05$). Inactivation of AKT

directly activates the FoxO family of proteins, which reduces oxidative stress, repairs DNA damage, and inhibits premature aging and cellular senescence (Zhang et al., 2011). Therefore, we tested the changes of FoxO protein, and the results showed that the expression of FoxO was increased ($p < 0.05$). The findings indicated that RG prolonged the life span of male *D. melanogaster* by regulating the PI3K/AKT/FoxO pathway. The mTOR pathway remained relatively balanced and was not overactivated. In addition, Rheb can reduce reactive oxygen species (ROS) and oxidative damage independently of the mTOR pathway (Ashraf et al., 2019). On feeding *D. melanogaster* RG, Rheb may reduce the oxidative damage and prolong the life span.

The differentially expressed protein Lpin, a homolog of human lipin, is a lipid protein regulated by the mTOR pathway. Aging is accompanied by the accumulation of Lpin (Romic et al., 2017). The expression of Lpin can be decreased by inhibiting mTOR and upregulating the expression of 4E-BP (Guo et al., 2019; Reue and Wang, 2019). The iTRAQ, qRT-PCR, and western blotting results showed that the expression of Lpin was decreased and 4E-BP was increased. The decreased expression of Lpin indicated that there was no excessive accumulation of lipids in the senile flies.

The differentially expressed protein Fk506-bp2 changed significantly, which is a binding protein. Fk506-bp2 is sensitive to oxidative stress and easily decomposes with the ryanodine receptor (Kreko-Pierce et al., 2016). The results showed that the expression of FK506-bp2 decreased, which may reduce the sensitivity to oxidative stress and oxidative damage.

The differentially expressed protein CG31674 is an oxidoreductase involved in oxidative stress (Yi et al., 2007; Fernandez-Ayala et al., 2010). Our results showed that the expression of CG31674 decreased. It was speculated that the activity of the redox reaction and oxidative damage may have decreased. Oxidative damage can induce aging. Significant prolongation of a healthy life span requires a reduction of all aging processes of an organism (Avril, et al., 1984).

Red ginseng prolonged the life span of male *D. melanogaster* through a complex biological process and may be used as a potential anti-aging drug. The iTRAQ examination revealed that a total of 94 proteins were differentially expressed in the RG group. Many proteins do not have primary antibodies and western blotting confirmatory experiments were not executed. In addition, *D. melanogaster* is a model organism and non-mammal. We will continue to study RG's effect on prolonging life span and PI3K/AKT/FoxO pathway in mammals.

CONCLUSION

In this study, we explored the pathways that RG may participate in when extending the life span of *D. melanogaster*, and the results showed that the PI3K/AKT/FoxO pathway was involved. In addition, 4E-BP expression increased and participated in the regulation of life span.

DATA AVAILABILITY STATEMENT

Raw mass spectrometry data were submitted to China National Genomics Data Center (CNCB-NGDC,

<https://bigd.big.ac.cn/omix/>) under BioProject accession number PRJCA004725.

AUTHOR CONTRIBUTIONS

JP conceived and designed the experiments. WH performed the experiments, analyzed the data, and contributed reagents/materials/analysis tools. WH and JP wrote the paper.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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