Age-related ceRNA networks and the mRNA/protein correlation in *Drosophila* ageing

Deying Yang

Sichuan Agricultural University

Ya Yuan

Sichuan Agricultural University

Zhi He

Sichuan Agricultural University

Jianbo Tu

Sichuan Agricultural University

Siqi Wang

Sichuan Agricultural University

Jiamei Li

Sichuan Agricultural University

Pu Zhang

Sichuan Agricultural University

Xueping Mao

Sichuan Agricultural University

Xiaolan Fan

Sichuan Agricultural University

Diyan Li

Sichuan Agricultural University

Bo Zeng

Sichuan Agricultural University

Qingyong Ni

Sichuan Agricultural University

Yan Li

Sichuan Agricultural University

Mingwang Zhang

Sichuan Agricultural University

Taiming Yan

Sichuan Agricultural University

Mingyao Yang (✉ yangmingyao@sicau.edu.cn)

Sichuan Agricultural University

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Abstract

Background

As *Drosophila* is a classic model organism, understanding of the regulatory networks has great significance in revealing the genetic mechanisms of ageing and human diseases. Competing endogenous RNA (ceRNA)-mediated regulation is an important mechanism of circular RNA (circRNA) and long non-coding RNA (lncRNA) regulation in ageing and age-related disease. However, extensive analyses of the multi-omics (circRNA/miRNA/mRNA, lncRNA/miRNA/mRNA, and mRNA/protein) characteristics of adult *Drosophila* ageing have not been reported. Our results determine the ceRNA networks and the mRNA/protein correlations of key differentially expressed genes in adult *Drosophila* aging, and provide a solid foundation for understanding mechanisms of human ageing and ageing-related diseases.

Results

Here, differentially expressed circRNAs and microRNAs (miRNAs) between 7 and 42 day old *Drosophila* were screened and identified. Then, the differentially expressed mRNAs, circRNAs, miRNAs, lncRNAs, and proteins between day 7 and day 42 were used to analyse fly age-related circRNA/miRNA/mRNA and lncRNA/miRNA/mRNA networks and the mRNA/protein correlation in *Drosophila* ageing. Several key ceRNA networks were identified, such as the circ_0006913/miR-985-3p/*Abl*, circ_0009500/miR-14-5p/*SERCA*, XLOC_100429/miR-14-5p/*SERCA*, and XLOC_100429/miR-14-5p/*Vha100-4* networks. Subsequently, 16 pairs of differentially expressed interacting mRNAs/proteins were found, including 10 pairs with the same expression trends and 6 pairs with the opposite expression trends. Furthermore, real-time quantitative PCR (qPCR) was used to verify the expression level of those genes.

Conclusions

The discovery of these ceRNA networks and the mRNA/protein correlations in adult *Drosophila* ageing will provide new information for research on human ageing and age-related disease.

Background

Drosophila melanogaster is a classic and modern genetic model that has been used for more than 100 years to study various aspects of the life sciences. In particular, fruit flies have been widely utilized to study ageing [1] and multiple human diseases, such as cancer [2], neurodegenerative disease [3], obesity and diabetes [4], sterile inflammation [5], and regeneration [6]. *D. melanogaster* has also been utilized to study complex behavioural and developmental biology topics, including exercise [7], courtship [8], and foraging [9]. Recently, mounting evidence has suggested that *Drosophila* is an outstanding model for studying ageing and age-related disease [10–12]. Ageing is a physiologic/pathologic process featuring declines in normal physiological function and progressive impairment of cellular functions [13, 14]. The ageing phenomenon has been conserved during biological evolution; even yeast and other single-celled eukaryotes

experience ageing [13]. Thus, in-depth study of the regulatory mechanism of ageing in *Drosophila* will be helpful for the study of human ageing and disease.

Current studies suggest that non-coding RNAs (ncRNAs) are involved in organismal ageing [15–18]. With the development of sequencing technology, dynamic changes in the transcriptome (including changes in mRNA, long non-coding RNA (lncRNA), microRNA (miRNA), and circular RNA (circRNA) [19–22]; the proteome [23]; and the metabolome [24, 25]) have been described in the context of *Drosophila* ageing. Most miRNAs, mRNAs, and proteins in fruit flies are evolutionarily conserved up to humans and regulate similar signalling pathways across organisms, such as the NF-κB, AMPK, mTOR, P53, PGC1α, and FoxO pathways [21, 26, 27]. Large studies have demonstrated that the circRNA/miRNA/mRNA axis and lncRNA/miRNA/mRNA axis play vital roles in ageing and age-related disease [26, 28–30].

As *Drosophila* is a workhorse model organism, thoroughly studying the characteristics of adult *Drosophila* from a multiomics perspective is necessary. However, extensive analyses of the multiomics (circRNA/miRNA/mRNA, lncRNA/miRNA/mRNA, and mRNA/protein) characteristics of adult *Drosophila* ageing have not been reported. In the present study, we investigated the circRNA/miRNA/mRNA axis, lncRNA/miRNA/mRNA axis, and mRNA/protein axis in adult *Drosophila* at two age points (day 7 and day 42) and determined the regulatory network of key differentially expressed (DE) genes in *Drosophila* ageing. The results will improve knowledge regarding the gene regulation network of adult *Drosophila* ageing and provide a solid foundation for understanding mechanisms of human ageing and ageing-related diseases.

Results

Overview of multiomics data

The DE genes and proteins in *Drosophila* between day 7 and day 42 were screened, and their networks were analysed (Table 1). A total of 537 DE mRNAs, 348 DE proteins, and 43 DE lncRNAs were obtained from a previous study in our laboratory (Dataset S1). A total of 6003 circRNAs and 226 miRNAs were identified at day 7 and day 42 (Dataset S2). Ultimately, 29 DE circRNAs and 30 DE miRNAs were found (Dataset S1). The merged sequences of novel circRNAs (Dataset S3, Dataset S4, and Dataset S5) and lncRNAs (Dataset S6) are shown in supplementary files.

Table 1. DE mRNA, proteins, lncRNAs, circRNAs, and miRNAs at day 42 compared to day 7

	mRNA	Proteins	LncRNAs	CircRNAs	miRNAs
Total	537	348	43	29	24
Upregulated	194	134	15	21	11
Downregulated	343	214	28	8	13

DE circRNAs and miRNAs in *Drosophila* between day 7 and day 42

The DE circRNAs and miRNAs between 7 and 42-day-old *Drosophila* were analysed. Between day 7 and day 42, 29 DE circRNAs in *Drosophila* were identified, including 21 upregulated and 8 downregulated circRNAs at day 42 (Table 2). The circRNAs were derived from different source genes. Furthermore, 24 DE miRNAs (11 upregulated and 13 downregulated) were screened between day 7 and day 42 (Figure 1A). Dme-miR-9a-3p and dme-miR-985-3p were identified as canonical specific fruit fly miRNAs. Then, dme-miR-956-3p, dme-miR-284-3p, and dme-miR-289-5p were identified as non-canonical miRNAs. The rest of 19 DE miRNAs were canonically conserved miRNAs.

Furthermore, functional annotation was carried out for the source genes of DE circRNAs and DE miRNAs. These source genes were found to be involved in multiple molecular functions (Table 2). Evidently, the biological processes of the short lifespan-related source genes *arm* and *pan* were involved in the Wnt signalling pathway and had similar molecular functions, such as binding, protein binding, transcription factor binding, and transcription regulator activity. In addition, different circRNAs were observed to originate from the same mRNA transcript. For example, dme_circ_0008175 and dme_circ_0008173 were originated from *Nlg1* gene, and dme_circ_0009514 and d me_circ_0009500 were derived from *pan* gene. The Gene Ontology (GO) annotations of ageing-related DE miRNAs were investigated, and 17 DE miRNAs were clustered with 20 GO terms (Figure 1B), including cell fate commitment, programmed cell death, developmental growth, and regulation of circadian rhythm terms.

Table 2. DE circRNAs of *Drosophila* between day 7 and day 42

ID	CircBase ID	Fold change	Pvalue	Source gene	Portion of biological process term(s) from Flybase database
Dme_circ_0009372	Dme_circ_0005033	4.5034↑	0.007635	<i>Asator</i>	Protein serine/threonine kinase activity; ATP binding
Dme_circ_0006708	Dme_circ_0005241	4.2791↑	0.012415	<i>Dad</i>	TGF-beta signalling pathway; negative regulation of BMP signalling pathway
Dme_circ_0004259	Dme_circ_0002098	4.2083↑	0.014401	<i>shot</i>	Cytoplasmic microtubule organization; wound healing; branching involved in open tracheal system development; cilium organization
Dme_circ_0002070	Dme_circ_0002195	3.9032↑	0.026301	<i>Scp1</i>	-
Dme_circ_0010408	Dme_circ_0003710	3.8118↑	0.031385	<i>CoRest</i>	Negative regulation of transcription by RNA polymerase II; positive regulation of DNA methylation-dependent heterochromatin assembly; negative regulation of histone H4-K16 acetylation; negative regulation of histone H3-K27 methylation
Dme_circ_0003904	Dme_circ_0003738	3.5948↑	0.045349	<i>CG33144</i>	Ubiquitin-dependent protein catabolic process
Dme_circ_0008175	Dme_circ_0001709	3.5948↑	0.045412	<i>Nlg1</i>	Neuromuscular junction development; cellular process

Dme_circ_0005030	Dme_circ_0002884	3.5765↑	0.046714	<i>Ccn</i>	Negative regulation of cell death; signal transduction; cell adhesion
Dme_circ_0006667	Dme_circ_0001321	3.5765↑	0.046714	<i>gish</i>	Positive regulation of Wnt-TCF and hedgehog signalling pathways; negative regulation of Hippo signalling pathway
Dme_circ_0010536	Dme_circ_0000629	3.559↑	0.048096	<i>slgA</i>	Arginine and proline metabolism
Dme_circ_0008383	Dme_circ_0004519	2.6054↑	0.043863	<i>mura</i>	Protein ubiquitination; long-term memory
Dme_circ_0010134	Dme_circ_0002087	1.1067↑	0.039466	<i>Stim</i>	Developmental process; cellular homeostasis
Dme_circ_0009358	-	0.74476↑	0.002481	<i>CaMKI</i>	Protein phosphorylation
Dme_circ_0000626	-	3.7656↑	0.03384	<i>CG17646</i>	Triglyceride metabolic process; transmembrane transport
Dme_circ_0011075	-	3.7618↑	0.03415	<i>Trf2</i>	Post-embryonic development; response to organic cyclic compound; programmed cell death; respiratory system development; response to oxygen-containing compound
Dme_circ_0006956	-	3.5884↑	0.045804	<i>GluClalpha</i>	Cellular process; transport; localization; establishment of localization; biological regulation

Dme_circ_0009667	-	3.559↑	0.048096	<i>dlg1</i>	Hippo signalling pathway-fly
Dme_circ_0009514	-	4.0161↑	0.021197	<i>pan</i>	Canonical Wnt signalling pathway
Dme_circ_0006334	-	3.8925↑	0.026799	<i>CG42402</i>	-
Dme_circ_0008173	-	3.881↑	0.027405	<i>Nlg1</i>	Neuromuscular junction development; cellular process
Dme_circ_0004404	-	0.61768↑	0.020721	<i>Dbp80</i>	Poly(A)+ mRNA export from nucleus
Dme_circ_0003891	Dme_circ_0001623	-3.8065↓	0.033572	<i>psq</i>	Anterior/posterior axis specification, embryo; DNA binding
Dme_circ_0006619	Dme_circ_0003501	-4.3746↓	0.010954	<i>srp</i>	Autophagy; cell fate commitment; midgut development
Dme_circ_0004913	Dme_circ_0004913	-0.52833↓	0.047568	<i>CG34347</i>	Actomyosin structure organization
Dme_circ_0004843	-	-0.48662↓	0.02228	<i>CG15715</i>	
Dme_circ_0010498	-	-0.73454↓	0.04614	<i>CG1304</i>	Proteolysis
Dme_circ_0006913	-	-2.7169↓	0.042802	<i>Cyp12a5</i>	Oxidation-reduction process
Dme_circ_0010310	-	-3.8065↓	0.033572	<i>arm</i>	Wnt signalling pathway
Dme_circ_0009500	-	-4.0543↓	0.021183	<i>pan</i>	Canonical Wnt signalling pathway

Notes: “↑” and “↓” mean upregulation and downregulation on day 42 compared to day 7, respectively.

DE circRNA/miRNA/mRNA networks in *Drosophila* ageing

In this section, the DE circRNA/miRNA/mRNA networks were analysed. Through the ceRNA mechanism, miRNAs can negatively regulate mRNA expression. Overall, 12 DE circRNAs, 21 DE miRNAs, and 30 DE

mRNAs had interactions (Figure 2A). According to the trends in expression quantity changes, three DE circRNAs (dme_circ_0006913, dme_circ_0008173, and dme_circ_0009500) targeted the DE miRNAs with opposite expression trends (Figure 2B). Based on qPCR results, the expression patterns of two circRNAs (dme_circ_0008173 and dme_circ_0009500), four miRNAs (dme-miR-289-5p, dme-miR-985-3p, dme-miR-286-3p, and dme-miR-14-5p), and four mRNAs (*frizzled*, CG31064, *Abi*, and *SERCA*) were consistent with RNA-seq data (Figure 2C). Importantly, the expression trend of dme_circ_0009500/dme-miR-14-5p/*SERCA*, dme_circ_0009500/dme-miR-289-5p/*frizzled*, and dme_circ_0009500/dme-miR-289-5p/CG31064 conformed to the ceRNA mechanism.

DE lncRNA/miRNA/mRNA networks in *Drosophila* ageing

According to the functional patterns of lncRNAs competing with mRNAs for binding to miRNAs, the interaction networks of DE lncRNAs/miRNAs/mRNAs were identified. In addition, DE miRNAs targeted DE mRNAs with opposite expression trends. Based on the DE genes in our database, 15 lncRNAs, 15 miRNAs, and 32 mRNAs had interactions (Figure 3A). Several specific lncRNA/miRNA/mRNA networks were found, such as XLOC_100429/dme-miR-14-5p/*SERCA*, XLOC_100429/dme-miR-14-5p/*Vha100-4*, and XLOC_073604/dme-miR-985-3p/*Abi* (Figure 3B). In XLOC_027736/dme-miR-985-3p/*Abi* and XLOC_189909/dme-miR-985-3p/*Abi* networks, the expression trend of these genes by qPCR analysis is consistent with the RNA-seq data from our study (Figure 3C).

Functional annotation of DE circRNA/lncRNA-associated networks

GO functional annotation of DE circRNAs/lncRNAs/mRNAs was carried out based on 74 target mRNA genes (Dataset S7). The first 30 GO terms based on the lowest *P* values are listed (Figure 5). In circRNA/mRNA GO terms, there were just two major GO categories in circRNA-associated networks, including biological processes (29 GO terms) and cellular components (1 GO term, perinuclear region of cytoplasm). Similarly, GO annotations of lncRNA-associated networks consisted of 25 GO terms in biological processes and 5 GO terms in cellular component.

In GO annotations of circRNA-associated networks, there were several aging-related biological processes in GO terms, such as homeostatic process (10 DE genes), cellular homeostasis (7 DE genes), cation homeostasis (5 DE genes), ion homeostasis (5 DE genes), cell-cell signalling (9 DE genes), cell fate determination (5 DE genes), and developmental growth (7 DE genes) (Figure 4A). Furthermore, the 13 out of 30 first GO terms of lncRNA-associated networks were related to homeostatic process, including cellular homeostasis, retina homeostasis, ion homeostasis, calcium ion homeostasis, cellular cation homeostasis, etc (Figure 4B).

mRNA/protein correlation in *Drosophila* ageing

To reveal the post-transcriptional regulatory network of gene expression in *Drosophila* at day 7 and day 42, the expression trends between proteins and mRNA were analysed. A relatively weak correlation (Pearson value 0.049) between mRNA and proteins was observed. A total of 2753 mRNAs and mapped proteins were found in *Drosophila* at day 7 and day 42 (Dataset S8). 16 pairs of interacting DE mRNAs/proteins were

identified, including 10 pairs with the same expression trends and 6 pairs with the opposite expression trends (Figure 5A, Dataset S8). GO annotation was performed for the 16 pairs of interacting mRNAs/proteins (Figure 5A). FBgn0035542 (*Diabetes and obesity regulated, DOR*) was enriched for the RNA processing term, FBgn0012042 (*Attacin-A, AttA*) was enriched for the extracellular region term, and FBgn0036948 (*CG7298*) was enriched for the carbohydrate derivative metabolic process term; the rest of the proteins/mRNAs were enriched for the response to biotic stimulus and organonitrogen compound metabolic process terms. Evidently, the mRNA levels of FBgn0001624 (*discs large 1, dlg1*), FBgn0052699 (*lysophosphatidylcholine acyltransferase, LPCAT*), FBgn0002707 (*meiotic 9, mei-9*), FBgn0086907 (*cytochrome c distal, Cyt-c-d*), and FBgn0265101 (*suppressor-of-G2-allele-of-skp1, Sgt1*) were not changed between day 7 and day 42; however, the proteins encoded by these mRNAs were DE between day 7 and day 42. Furthermore, *dlg1*, *LPCAT*, *mei-9*, *Cyt-c-d*, and *Sgt1* were clustered into different KEGG pathways (Figure 5B).

10 mRNA genes were randomly selected for expression level detection via qPCR at day 7 and day 42. FBgn0034335 (*Glutathione S transferase E1, GstE1*), FBgn0034480 (*CG16898*) and FBgn0035542 (*DOR*) exhibited the same expression patterns as observed in the RNA-seq data (Figure 5C). When compared to 7 day-old fruit flies, *GstE1* and *CG16898* were upregulated in 42 day-old fruit flies, while *DOR* was downregulated at day 42.

Discussion

Increasing evidence has suggested that ceRNA networks play key roles in a variety of biological processes, such as cancer [31, 32], Alzheimer's disease [33], skeletal muscle myogenesis[34], and ageing [35, 36]. *Drosophila* models are used to study multiple biological phenomena and various human diseases [7, 37-40]. Thus, identification and analysis of gene regulatory networks in *Drosophila* are very important and are necessary to improve the applicability of research on ageing and age-related diseases. In this study, DE circRNA/miRNA/mRNA networks, DE lncRNA/miRNA/mRNA networks, and the mRNA/protein correlation were analysed in 7 and 42-day-old *Drosophila*.

Most of the DE circRNAs were accumulated in elder age day 42 in *Drosophila*, which are similar to other findings in model organisms [16, 17]. circRNAs are highly stable molecules and have been demonstrated to play important roles in ageing [41] and age-related diseases [42, 43]. Additionally, six source genes of circRNAs were related to fruit fly ageing, including *asator* (short-lived, *Asator*^{GD7323}) [44], *dad* (short-lived, *Dad*^{GD1335}) [45], *gish* (short-lived, *gish*^{GD10588}) [44, 46], *pan* (short-lived, *pan*^{AN.UAS}) [47, 48], *psq* (long-lived, *psq*^{BG01031}) [49, 50], and *arm* (short-lived, *arm*^{S10.UAS.Tag:MYC}) [51]. Previous studies have demonstrated that lncRNAs are also involved in the regulation of ageing and multiple diseases [52, 53]. Furthermore, the ceRNA mechanism is a very important mechanism of circRNA and lncRNA regulation in the context of age-related disease [43, 54, 55]. miRNAs are the core molecules of the ceRNA regulatory system [34, 35]. Thus, screening of DE miRNAs is essential for research on ceRNA networks related to *Drosophila* ageing. miRNAs generally induce mRNA degradation or repress translation of target transcripts through sequence-specific binding to the transcript 3'UTR [56, 57]. Each transcript can be targeted by multiple miRNAs, and each

miRNA can target hundreds of different transcripts (mRNA, circRNA, and lncRNA transcripts) [21, 58, 59]. Thus, the miRNA regulatory network is far-reaching [21]. Previous studies have verified that miRNAs are important small regulatory ncRNA molecules that control a fairly large number of biological processes; their important functions have generated interest in their use as biomarkers and their roles as regulators of ageing [21, 60] and a number of cancer types [61-63]. Recent studies have also suggested that miRNAs are involved in the regulation of age-associated processes and pathologies in multiple mammalian tissues, including the brain, heart, bones, and muscles [21, 64-66]. In our study, 60% (18 out of 30 total miRNAs) of DE miRNAs between day 7 and day 42 were found to affect fruit fly ageing, including dme-miR-14 (short-lived, miR-14^{Δ1}) [67] and dme-miR-276a/b-5p (short-lived, miR-276a^{K0}) [68, 69], etc (Table S1). The results indicate that these miRNAs may act as sponges of circRNAs and lncRNAs involved in regulation of *Drosophila* ageing.

CircRNA/miRNA/mRNA and lncRNA/miRNA/mRNA interactions have essential roles in fly ageing. A previous study performed global identification of functional miRNA-mRNA interactions in *Drosophila* [70]. However, the circRNA/miRNA/mRNA and lncRNA/miRNA/mRNA interactions in fly ageing have remained unclear. In the present study, multiple networks of DE circRNAs/miRNAs/mRNAs and DE lncRNAs/miRNAs/mRNAs in *Drosophila* between day 7 and day 42 were identified. In previous studies, mutation of four miRNAs (dme-miR-276a/b-5p [68, 69], dme-miR-286-3p [68], and dme-miR-14-5p [67]) and two mRNA targets (*Abi* [44, 71] and *SERCA* (short-lived, *SERCA*^{GD436}) [44]) within these DE circRNA/miRNA/mRNA networks was found to affect the lifespan of *Drosophila*. Thus, we determined that the circ_0006913/miR-985-3p/*Abi* and circ_0009500/miR-14-5p/*SERCA* networks merited further investigation. Two circRNAs (dme_circ_0006913 and dme_circ_0009500) and the *frizzled* gene were predicted to competitively bind to dme-miR-289-5p. Furthermore, *frizzled* has been reported to be involved in regulation of the mTOR signalling pathway and Wnt signalling pathway [72]. In XLOC_100429/miR-14-5p/*SERCA* and XLOC_100429/miR-14-5p/*Vha100-4* networks, including mutants of miR-14 [67], *SERCA* [44], and *Vha100-4* (long-lived, *Vha100-4*^{dsRNA.shRNA.UAS}) [73], were predicted to affect fruit fly ageing. Furthermore, the lncRNAs XLOC_073604, CR44922, XLOC_027736, XLOC_189909, and XLOC_189798 and the mRNA *Abi* (FBgn0000017) were predicted to competitively bind to miR-985-3p. The ageing-related phenotype associated with the *Abi* mutant led to a shorter lifespan for flies with three specific alleles (*Abi*^{l2}, *Abi*^{l3}, and *Abi*^{GD1344}) [44, 71, 74].

Several of the DE miRNAs in those networks have been predicted to interact with both DE circRNAs and DE lncRNAs, such as dme-miR-14-5p, dme-miR-276a-5p, and dme-miR-276b-5p. Based on our study findings, dme-miR-14-5p may target XLOC_100429 and thereby regulate *SERCA* and *Vha100-4* mRNA expression. Recent studies have demonstrated that miR-14 is an abundant miRNA in S2 cells [75], and it is one of the most engaged miRNAs in AGO1-RNP complexes [70]. Furthermore, the expression trend of dme_circ_0009500/dme-miR-14-5p/*SERCA* network determined by qPCR was consistent with the RNA-seq data in our study. miR-14-mutant flies present defects related to apoptosis, the stress response, survival, and metabolism [76] and are more sensitive to nutrient deprivation than wild-type flies [67]. *Drosophila* miR-14 affects ageing by regulating insulin production and metabolism through its target, *sugarbabe* [67]. In male flies, miR-14 mutation can reduce survival during nutrient deprivation [67]. Therefore, miR-14 may be a

sponge of dme_circ_0009500 and XLOC_100429 to regulate *Drosophila* ageing. The specific regulatory mechanism will be the subject of future research.

GO annotations of the DE circRNA/miRNA/mRNA and DE lncRNA/miRNA/mRNA networks demonstrated that those DE circRNAs and lncRNAs may play an important part in *Drosophila* aging via various biological processes. Apparently, multiple GO terms of DE circRNA/miRNA/mRNA networks are related to aging, such as cellular homeostasis [77] and developmental growth [78]. Specifically, multiple GO terms are involved in regulation of sequestering of calcium ion, including inorganic ion homeostasis, cation homeostasis, and cellular calcium ion homeostasis, etc. Ca^{2+} dyshomeostasis is associated with several aging-related neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) for altered Ca^{2+} buffering capacity, altered regulation of Ca^{2+} channels and pumps, and altered neuronal excitability [79]. Previous study demonstrated that aging was closely linked to dysregulation of Ca^{2+} homeostasis resulting in a chronically elevated level of cytosolic Ca^{2+} in experimental models of neuronal aging [80, 81]. As *Drosophila* aging-related DE mRNA targets of lncRNA XLOC_100429, *Vha100-4* and *SERCA* were involved in inorganic ion homeostasis, cation homeostasis, ion homeostasis, cellular cation homeostasis, and cellular ion homeostasis. The activity of *Vha100-4* and *SERCA* protein were related to the restoration of resting cytoplasmic Ca^{2+} concentrations and termination of such intracellular Ca^{2+} transients[82]. The results suggest that those DE circRNA/miRNA/mRNA and DE lncRNA/miRNA/mRNA networks may play a significant role in in *Drosophila* aging.

In our present study, the mRNA/protein correlation in *Drosophila* ageing was analysed. The relatively weak correlation (Pearson value 0.049) may have been due to different post-translational protein regulatory mechanisms, such as protein modification (e.g., phosphorylation and ubiquitination)[83]. Explaining the mechanism of the same expression patterns between mRNAs and proteins is relatively simple. Ten pairs of DE mRNAs and proteins with the same expression were identified in this study. The mRNA and protein expression levels of *AattA* (short-lived with allele *AttA*^{GD17307}) [84], FBgn0034511 (*GGBP-like3*, short-lived with alleles *GGBP-like3*^{GD503}, *GGBP-like3*^{KK106085}, and *GGBP-like3*^{NIG.13422R}) [84], and FBgn0038074 (*Gnmt*, long-lived with allele *Gnmt*^{UAS.c0a}) [85] were high in *Drosophila* at day 7 and day 42, which affected the lifespan of the flies. In contrast, in the present study, *DOR* exhibited relatively low expression in flies on day 7 and day 42. *DOR* is antagonized by insulin signalling via the product of *foxo* in the fat body and forms part of a feed-forward mechanism whereby ecdysone potentiates its own signalling[86], which also regulates autophagosome formation and protein degradation via ecdysone signalling [87, 88]. Therefore, the mRNA/protein correlation analysis provides an important foundation to parse the genetic process of *Drosophila* ageing.

Methods

Sample collection and preparation

Female flies (*Dahomey*^{WT}) were bred under a 12 hr on/off light cycle at 25°C in 50% humidity. Sample collection of adult flies at day 7 and day 42 was performed as described by Yang *et al.* (2016)[20]. RNA-seq (of mRNA, lncRNA, and circRNA) and proteome sequencing was conducted on two biological replicates, while miRNA sequencing was conducted on three biological replicates. All samples were stored at -80°C until use.

lncRNA, mRNA, and protein data for *Drosophila* at day 7 and day 42

RNA-seq data of lncRNAs and mRNAs from *Drosophila* at day 7 and day 42 was carried out by Yang *et al.* (2016)[20]. The proteins from *Drosophila* at day 7 and day 42 were sequenced by Zhu (2017)[89]. These original data were from our laboratory.

CircRNAs in *Drosophila* at day 7 and day 42

The circRNA analysis was based on the RNA-seq data for *Drosophila* at day 7 and day 42 from Yang *et al.* (2016)[20]. Overall, 95.79% clean reads were obtained from 26.7 GB of raw sequence data (SRP073695) and then aligned to the *D. melanogaster* genome from FlyBase (Dmel_Release_6, <http://FlyBase.org/>). The find_circ [90] and CIRI2[91] software tools were utilized to identify circRNAs. Then, the overlapping *Drosophila* circRNAs identified by both software programs were selected. The input data for circRNA differential expression analysis were readCount data obtained from the circRNA expression level analysis. Then, paired differential expression analysis of circRNAs between day 7 and day 42 was conducted with DESeq2[92] based on a negative binomial distribution. The *P* value was adjusted using Hochberg and Benjamini's methods[93] to control the error discovery rate. A *P* value < 0.05 was considered to indicate a DE circRNA.

MiRNAs in *Drosophila* at day 7 and day 42

TRIzol Reagent (Invitrogen, CA) was used to extract total RNA from fruit flies at day 7 and day 42. Agarose gel electrophoresis was performed to verify the integrity of the total RNA samples. A NanoDrop ND-1000 instrument was used for accurate measurement of the concentrations and protein contamination of the total RNA samples. miRNA sequencing libraries were generated using rRNA-depleted RNA with a NEBNext® Ultra™ Multiplex Small RNA library Prep Set Kit for Illumina® (New England Biolabs, USA) following the manufacturer's recommendations. Subsequently, an Agilent 2100 Bioanalyzer and an Agilent DNA 1000 chip kit (Agilent, part #5067-1504) were utilized for accurate assessment of the quality and concentration of the sequencing libraries. The libraries were sequenced using an Illumina NextSeq 500. MiRNA Fragment Sequencing service provided by Aksomics company.

Clean reads were generated from the raw sequence data from the Illumina NextSeq instrument through real-time base calling and quality filtering. The clean reads were recorded in FASTQ format and contained the read information, sequences and quality encoding. Subsequently, the 5'- and 3'-adapter sequences were trimmed from the clean reads by Cutadapt, and reads with lengths shorter than 14 nt or longer than 40 nt were discarded. The trimmed reads were collapsed into FASTA format. The trimmed reads that did not map to mature or precursor tRNA sequences were aligned with an allowance of only one mismatch to miRNA

reference sequences with miRDeep2[94]. The expression profiles of miRNAs were determined based on the counts of the reads mapped. The DE miRNAs were screened with the R package EdgeR based on the count values [95]. A fold change cutoff of 1.5 and a *P* value cutoff of 0.05 were applied only when replicates were used for screening of DE miRNAs. GO enrichment analysis of the DE miRNAs was implemented with the GOseq R package[96].

CeRNA analysis of lncRNA/circRNA-miRNA-mRNA pathways

lncRNAs, circRNAs, and miRNAs showed significantly different expression levels between day 7 and day 42 and thus were analysed. The potential ceRNAs were searched based on the sequences of the lncRNAs, circRNAs, and mRNAs. Offline software of MiRanda (<http://cbio.mskcc.org/miRNA2003/miranda.html>) was utilized to predict miRNA binding seed sequence sites, and overlap of the same miRNA binding sites on lncRNAs-miRNAs and circRNAs-mRNAs was taken to indicate a lncRNA/circRNA-miRNA-mRNA interaction.

Network analysis of mRNA/protein correlation

DE mRNAs and proteins between day 7 and day 42 were analysed. Expression regulation analysis was performed by integrating the mRNA and protein data with the Perl language. The Python program Matplotlib was used to integrate the mRNA and protein expression levels, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology-Based Annotation System (KOBAS) online database[97] was used to analyse the statistical enrichment of the DE mRNAs and proteins in KEGG pathways (<http://www.genome.jp/kegg/>). GO enrichment analysis of DE mRNAs and proteins was implemented with the GOseq R package[96]. GO terms with corrected *P* values less than 0.05 were considered significantly enriched for the DE genes.

qPCR detection

To evaluate the quality of ceRNA networks and mRNA/protein correlation, 8 miRNAs, 19 mRNAs, 6 lncRNAs, and 3 circRNAs were selected to qualify the expression using qPCR. The samples from fruit flies at 7 days (three biological duplicates) and 42 days (three biological duplicates) were used to isolated total RNA using TRIzol™ LS Reagent (ThermoFisher) according to the manufacturer's instructions. Total RNA (1 mg) from each sample was reverse transcribed with random primers using a RevertAid First-strand cDNA Synthesis Kit (ThermoFisher) according to the manufacturer's protocol, which was utilized to detect the expression level of mRNA, lncRNA, and circRNA. In addition, total RNA (1 mg) for miRNA expression detection in each sample was reverse transcribed using a TaqMan™ MicroRNA Reverse Transcription Kit (ThermoFisher) according to the kit instructions. All gene primers (Dataset S9) were designed using primer 5.0 software and purchased from Sangon Biotech. The SYBR green method was used for qRT-PCR with TransStart® Green qPCR SuperMix (TransGen Biotech) following the manufacturer's instructions. Gene expression levels were calculated by the cycle threshold values that were identified as 2-DDCT. Ribosomal protein L32 (*rp 49*) was used as the reference gene to calculate the relative mRNA, miRNA, lncRNA, and circRNA levels.

In summary, we screened and identified DE circRNAs and miRNAs in *Drosophila* between days 7 and 42. Then, we used the DE mRNAs, circRNAs, miRNAs, lncRNAs, and proteins between day 7 and day 42 to

analyse fly age-related circRNA/miRNA/mRNA and lncRNA/miRNA/mRNA networks and the mRNA/protein correlation in the context of *Drosophila* ageing. These regulatory networks have the potential to reveal the genetic mechanisms of fly ageing and to eventually enhance our understanding of human ageing and age-related diseases.

Declarations

Supplementary Information

The online version contains supplementary material available at <https://doi.org/xxxx/xxxx>.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Raw read data of miRNA sequencing is available in the Sequence Read Archive (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=search_obj, accession number SRP311738).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DY and MY conceived and designed the experiments. ZH, JT, SW, JL performed the experiments. YY and PZ analyzed the data. XM, XF, DL, BZ, QN, YL, MZ, TY contributed reagents, materials and tools and collected the samples. DY and YY wrote the manuscript and revised the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Expression and GO annotations of DE miRNAs in *Drosophila* between day 7 and day 42. A, the expression level of 30 DE miRNAs in *Drosophila* between day 7 and day 42; “↑” and “↓” mean upregulation and downregulation on day 42 compared to day 7 in RNA-seq data. B, GO annotations of 17 DE miRNAs in *Drosophila* between day 7 and day 42. Grey, no target genes of miRNA were enriched in GO terms; Color, the target genes of these miRNAs were significantly enriched in GO terms ($P \leq 0.01$), and the darker presents the higher of the enrichment degree.

Figure 2

DE circRNA/miRNA/mRNA networks of *Drosophila* at day 7 and day 42. A, circRNA/miRNA/mRNA interaction networks; yellow, circRNAs; red, mRNAs; green, miRNAs. B, specific circRNA/miRNA/mRNA networks; “↑” and “↓” mean upregulation and downregulation on day 42 compared to day 7 in RNA-seq data, respectively. C, data on relative expression of circRNA, miRNA, and mRNA detected by qPCR analysis.

Figure 3

DE lncRNA/miRNA/mRNA networks in *Drosophila* at day 7 and day 42. A, lncRNA/miRNA/mRNA interaction networks; yellow, lncRNAs; red, mRNAs; green, miRNAs. B, specific lncRNA/miRNA/mRNA networks; “↑” and “↓” indicate upregulation and downregulation on day 42 compared to day 7, respectively. C, data on relative expression of lncRNA, miRNA, and mRNA by qPCR analysis. 7 d, 7 days; 42 d, 42 days.

Figure 4

GO annotations of DE circRNA-/lncRNA-associated networks. A, GO annotations of DE circRNA/miRNA/mRNA networks. B, GO annotations of DE lncRNA/miRNA/mRNA networks. LogP values indicate the enrichment degree of targets in corresponding GO term, and the smaller value represents the higher of the enrichment degree. Number of genes represents the number of target genes enriched in GO terms.

Figure 5

Expression trends between proteins and mRNAs in *Drosophila* at day 7 and day 42. A, GO annotations of DE proteins and DE mRNAs. B, KEGG pathways of DE proteins and DE mRNAs. C, data on relative expression of DE mRNA by qPCR analysis. 7 d, 7 days; 42 d, 42 days. Protein and mRNA expression level were presented in different colour in Figure 5A and 5B. A positive number indicates upregulation and a negative number means downregulation in 42 day when compared to 7 day. The "+" in Figure 5C means the same expression patterns between qPCR results and RNA-seq data.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [DatasetS1informationofDEmRNAslncRNAscircRNAproteinsandmiRNAsbetween7dayand42day.xlsx](#)
- [DatasetS2circRNAandmiRNASequencebetween7dayand42day.xlsx](#)
- [DatasetS3novelcircRNAseqmerged1.pdf](#)
- [DatasetS4novelcircRNAseqmerged2.pdf](#)
- [DatasetS5novelcircRNAseqmerged3.pdf](#)
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