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Knockdown expression of eukaryotic initiation factor 5 C-terminal domain containing protein extends lifespan in *Drosophila melanogaster*

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ABSTRACT

Inhibition of translation by mutations of a growing number of genes involved in protein synthesis could extend healthy lifespan in yeast, worm, fly and mouse as well. These genes vary from translation initiation factors to structural components of ribosomes and ribosomal RNA processing factors. ECP is a novel ribosome associated protein. Previous data supports the involvement of this gene in long term memory formation and exon guidance in *Drosophila* probably through its still unconfirmed functions in protein synthesis. However, the exact molecular function of ECP is still largely unknown. Our findings here show that fly lifespan could be significantly extended in ECP RNAi flies. Meanwhile, the locomotion ability of elder ECP RNAi flies was also improved remarkably. Further studies revealed an increase of mitochondria Complex IV activity in these ECP RNAi flies. A decrease of AKT and S6K phosphorylation level in contrast to an increase of AMPK phosphorylation level could also be detected in these flies. Together, these findings support a positive effect of ECP on longevity and delaying age-related impairment in locomotor behavior probably through activation of AMPK and enhancement of mitochondrial function via insulin/IGF-1 and TOR pathway.

1. Introduction

The insulin/IGF-1 signaling was the first signaling pathway shown to influence aging in animals [1]. In this signaling cascade, binding of insulin and IGF-1 to the receptor triggers the activation of AKT/PKB. Three main targets of AKT are GSK3β (glycogen synthase kinase 3β), FOXO (Forkhead box-O class) transcription factors and mTOR. AKT phosphorylates and inactivates GSK3β and FOXO, while mTOR is activated by this kinase on phosphorylation [2,3]. FOXO activity is necessary for the transcription of stress response genes and repair systems which are of course important for longevity [4]. mTOR acts through TORC1 to promote global mRNA translation and ribosome synthesis by direct phosphorylation of the ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E binding proteins (4E-BP) [5–7]. Inhibition of S6K extends lifespan in *Caenorhabditis elegans* [8,9] and *Saccharomyces cerevisiae* [10]. In Drosophila, overexpression of a dominant-negative S6K leads to increased lifespan while conversely overexpression of a constitutively active form of S6K results in reduced lifespan [11].

It is already known that inhibition of protein translation can promote longevity in several model organisms [12,13]. Mutations of a growing number of genes involved in protein synthesis have been identified as being able to extend healthy lifespan in yeast, worm, fly and mouse as well. These genes vary from translation initiation factors to structural components of ribosomes and ribosomal RNA processing factors [14]. Eukaryotic Initiation Factor 5 C-terminal Domain Containing Protein (ECP, also known as KRA or eIF5C) was found to be involved in the biological processes of long term memory formation and axon guidance in Drosophila [15,16]. It is a novel ribosome associated protein which suggests its potential role in translation [17]. However, the exact molecular function of ECP is still largely unknown. In this study, we find that fly lifespan could be significantly extended by knockdown ECP expression. The possible underlying mechanism might be related to altering of AMPK and IIS/TOR signaling.

2. Materials and methods

2.1. Fly strains and maintenance

da-Gal4 was obtained from the Bloomington stock center. Transgenic *ECP* RNAi-1 was obtained from VDRC. Transgenic flies

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UAS-ECP and *ECP* RNAi-2 were generated in our lab. For all experiments, w^{1118} was crossed to the same daGAL4 driver, and offspring were used as control. Flies were kept on standard cornmeal sucrose food at 25 °C, 50% humidity with a 12-h light/dark cycle.

2.2. Lifespan determinations

Newly enclosed adults were collected under brief CO_2 anesthesia. 20 males and 20 females were placed into each vial with normal food. three vials of each genotype and gender were counted in parallel, and the data obtained was combined together for making survival curves. Lifespans were determined at 29 °C, 50% humidity with a 12-h light/dark cycle. The number of dead flies was recorded every 2–3 days, when flies were transferred to new vials containing fresh food.

2.3. Paraquat resistance

For paraquat resistance assays, 2- to 3-day-old flies reared on normal food were collected. Flies were first starved in empty vials for 6 h at 25 °C and then placed into the vials (20 flies in each vial) with a filter paper soaked with 200 μl of 20 mM paraquat in 5% sucrose. Dead flies were counted every 4–8 h.

2.3.1. Real-time PCR

Gene expression of SOD1, SOD2, CAT was detected by real-time PCR according to Ref. [32]. The Trizol, reverse transcriptase and SYBR Green I containing real-time PCR reagent are all from TAKARA.

2.4. Cytochrome C oxidase activity

Ten whole male flies were ground in 400 μL PBST (PBS with 0.05% TWEEN-80), centrifuged at 8000*g* for 5 min at 4 °C, and supernatant was collected. 0.22 mM Cytochrome *C* was made fresh and reduced with 0.1 M DTT. Assay buffer contained 0.01 M Tris–HCl, 2.5 mM $MgCl_2$ and 10 μM reduced Cytochrome *C*. 10 μL of extract was added to 165 μL assay buffer and monitored at 550 nm over 10 s intervals for 1 min. Cytochrome *C* oxidase activity was normalized to total protein content quantified using Pierce BCA Protein Assay kit.

2.5. Climbing assay

20 Young (3 days) or old flies (30 days) were collected from each genotype and placed in a glass tube 10 cm high and 3 cm in diameter. The glass tubes were sealed at the top with parafilm to prevent flies from escaping. The flies were gently tapped down to the bottom of the tube and then observed for their climbing performance by counting the number of flies that could climb across the 8 cm line marked on the outside of the tube. The percentage of flies that crossed over the 8 cm line of each genotype was graphed.

2.6. Western blot

About 10–20, 6-day old, flies were ground in PBST supplemented with proteinase inhibitor cocktail and phosphotase inhibitor, centrifuged at 10,000*g* for 5 min at 4 °C and the supernatant was isolated. Total protein concentration was measured using Pierce BCA protein assay kit. Equal amounts of protein were run on SDS–PAGE under reducing conditions and then transferred to PVDF membrane. Proteins of interest were probed with specific primary antibodies: mouse anti ECP described in [17] at 1:2000, mouse anti α-tubulin at 1:5000 (Sigma), rabbit anti-phospho-Drosophila S6K (Cell signaling #9209) at 1:1000, rabbit anti phospho-AMPKα (Thr172, Cell signaling #2535) at 1:1000, rabbit anti-phospho-p44/42MAPK (Thr202/Tyr204, Cell signaling #9101) and rabbit anti-Phospho-Drosophila AKT (Ser505, Cell signaling #4054) at 1:1000. Blots were incubated in appropriate horseradish peroxidase conjugated secondary antibody, and then detected using ECL reagents (Pierce). Western blot results were imaged by exposing the blots to X-ray films and signal intensity was quantified using the Image J software. Three independent experiments were performed.

2.7. Statistical analysis

Quantitative data are expressed as mean ± S.E.M. Statistical significance of all the quantitative data obtained was analyzed using the Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3. Results

3.1. Ubiquitous knockdown of ECP results in extended lifespan and better locomotor performance

Bioinformatical analysis and previous data [17] support the involvement of ECP in protein synthesis. In order to observe the effect of ECP on translation, ECP was down regulated by RNAi in Drosophila KC cell line, then global translation efficiency was observed by quantification of S^{35}-Methionine incorporation. Results from this experiment show that when expression of ECP was effectively inhibited, S^{35}-Methionine incorporation decreased by about 45% (Supplementary Fig. 1).

Since protein synthesis, a complex and elegantly regulated process, was closely linked to extension of healthy lifespan [12,13], next, we wanted to test the effect of ECP on longevity in *Drosophila*. To this end, transgenic UAS-*ECP* or RNAi lines were driven by ubiquitous daGAL4 driver. Compared to control w^{1118}, median lifespan of da > UAS-*ECP* flies decreased by 25% for males and 6% for females, while that of da > RNAi-1 flies increased by 18% and 16% for males and females, respectively. Similarly, median lifespan of da > RNAi-2 flies increased by 25% and 21% for males and females, respectively (Fig. 1A and B).

There exists accumulating evidence to show that extension of lifespan also improves the declining of locomotor ability and delays the occurrence of age-related diseases [18,19]. Then, will this extension of lifespan by reducing ECP expression be accompanied by a slower decreasing of locomotor ability? To address this question, negative geotaxis assay was employed. Our findings show that all of the young flies (3-day) irrespective of ECP expression level could climb across the 8 cm line in 10 s. In contrast, for those old flies (30-day), only 11.1% of the *ECP* overexpression males were able to climb across the 8 cm line compared to 29.5% of control w^{1118} flies, while the number for *ECP* RNAi-1 and RNAi-2 flies is 44.4% and 50.1%, respectively. Similar trends could also be observed in females, though locomotion ability in females aged faster than in their male counterparts (Fig. 1C and D). Therefore, *ECP* RNAi can not only prolong the lifespan, but can also slow down the decline in climbing ability, while *ECP* overexpression will lead to the acceleration of this process.

3.2. ECP knockdown flies do not exhibit resistance to paraquat

More and more evidence in *C. elegans* and *Drosophila* supports that mutants selected for postponed senescence also display elevated resistance to various kinds of stress including oxidative stress [20]. Therefore, the effect of ECP on oxidative stress was examined next. Unexpectedly, the ECP overexpression flies were found to have a slight resistance to oxidative stress compared to control flies. In contrast, *ECP* RNAi flies do not exhibit obvious

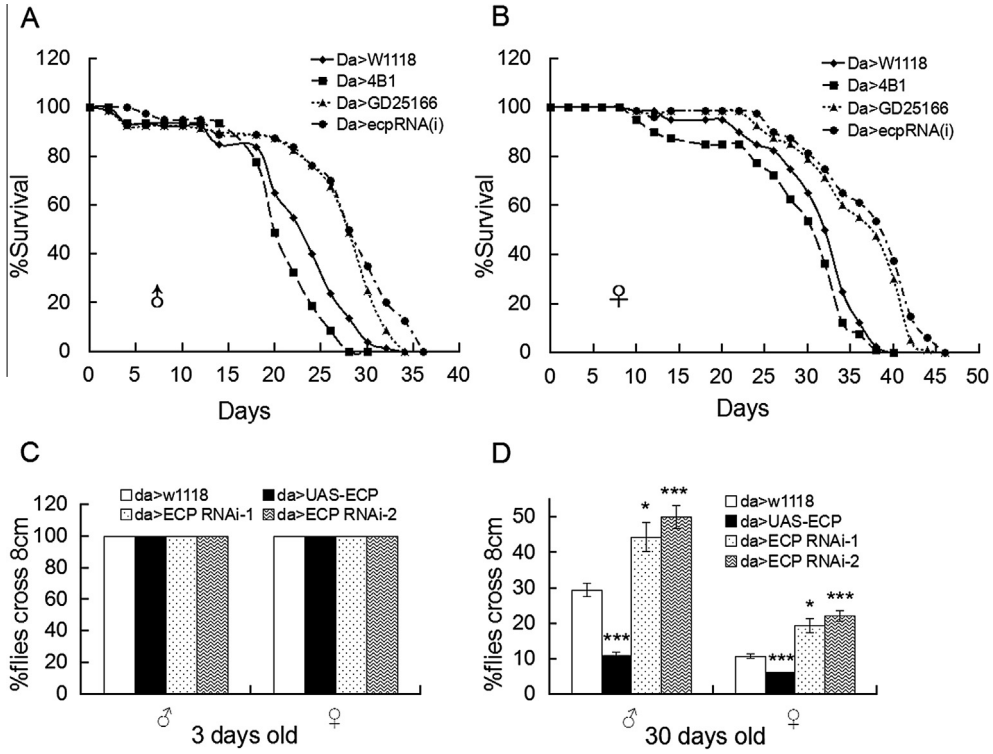

Fig. 1. Ubiquitous knockdown of ECP results in longer life-span and better locomotor performance. Survival of ECP overexpression and RNAi flies driven by daGAL4, (A) males and (B) females. (C) The percentages of 3-day-old flies of indicated genotypes that could climb up across the 8 cm line in 10 s. (D)The percentages of 30-day-old flies of indicated genotypes that could climb up across the 8 cm line in 10 s. (Student's *t* test,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

resistance to oxidative stress conferred by paraquat (Fig. 2). Superoxide dismutase (SOD1 and SOD2) and catalase (CAT) are the main antioxidant enzymes in Drosophila. No significant difference of the mRNA level of these genes was revealed by real-time PCR among the wild type, ECP overexpression and RNAi flies (Fig. 2C). This was consistent with the above paraquat resistance results.

Considering that *ECP* RNAi flies have a positive effect on lifespan extension, we think that the extension of lifespan by *ECP* RNAi might not result from activation of oxidative stress resistant genes.

3.3. Mitochondria complex IV activity is enhanced in ECP knockdown flies

Mitochondria have been identified as key players in aging process [21,22]. Significant age-related decline in COX (mitochondrial complex IV) activity, but not of the other mitochondrial oxidoreductases, was observed [23]. Zid and coworkers found that mitochondrial protein level and activity was enhanced significantly on DR while general cellular translation efficiency decreased due to elevated activity of 4EBP [24]. Similarly, knockdown of ECP also leads to reduced general protein synthesis (our unpublished data) and extension of lifespan. Then, does there exist a similar change of mitochondria function on *ECP* RNAi? To address this question, mitochondria complex IV activity was measured. Compared to control, both lines of ECP-RNAi flies driven by daGAL4 have an elevated complex IV activity at 11-days and 25-days as well, while ECP overexpression by the same driver has no obvious effect on complex IV activity (Fig. 3). These results suggest that lifespan extension by *ECP* RNAi might partially be mediated by the enhancement of mitochondria function.

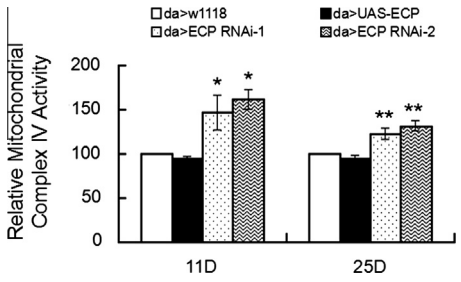

Fig. 2. Mitochondrial complex IV activity is enhanced in ECP knockdown flies. Mitochondria complex IV activity was measured on crude homogenates from whole males of different genotypes as indicated, and normalized to total protein content. Shown in this Figure is relative activity compared to control da > w^{1118} (as100%). (Student's *t* test,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3.4. Effect of ECP on AKT, TOR, AMPK and MAPK phosphorylation

Up to now, the clearest cellular signaling pathway tightly associated with longevity is the insulin/insulin-like growth factor (IGF) and Target of Rapamycin (TOR) network [2,6]. In addition, AMPK is an important nutrient and energy sensor that is required for insulin/IGF mutations to extend lifespan [25]. In order to investigate the possible interaction of ECP with the known longevity signaling pathways, the activities of AKT, AMPK and S6K, represented by their phosphorylation status were tested by Western blot using phosphorylation specific antibodies. As a result, a decrease of AKT and S6K phosphorylation level in contrast to an increase of AMPK phosphorylation level in those *ECP* RNAi flies was detected. Conversely, ECP overexpression has an opposite effect on the phosphorylation level of these kinases (Fig. 4). Given that insulin/IGF and TOR signaling has a positive effect on translation through S6K, thus knockdown of ECP expression might result in a decrease of cellular general translation efficiency which might contribute to longevity.

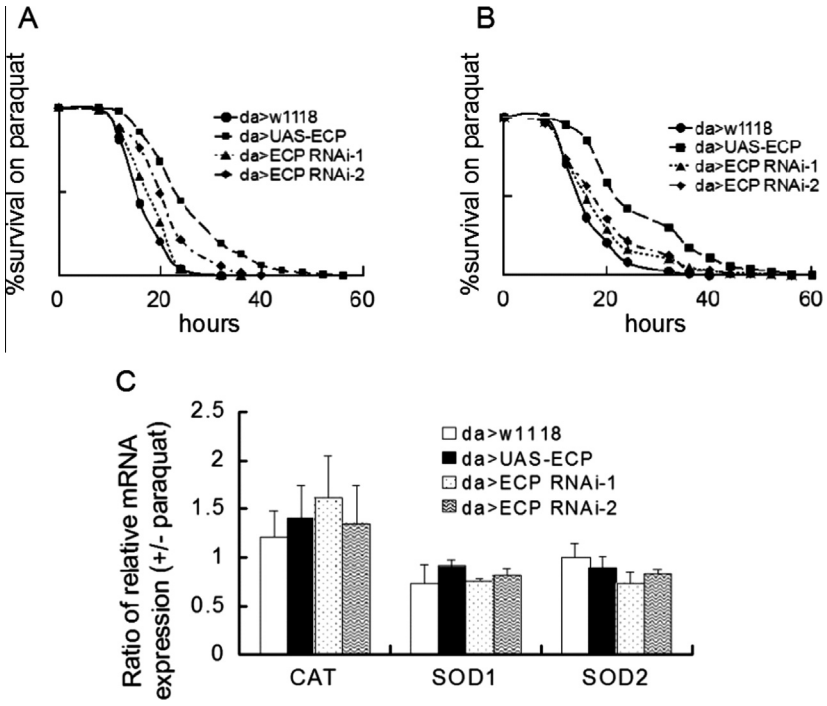

Fig. 3. ECP knockdown flies do not exhibit resistance to Paraquat. Survival of 3-day old males (A) and females (B) of different genotypes on 20 mM paraquat in 5% sucrose. (C) mRNA level of CAT, SOD1 and SOD2 respectively was detected by real-time PCR and normalized to RP49. Total RNA of about one week old male adults of the specified genotypes was used for reverse transcription. Shown here are the ratios of mRNA level of each gene under conditions with and without paraquat.

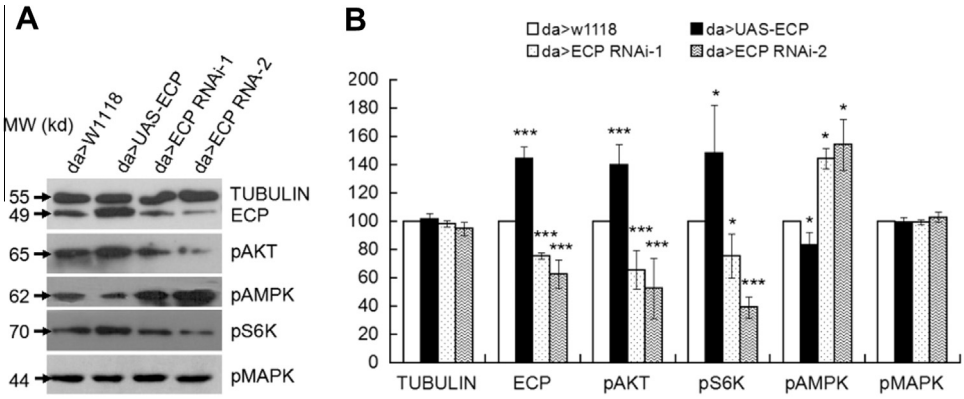

Fig. 4. Effect of ECP on AKT, TOR, AMPK and MAPK phosphorylation. Homogenates from adults of control w^{1118}, *ECP* RNAi or overexpression flies driven by daGAL4 were subjected to immunoblot analysis with indicated antibodies. (A) Representative Western blot results. (B) Relative band intensities of three independent experiments were quantified and are presented as bar graphs. (Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

4. Discussion

It is now widely accepted that inhibition of protein translation can promote longevity in different model organisms [12,13]. Mutations of a growing number of genes involved in protein synthesis have been identified as being able to extend healthy lifespan in yeast, worm, fly and mouse as well [14]. ECP/KRA was found to be a novel ribosome associated protein [17] which suggests its potential role in protein synthesis and thus might be related to longevity. Complete knockout of ECP or RNAi of this gene by strong ubiquitous actin-GAL4 results in developmental arrest at early pupa stage. Therefore, *ECP* is essential to fly development. However, another ubiquitous daGAL4 driver could effectively knockdown ECP expression while having no obvious effect on fly development. Our findings here show that both female and male *ECP* RNAi flies with this driver exhibit a significant longer lifespan compared to control flies, while overexpression of this gene leads to a slight decrease of lifespan (Fig. 1). Meanwhile, the old *ECP* RNAi flies also show a much better locomotor performance compared to control flies (Fig. 1). Together, the above data supports a positive effect of ECP on longevity and delaying age-related impairment in locomotor behavior. *ECP* was an evolutionarily highly conserved gene. Homologs of *ECP* exist in a variety of organisms including human. Two homologs of ECP named Bzw1 and Bzw2 exists in humans with about 50% sequence identity to its Drosophila counterpart ECP. But no homologs were found in yeasts and worms, from which most of the known longevity and aging related genes were first identified. Thus, the conservation of this gene from fly to humans suggests that its mammalian homologs, Bzw1 and/or Bzw2, might also have similar effects on longevity and delaying of aging related diseases.

IIS and TOR pathways are the most well-known signaling linked to longevity. Since these pathways are essential to life, strong alterations of either of the two signaling pathways can cause adverse effects, including embryonic lethality, cancer, and diabetes. On the other hand, milder down regulation is beneficial not only for longevity but also for delaying the progression of numerous aging related diseases such as cancer, neurodegeneration, cardiovascular disease, and diabetes [26,27]. TOR kinase acts through two protein complexes: TORC1 and TORC2. Both TORC1 and TORC2 interact with components of the IIS pathway: the TORC2 complex phosphorylates and activates AKT kinase. In addition, S6K, a downstream target of TORC1, could inhibit IRS at different levels. AMPK is a sensor of cellular energy status that is activated when ATP becomes limiting [28]. Overexpression of AMPK is sufficient to extend lifespan in *C. elegans* [29]. AMPK also interacts with TOR signaling by phosphorylating TSC2 on conserved serine sites, resulting in down regulation of mTORC1 activity [30]. Besides, mitochondria function has also been identified as a key player of aging process [20,21]. Here, we found a decrease of AKT and S6K activity accompanied by an increase of AMPK activity in *ECP* RNAi flies, while *ECP* overexpression has an opposite effect (Fig. 4). In addition, an elevated activity of mitochondrial complex IV was also revealed in those ECP RNAi flies (Fig. 2). It is important to note that knockdown ECP alone will result in several different cellular changes towards longevity. Therefore, ECP could become a novel effective target for fighting aging and aging related diseases in the future.

There is one possibility to explain the underlying mechanism about the complicated cellular changes in ECP RNAi flies. ECP down regulation might first lead to AMPK activation by yet unknown mechanism. Then, on one hand, activated AMPK will activate TSC2 by direct phosphorylation thus inhibiting TORC1, which eventually leads to the down regulation of S6K phosphorylation. On the other hand, AMPK will activate PGC-1α which is a transcriptional coregulator that orchestrates the mitochondrial biogenesis, therefore mitochondria function will be enhanced at transcriptional level [31]. Alternatively, mitochondria function might be activated at translational level. Mitochondrial ETC and mitochondrial ribosomal proteins have significantly shorter 5′UTRs with weaker secondary structure. This will lead to their translational up regulation upon activation of 4EBP when TOCR1 is inactivated by AMPK phosphorylation [24]. However, it is hard to explain how AKT activity was down regulated by ECP knockdown.

Taken together, our findings here reveal a novel aging related gene ECP. It is involved in regulation of the most well-validated antiaging pathway, the IIS/TOR signaling. Since milder down regulation of IIS/TOR signaling is beneficial not only for longevity but also for delaying the progression of numerous aging related diseases, it is possible that knock down ECP expression might also have beneficial effects on delaying the progression of these diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.133.

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