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Folic acid supplementation rescues anomalies associated with knockdown of *parkin* in dopaminergic and serotonergic neurons in *Drosophila* model of Parkinson's disease

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ABSTRACT

parkin loss associated early-onset of Parkinson's disease, involves mitochondrial dysfunction and oxidative stress as the plausible decisive molecular mechanisms in disease pathogenesis. Mitochondrial dysfunction involves several up/down regulation of gene products, one of which being p53 is found to be elevated. Elevated p53 is involved in mitochondrial mediated apoptosis of neuronal cells in Parkinson's patients who are folate deficient as well. The present study therefore attempts to examine the effect of Folic acid (FA) supplementation in alleviation of anomalies associated with *parkin* knockdown using RNAi approach, specific to Dopaminergic (DA) neurons in *Drosophila* model system. Here we show that FA supplementation provide protection against *parkin* RNAi associated discrepancies, thereby improves locomotor ability, reduces mortality and oxidative stress, and partially improves Zn levels. Further, metabolic active cell status and ATP levels were also found to be improved thereby indicating improved mitochondrial function. To corroborate FA supplementation in mitochondrial functioning further, status of p53 and spargel was checked by qRT-PCR. Here we show that folic acid supplementation enrich mitochondrial functioning as depicted from improved spargel level and lowered p53 level, which was originally vice versa in *parkin* knockdown flies cultured in standard media. Our data thus support the potential of folic acid in alleviating the behavioural defects, oxidative stress, augmentation of zinc and ATP levels in *parkin* knock down flies. Further, folic acid role in repressing mitochondrial dysfunction is encouraging to further explore its possible mechanistic role to be utilized as potential therapeutics for Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, comprising muscle rigidity, slowness of movement, resting tremor, and postural instability. PD is largely characterized by profound and progressive loss of DA neurons in the substantia nigra pars compacta region of the brain, although the mechanism underlying DA neurons loss is still unclear. In context to disease pathogenesis, mitochondrial dysfunction and oxidative stress act as key players, although what initiates disease progression involving dopamine producing neuron loss is still a big question to be decrypted. Genetic studies have identified gene candidates potentially involved in disease. To date many PD associated candidate genes have been identified, one of them being Parkin, a class of E3 ubiquitin ligases [1], whose loss of function mutation is associated with familial forms of autosomal–recessive juvenile Parkinsonism (AR-JP) [2]. Parkin is a pleiotropic protein playing crucial role in clearing unfolded protein stress through ubiquitin mediated protein degradation [3], transcriptional regulation [4], mitochondrial fission-fusion [5], mitophagy [6], mitochondrial aerobic respiration [7]. JNK down regulation [8], and also have a role in fertility [9,10].

Studies involving *Drosophila* model with *parkin* mutation/knockdown, has revealed various insights including the mitochondrial dynamics [9,10], which being a key ingredient in disease onset and progression. *parkin* mutation/knockdown in a fly model has decrypted its role in development and neurodegeneration, which involves progressive loss of DA neurons [11], indirect flight

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muscle degeneration [9], altered mitochondrial dynamics and mitophagy, susceptibility to oxidative stress, male infertility involving defective spermatogenesis, reduced longevity, flight disability with many other developmental glitches and Zn homeostasis [9,10,12–14].

Currently, researchers are focussing in deciphering role of Parkin in mitochondria related bioenergetics, as it is critical for functioning and survival of neuronal cells with high energy demands. In this context mitochondrial biogenesis is crucial and could be protective against mitochondrial dysfunction. Various approaches are targeted for enhancing mitochondrial biogenesis, one of the studies showed that enhancing nucleotide metabolism mends mitochondrial biogenesis which alleviate mitochondrial dysfunction in PINK-1 model of PD [15]. Moreover, folate involvement in nucleotide metabolism as a cofactor in transfer of 1-carbon unit is well established. Further, Parkinson's disease often involves certain micronutrient deficiency like folic acid [16,17] and zinc [18,19]. Folic acid deficiency in PD is also the cause of hyperhomocysteinemia [17] which in turn is responsible for DNA damage and p53 dependent mitochondrial mediated apoptosis [16]. p53 role is important as it's being a common target in both sporadic and familial forms of PD [20]. Therefore approaches targeting these aspects will provide a better understanding of the disease.

In the present study, effect of folic acid supplementation in specific knockdown (KD) of *parkin* in DA neurons of *Drosophila* was undertaken. Here we show that FA supplementation to *parkin* KD flies alleviates behavioural and locomotor defects with reduction in oxidative stress and the amelioration of ATP levels. We also report that *parkin* KD flies have reduced Zn levels and elevated p53 transcript levels. FA supplemented *parkin* KD flies have reduced p53 transcript level. Interestingly, Zn level was partially ameliorated in FA supplemented flies. Further, FA supplemented *parkin* KD flies improved spargel (a human PGC-1α homologue) transcript level, which is involved in mitochondrial biogenesis. Enhanced ATP level on account of FA supplementation could be associated with improved spargel levels and thus mitochondrial biogenesis. Therefore, FA supplementation could be beneficial in alleviating the anomalies associated with age-related neurological disorders like PD.

2. Material and methods

2.1. Fly stocks and culture conditions

Wild type (Oregon R^+), *parkin* RNAi stock (*P*{*UAS-parkin*^{RNAi}), DA GAL4 (w[*]; P{w[+mC] = ple-GAL4.F}3), and a DS GAL4 (w[1118]; P{w[+mC] = Ddc-GAL4.L}4.36), were used. All stocks were obtained from Bloomington *Drosophila* Stock Centre, Indiana, USA. Culturing of flies was done in standard cornmeal agar media and stocks were maintained at 24 ± 2 °C in B.O.D incubator.

2.2. Driving *parkin*^{RNAi} in DA and DS neurons

Knockdown of *parkin* was done specifically in DA and DS neurons using UAS-*parkin*^{RNAi}, Dopeminergic (DA) and Serotonergic (DS) GAL4 driver lines, respectively. For this, virgin female flies of *UAS-parkin*^{RNAi} were crossed to males of DA and DS GAL4 separately. UAS-*parkin*^{RNAi}/DA GAL4 and UAS-*parkin*^{RNAi}/DS GAL4 obtained from F1 generation were used for the study. Twenty each of male and female flies were taken for the cross. (Cross scheme shown in Fig. S1, Supplementary Information).

2.3. Folic acid treatment

FA was added to the food to achieve a final concentrations of 125 μM (as per best effective dosage determined ranging between 10 and 250 μM) in the food after it had cooled below 60 °C. Comparative analysis was done between FA treated food versus standard food in flies of each genotype (*UAS-parkin*^{RNAi}/DA GAL4 and *UAS-parkin*^{RNAi}/DS GAL4).

2.4. Climbing assay

To study the locomotor activity status, comparative climbing assay was carried out in 10 days old flies of each genotype (*UAS-parkin*^{RNAi}/DA GAL4 and *UAS-parkin*^{RNAi}/DS GAL4) in both FA treated versus standard food. DA GAL4 flies were used as control. Flies of each genotypes were dropped simultaneously in separate twin towers (two transparent measuring cylinders as a unit) having marking of 10 cm. Flies were knocked down to bottom by tapping and allowed to climb for 20 s, climbing distance with respect to ≤10 cm and >10 cm was observed for both genotypes. Data was analyzed based on triplicate experiments.

2.5. Mortality assay

Comparative study on mortality between flies of each genotype (*UAS-parkin*^{RNAi}/DA GAL4 and *UAS-parkin*^{RNAi}/DS GAL4) in both FA treated versus standard food was done from post eclosion till death. DA GAL4 flies were used as control. Six vials each containing twenty flies for each genotype were maintained in their respective media. Flies were transferred to fresh food vials every day and number of dead flies was counted. Data was analyzed for mortality in triplicate.

2.6. Oxidative stress analysis

Lipid peroxidation (LPO) involving TBARS (Thiobarbituric acid reactive substances) content estimation using method as described [21] and H_2O_2 content analysis using method as described [22] were comparatively assayed to look into the stress response status in FA supplemented versus standard food cultured flies of each genotype. DA GAL4 flies were used as control. Fly heads were taken for the study. Data was analyzed based on the triplicate experiments.

2.7. RNA extraction and quantitative real time-PCR (qRT-PCR)

parkin, p53 and spargel mRNA levels were analysed using qRT-PCR. Total RNA from 100 fly heads of each genotype (DA GAL4, *UAS-parkin*^{RNAi}/DA GAL4 and *UAS-parkin*^{RNAi}/DS GAL4) was isolated using TRI reagent (Sigma) as per manufacturer's protocol. High quality RNA from both genotypes (as estimated by absorbance ratio A260/280) was reverse transcribed to their respective cDNA through reverse transcriptase PCR. Resulting cDNA of each genotype was used as a template for quantification of parkin, p53 and spargel normalised against gapdh using specific primer set (shown in 5′-3′):

parkin: FP-TTGCAGCCAATGCGATAAGC; RP- TGGAGCCGCAAA ATCCTTCT.

p53: FP- TGGGATATCGGCAACGAAGT; RP- ATCTTGTTGGGGAAG AGGC.

spargel: FP- AAAGTGATTGCGGGTTGCTT; RP- GTTGTGGTTCCGG ATCACCT.

gapdh: FP- CCACTGCCGAGGAGGTCAACTAC; RP- ATGATGCT-CAGGGTGATTG CGTATGC by SYBR(R) GREEN JUMPSTART TAQ READYMIX (Sigma) using real-time thermal cycler (Applied

Biosystems, 7500 real time PCR). Samples were run in triplicates and the mRNA relative levels were standardized by cycling threshold analysis (ΔC_T).

2.8. Zinc homeostasis

In this case, age matched adult flies of each genotype cultured in FA supplemented food versus standard food, with DA GAL4 flies as control were taken for comparative zinc metal homeostasis analysis. 200 adult fly heads of each genotype were taken for the study. Fly heads were subjected to digestion for mineralization in 10 ml of 20% conc. HNO_3 (Merck) at 150 °C for 30 min. Samples were cooled and filtered by Whatman paper, filtrate collected was maintained to a net volume of 50 ml with Milli-Q water (Millipore). The sample was analyzed against their respective standards (Transition metal mix 1 for ICP, Sigma) in a Perkin–Elmer Optima 7000 DV ICP optical emission spectrometer. Data was analyzed based on the triplicate experiments.

2.9. Metabolic active cell status

In this study, metabolically active cell status in larval brain of DA GAL4, *UAS-parkin*^{RNAi}/DA GAL4 and *UAS-parkin*^{RNAi}/DS GAL4was comparatively analysed in FA supplemented food versus standard food using MTT assay [23] with slight modifications. Ten 3rd instar larvae of each genotype with respective treatments were taken separately in triplicate for the study. Larvae were dissected in 1x PBS and whole larval brain tissues were incubated in 100ul of 0.5 mg/ml MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide] (MP Biomedicals, France) for 2 h at 37 °C. MTT solution was removed and tissues were washed twice with 1xPBS. Further 200 ul DMSO (Sigma) was added (1 h, 37 °C) to the tissue sample in order to solubilize the insoluble purple formazan crystals. The coloured solution was then quantified by spectrophotometric absorbance at 570 nm wavelength on a microtiter plate reader (Bio-Rad model 680 microplate reader). Data was analysed based on triplicate experiments.

2.10. ATP quantitation assay

Age matched 10 days old fly heads of each genotype cultured in FA supplemented food versus standard food were taken for ATP quantification. ATP content was determined using ATP colorimetric assay kit (Biovision, USA) as per manufacturer's protocol. Absorbance at 570 nm was taken for each sample on a microtiter plate reader (Bio-Rad model 680 microplate reader) and was analysed with ATP standard reference curve. Finally, the amount of ATP was standardized against protein concentration determined by the Bradford method. Data was analysed based on triplicate experiments.

2.11. Statistical analysis

Graph Pad Prism 5 software was used for statistical analysis. Data obtained from the experiments were compared with either the student t–test or one way anova depending on the number of groups involved. Error bar represents SEM (Standard Error of Mean).

3. Results and discussion

3.1. Folic acid alleviates mortality, locomotory defect and oxidative stress in parkin^{RNAi} flies

Folic acid supplementation to *parkin* knockdown flies, alleviates mortality and locomotory defects involving reduced average life span and climbing ability, respectively (Fig. 1A and B). In climbing assay, majority of FA supplemented *parkin* knockdown flies were able to climb above 10 cm mark (Fig. 1B), unlike *parkin* knockdown flies cultured in standard food where majority of flies was failed to climb beyond 10 cm mark (Fig. 1B). FA supplemented *parkin* knockdown flies were found to be more active contrary to *parkin* knockdown flies without FA supplementation, thereby suggesting a possibility of improved energetics on account of FA supplementation. Further, elevated level of TBARS (Fig. 1C) and H_2O_2 (Fig. 1D) was observed on account of parkin suppression using RNAi approach. FA supplementation attenuates TBARS and H_2O_2 levels (Fig. 1C and D respectively) thereby reducing oxidative stress and thus complementing improved locomotor activity and longevity.

3.2. Folic acid partially ameliorates zinc levels in DA neuron specific parkin knockdown flies

Zinc is one of the essential trace elements and its imbalance is associated with oxidative stress and neurodegenerative diseases like Alzheimer's and Parkinson's [24]. A study conducted to check zinc status in DA neurons specific *parkin* knockdown fly heads revealed lower Zn level as compared to control (Fig. 2A). Further, Folic acid supplementation partially improves Zn levels as compared to flies with *parkin* RNAi background cultured in standard food (Fig. 2A). Amelioration of Zinc levels is crucial as it associates with a variety of proteins and imparts complex roles in various biological processes, specifically in reproduction, development, synaptic plasticity, antioxidant activity and DNA damage/repair [24].

3.3. Folic acid ameliorates metabolic active cell status and ATP level

Folic acid supplemented *UAS-parkin*^{RNAi}/DA GAL4 and *UAS-parkin*^{RNAi}/DS GAL4 larvae displayed improved level of metabolically active cells as revealed by MTT assay, unlike larvae of same genotype cultured in standard food (Fig. 2B). MTT is a yellow tetrazolium salt which on reduction forms insoluble formazan by the action of cytosolic NAD(P)H-dependent cellular oxido-reductase enzymes present in metabolically active cells [23]. Greater the metabolic activity of cells, higher will be the activity of these enzymes thus leading to increased reduction of MTT. Further, low ATP levels associated with adult flies of *UAS-parkin*^{RNAi}/DA GAL4 and *UAS-parkin*^{RNAi}/DS GAL4 as compared to control was improved in Folic acid supplemented flies of same genotype (Fig. 2C), suggesting improved energetics.

3.4. FA supplementation reduces p53 and improves spargel transcript levels

Parkin is known to regulate repression of p53 transcription and oxidative stress [20], and may mediate PGC-1α dependent mitochondrial biogenesis [25]. We therefore checked status of *parkin* transcript level in *UAS-parkin*^{RNAi}/DA GAL4 and *UAS-parkin*^{RNAi}/DS GAL4 flies against its control by qRT-PCR analysis and low relative mRNA fold change for parkin was found in *parkin* KD flies (Fig. 3A). Further, p53 and spargel (*Drosophila* PGC-1α) transcript levels were checked by qRT-PCR to compare the status of mitochondrial biogenesis in adult brains of *parkin* KD flies cultured in folic acid supplemented versus standard food. Elevated p53 with reduced spargel transcript level in adult brain of *parkin* KD flies was reversed in FA supplemented flies (Fig. 3B and C respectively). Therefore, FA supplementation improves mitochondrial biogenesis as is reflected from improved spargel and ATP levels in brain of folic acid supplemented UAS-*parkin*^{RNAi}/DA GAL4 and UAS-*parkin*^{RNAi}/DS GAL4 flies.

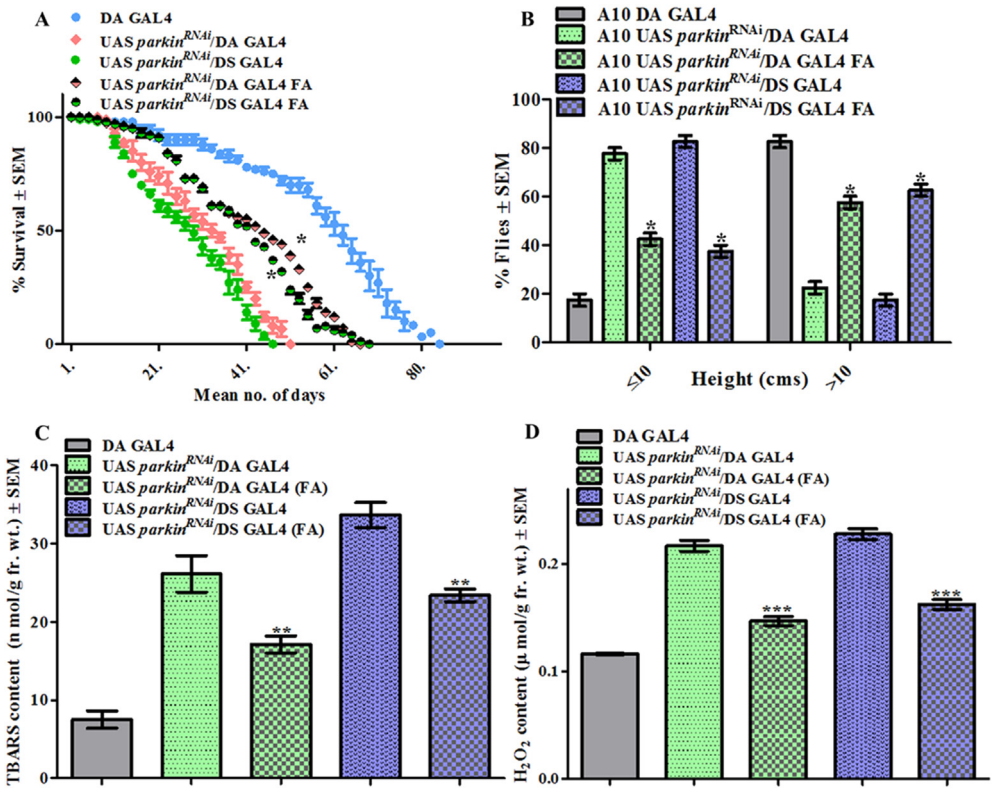

Fig. 1. Folic acid alleviates locomotor defect, reduces mortality and oxidative stress associated with *parkin* knockdown in DA neurons. A. Mortality rate reduction in FA supplemented *parkin* knockdown flies. B. FA supplementation improves climbing ability in *parkin* knockdown flies. Fly age is shown as A10 = 10 days old. C and D. FA reduced TBARS level and H_2O_2 content in *parkin* knockdown flies. ***P < 0.0001, **P < 0.05, *P < 0.05.

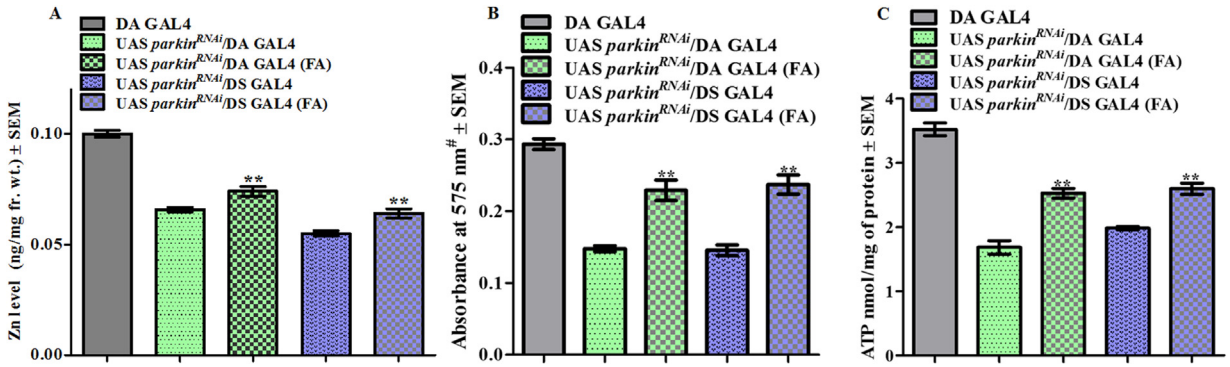

Fig. 2. Comparative illustration of Zn level, metabolic active cell status and ATP level. A, FA supplementation in *parkin* KD flies partially ameliorates Zn levels. B, FA ameliorates metabolic active cell status as observed from MTT assay of 3rd instar larvae brain with *parkin* KD background. C, comparative ATP level in adult fly brain of each genotypes. #: Absorbance corresponds to metabolically (viable) active cells. **P < 0.005.

4. Discussion

Folic acid deficiency is often associated with neurodegenerative diseases like Alzheimer's and Parkinson's [16], leading to hyperhomocysteinemia [17] that damages DNA, initiating mitochondrial mediated apoptosis via p53 induction [16]. Therefore, FA supplementation could be beneficial in alleviating the PD associated pathology. In the present study, effect of FA supplementation was undertaken in DA and DS neuron specific *parkin* KD in *Drosophila* model of Parkinson's disease. This study clearly shows that FA supplementation to *Drosophila* with DA neurons specific *parkin* KD, improves longevity as is depicted from low mortality rate. Locomotory defect as manifested from depreciated climbing ability in *parkin* KD flies was alleviated by FA supplementation. Further, elevated level of oxidative stress associated with *parkin* KD flies was also melted down by FA. Oxidative stress and mitochondrial dysfunction both being a key player in disease pathogenesis are commonly associated to each other [26–28]. Further, *parkin* is associated in mitochondrial maintenance, and its loss of function leads to mitochondrial dysfunction with low ATP levels as reviewed elsewhere [20]. In this context, metabolic active cell status and ATP levels were analysed and we found that FA supplementation improve metabolic active cell status and ATP levels in *parkin* KD flies, thereby indicating improved status of mitochondrial functioning, which was also supported from alleviated level of oxidative stress in FA supplemented *parkin* KD flies.

Further, p53 is a key apoptotic modulator in several neurodegenerative disorders and is linked to genetic and sporadic forms of

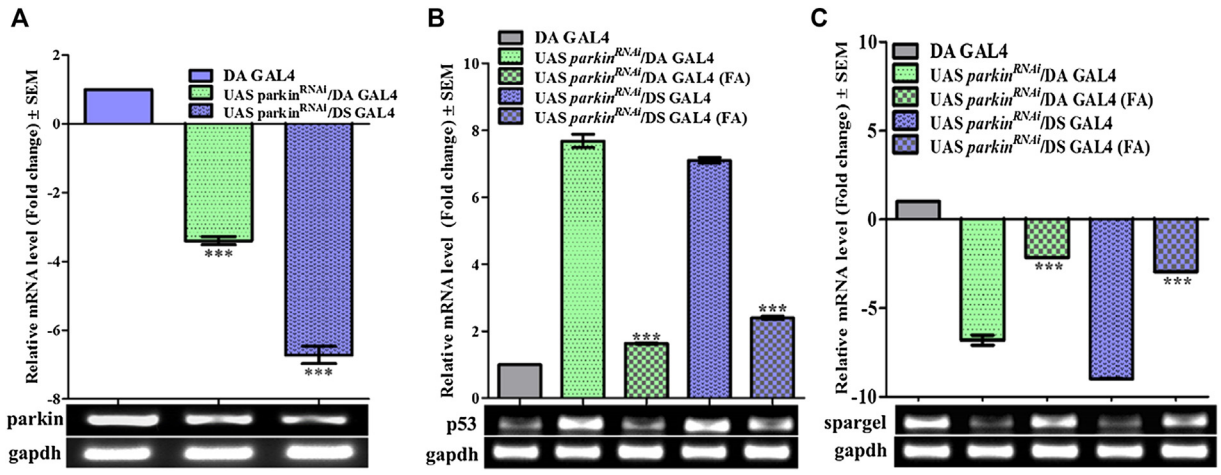

Fig. 3. qRT-PCR analysis of the parkin, p53 and spargel (pgc-1α) transcript levels in *parkin* KD flies supplemented with or without FA. A, relative mRNA status of parkin. B, comparative illustration of relative mRNA levels of p53 and C, spargel mRNA level. FA supplementation to *parkin* KD flies represses p53 while upregulate spargel level, thereby indicating improved mitochondrial functioning.gapdh was taken as internal control. Relative mRNA fold change was derived from $\Delta\Delta C_T$ values. ***P < 0.0001.

parkinson's disease. Parkin is known to regulate repression of p53 transcription and oxidative stress [20], and may mediate PGC-1α dependent mitochondrial biogenesis [25] (Fig. 4). Also, *parkin* loss leads to p53 mediated alteration in energy metabolism leading to Warburg effect [7]. In this milieu, qRT-PCR analysis of p53 and spargel (*Drosophila* PGC-1α) showed elevated p53 with reduced spargel transcript level in *parkin* KD flies. Further, it was also found that FA supplementation reverses the p53 and spargel transcript status, an indicative of improved mitochondrial biogenesis which was also reflected from improved ATP level in folic acid supplemented *parkin* KD flies (Fig. 4). Also, spargel is known to regulate oxidative stress therefore increased transcript level of spargel further corroborates the reduction of oxidative stress in folic acid supplemented *parkin* KD flies.

p53 mediated apoptosis involving mitochondria is critical for cell and one of the trace metal ion Zn, plays a worthy role as Zn deficiency induces p53 expression, thereby initiating apoptotic signal causing loss of mitochondrial membrane potential and p53 dependent caspase activation leading to apoptosis [29]. Also, Zn deficiency induces p53 and oxidative DNA damage [30]. As *parkin* KD flies were having increased p53 transcript level and oxidative stress, we also analysed Zn level in *parkin* KD flies. Here we show that *parkin* KD flies have low Zn level, thereby supporting p53 mediated mitochondrial dysfunction. However, FA supplemented *parkin* KD flies were having partially improved Zn level, which could possibly suggest that FA is somehow maintaining the Zn level, although FA role in Zn absorption and excretion is not yet clear. It will be interesting to decrypt complementary role of FA and Zn in context to *parkin* and *p53*. Zn deficiency on the other hand could also be the reason for FA deficiency, as Zn based enzymes are known to be involved in folate absorption from food.

To summarize, we confirm that FA dietary supplementation to DA neuron specific *parkin* KD model of *Drosophila* is protective against disparities involving increased mortality, locomotor defect involving reduced climbing ability, elevated oxidative stress, low metabolic active cell status and energy metabolism with reduced ATP level. Further, FA supplementation also resulted in down regulation of p53 and up regulation of spargel transcript level

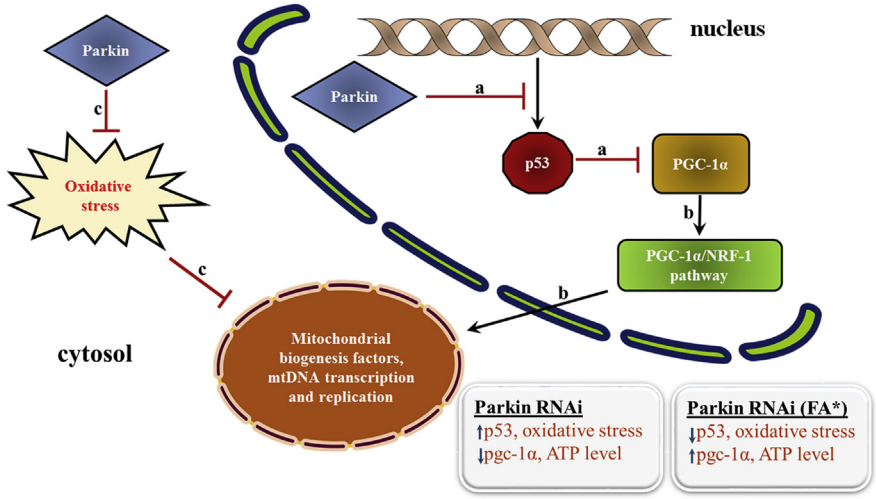

Fig. 4. Schematic illustration showing the effect of FA supplementation on oxidative stress, p53 and pgc1-α mRNA level, and ATP level in DA neuron specific parkin RNAi flies. Parkin RNAi elevates the p53 transcription and oxidative stress while down regulating spargel (shown as pgc-1α) and ATP levels. FA supplementation to the *parkin* RNAi flies shows the repression of p53 and up regulation of spargel transcription. Further, oxidative stress was reduced while ATP levels were ameliorated thereby indicating improved mitochondrial function. Schematic illustration of Parkin role: a, Parkin acts as a transcriptional repressor of p53 thereby enhancing PGC-1α activity. b, PGC-1α co-activates the pathway leading to mitochondrial biogenesis and cellular energetics. c, Parkin regulates oxidative stress thereby keeping mitochondrial stress in check. * Folic acid supplemented parkin RNAi flies.

suggesting its role in alleviating oxidative stress, and also indicates the improvement in mitochondrial biogenesis as reflected from improved ATP levels. Low Zn level in *parkin* KD flies, also indicates oxidative damage and further corroborates increase in p53 transcript level thereby causing mitochondrial dysfunction leading to reduced ATP levels. Therefore, role of FA in context to *parkin* loss, Zn homeostasis and p53 needs attention as it would provide a better insight to understand the mechanistic role in mitochondrial maintenance and disease pathogenesis and to design a better therapeutic approach for complex neurological disorders. To the best of our knowledge this is the first report showing protective role of FA in alleviating the anomalies associated with *parkin* loss of function in dopaminergic neurons in Parkinson's model of *Drosophila*.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.106.

Transparency document

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