Oxidative Stress Promotes Eating Behavior and Obesity in *C. elegans* via EGL-4 / DAF-16 Signaling

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Abstract: Oxidative stress is associated with pathophysiological progress of many diseases. The objective of study was to investigate whether increased environmental oxidative stress stimulation can promote excessive eating behavior, a common cause of obesity, and to identify the molecular mechanism. The cGMP-dependent kinase (PKG) activator 8-pCPT-cGMP was applied in worm swimming assay to study behavior shifting between quiescence and foraging in *C. elegans*. Genetically modified *C. elegans* (*egl-4* loss or gain of function, and *daf-16* mutant) were treated with paraquat, an oxidative stress inducer. Worm's foraging behavior, body fat accumulation and body length were determined. The *foxo1*::*gfp*-transfected HEK293 cells and *C. elegans* (*daf-16*::*gfp*TJ356) were further used to examine the effect of paraquat on PKG expression and FOXO nuclear translocation. A novel swimming assay using PKG activator stimulation was developed, which allows the rapid and effective study of foraging behavior in *C. elegans*. Paraquat treatment significantly inhibited quiescence, promoted foraging behavior, increased body fat accumulation and body growth. These responses were associated with diminished PKG expression/activation and increased FOXO (DAF-16) nuclear translocation in both transfected *C. elegans* and HEK293 cells. Our data suggest that PKG/FOXO signaling may plays an important role in mediating oxidative stress-induced excessive eating behavior and obesity development.

Keywords: Oxidative stress, Eating behavior, Obesity, EGL4/cGMP-dependent protein kinases, DAF-16/FOXO, *C. elegans,* HEK293 cells.

INTRODUCTION

The incidence of obesity is increasing at an alarming rate in the developed world [1, 2]. A recent study by the National Health and Nutrition Examination Survey has suggested that 17% of the youth and 35% of the adults in the United States are obese [2]. The reasons for the growth in the number of obese are not currently clear, likely complicated, and probably multifactorial in nature, however, it is probable that excessive food energy intake plays a major role [3].

Eating behavior and energy homeostasis are thought to be tightly regulated by an intricate feed-back system, which involves sensing and integrating numerous types of information regarding energy availability and demand. Although the signaling cascades involved in the integration process are not entirely understood, recent data suggest that cGMP-dependent protein kinases or protein Kinase G (PKG) may be involved [4-6]. The PKGs are serine / threonine kinases that are activated by cGMP [4, 5]. These proteins are present in most eukaryotic organisms and have been posited to play roles in a number of different processes, including the regulation of foraging behavior, food acquisition and energy balance [7, 8]. In Caenorhabditis elegans (*C. elegans*), it has been reported that a PKG homolog, *egl-4*, regulates quiescence, a state which is thought to mimic satiety in mammals [9, 10]. On the basis of these data and previous reports suggesting that oxidative stress can impair PKG activity [11-14], we hypothesized that increases in oxidative stress would be associated with diminished *C. elegans* quiescence, increased eating behavior, and body size (obesity). To test this possibility, we measured the foraging behavior under conditions of elevated oxidative stress in different *C. elegans* strains containing various mutations in the *egl-4* gene. Our data demonstrate that increased oxidative stress stimulates worm food seeking behavior which is associated with increased body growth and fat deposition. Additional work using other constructs and transfected HEK293 cells suggests that these responses were mediated, at least in part, by EGL-4 (PKG) and the inhibition of DAF-16 (FOXO) signaling.

MATERIALS AND METHODS

Materials

C. elegans strains, including N2 (wild type), *daf-16* (CF1038, DAF-16 loss of function), *egl-4* (lf) (FK223, PKG loss of function), *egl-4* (gf) (DA521; PKG gain of function), *daf-16*::*gfp* (TJ356), were obtained from University of Minnesota Caenorhabditis Genetics Center (Minneapolis, MN). The PKG activator 8-(4-

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chlorophenylthio)-cGMP (8-pCPT-cGMP) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sudan black B and paraquat (PQ) were from Sigma Aldrich (Saint Louis, MO). M9 buffer, S-basal complete medium (S medium) and nematode growth medium (NGM) were from IPM Scientific (Eldersburg, MD). The Lipofectamine 2000, Lipofectamine plus reagents and G418 were from (Invitrogen, Carlsbad, CA). The PKG antibody was from LSBIO (Seattle, WA), while β-tubulin antibody was obtained from Thermo Scientific (Waltham, MA). Enhanced chemiluminescence detection system was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Swimming Assays

Swimming assays were performed as described earlier [10, 15]. Briefly, one day old worms were transferred to a fresh unseeded plate and allowed to forage for 2 minutes before being transferred individually into the wells of a microtiter plate filled with 200 µL of S medium containing *E. coli* OP50. The optical density at 600 nm of the S medium was 0.6 units. Assays were carried out at 25 ± 1 °C. Worms that exhibited less than two body bends over a 5 second period of observation were considered quiescent [10, 15]. Swimming assays were conducted over a 10 minute time period.

PKG Resistance Assay

The PKG activator 8-pCPT-cGMP was used to determine the role of PKG activation in regulating eating behavior. Briefly, two days old worms were transferred to a fresh unseeded plate and allowed to forage for 2 minutes before being transferred individually into the wells of a microtiter plate filled with 200 µL of S medium containing 10 mM of 8-pCPT-cGMP. Assays were carried out at 25 ± 1 °C. Worms that exhibited less than two body bends over a 5 second period of observation were considered quiescent [10, 15]. PKG resistance was examined over a 10 minute time period.

Cell Culture

HEK293 cells were transfected in 6 cm^2 dishes with *foxo1::gfp* DNA using Lipofectamine Plus reagents as directed by the manufacturer. Forty-eight hours after transfection the cells were split at 1:10, 1:20, and 1:40 and selected in G418. Surviving colonies exhibiting GFP fluorescence were picked by pipette and expanded for further use.

Immunoblotting

Transfected HEK293 cells were pelleted and resuspended in 1× Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM NaPiP, 2 mM Na_3VO_4, and 1× protease inhibitor cocktail). Cells were lysed for 10 min at 4 °C, and centrifuged at 15,000 × g for 10 min at 4 °C to pellet debris [16]. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis analysis and electrotransferred onto polyvinylidene difluoride membranes. Membranes were probed with the indicated primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive signals were visualized after washing with tris buffer saline containing 0.5% Tween 20 (TBS-T) with an enhanced chemiluminescence detection system as described previously [16]. Where appropriate, membranes were stripped by incubation in stripping buffer (62.5 mM Tris-HCl, pH 5.7, 100 mM 2-mercaptoethanol, and 2% SDS) for 1 h at 70 °C with constant agitation, washed, and then re-probed with additional antibodies as indicated.

Sudan Black B Staining

Worms were synchronized after three days in culture, washed in S medium for 30 min, fixed with 1% paraformaldehyde in S medium, and subjected to three freeze-thaw cycles. The animals were then dehydrated through consecutive washes with 25%, 50%, and 70% ethanol. Staining was performed overnight (approximately 16 hours) in a 50% saturated solution of Sudan black B in 70% ethanol. After staining was complete, worms were washed for 4 × 10 min with M9 buffer and randomly chosen fields were photographed under a bright field microscope equipped with Olympus WH 10x widefield eyepiesces and an Olympus UPlan F1 40x/0.75 objective lens (Olympus, BFX51, Melville, NY) [16].

Statistical Analysis

Results are presented as mean ± SEM. Comparisons between groups were performed using the *Students t-tests* or one-way analysis of variance (ANOVA) and *post hoc* testing as appropriate. The level of significance accepted *a priori* was $P \leq 0.05$.

RESULTS

Development of EGL-4/PKG-Dependent *C. elegans* Quiescence Assay

Previous reports have demonstrated that *C. elegans* typically exhibits an initial 1-2 hour phase of continuous

swimming after transfer from a solid surface into a liquid solution that is followed by quiescence for several minutes [10, 15]. Here we developed a new assay system, which appeared to significantly shorten this course of events. One day old *C. elegans* were transferred to a 96-well plate containing S medium with 10 mM PKG activator (8-pCPT-cGMP). Swimming assays were performed to determine the quiescence of worms, which exhibited less than two body bends over 5 seconds of observation. After transferring from a solid surface into a liquid solution containing the PKG activator 8-pCPT-cGMP, about half of the N2 (wild type) worms became quiescent within ~ 2.5 minutes and that almost all were quiescent within about 4 minutes (Figure **1**). When repeating this swimming assay with *egl-4*(gf) worms (DA521; gain of function mutation) we observed that about 50% of the worms were quiescent within ~1.5 minutes and that almost all of the worms became quiescent within ~2.5 minutes (Figure **1**). Conversely, using the *egl-4*(lf) worms (loss of function mutation) the presence of quiescence appeared to be much lower as almost 95% of the worms were still active after 10 minutes of exposure to the PKG activator. Taken together, these data suggest that exposure to the PKG activator 8-pCPT-cGMP can be used as an effective assay to study quiescence and foraging behaviors in *C. elegans*, and that this response appears to be regulated through PKG-dependent signaling.

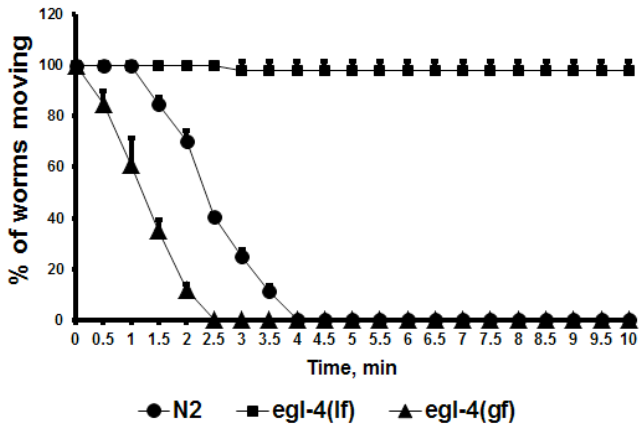

Figure 1: PKG-dependent quiescence assay.

Paraquat Treatment Decreases EGL-4/PKG-Dependent Quiescence, and Increases Body Length and Fat Accumulation

Recent data has posited that reactive oxygen species may play an important role in the regulation of obesity [16, 17]. It is also thought that over-eating is a primary cause of obesity however it is unclear whether reactive oxygen species (ROS) are involved in the regulation of eating behavior. To investigate the relationship between elevations in ROS and eating behavior we next examined how paraquat exposure, an oxidative stress inducer [18], affected worm quiescence, and swimming assays in response to PKG activator (10 mM 8-pCPT-cGMP) were performed in N2 worms without or pre-treated with paraquat (PQ; 0.25 mM) for 1 day. As shown in Figure **2A**, worms grown in the absence of paraquat exhibited 100% quiescence after approximately 4 minutes in the swimming assay when exposed to the PKG activator 8-pCPT-cGMP. Conversely, about 85% of the worms that had been grown in the presence of 0.25 mM paraquat failed to become quiescent even after ten minutes of exposure to 8-pCPT-cGMP (Figure **2A**).

To extend these data, we next examined the effects of ROS on the growth of *C. elegans* by measuring body length and body fat accumulation of N2 worms after 3 days without or with 2.5 mM paraquat (PQ), as estimated by Sudan Black B staining. Compared to untreated controls, paraquat exposure was associated with increased body length ($P \leq 0.05$; Figure **3**) and body fat accumulation in wild type N2 worms (black spot; Figure **2B**). Taken together, these data support the contention that exposure to ROS is associated with increased feeding behavior and the subsequent development of obesity.

EGL-4/PKG Mediates Paraquat-Induced Body Growth

To determine whether PKG might be involved in mediating the effect of ROS on body growth, we next investigated the effects of *egl-4* loss of function (FK223) and gain of function (DA521) on worm growth. Compared to N2 worms, body length was shorter in the *egl-4* (gf) mutants ($P \leq 0.05$; Figure **3**) and conversely, longer for *egl-4* (lf) mutants ($P \leq 0.05$; Figure **3**). Taken together, these data support the notion that body size may be controlled, at least in part, through EGL-4 signaling.

In an effort to assess whether these molecules play a mechanistic role in paraquat-induced animal growth, we next examined how mutation of *egl-4* may influence body length in response to paraquat treatment. Worms were collected at L1 stage and cultured on NGM medium with 2.5 mM PQ at 20 ^{o}C, and body length was measured after four days of treatment. Similar to that observed in N2 wild type worms, paraquat exposure was associated with robust increases in body size for the *egl-4* (lf) ($P \leq 0.05$; Figure **3**) but not the *egl-4* (gf) mutants (Figure **3**) which suggests that diminished

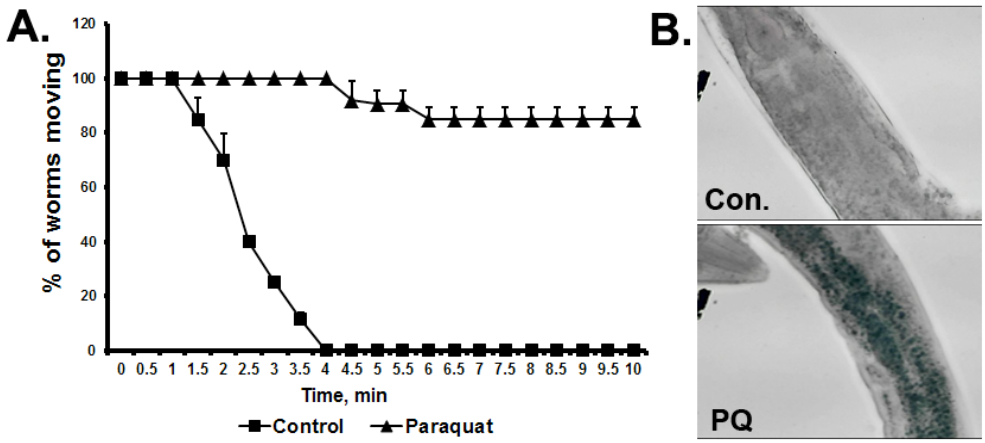

Figure 2: Paraquat decreases quiescence (**A**) and increases body fat accumulation (**B**) in *C elegans.*

PKG enzymatic function may be involved in mediating ROS-induced body growth.

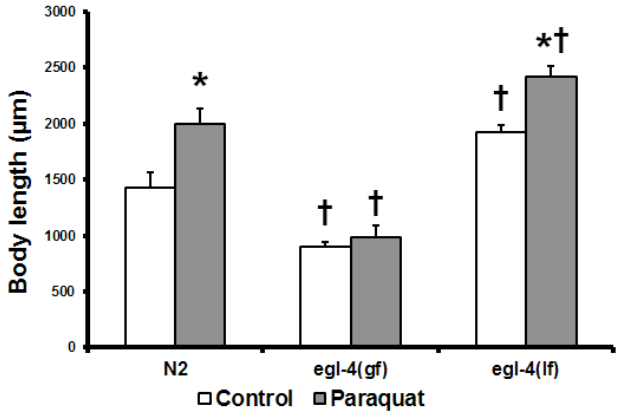

Figure 3: EGL-4/PKG mediates paraquat-induced body growth.

ROS Decreases PKG Expression

In an effort to further understand the interaction between PKG and cellular ROS levels, we next investigated whether paraquat treatment results in decreased PKG expression using cultured HEK293 cells. Consistent with the possibility that ROS levels may function to modulate PKG, we found that treatment with increased paraquat concentration (1-10 mM) for 8 hours was capable of decreasing PKG expression (Figure **4**).

DAF-16/FOXO may Function as a Downstream Mediator of EGL-4/PKG

Despite its potential importance in regulating energy homeostasis, little is known regarding how PKG may function. Recent studies suggested that FOXO can be phosphorylated by PKG [19-21], and that the over-activation of FOXO can cause increased food intake and the development of obesity [22-24]. To investigate the potential role of FOXO in mediating EGL-4/PKG-dependent quiescence we next exposed wild type (N2) and *daf-16* mutants (CF1038) to the PKG activator 8-pCPT-cGMP (10 mM) and measured the number of animals either moving (foraging) or in quiescence. We found that mutation of *daf-16* was associated with increased quiescence when compared to wild type, while it had similar quiescent response as those *egl-4* (gf) worms (Figure **5A**). To investigate the molecular events of FOXO in mediating the quiescence/forage shift in response to ROS, *C. elegans* (TJ356) worms that stably expressed *daf-16::gfp* were exposed to 10 mM paraquat for two hours and then imaged. We found that paraquat treatment increased nuclear translocation of DAF-16/GFP (Figure **5B**, bottom panel). To extend these findings, we next repeated these experiments using HEK293 cells that stably expressed FOXO1::GFP. As expected, we found that FOXO1 proteins were largely confined to the cytoplasm in control cells (Figure **5C**, top panel) but that it

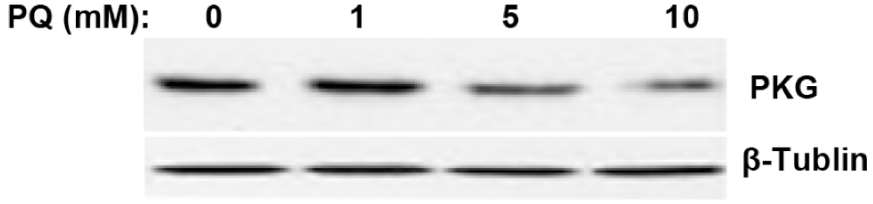

Figure 4: Paraquat decreases PKG protein expression in HEK293 cells.

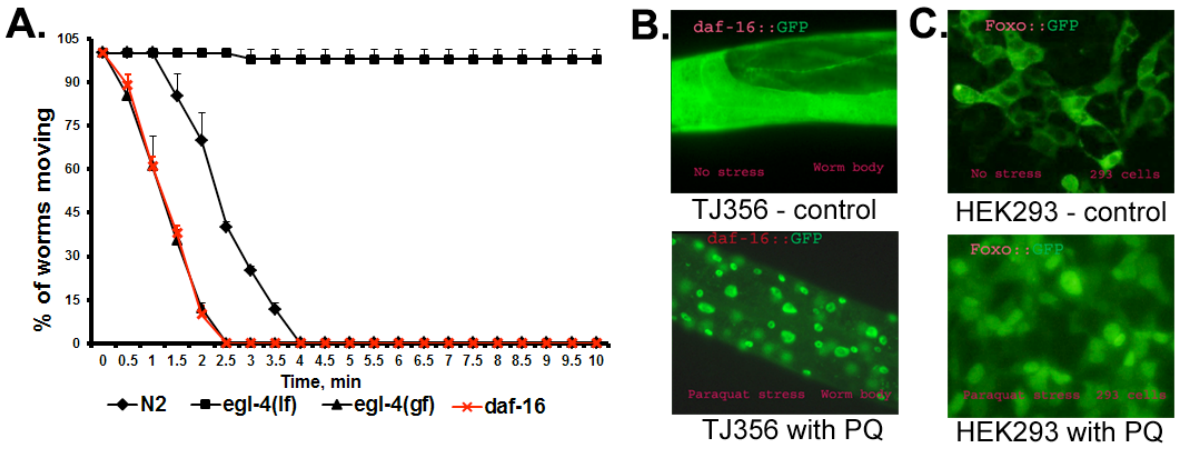

Figure 5: A. Impaired DAF-16 /FOXO function increases quiescence. **B**. Paraquat treatment increases nuclear translocation of DAF-16 in *C. elegans* (TJ356; *daf16*::*gfp*). **C**. Paraquat treatment increases nuclear translocation of FOXO1 (bottom panel) in HEK293 cells stably expressing FOXO1::GFP.

underwent nuclear translocation following paraquat exposure (10 mM) for 2 hours (Figure **5C**, bottom panel). Taken together, these data support the notion that DAF-16/FOXO may function as a downstream mediator of egl-4/PKG in regulating ROS-associated changes in eating / quiescence behavior and body growth.

DISCUSSION

Obesity due to excessive food intake is a global epidemic that affects virtually all age and socioeconomic groups, and threatens to overwhelm both developed and developing countries [1, 2]. Previous studies have reported that quiescence in the worm resembles the behavioral sequence of satiety and sleeping in mammals and that this behavior is PKG dependent [6-9]. The factor(s) regulating PKG-associated alterations in eating behavior are currently unclear however recent studies have posited that elevations in oxidative stress can impair PKG activation [11-14]. Whether increases in oxidative stress are associated with increased food intake and if this response is regulated by changes in PKG activation have, to our knowledge, not been investigated. Herein, we examine whether increases in ROS are linked to decreased quiescence and if this response, if present, is mediated by PKG dependent signaling. Our data suggest that increases in ROS are associated with the down-regulation of PKG expression, decreased quiescence, enlarged body size, and increased body fat accumulation. In addition, our data also suggests that the decreased quiescence we observed with exposure to ROS appears to be mediated by egl-4/daf-16 (PKG / FOXO) signaling. Taken together, these data support the possibility that over eating may be linked to changes in oxidative stress levels.

Increased Oxidative Stress Stimulation can Diminish Quiescence and Promote Obesity

Several assays have been developed to study the quiescence (sleep) and roaming (forage) of *C. elegans*, including change from high-quality food to low-quality food [6], and from a solid surface culture medium to a liquid solution [10, 15]. However, these assays usually take several hours to complete. Therefore, a rapid but effective assay will be very helpful for researchers in the field. Given our postulate that the PKG homolog, *egl-4*, can regulate quiescence in *C. elegans* [6, 9, 10], we developed a PKG activator-based swimming assay (Figure **1**). N2 wild-type worms were incubated with 10 mM of PKG activator 8-pCPT-cGMP, and the number of worms undergoing foraging behavior (locomotion) was determined. Consistent with previous findings [6], we found that exposure to PKG activator in N2 worms caused a rapid decrease in the number of worms exhibiting locomotion. Supporting these data, we also found that this response to PKG was absent in the *egl-4* loss of function mutants and that quiescence was potentiated in the *egl-4* gain of function mutants (Figure **1**).

One of most common causes of obesity is excessive food intake. On the basis of our previous work examining deteriorative effects of oxidative stress on the pathophysiological progress of diseases [16, 25, 26], we speculated that increases in oxidative stress would be associated with diminished quiescence and increased eating behavior and body size (indicator of obesity) in *C. elegans*. To examine this possibility, we chose to expose *C. elegans* to paraquat, an herbicide that is well known to cause increases cellular ROS levels [18]. As predicted from our hypothesis, we found that paraquat exposure was associated with diminished

quiescence (Figure **2**), which resulted in increased body length and body lipid accumulation (Figures **2** and **3**). These data indicate that oxidative stress is likely a contributing factor for the development of obesity related to over eating. Indeed, recent studies in humans have suggested that physical inactivity, environmental pollution and an unhealthy diet can lead to increases in tissue ROS levels and oxidative stress [27-29]. Although far less studied, it has also been reported increases in tissue ROS levels precede the onset of diet-induced obesity [30, 31] and that interventions aimed at decreasing oxidative stress are associated with decreases in excessive food intake [32, 33]. Therefore, recognizing the possible role of oxidative stress in stimulating eating behavior will provide many therapeutic strategies in combating the increasing prevalence of obesity.

Role of PKG in Medicating Oxidative Stress-Induced Foraging Behavior and Obesity

Given that exposure to paraquat was associated with decreased quiescence (Figure **2**) and that this response may be mediated by PKG activation (Figure **1**), we next wondered if increases in cellular ROS were associated with changes in PKG protein abundance. To address this possibility, we treated HEK293 with different concentrations of paraquat and then examined the expression of PKG protein. Our findings demonstrated that increased ROS exposure was associated with diminished PKG protein levels (Figure **4**). This finding is consistent with previous data showing that increased ROS can decrease PKG protein expression and impair PKG activation [11-14], suggesting that increased foraging behavior and body fat accumulation seen in the paraquat-treated worms (Figure **2**) was could be related to changes in the degree of PKG activation. To further test this possibility, we treated different strains of *C. elegans*, including wild type N2, *egl-4* gain of function (gf) and *egl-4* loss of function (lf), with paraquat over a prolonged period of time. As predicted, we found that compared to the wild type worms, loss of *egl-4* function significantly increased body length (indicator of obesity), while gain of *egl-4* function had less body growth when worms were cultured in the normal NGM medium (Figure **3**). However, when chronically exposed to paraquat, both *egl-4*(lf) and wild type worms grew more than that in normal medium, while the body growth of *egl-4*(gf) worms was not affected by the paraquat. These data support the possibility that impaired PKG function (due to increased oxidative stress and/or loss-of-function mutation) can result in excessive eating behavior and the development of obesity.

The daf-16 (FOXO) Pathway Appears to Mediate PKG Activation and Foraging Behavior

Recent studies have suggested that transcription factor FOXO protein is a downstream target of PKG signaling [19-21]. The FOXO protein coordinates a wide-range of cellular outputs including cell growth, organismal longevity and appetite [22-24]. The transcription activity and nuclear residence of FOXO is negatively regulated by phosphorylation [22-24]. Therefore, elevated PKG enzymatic function would be expected to increase the phosphorylation of FOXO and hence diminish its transcription activity (Figure **6**). To examine the potential role of FOXO in oxidative stress-diminished quiescence, we first investigated how mutation of *daf-16* (the ortholog *foxo* in *C. elegans*) can affect quiescence following exposure to the PKG activator 8-pCPT-cGMP. Our data revealed that similar to the *egl-4*(gf) worms, mutation of *daf-16* (deficiency of FOXO function) was associated with the induction of quiescence (Figure **5A**). Next we used a two pronged approach to investigate the effect of oxidative stress on FOXO nuclear translation. In the first set of experiments, *C. elegans* (TJ356 *daf-16::gfp*) stably expressing FOXO::GFP were imaged in the absence or presence of paraquat. We found that paraquat exposure (increased ROS) was associated with DAF-16 localization to the nucleus (Figure **5B**). To extend these findings, we next constructed a HEK293 cell line that stably expressed a GFP-labeled FOXO1 construct. Similar to that found in *C. elegans* TJ356 (*daf-16::gfp*), incubation of HEK293 cells with paraquat was

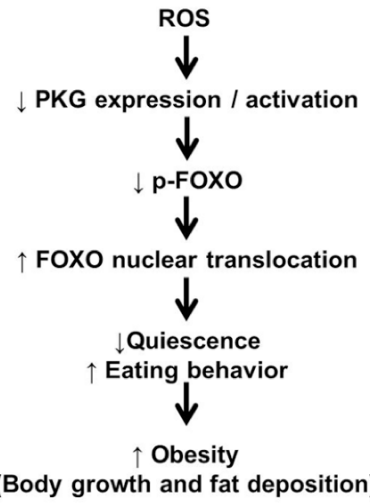

Figure 6: Proposed mechanisms of oxidative stress-stimulated eating behavior and obesity in *C. elegans*.

associated with increased nuclear localization of the FOXO1::GFP (Figure **5C**). Taken together, our data supported that FOXO likely serves as the mediator of PKG in regulating excessive foraging behavior in response to oxidative stress.

In conclusion, our study suggests that oxidative stress down-regulates PKG activity, subsequently increases FOXO transcription activity (nuclear location), and hence stimulates eating behavioral activity, which if allowed to proceed unchecked can lead to the development of obesity (increased body size and fat accumulation) (Figure **6**). These data also suggest a link between environmental stressors and eating behavior.

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