

PAK1-deficiency/down-regulation reduces brood size, activates HSP16.2 gene and extends lifespan in *Caenorhabditis elegans*

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ABSTRACT: There is an increasing evidence that the oncogenic kinase PAK1 is responsible not only for malignant transformation, but also for several other diseases such as inflammatory diseases (asthma and arthritis), infectious diseases including malaria, AIDS, and flu, as well as a series of neuronal diseases/disorders (neurofibromatosis, tuberous sclerosis, Alzheimer's diseases, Huntington's disease, epilepsy, depression, learning deficit, etc.) which often cause premature death. Interestingly, a few natural PAK1-blockers such as curcumin, caffeic acid (CA) and rosmarinic acid (RA) extend the lifespan of the nematode *Caenorhabditis elegans* or fruit flies. Here, to explore the possibility that *C. elegans* could provide us with a quick and inexpensive *in vivo* screening system for a series of more potent but safe (non-toxic) PAK1-blocking therapeutics, we examined the effects of PAK1-deficiency or down-regulation on a few selected functions of this worm, including reproduction, expression of HSP16.2 gene, and lifespan. In short, we found that PAK1 promotes reproduction, whereas it inactivates HSP16.2 gene and shortens lifespan, as do PI-3 kinase (AGE-1), TOR, and insulin-like signalling / ILS (Daf-2) in this worm. These findings not only support the "trade-off" theory on reproduction versus lifespan, but also suggest the possibility that the reduced reproduction (or HSP16.2 gene activation) of this worm could be used as the first indicator of extended lifespan for a quick *in vivo* screening for PAK1-blockers.

Keywords: PAK1, HSP16, GFP, CAPE, ARC, nematode, lifespan, RB689, trade-off, reproduction

1. Introduction

Calorie restriction (CR) is well known to extend the lifespan of mice by activating the tumor-suppressing kinase (LKB1-AMPK) cascade which eventually activates the longevity and tumor suppressing transcription factor (FOXO) family by its phosphorylation and nuclear translocation (1-3). Interestingly, LKB1 inactivates an oncogenic kinase called PAK1, whereas it activates AMPK (4). In fruit flies (*Drosophila*) the gene "Methuselah" (*MTH*), encoding a G protein-coupled receptor (GPCR) of secretin receptor family, shortens the lifespan (5,6). Dysfunction of this gene (6) or the inhibition of MTH protein by its ligand antagonists (7) extends the lifespan by 35%. The MTH protein eventually activates the oncogenic RAS-PI-3 kinase cascade (8), leading to the activation of two oncogenic kinases, PAK1 and AKT. Thus, in both mammals and insects, PAK1 or AKT appears to be involved in the regulation of their lifespan somehow. In mammals both PAK1 and AKT are known to inactivate the longevity protein "FOXO" by the specific phosphorylation (9,10).

In the nematode *Caenorhabditis elegans* several distinct genes were identified as lifespan modulators. Among the positive regulators, are "FOXO" (*DAF-16*), "HSP16.2", and "PTEN" (*DAF-18*), whose dysfunction shortens the lifespan (11-13). Among the negative regulators, are insulin-like signalling (*ILS/DAF-2*), PI-3 kinase (*AGE-1*), *AKT*, and *TOR*, whose dysfunction extends the lifespan (14-17). If we understand correctly, these negative regulators shorten the lifespan mainly by inactivating the FOXO-HSP16.2 signalling pathway (14-17).

In this worm there are three distinct members of PAK family, PAK1, PAK2, and MAX-2 (18-20). Among them, the nematode PAK1 is most closely related to (and functionally same as) mammalian PAK1 which is essential for malignant growth of cells and several other diseases such as neurofibromatosis (NF) and Alzheimer's disease (AD) which often cause the premature death of human beings (21,22). So far no phenotype of PAK1-deficient mutant of *C. elegans* (RB689) has been reported, except that the PAK1 and MAX-2 double mutant is

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embryonically lethal, whereas *PAK1*-deficient mutant shows no apparent defect, suggesting that *PAK1* and *MAX-2* are functionally redundant in part (19). *MAX-2* is similar to *Drosophila PAK3*, whereas *PAK2* in this worm belongs to group II of PAK family (*PAK4-6*).

During our searching the potential phenotypes of RB689, or effects of PAK1-blockers, we found that RB689 shares a few common phenotypes with dysfunction of ILS, PI-3 kinase, and TOR in this worm and MTH in *Drosophila*: reduced brood size, increase in stress-resistance, and longer lifespan. Furthermore, we demonstrated the first example that *HSP16.2-GFP* (green fluorescent protein) fusion gene in this worm (CL2070) could be used as a potent visible indicator for a quick and inexpensive screening for "non-toxic" PAK1-blockers *in vivo* (combined with heat-shock).

2. Materials and Methods

2.1. Strains of *C. elegans* and reagents

Strains (RB689 and CL2070) of *C. elegans* were kindly provided by *C. elegans* Genomic Center (CGC). Caffeic acid phenethyl ester (CAPE) was purchased from Sigma Chemicals and artemisinin (ARC) was kindly provided by Dr. Hitoshi Hori of Tokushima University.

2.2. Measurement of brood size

The wild-type (N2) and RB689 of *C. elegans* were fed by the lawn culture of *Escherichia coli* (OP50) which was grown in the presence or absence of 200 μ M CAPE or ARC on the standard nematode growth medium (NGM) agar plate for 2 days shortly after the hatching at 23°C. Then the number of eggs laid by each group of around 40 adult worms overnight (for 10 h) was counted. The brood size was calculated as the number of eggs per mother (female worm).

2.3. Heat-shock-induced paralysis

The wild-type (N2) and RB689 were fed by *E. coli* which was grown in the presence or absence of 200 μ M CAPE or ARC for 2 days as described above. Then each group of 100 adult worms was heat-shocked at 35°C for 2 h. Then each group was cultured at 22°C for 4 h recovery, and then heated again at 35°C over 4 h. Every hour after the 2nd heat challenge, the number of worms in each group, which are "paralyzed" (not moved by a gentle touch with a platinum rod), was counted for scoring the survivors. The whole population of control wild-type group usually perished after 4 h, whereas more than a half of RB689 or CAPE/ARC-treated wild-type survived the 2nd heat-challenge.

2.4. *HSP16.2* dependent GFP expression

The strain CL2070 which carries a transgenic reporter

gene called "*HSP16.2-GFP*" fusion gene (23) was fed by *E. coli* (OP50) which was grown in the presence or absence of 200 μ M CAPE overnight (12 h) at 22°C. Then each group of around 20 worms was heat-shocked at 35°C for 2 h, and then kept at 22°C for the recovery. After 4 h, each group was fixed with a drop (10 μ L) of sodium azide (1 M) on slides for microscopy. Under blue light which stimulates the green fluorescence emission from GFP produced in each worm, the fluorescence images were acquired at the same exposure parameters, using a 40 \times objective of the microscope (BX60; Olympus, Tokyo, Japan) equipped with a digital camera (Micropublisher 5.0; QImaging, Burnaby, British Columbia, Canada).

2.5. Measurement of lifespan

The gravid hermaphrodites of the N2 and RB689 from the standard agar plates were washed, respectively. Then worms were dissolved in alkaline sodium hypochlorite in order to collect the eggs in utero. The released eggs were allowed to hatch by overnight incubation at 20°C in S-basal (24) to the age synchronous cultures of the L1 stage larvae. The lifespan of the hermaphrodites at 20°C was measured on the agar plates with the lawn culture of *E. coli* (OP50). In order to prevent progeny production, 5-fluoro-2'-deoxyuridine (FUDR; Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to the agar plate at the final concentration of 40 μ M after the animals had reached adulthood as described previously (25).

2.6. Nuclear localization of FOXO (*Daf-16*)

To detect the intracellular DAF-16 localization, pGP30 vector (26), in which the *Daf-16* gene transcript a2 (*daf-16a2*) is fused to GFP cDNA, was microinjected into each gonad of the N2 and RB689 animals at 100 ng/ μ L with pRF4 containing the *rol-6* (*su1006*) gene as described previously (25). The presence or absence of DAF-16 localization into the nucleus of the transgenic 4- and 10-day old animals was observed using a fluorescence microscope with digital imaging system (BX51TRF; Olympus Co., Tokyo, Japan).

3. Results

3.1. *PAK1*-deficient strain (RB689) has smaller brood size than the wild-type (N2)

When we started culturing the *PAK1*-deficient strain (RB689) on a plate for a few days, we noticed that eggs this strain laid are clearly fewer than those the wild-type produced, although all eggs are hatched and none of mutant adults shows any other detectable defect. Since the long-lived *C. elegans* strains, in which ILS, PI-3 kinase, or TOR dysfunctions, also show a significantly

smaller brood size (14-16), as does the longevity mutant (Methuselah) of *Drosophila* in which the *MTH* gene dysfunctions (5,6), we decided to measure the brood size of RB689, compared with those of the wild-type (N2) in the presence or absence of two distinct PAK1-blockers, CAPE and ARC from a bee product called propolis (27,28). As shown in Figure 1, the brood size of RB689 is only one 7th of that of N2 (non-treated wild-type strain), whereas both CAPE and ARC-treated wild-type populations produced basically same number of eggs as RB689, suggesting strongly that this phenotype is closely associated with reduced PAK1 activity in this worm. In other words PAK1 promotes the reproduction in this worm, as do RAS, PI-3 kinase, and FYN, the three upstream activators of PAK1 in this worm or mice (14,29,30).

3.2. The PAK1-blocker CAPE activates *HSP16.2* gene

In mammals as well as yeasts, the oncogenic RAS-PI-3 kinase signalling cascade blocks a series of heat-shock genes, and as a consequence mammalian (or yeast) cells are rendered highly sensitive to heat shock treatment (31). Interestingly, two mammalian oncogenic kinases,

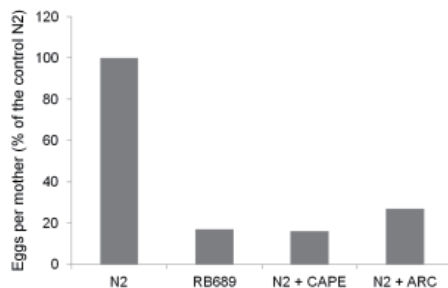


Figure 1. Brood size of RB689 compared with wild-type (N2) in the presence or absence of CAPE/ARC. Either *PAK1*-deficiency (RB689) or treatment of N2 with CAPE/ARC (200 μ M) reduces the brood size (number of eggs/mother).

PAK1 and AKT, phosphorylate distinct members of FOXO family, and inactivate their transcriptional function essential for the expression of heat-shock genes (9,10). In the nematode, FOXO is also essential for the transcription of heat-shock genes including *HSP16.2*, and the ILS-PI-3 kinase signalling cascade and TOR are known to inactivate the longevity (FOXO-*HSP16.2*) signalling cascade (14-16). However, it still remains to be clarified if AKT or PAK1 is involved in this PI-3 kinase mediated inactivation of FOXO-*HSP16.2* signalling cascade. To monitor the possible role of PAK1 in the heat-sensitivity of this worm, we compared the heat-induced paralysis of this worm, between the wild-type and RB689 as well as the ARC/CAPE-treated wild-type. We observed that both RB689 and ARC/CAPE-treated worm are significantly resistant to the heat challenge than the non-treated wild-type population which perished in 4 h at 35°C (data not shown), suggesting that PAK1 appears to mediate the suppression of heat-shock genes in this worm as well.

Thus, we examined the effect of CAPE, the PAK1-blocker which does not affect AKT, on the expression of *HSP16.2* gene, in an early stage of post heat-shock treatment of the strain CL2070, which carries a reporter gene, *i.e.*, the promoter of *HSP16.2* gene fused to *GFP* gene (cDNA). This fusion gene gradually produces a green fluorescent protein, only after heat shock, in this transparent worm over 24 h (23). If PAK1 suppresses the *HSP16.2* gene, CAPE (or any other "non-toxic" PAK1-blockers) could promote a rapid production of GFP shortly after the heat shock, and this fluorescent nematode system would provide us with a rapid and inexpensive *in vivo* screening for safe PAK1-blockers potentially useful for clinical application.

As shown in Figure 2, the strain CL2070 which has been treated with 200 μ M CAPE overnight, started to glow rapidly even in 4 h after 2 h-heat shock, whereas the non-treated worm glowed only dimly in the same

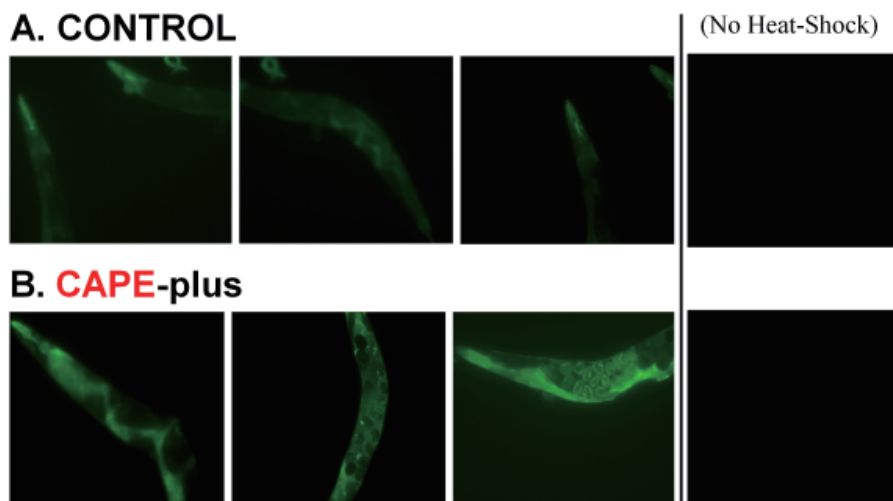


Figure 2. CAPE activates *HSP16.2* gene in *C. elegans*. 200 μ M CAPE stimulates *HSP16.2*-GFP production in 4 h after the heat shock of *C. elegans* (CL2070). (A) The control CL2070; (B) CAPE-treated CL2070. Without heat shock, no GFP is produced.

period. The GFP expression occurred only after heat shock, and no GFP expression was detected without heat shock. In other words the *HSP16.2* gene promoter is regulated by at least two distinct factors. It is activated by heat shock, and inactivated by PAK1.

3.3. RB689 lives longer than the wild-type

According to numerous studies by Tom Johnson and his colleagues during last two decades or so, it was firmly established that the expression level of *HSP16.2* gene in this worm is well correlated to its lifespan (32-34). Thus, we compared the lifespan between RB689 (*PAK1*-deficient) and the wild-type under the standard conditions. As shown in Figure 3, RB689 lives significantly longer than the wild-type. Fifty percent of RB689 survived the first 27 days, whereas 50% of the wild-type survived only 17 days. In other words the *PAK1*-deficient worm could live 10 days (by around 60%) longer than the control worm, clearly indicating that PAK1 shortens lifespan of this worm, as do its upstream activators PI-3 kinase and ILS as well as TOR.

Does PAK1 inactivate FOXO in this worm, as it does in mammals? We think it does, based on our observation that nuclear localization of FOXO in RB689 is significantly increased in comparison with that in the wild-type, in particular at the early age (see Table 1). In addition, the PAK1-blocker CAPE activates *HSP16.2* gene expression, which depends on the transcription activity of nuclear FOXO.

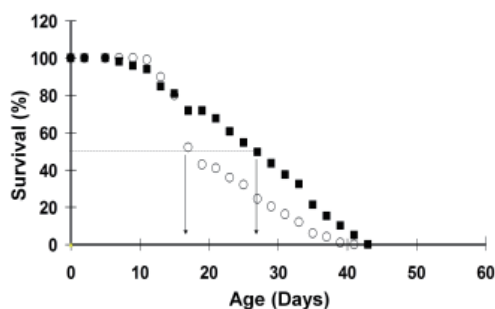


Figure 3. The effect of *PAK1*-deficiency on the lifespan. The survival rate between N2 (the control, circle) and RB689 (*PAK1*-deficient, square) strains of *C. elegans* was compared at 20°C on the standard agar plate. Fifty percent of RB689 survived on day 27, 10 days longer than the N2 counterparts.

Table 1. Effect of *PAK1*-deficiency on nuclear localization of FOXO

Strain	Age (days)	Nuclear FOXO/unit area
N2	4	1.6 ± 0.7
RB689	4	17.4 ± 1.6*
N2	10	11.0 ± 1.4
RB689	10	38.0 ± 7.4*

Numbers of GFP-stained nuclei were scored mainly in epithelia, muscles and intestine, and indicated as mean ± SD from more than three independent transgenic (FOXO-GFP) worms of each strain. * The numbers in RB689 are significantly larger than those in the control (N2) on both days 4 and 10 (by *t*-test, $p < 0.001$).

4. Discussion

Here we have shown three distinct phenotypes of *C. elegans* which lacks PAK1 or whose PAK1 is down-regulated by the PAK1-blocker CAPE. These phenotypes clearly indicate that PAK1 promotes the reproduction, inactivates *HSP16.2* gene and shortens the lifespan of this worm. These phenotypes are basically same as those of a few other "longevity" mutants of this worm, in which either ILS, PI-3 kinase, or TOR dysfunctions (14-17). Interestingly, these three phenotypes are also shared by the "longevity" mutant (Methuselah) of *Drosophila* in which *MTH* gene dysfunction (5,6). Since dysfunction of ILS, PI-3 kinase, and *MTH* eventually leads to the inactivation of PAK1, it is not a big surprise that these "longevity" mutants share the same phenotypes with RB689 or the CAPE-treated worm.

How about dysfunction of TOR? There is an evidence for a cross-talk between PAK1 pathway and TOR pathway (35,36). As shown in Figure 4, TOR requires another protein called Raptor for the full activation, and their interaction requires PAK1 (35). Conversely, TOR activates S6 kinase which in turn activates PAK1 (36). Thus, PAK1 and TOR form a vicious oncogenic cycle, and if TOR dysfunctions, PAK1 could be down-regulated accordingly.

In this context it should be worth noting that treatment of mice or fruit flies with rapamycin (Rapa), which directly inhibits TOR, also live longer than the control mice or fruit flies, respectively (37,38). To our great surprise, it was found recently that transgenic *RAC*-deficient fruit flies (*Drosophila*), in which the dominant negative (DN) mutant of *RAC* is expressed to block the oncogenic *RAC*-PAK1 signaling pathway, are extremely resistant to a variety of stresses such as heat, oxidants, desiccation, and starvation, and also can live for 40 days at 30°C, almost twice as long as the control flies which live for only 22 days (39).

Thus, a group at NIA in Baltimore recently started measuring the lifespan of PAK1-deficient (inflammation-resistant) mice (40) compared with that of controls. If PAK1 plays a similar role in the lifespan of mice, this study is expected to take around 5 years since the average lifespan of control mice is around 3 years. Nevertheless

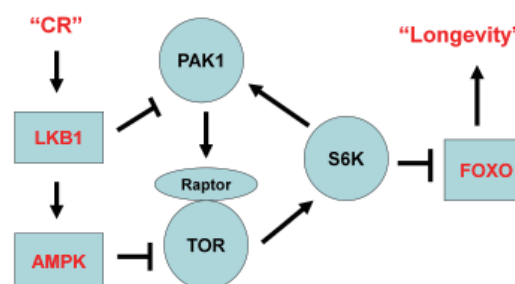


Figure 4. Cross-talk between PAK1 and TOR pathways. PAK1 and TOR form a vicious oncogenic cycle through Raptor and S6 kinase, respectively, to cause cancers in mammals and shorten lifespan of *C. elegans*.

it is most likely that *PAK1*-deficient mice live longer than controls, as do the Rapa-treated mice in which both TOR and PAK1 are down-regulated.

Interestingly, in 1999 Cynthia Kenyon's group at UCSF discovered that removal of germ-line cells by a laser micro-beam from *C. elegans* extends the lifespan by 60%, and this effect depends on the transcriptional function of FOXO (41). Along with this finding came an evolutionary "trade-off" theory noting that reduction in reproduction (fecundity) leads to extension of lifespan (5,42). In fact, over-expression of FOXO in adult *Drosophila* fat body caused an increased lifespan and reduced fecundity (43).

In other words, any compounds that reduce the brood size could extend the lifespan of *C. elegans* or *vice versa* (any "elixirs" could reduce the reproduction). However, curcumin was recently reported to extend lifespan of this worm, without affecting the reproduction, and even in the absence of FOXO (44). Curcumin is known to inhibit directly PAK1 as well as activates AMPK (45,46), and AMPK activates FOXO. Thus, it is likely that curcumin's life-extending property has nothing to do with its effect on either PAK1 or AMPK.

Nevertheless, we hypothesized that a PAK1-dependent "reproductive" signalling cascade from germ-line cells shortens the lifespan of this worm by inactivating FOXO. Then, based on our preliminary observation that ivermectin reduces the brood size of this worm by 90% at its sublethal dose (Grant W, *et al.*, unpublished observation), we recently found that this old anti-parasitic drug also blocks PAK1 (47). Moreover, a natural PAK1-blocker called caffeic acid (CA) and its dimer called rosmarinic acid (RA) extend the lifespan of this worm (48). Thus, we are prompted to test this "trade-off" theory further by examining effects of several distinct PAK1-blockers such as CAPE, ARC, ivermectin, bitter anti-malaria drugs including berberine, glaucarubinone and "king of bitters" extract on *HSP16.2* gene expression and lifespan of this worm in no distant future. It is our "optimistic" hope that either reproduction assay or *GFP-HSP16.2* gene expression assay (or combination of these two), which can be done in a few days, may serve a quick *in vivo* screening for life-extending products (so-called "elixirs"), which would also be useful for therapy of cancers and several other PAK1-dependent diseases such as NF and AD without any side effect.

Lastly, it would be of great interest to test if *FYN*-deficient (or PPI-treated) mice live significantly longer than the control counterparts. Why? *FYN* is up-regulated by RAS, the major target of PPI, the SRC family-specific inhibitor, and contributes to the activation of PAK1 by 50% in RAS-transformed cells (49). Furthermore, the *FYN*-deficient mice are less fertile (up to 50%) compared with controls (30). Thus, if the "trade-off" theory is also applicable to mammals, *FYN*-deficient mice could be among the so-called "longevity" mouse family including

"snell" dwarf mice in which the transcription factor Pit-1 dysfunctions, leading to growth hormone (GH) deficiency among others (50).

Interestingly, treatment of these dwarfs with GH and thyroxine increased their weight by approximately 45%, although they remained much smaller than controls. The hormone treatment also restored fertility to male dwarfs. However, this treatment did not diminish lifespan or lower their resistance to cataracts and kidney disease. Moreover, this dwarf is resistant to cancers and a variety of other diseases such as inflammation (51), as well as oxidative stresses such as 3-nitropropionic acid (3-NPA). In normal mice, 3-NPA activates MEK/ERK kinases and stimulates a robust phosphorylation of JNK at Ser63, whereas no phosphorylation takes place in snell mice, in response to 3-NPA (52). Since both MEK/ERK and JNK are the direct targets of PAK1, it is almost certain that in snell mice, PAK1 is somehow blocked or down-regulated. Again, these findings also strongly suggest, if not proven as yet, that *PAK1*-deficient (inflammation-resistant) mice could live significantly longer than controls, as does the *PAK1*-deficient nematode (RB689).

Acknowledgements

This study was supported in part by DFG (Deutsche Forschungsgemeinschaft) visiting professorship to HM during his 2006-2007 research at UKE (Hamburg University Hospital) and UMB (University of Maryland at Baltimore). One of the authors (HM) is very grateful to Dr. Warwick Grant (La Trobe University in Melbourne) and his colleagues for his laboratory space and kind support during a follow-up study on the *PAK1*-deficient (RB689) and *HSP16.2-GFP* transgenic (CL2070) strains of *C. elegans* in the fall of 2007. Also we are indebted to Dr. Chris Link for his critical reading/improvement of this manuscript, to Dr. Tom Johnson and Ms. Patricia Tedesco (University of Colorado at Boulder) for their kindly providing us with pGP30 vector, to Dr. Masamitsu Fukuyama (University of Tokyo) and CGC (*C. elegans* Genome Center) for their kindly providing us with RB689 and CL2070 strains.

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(Received December 30, 2012; Revised January 16, 2013; Accepted January 25, 2013)