A novel kinase regulates dietary restriction-mediated longevity in Caenorhabditis elegans

Manish Chamoli, Anupama Singh, Yasir Malik and Arnab Mukhopadhyay
Molecular Aging Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 10067, India

Summary
Although dietary restriction (DR) is known to extend lifespan across species, from yeast to mammals, the signalling events downstream of food/nutrient perception are not well understood. In Caenorhabditis elegans, DR is typically attained either by using the eat-2 mutants that have reduced pharyngeal pumping leading to lower food intake or by feeding diluted bacterial food to the worms. In this study, we show that knocking down a mammalian MEKK3-like kinase gene, mekk-3 in C. elegans, initiates a process similar to DR without compromising food intake. This DR-like state results in upregulation of beta-oxidation genes through the nuclear hormone receptor NHR-49, a HNF-4 homolog, resulting in depletion of stored fat. This metabolic shift leads to low levels of reactive oxygen species (ROS), potent oxidizing agents that damage macromolecules. Increased beta-oxidation, in turn, induces the phase I and II xenobiotic detoxification genes, through PHA-4/FOX, NHR-8 and aryl hydrocarbon receptor AHR-1, possibly to purge lipophilic endotoxins generated during fatty acid catabolism. The coupling of a metabolic shift with endotoxin detoxification results in extreme longevity following mekk-3 knock-down. Thus, MEKK3 may function as an important nutrient sensor and signalling component within the organism that controls metabolism. Knocking down mekk-3 may signal an imminent nutrient crisis that results in initiation of a DR-like state, even when food is plentiful.

Key words: beta-oxidation; Caenorhabditis elegans; dietary restriction; fat storage; lifespan; xenobiotic detoxification.

Introduction
Dietary restriction (DR) is known to increase lifespan in almost all model systems tested. In mammals, it is associated with health benefits including reduced risk of cancer, neurodegenerative disorders, autoimmune diseases, cardiovascular diseases and type II diabetes (Fontana et al., 2010; Omodei & Fontana, 2011; Speakman & Mitchell, 2011). In spite of having positive impact on longevity and quality of life, DR is easier to implement in an experimental model as compared to human beings. DR in humans is challenging in terms of compliance, as we frequently succumb to gastronomic delights which are invariably high in fat and carbohydrates. In this context, it will be beneficial to have a model of DR where, irrespective of the calorie intake, an organism may enjoy its beneficial effects. In this study, we present such a model in Caenorhabditis elegans.

Although the beneficial effects of DR are known for decades, the molecular mechanisms and genetic pathways that mediate DR have remained elusive. In this direction, studies using model systems such as Saccharomyces cerevisiae, C. elegans and Drosophila have provided tremendous insights into the mechanisms of DR and longevity (Bishop & Guarente, 2007a; Mair & Dillin, 2008; Fontana et al., 2010; Kenyon, 2010). In C. elegans, molecular components working downstream of DR are studied after restricting food intake by using either genetic or nongenetic manipulations (Lakowski & Hekimi, 1998; Walker et al., 2005; Kaebelien et al., 2006; Bishop & Guarente, 2007b; Panowski et al., 2007; Greer & Brunet, 2009). The eat-2 mutants represent a well-studied genetic model of DR. Due to a mutation in a nonalpha nicotinic acetylcholine receptor subunit, the eat-2 mutant worms have defective pharyngeal pumping and ingest fewer bacteria (Lakowski & Hekimi, 1998). Several nongenetic methods for DR have also been developed. While some protocols use serial dilutions to essentially limit the amount of bacteria available to the worms, others resort to complete bacterial deprivation (Walker et al., 2005; Kaebelien et al., 2006; Bishop & Guarente, 2007b; Panowski et al., 2007; Greer & Brunet, 2009). However, these different DR regimes seem to activate distinct pathways. For example, eat-2 mutants and liquid DR regimes require pah-4 and skn-1 transcription factors and are independent of the FOXO transcription factor, daf-16 (Bishop & Guarente, 2007b; Panowski et al., 2007; Greer & Brunet, 2009). On the other hand, bacterial dilution protocol on solid media requires daf-16 (Greer & Brunet, 2009). In this context, identification of genes that would induce a DR-like state when manipulated, without the confounding effects of dietary intake, is likely to provide fundamental insights into the mechanisms of DR.

Evolutionary advantages of unpredictable short-term shortage of food may have given rise to the DR response (Masoro, 1996). Molecularly, signalling components within an organism sense low nutrient availability to signal onset of a DR response. In this study, we characterize a novel mammalian MEKK3 (mMEKK3)-like serine-threonine kinase that qualifies as an important component of a nutrient sensing pathway and a DR response initiator. Knocking down mekk-3 may signal an imminent nutrient crisis that initiates a DR-like state, although food is plentiful and intake is not compromised. This DR-like process dramatically increases lifespan and health span. The increased lifespan is dependent on pah-4/FOXA and partially dependent on skn-1/NRF2. Importantly, using this model, we further show that mekk-3 knock-down increases the expression of beta-oxidation genes through the nuclear hormone receptor, NHR-49/HNF4, leading to depletion of stored fat. This metabolic shift towards fatty acid oxidation results in lower reactive oxygen species (ROS) generation without activation of the superoxide dismutases. However, increased fatty acid oxidation, which may lead to the formation of lipophilic endotoxins, activated the xenobiotic detoxification genes such as cytochrome P450 and UDP-glucuronosyltransferase (UGT). We show that this is achieved through the conserved transcription factors, PHA-4/FOX, NHR-8 and aryl hydrocarbon receptor AHR-1. Thus, our study elucidates a novel mechanism.
mechanism of lifespan extension where a metabolic shift to fatty acid oxidation is coupled to xenobiotic detoxification, leading to dramatic increase in longevity.

Results

mekk-3 dramatically affects lifespan and health span

In an effort to find kinases that genetically interact with the insulin/IGF-like signalling (IIS) pathway, we performed an RNAi screen to identifydaf-2(e1370) dauer enhancers (M. Chamoli, A. Mukhopadhyay, unpublished data). We identifiedmekk-3as one of the top candidates (data not shown). Interestingly, mekk-3 RNAi effectively knocked down the expression ofmekk-3 mRNA (Fig. S1A, Supporting Information) and consistently increased the lifespan of wild-type (WT) N2 Bristol strain by an average of ~60% [mean lifespan (MLS) of WT on control RNAi is 16.36 ± 0.34 days, on mekk-3 RNAi is 26.27 ± 0.52 days, P < 0.0001; Fig. 1A, also see Tables 1 and S1, Supporting Information] without affecting development significantly (data not shown). Worms fed RNAi constructs containing either the full-lengthmekk-3or subcloned fragments (Fig. S1C) also showed increased lifespan. The lifespan extension was not dependent either on addition of FUDR (Fig. S1D), a DNA synthesis inhibitor routinely supplemented during lifespan analysis, or on exposure of worms to OP50 prior to RNAi knock-down using HT115 (Fig. S1E). MEKK-3, encoded by F18F11.5, is a serine-threonine kinase that has 44% homology (26% identity) to mammalian MAP kinase kinase kinase (MAPKKK or MEKK), mMEKK3 and is able to function as a kinase in vitro (Fig. S2A,B). The increased longevity inmekk-3 RNAi worms was distinctly associated with better health. These worms showed delayed as well as lower accumulation of lipofuscin, an age pigment (Fig. 1B). They also maintained healthier musculature, indicated by delayed age-onset nuclear membrane disintegration in muscles (Fig. 1C) and loss of mobility (Fig. S2C), when compared to control RNAi-treated worms. Together, mekk-3 regulates lifespan and health span inC. elegans.

To increase lifespan, components of the major longevity pathways have to be knocked down at temporally distinct points in the life cycle. For example, reducing insulin/IGF signalling during adulthood or at an advanced age is sufficient to increase lifespan (Dillin et al., 2002a). On the other hand, mitochondrial genes have to be knocked down early during the development to positively influence longevity (Dillin et al., 2002b). In case of mekk-3, knocking the gene down early in life resulted in increased longevity; RNAI initiated at L4 or later had no effect (MLS of WT on control RNAi is 18.99 ± 0.44 days, on mekk-3 RNAi from L1 is 31.40 ± 0.55 days, P < 0.0001, while from L4 is 19.16 ± 0.38 days P = 0.869, Fig. 1D, Table 1). Thus, mekk-3 has temporal requirements.

Fig. 1mekk-3 is an important regulator of lifespan and health span. (A) Lifespan was significantly increased when mekk-3 was knocked down using RNAI. Mean lifespan (MLS) [days ± SEM, (n)] of wild-type (WT) on control RNAI was 16.36 ± 0.34 days (n = 78), while on mekk-3 RNAI it was 26.27 ± 0.52 days (n = 74), P < 0.0001 by log rank test. (B) mekk-3 knock-down delayed accumulation of lipofuscin pigment in the intestine. (C) Muscle nuclei degeneration was delayed inmyo-3::gfp transgenic worms grown on mekk-3 RNAI. Nuclei were categorized as intact, moderately or severely degraded according to representative photographs in the inset. Error bars indicate standard deviation; n > 20, Student’s t-test. (D) Temporal requirements of mekk-3 knock-down. Maximum extension of lifespan was observed when mekk-3 RNAI was initiated at L1 (31.40 ± 0.55 days, n = 83, P < 0.0001), while no extension was seen when it was initiated at L4 (19.16 ± 0.38 days, n = 82, P = 0.869). As a control, worms were transferred to control RNAI at L1. (E) Knocking down mekk-3 in daf-2(e1370) further increased lifespan. MLS on control RNAI was 46.39 ± 0.85 days (n = 165), while on mekk-3 RNAI was 60.42 ± 1.08 days (n = 158), P < 0.0001. The grey lines indicate WT on control or mekk-3 RNAI. (F) mekk-3 RNAI increased lifespan ofdaf-16(mgDf50). Mean lifespan on control RNAI was 13.86 ± 0.26 days (n = 79), whereas on mekk-3 RNAI it was 24.06 ± 0.59 days (n = 70), P < 0.0001. pdk-1 RNAI, which fails to extend life-span indaf-16(mgDf50), was used as control. All life-spans were performed at 20°C.
Table 1 Summary of lifespan analyses reported in this study

| Genetic background | RNAi/used          | Mean ± SEM (days) | N  | % change w.r.t control | P value |
|--------------------|--------------------|------------------|----|------------------------|---------|
| Wild-type          | Control            | 16.36 ± 0.34     | 78 |                        |         |
|                    | mekk-3′            | 26.27 ± 0.52     | 74 | (+60.57)               | < 0.0001|
| Wild-type          | Control            | 17.37 ± 0.26     | 111|                        |         |
|                    | mekk-3 DNA construct| 30.77 ± 0.44     | 106| (+77.14)               | < 0.0001|
| daf-2(e1370)       | Control            | 46.39 ± 0.85     | 165|                        |         |
|                    | mekk-3             | 60.42 ± 1.08     | 158| (+30.24)               | < 0.0001|
| daf-2(e1368)       | Control            | 28.34 ± 0.69     | 119|                        |         |
|                    | mekk-3             | 40.43 ± 0.84     | 120| (+42.66)               | < 0.0001|
| daf-16(mgDF50)     | Control            | 13.86 ± 0.26     | 79 |                        |         |
|                    | mekk-3             | 24.06 ± 0.59     | 70 | (+73.59)               | < 0.0001|
|                    | pdk-1              | 13.07 ± 0.16     | 82 | (~5.69)                | ≤ 0.001 |
| daf-3(mgDF90)      | Control            | 15.93 ± 0.33     | 90 |                        |         |
|                    | mekk-3             | 25.65 ± 0.48     | 158| (+61.01)               | < 0.0001|
| daf-5(e1386)       | Control            | 14.71 ± 0.36     | 76 |                        |         |
|                    | mekk-3             | 27.00 ± 0.74     | 76 | (+83.54)               | < 0.0001|
| daf-2(e1370); daf-3(mgDF90) | Control | 49.86 ± 1.42 | 78 |                        |         |
|                    | mekk-3             | 73.85 ± 1.18     | 106| (+48.11)               | < 0.0001|
| eat-2(ad1116)      | Control            | 28.94 ± 0.65     | 90 |                        |         |
|                    | mekk-3             | 27.35 ± 0.61     | 48 | (~5.49)                | 0.0453  |
| eat-2(ad1113)      | Control            | 24.16 ± 0.48     | 88 |                        |         |
|                    | mekk-3             | 21.66 ± 0.67     | 62 | (~10.34)               | 0.0089  |
| eat-2(ad465)       | Control            | 28.76 ± 0.40     | 94 |                        |         |
|                    | mekk-3             | 25.64 ± 0.43     | 100| (~10.84)               | < 0.0001|
| clk-1(qm30)        | Control            | 33.04 ± 0.85     | 67 |                        |         |
|                    | mekk-3             | 34.24 ± 0.83     | 76 | (~3.63)                | 0.4198  |
| skn-1(2u169)       | Control            | 17.86 ± 0.49     | 76 |                        |         |
|                    | mekk-3             | 26.39 ± 0.79     | 67 | (+47.76)               | < 0.0001|
| nhr-49(ok2165)     | Control            | 16.22 ± 0.40     | 78 |                        |         |
|                    | mekk-3             | 15.49 ± 0.58     | 45 | (~4.5)                 | 0.5572  |
| nhr-49(nr2041)     | Control            | 12.91 ± 0.12     | 141|                        |         |
|                    | mekk-3             | 13.67 ± 0.29     | 140| (+5.88)                | < 0.0006|
| daf-22(m30)        | Control            | 20.34 ± 0.33     | 73 |                        |         |
|                    | mekk-3             | 20.59 ± 0.36     | 51 | (+1.22)                | 0.6539  |
| mev-1(kn1)         | Control            | 14.36 ± 0.40     | 66 |                        |         |
|                    | mekk-3             | 15.06 ± 0.55     | 52 | (~4.87)                | 0.2868  |
| gas-1(fc21)        | Control            | 19.27 ± 0.28     | 89 |                        |         |
|                    | mekk-3             | 29.74 ± 0.42     | 66 | (+54.33)               | < 0.0001|
| nhr-8(ok186)       | Control            | 17.53 ± 0.34     | 85 |                        |         |
|                    | mekk-3             | 12.71 ± 0.30     | 42 | (~27.49)               | ≤ 0.001 |
| aha-1(ok1396)      | Control            | 16.69 ± 0.33     | 41 |                        |         |
|                    | mekk-3             | 12.89 ± 0.34     | 148| (~22.76)               | < 0.0001|
| ahr-1(ju145)       | Control            | 16.16 ± 0.45     | 49 |                        |         |
|                    | mekk-3             | 14.92 ± 0.38     | 51 | (~7.67)                | 0.0561  |
| ahr-1(a3)          | Control            | 18.43 ± 0.27     | 134|                        |         |
|                    | mekk-3             | 20.96 ± 0.41     | 95 | (+13.72)               | < 0.0001|
| pgp-3(ok3091)      | Control            | 17.16 ± 0.47     | 69 |                        |         |
|                    | mekk-3             | 18.63 ± 0.51     | 72 | (+8.56)                | 0.0915  |
| pgp-3(pk18)        | Control            | 17.56 ± 0.30     | 81 |                        |         |
|                    | mekk-3             | 19.67 ± 0.32     | 83 | (+12.01)               | < 0.0001|
| fer-15(b26); fem-1(hc17) | Control | 17.82 ± 0.47 | 115 |                        |         |
|                    | mekk-3             | 24.19 ± 0.63     | 104| (+35.74)               | < 0.0001|
| Wild-type (15 °C)  | Control            | 19.69 ± 0.46     | 105|                        |         |
|                    | mekk-3             | 35.89 ± 1.26     | 117| (+82.27)               | < 0.0001|
| hsf-1(yd41) (15 °C) | Control            | 17.85 ± 0.66     | 73 |                        |         |
|                    | mekk-3             | 31.78 ± 1.69     | 74 | (+78.03)               | < 0.0001|
| Wild-type          | Control            | 18.99 ± 0.44     | 71 |                        |         |
|                    | mekk-3(post-L1)    | 31.40 ± 0.55     | 83 | (+65.35)               | < 0.0001|
|                    | mekk-3(post-L2)    | 24.87 ± 0.50     | 99 | (+30.96)               | < 0.0001|
|                    | mekk-3(post-L3)    | 23.40 ± 0.64     | 70 | (~23.22)               | < 0.0001|
|                    | mekk-3(post-L4)    | 19.16 ± 0.38     | 82 | (~0.89)                | 0.869   |
|                    | mekk-3(post-YA)    | 17.81 ± 0.36     | 70 | (~6.21)                | 0.0336  |

(Continued)
Table 1 (Continued)

| Genetic background | RNAi used       | Mean ± SEM (days) | N  | % change w.r.t control | P value |
|---------------------|-----------------|-------------------|----|------------------------|---------|
| smg-1(cc546)        | Control         | 25.92 ± 0.89      | 78 |                        |         |
|                     | mekk-3          | 34.60 ± 1.03      | 77 | (+)33.48               | < 0.0001|
| smg-1(cc546) pha-4(2zu225) | Control | 25.94 ± 0.63      | 87 |                        |         |
|                     | mekk-3          | 27.84 ± 0.66      | 79 | (+)7.32                | 0.2621  |
| Wild-type           | Control         | 17.48 ± 0.28      | 95 |                        |         |
|                     | nhr-49          | 16.80 ± 0.26      | 125| (-)3.89                | 0.0944  |
| eat-2(ad1116)       | Control         | 24.38 ± 0.41      | 117|                        |         |
|                     | nhr-49          | 12.55 ± 0.29      | 88 | (-)48.52               | < 0.0001|
| Wild-type           | Control         | 18.37 ± 0.20      | 150|                        |         |
|                     | nhr-8           | 14.44 ± 0.26      | 106| (-)21.39               | < 0.0001|
| eat-2(ad1116)       | Control         | 24.40 ± 0.48      | 120|                        |         |
|                     | nhr-8           | 22.52 ± 0.26      | 124| (-)7.70                | < 0.0001|
| rde-1(ne219)        | Control         | 20.01 ± 0.43      | 106|                        |         |
|                     | mekk-3          | 20.96 ± 0.45      | 93 | (+)4.74                | 0.165   |
| rde-1(ne219); kzb9  | Control         | 18.14 ± 0.36      | 96 |                        |         |
|                     | mekk-3          | 23.37 ± 0.46      | 93 | (+)28.83               | < 0.0001|
| rde-1(ne219); kzb20 | Control         | 16.09 ± 0.51      | 85 |                        |         |
|                     | mekk-3          | 20.23 ± 0.60      | 95 | (+)25.73               | < 0.0001|
| rde-1(ne213); kbis7 | Control         | 20.16 ± 0.45      | 86 |                        |         |
|                     | mekk-3          | 19.65 ± 0.50      | 84 | (-)0.02                | 0.554   |
| Wild-type           | Control         | 18.76 ± 0.35      | 100|                        |         |
|                     | mekk-3 cDNA construct 1 | 27.98 ± 0.47 | 117| (+)49.15               | < 0.0001|
|                     | mekk-3 cDNA construct 2 | 27.88 ± 0.39 | 162| (+)32.71               | < 0.0001|
|                     | mekk-3 cDNA construct 3 | 26.77 ± 0.41 | 140| (+)42.69               | < 0.0001|
| Wild-type (25 °C)   | Control         | 12.76 ± 0.25      | 100|                        |         |
|                     | mekk-3          | 17.68 ± 0.39      | 108| (+)38.56               | < 0.0001|
| daf-2(e1370) (25 °C) | Control       | 35.20 ± 0.59      | 120|                        |         |
|                     | mekk-3          | 44.41 ± 0.76      | 120| (+)26.16               | < 0.0001|
| Wild-type (without FUDR) | Control | 18.37 ± 0.33      | 120|                        |         |
|                     | mekk-3          | 29.19 ± 0.42      | 120| (+)58.90               | < 0.0001|
| Wild-type (grown on HT115) | Control | 18.11 ± 0.33      | 120|                        |         |
|                     | mekk-3          | 28.42 ± 0.44      | 120| (+)56.62               | < 0.0001|
| Wild-type           | Control         | 20.34 ± 0.30      | 119|                        |         |
|                     | mekk-3          | 27.01 ± 0.44      | 107| (+)32.79               | < 0.0001|
| glp-1(e2141)        | Control         | 24.83 ± 0.42      | 120|                        |         |
|                     | mekk-3          | 25.65 ± 0.55      | 116| (+)3.30                | 0.0003  |
| Wild-type           | Control         | 18.74 ± 0.43      | 97 |                        |         |
|                     | mekk-3          | 26.57 ± 0.52      | 136| (+)41.78               | < 0.0001|
| Control (5 mwa DOG) | Control (OD 3.50) | 23.91 ± 0.63 | 135| (+)11.20               | 0.0009  |
|                     | mekk-3 (5 mwa DOG) | 26.59 ± 0.65 | 150|                        |         |
| Wild-type           | Control (OD 1.75) | 29.68 ± 1.11      | 47 |                        |         |
|                     | mekk-3 (OD 1.75) | 41.47 ± 1.05      | 49 | (+)39.72               | < 0.0001|
|                     | Control (OD 0.87)| 47.92 ± 1.51      | 51 | (+)61.45               | < 0.0001|
|                     | Control (OD 0.44)| 41.73 ± 1.93      | 49 | (+)40.59               | < 0.0001|
|                     | Control (OD 0.22)| 40.89 ± 1.79      | 46 | (+)37.76               | < 0.0001|
|                     | Control (OD 0.03)| 29.27 ± 1.27      | 49 | (-)1.38                | 0.8725  |
|                     | mekk-3 (OD 3.50) | 34.08 ± 1.73      | 38 |                        |         |
|                     | mekk-3 (OD 1.75) | 38.07 ± 1.89      | 43 | (+)11.70               | 0.0296  |
|                     | mekk-3 (OD 0.87) | 28.40 ± 1.30      | 45 | (-)16.66               | 0.0027  |
|                     | mekk-3 (OD 0.44) | 31.10 ± 1.62      | 41 | (-)8.74                | 0.1619  |
|                     | mekk-3 (OD 0.22) | 37.14 ± 1.82      | 37 | (-)8.97                | 0.1251  |
|                     | mekk-3 (OD 0.03) | 25.86 ± 1.28      | 44 | (-)124.12              | < 0.0001|

Expanded table for these experiments as well as an independent biological repeat is provided in Table S1.

1All experiments were performed using genomic RNAi construct from Ahringer RNAi library, unless mentioned otherwise.
2The full-length cDNA was cloned into pL4440. The cDNA sequence does not match any other sequence in the worm genome.
3hsf-1(y441) is lethal at higher temperatures. So, the strain and respective control wild-type were grown at 15 °C. Lifespan performed at 15 °C.
4smg-1(cc546), pha-4(2zu225) was grown at 25 °C to deactivate smg-1, a component of the NMD pathway. At L4 stage, the strain was shifted to 15 °C for lifespan analysis. At 15 °C, smg-1 is active and degrades pha-4 by NMD. The smg-1(cc546) was grown under similar conditions.
5Eggs were hatched on control RNAi, and worms were then transferred to mekk-3 RNAi at different larval stages indicated in brackets.
6The worms were grown at 20 °C till L3 and then transferred to 25 °C for lifespan analysis.
7Wild-type and glp-1(e2141) worms were hatched at 25 °C and transferred to 20 °C after 24 h. Lifespans were performed at 20 °C.
that are different from IIS pathway and similar to mitochondrial genes that affect lifespan.

**mekk-3 works independently of IIS and TGF-beta-like signalling**

In *C. elegans*, lifespan and dauer diapause are controlled by two parallel and overlapping signalling cascades, the IIS pathway and the noncanonical TGF-beta-like pathway (Shaw et al., 2007; Fielenbach & Antebi, 2008; Kenyon, 2010). Mutation in IIS receptor daf-2 leads to extended lifespan that is dependent on the FOXO transcription factor DAF-16 and the heat shock transcription factor HSF-1 (Kenyon, 2010). When daf-2 (e1370) worms were grown on mekk-3 RNAi at 20 °C, a dramatic increase in lifespan was observed over control RNAI-grown worms, suggesting that MEKK-3 may work in a parallel pathway (MLS of *daf-2* (e1370) on control RNAI is 46.29 ± 0.85 days, on mekk-3 RNAI is 60.42 ± 1.08 days, *P < 0.0001*; Fig. 1E, Table 1). RNAI knock-down of mekk-3 increased lifespan of *daf-2* (e1370) shifted to 25 °C at L3 when IIS completely shuts down, as well as in *daf-2* (e1368), a weaker allele (Fig. S2D,E). It is also possible that mekk-3 and daf-2 signalling pathways may overlap. Additionally, the lifespan extension observed on knocking down mekk-3 requires neither daf-16 nor hsf-1 (MLS of *daf-16* (mgDf50) on control RNAI is 13.86 ± 0.26 days, on mekk-3 RNAI is 24.06 ± 0.59 days, *P < 0.0001*; hsf-1 (sy441) on control RNAI is 17.85 ± 0.66 days, on mekk-3 RNAI is 31.78 ± 1.69 days, *P < 0.0001*; Figs 1F and S3A, Table 1). mekk-3 RNAI was also able to extend the lifespans of *daf-3* (e1390) and *daf-5* (e1386) (Fig. S3B,C and Table 1). DAF-3 is a SMAD factor, while DAF-5 is a SNO/SKI transcription factor downstream of the TGF-beta-like pathway (Fielenbach & Antebi, 2008). Further, mekk-3 RNAI also increased the lifespan of *daf-2* (e1370); *daf-3* (mgDf90) where the inputs from the DAF-7 pathway into IIS pathway have been eliminated (Padminabhan et al., 2009) (Fig. S3D, Table 1). Taken together, these experiments show that MEKK-3 affects longevity independent of the IIS and TGF-beta-like pathways, possibly using a different mechanism.

**mekk-3 knock-down induces a DR-like state**

As mekk-3 works independently of the IIS and TGF-beta-like pathway to affect lifespan, we investigated its role in other longevity pathways. In *C. elegans*, the eat-2 mutants represent a robust genetic model of DR (Lakowski & Hekimi, 1998), that are characterized by long lifespan and reduced fat storage (Lakowski & Hekimi, 1998; Brooks et al., 2009). We determined the levels of stored fat in the long-lived mekk-3 RNAI worms using Oil Red O and Nile Red staining (Yen et al., 2010) as well as biochemically by quantifying the triglyceride levels. We found that similar to eat-2 mutants, mekk-3 RNAI worms possess considerably fewer fat droplets in the intestinal cells and the hypodermis (Figs 2A and S10A) and had significantly lower triglyceride levels (Fig. S2E). The suppression of fat storage was also substantial when we grew *daf-2* (e1370), having intrinsically higher fat content, on mekk-3 RNAI (Figs S3F and S10D). Thus, similar to eat-2 mutants, mekk-3 knock-down worms have increased lifespan and low fat reserves.

The eat-2 mutants (Lakowski & Hekimi, 1998; Brooks et al., 2009) as well as mekk-3 knock-down worms have enhanced longevity and reduced fat storage. So, next we investigated their genetic interactions. We found that mekk-3 RNAI failed to increase the long lifespans of eat-2 (ad1116), eat-2(ad1113) and eat-2(ad465) to the same extent as in WT (MLS of eat-2(ad1116) on control RNAI is 28.94 ± 0.65 days, on mekk-3 RNAI is 27.35 ± 0.61 days, *P = 0.0453*; Figs 2B and S4A,B, Table 1), suggesting that they may function in the same genetic pathway for lifespan regulation. RNAI of mekk-3 decreased the low fat storage of eat-2(ad1116), suggesting that metabolic changes precede longevity mechanisms (Figs S4C and S10E). Similar to eat-2 mutants (Crawford et al., 2007; Jia & Levine, 2007), the mekk-3 RNAI worms have low brood size, longer reproductive span, increased autophagosome formation in the seam cells and smaller body size compared to control RNAI worms (Figs 2C,D and S4D). Additionally, like eat-2 mutants (Lakowski & Hekimi, 1998), mekk-3 RNAI did not significantly increase the lifespan of *clk-1* (qm30), a mutant in the highly conserved demethoxyubiquinone (DMQ) hydroxylase gene that is necessary for the biosynthesis of ubiquinone (*MLS of clk-1* (qm30) on control RNAI is 33.04 ± 0.85 days, while on mekk-3 RNAI is 34.24 ± 0.83 days, *P = 0.419*, Fig. S4E). However, the eat-2 mutants have low pharyngeal pumping rate and as a result ingest less bacteria (Lakowski & Hekimi, 1998). Surprisingly, distinct from eat-2 mutants, mekk-3 RNAI did not significantly affect pharyngeal pumping and the worms had normal feeding rate as measured by ingestion of fluorescent beads, GFP-expressing bacteria or by uptake of C12 BODIPY (Figs 2E, S12 and S13). Together, mekk-3 knock-down may initiate a DR-like process by a novel mechanism independent of pharyngeal pumping and feeding defects. It may thus represent a new model of DR.

Dietary restriction may be implemented in *C. elegans* by nongenetic means either by serially diluting the bacteria that the worms feed on (Panowski et al., 2007) or by using a nonhydrolysable analogue of glucose (Schulz et al., 2007). Next, we studied the genetic interaction of these two DR regimes with the new model of DR. Bacterial dilution-induced DR typically produces a bell-shaped curve when MLSs are plotted against the decreasing values of bacterial concentration (Panowski et al., 2007). However, we found that when mekk-3 RNAI-treated worms were exposed to bacterial dilution starting at adulthood, the MLSs failed to produce a bell-shaped curve (Fig. S5A, Table 1). Additionally, mekk-3 RNAI failed to extend the lifespan of worms exposed to 2-deoxyglucose (DOG), to the same extent as in untreated worms (Fig. S5B, Table 1). Together, these observations point to the fact that the DR-like state initiated by either knocking down mekk-3 or through nongenetic means may utilize similar pathways to affect longevity positively.

Genetic and nongenetic models of DR specifically require a FoxA transcription factor, PHA-4 (Panowski et al., 2007), to increase lifespan. PHA-4 is not required for IIS pathway-mediated longevity effects. So, next we asked whether our new model of DR requires pha-4. We used the temperature-sensitive mutant smg-1 (cc546); pha-4 (zu225) and evaluated whether mekk-3 RNAI can extend lifespan when pha-4 is absent; in this mutant, smg-1 is WT at 15 °C and pha-4 is degraded by non-sense-mediated decay (NMD) pathway. Under this condition, mekk-3 failed to increase lifespan significantly (*MLS of smg-1 (cc546); pha-4 (zu225) on control RNAI is 25.94 ± 0.63, on mekk-3 RNAI is 27.84 ± 0.66, *P = 0.262*, Fig. 2F, Table 1). Thus, similar to eat-2 mutants and nongenetic liquid DR, knocking down mekk-3 requires PHA-4 to enhance longevity.

The stress-protective transcription factor SKN-1/NRF2 is important in the ASI neurons to regulate DR-induced longevity, whereas it works downstream of the IIS pathway, in the intestine, to regulate oxidative stress tolerance (Bishop & Guarente, 2007b; Tullet et al., 2008; Park et al., 2010). We asked whether the new model of DR requires SKN-1. We found that when null mutant skn-1 (zu169) was grown on mekk-3 RNAI, lifespan was significantly extended (*MLS of skn-1 (zu169) on control RNAI is 17.86 ± 0.49 days, on mekk-3 RNAI is 26.39 ± 0.79 days, *P < 0.0001*, Fig. S5C, Table 1), but not to the same extent as in the eat-2 mutants.
Novel kinase in DR-mediated longevity, M. Chamoli et al.

Taken together, multiple lines of evidence suggest that knocking down the novel kinase mekk-3 extends longevity by initiating a DR-like state, similar to the genetic and nongenetic models of DR. However, distinct from the eat-2 mutants, the induction of this DR-like state is independent of food intake.

mekk-3 works in the muscle and hypodermis to regulate lifespan

To study the distribution of mekk-3 expression, its promoter was cloned in the promoter-less gfp vector, pPD95.75. The mekk-3 promoter drives expression of gfp in vulval muscles, body wall muscles, hypodermis, seam cells and tissues adjoining the pharynx and anus (Fig. 3A; Supplementary confocal videos). Expression was also noticed in some neurons but is excluded from the intestine. To find tissues where knocking down of mekk-3 is sufficient to extend lifespan, we used tissue-specific RNAi systems (Espelt et al., 2005; Qadota et al., 2007). Interestingly, we found that mekk-3 RNAi did not extend lifespan when specifically knocked down in the intestine, the major metabolic tissue in...
the worm. However, partial lifespan extension was observed when expression is reduced in muscles or hypodermis (Fig. 3B–E, Table 1). This suggests that mekk-3 functions in a cell nonautonomous manner and cooperation between multiple tissues may be required for its normal function. It also establishes hypodermis as an important energy sensing and transducing tissue.

A metabolic shift towards fatty acid oxidation on mekk-3 knock-down

mekk-3 knock-down resulted in increased longevity and decreased fat storage. The decreased fat storage observed may be a result of increased degradation or decreased assimilation of fat, and a gene expression profile may shed light into the mechanism of mekk-3 knock-down-mediated longevity. We performed a microarray analysis to compare the gene expression profiles of control and mekk-3 RNAi-treated WT worms. We found that several genes involved in fatty acid degradation and subsequent utilization were upregulated following mekk-3 knock-down, apart from genes involved in proteolysis and organismal aging (Table 2 and Fig. S11). Genes that were upregulated more than twofold include lipases, components of the mitochondrial and peroxisomal beta-oxidation system (acyl-CoA synthetase, carnitine palmitoyl transferase, acyl-CoA oxidase, enoyl-CoA hydratase), glyoxylate pathway component (isocitrate/malate synthase) as well as lipid transfer proteins and retinol/fatty

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Fig. 3  MEKK-3 is required in the hypodermis and muscle for longevity. (A) Expression pattern of mekk-3p::gfp transgenic worms. Expression is observed in the hypodermis, vulva and regions adjoining the head and tail. (B) mekk-3 RNAi had limited effect on the lifespan of RNAi-deficient rde-1(ne219). Mean lifespan (MLS) on control RNAi was 20.01 ± 0.43 (n = 106), on mekk-3 RNAi was 20.96 ± 0.45 days (n = 93), P = 0.165, log-rank test. (C) mekk-3 RNAi partially extended the lifespan of rde-1(ne219); kzs20. The rde-1 gene is rescued in the muscles of rde-1(ne219). Mean lifespan on control RNAi was 20.23 ± 0.60 days (n = 95), P < 0.0001. (D) mekk-3 RNAi had limited effects on lifespan of rde-1(ne213); kbs7. The rde-1 gene is rescued in the intestine of rde-1(ne219). Mean lifespan on control RNAi was 20.16 ± 0.45 days (n = 86), on mekk-3 RNAi was 19.65 ± 0.50 days (n = 85), P < 0.554. (E) mekk-3 RNAi partially extended the lifespan of rde-1(ne219); kzs9. The rde-1 gene is rescued in the hypodermis of rde-1(ne219). Mean lifespan on control RNAi was 23.37 ± 0.46 days (n = 93), P < 0.0001. All lifespans were performed at 20 °C.
Table 2: Partial list of genes upregulated on mekk-3 knock-down as determined by microarray analysis. Genes that may function in lipid metabolism and xenobiotic biotransformation are shown.

| Gene name          | Wormbase ID   | Brief descriptions                        | Fold changes | P value |
|--------------------|---------------|-------------------------------------------|--------------|---------|
| acs-2              | WBGene00009221 | Fatty acyl-CoA synthetase                 | 3.7          | 0.0178  |
| ech-9              | WBGene0001158  | Enoyl-CoA hydratase                       | 170.4        | 0.0003  |
| cpt-3              | WBGene00021703 | Carnitine palmitoyl transferase           | 26.2         | 0.0118  |
| C48B4.1            | WBGene00008167 | Peroxisomal acyl-CoA oxidase              | 16.5         | 0.0005  |
| F25A2.1            | WBGene00017764 | Lipase                                    | 2.6          | 0.0012  |
| Y49E10.18          | WBGene00013037 | Lipase                                    | 2.6          | 0.0404  |
| far-7              | WBGene00001391 | Fatty acid- /retinol-binding protein      | 4.5          | 0.0277  |
| gei-7              | WBGene00001564 | Isocitrate lyase/malate synthase          | 2.6          | 0.0174  |
| C31H5.6            | WBGene00007857 | Acyl-CoA thioesterase                    | 2.1          | 0.0020  |
| FA7A4.5            | WBGene00008901 | Involved in lipid catabolic processes     | 8.5          | 0.0280  |
| F25E2.3            | WBGene00017781 | Acyl-CoA thioesterase                    | 2.2          | 0.0018  |
| pmp-7              | WBGene00004058 | Peroxisomal long-chain fatty acyl transporter | 2.0       | 0.0351  |
| oac-29             | WBGene00018295 | O-Acyltransferase activity                | 2.8          | 0.0394  |
| oac-49             | WBGene00012068 | O-Acyltransferase activity                | 2.6          | 0.0243  |
| M01A8.1            | WBGene00010795 | Lipid storage                            | 2.6          | 0.0240  |
| C30F12.1           | WBGene00016260 | Lipid storage                            | 2.1          | 0.0477  |
| spp-20             | WBGene00005005 | Lipid degradation                         | 9.4          | 0.0010  |
| FA7A4.5            | WBGene00008901 | Lipid transport                           | 8.5          | 0.028   |
| F09C8.1            | WBGene00008621 | Phospholipase B1                         | 2.0          | 0.048   |

Xenobiotic biotransformation

cy-32B1            | WBGene00021167 | Phase I detoxification, cytochrome P450   | 3.3          | 0.0134  |
| cy-33C8            | WBGene00019967 | Phase I detoxification, cytochrome P450   | 2.6          | 0.0154  |
| cy-34A4            | WBGene00020386 | Phase I detoxification, cytochrome P450   | 3.1          | 0.0692  |
| cy-35A1            | WBGene00015399 | Phase I detoxification, cytochrome P450   | 2.9          | 0.0075  |
| cy-37B1            | WBGene00009226 | Phase I detoxification, cytochrome P450   | 9.7          | 0.0066  |
| ugt-16             | WBGene00013901 | Phase II detoxification, UDP-glucuronosyltransferase | 3.6  | 0.0186  |
| ugt-18             | WBGene00013900 | Phase II detoxification, UDP-glucuronosyltransferase | 820.4 | 0.0002  |
| ugt-43             | WBGene00008485 | Phase II detoxification, UDP-glucuronosyltransferase | 2.1  | 0.0195  |

Fold changes determined between control RNAi and mekk-3 RNAi-treated worms.

acid-binding proteins. These observations suggested that worms may reprogramme metabolism, following initiation of a DR-like state by mekk-3 knock-down, towards using beta-oxidation leading to prolonged lifespan. The eat-2 mutant worms also show similar switch in metabolism (Yuan et al., 2012).

Nuclear hormone receptor NHR-49, a mammalian HNF4 homolog, transcriptionally regulates many rate-limiting genes of beta-oxidation, including acyl-CoA synthetase, enoyl-CoA hydratase and carnitine palmitoyl transferase (Van Gilst et al., 2005; Pathare et al., 2012). The lack of beta-oxidation leads to higher amount of stored fat in nhr-49 mutant worms, as seen in nhr-49(ok2165) (Figs S6A and S10F). We reasoned that if mekk-3 decreases fat storage by upregulating beta-oxidation, it should fail to do so in nhr-49(ok2165). As expected, we found that mekk-3 RNAi did not affect fat storage in nhr-49(ok2165) as determined by Oil Red O, Nile Red and triglyceride quantification (Figs 4A and S10B,G). Consequently, mekk-3 RNAi failed to extend its lifespan significantly [MLS of nhr-49(ok2165) on control RNAi was 16.22 ± 0.40 days, on mekk-3 RNAi was 15.49 ± 0.58 days, P = 0.56, Fig. 4B, Table 1]. The mekk-3 RNAi was also not able to increase lifespan in another allele of nhr-49, namely nhr-49(nr2041) to the same extent as in WT (Fig. S5B). In worms,daf-22 codes for a thiolase required in the last step of peroxisomal beta-oxidation (Zhang et al., 2010), an important catabolic step before mitochondrial beta-oxidation of long-chain fatty acids. RNAi of mekk-3 was unable to increase lifespan in daf-22(m130) (Fig. S6C). Taken together, mekk-3 knock-down-induced DR-like state reprogrammes metabolism towards fatty acid oxidation through NHR-49 and DAF-22 to positively affect lifespan.

Next, we asked whether eat-2-mediated DR also requires nhr-49-dependent metabolic reprogramming. We grew eat-2(ad1116) worms on nhr-49 RNAi and found that the long lifespan of eat-2(ad1116) is significantly suppressed [MLS of eat-2(ad1116) on control RNAI is 24.38 ± 0.64 days, on mekk-3 RNAI was 12.55 ± 0.29 days, P < 0.0001; Fig. 4C, Table 1]. The nhr-49 RNAI had no effect on WT worms (Table 1, also see Table S1). On the other hand, nhr-49 knock-down increased the fat/triglyceride levels of eat-2(ad1116) (Figs 4D and S10C,H). Thus, nhr-49-mediated metabolic reprogramming-induced longevity is a common mechanism for lifespan extension during DR.

Knocking down mekk-3 generates low ROS and requires Complex II activity

DR is known to produce reduced levels of ROS, potent oxidizing agents that damage cellular macromolecules and may be the underlying cause of aging (Pamplona & Barja, 2007; Page et al., 2010). We measured the total cellular ROS in mekk-3 RNAI worms and found that the levels were significantly lower than that of control RNAI-treated worms, comparable to the ROS generated in eat-2(ad1116) (Fig. 4E). It appears that the reduced ROS levels are a result of metabolic shift to beta-oxidation and not due to active ROS detoxification. This is supported by gene
expression profiling by microarray and qRT-PCR analysis where none of the five worm superoxide dismutase genes were significantly upregulated when mekk-3 is knocked down (Fig. S6D). Also, the total cellular SOD activity did not increase when worms were grown on mekk-3 RNAi (Fig. S6E). Additionally in nhr-49(ok2165) worms, that are incapable of making the metabolic shift, mekk-3 RNAi was not able to significantly decrease the ROS (Fig. S6F). Also, these worms, unlike wild-type (WT) worms on mekk-3 RNAi and eat-2(ad1116) on control RNAi, have lower reactive oxygen species levels compared with WT grown on control RNAi. Average of three biological replicates; Student’s t-test. (A) mekk-3 RNAi worms were not resistant to external application of 100 mM paraquat that induces severe oxidative stress (mean survival of WT on control RNAi is 26.27 ± 2.74 h (n = 15) P = 0.0001. (E) Wild-type (WT) worms on mekk-3 RNAi were not resistant to paraquat in a daf-16-dependent manner. (G) mekk-3 knock-down failed to extend lifespan of mev-1(kn1). Mean lifespan on control RNAi was 14.36 ± 0.40 days (n = 66), while that on mekk-3 RNAI was 15.06 ± 0.55 days (n = 52), P = 0.2868. (H) mekk-3 RNAI extended lifespan of gas-1(fc21). Mean lifespan on control RNAI was 12.55 ± 0.42 days (n = 89), while that on mekk-3 RNAI was 14.36 ± 0.40 days, on mekk-3 RNAI is 15.06 ± 0.55 days, P = 0.29; Fig. 4G, Table 1. On the other hand, mekk-3 RNAI significantly increased the lifespan of gas-1(fc21), a mutant in the NADH:ubiquinone oxidoreductase 49 kD subunit of Complex I [MLS of gas-1(fc21)] on control RNAi is 19.27 ± 0.28 h, on mekk-3 RNAI is 29.74 ± 0.42 days (n = 66), P < 0.0001. All lifespans were performed at 20 °C.

Knock-down of mekk-3 activates the xenobiotic detoxification machinery through conserved transcription factors

As observed in dauer, biological systems that depend on fatty acid oxidation for their energy needs activate the xenobiotic detoxification machinery (Wang & Kim, 2003; McElwée et al., 2004; Lindblom & Dodd, 2006). Xenobiotic endotoxins are detoxified by a two-step procedure. The phase I detoxification enzymes like cytochrome p450 (CYP) chemically modify offending endotoxins that are then acted upon by phase II enzymes like UGT, making them more soluble (Lindblom & Dodd, 2006). Finally, these modified toxins are secreted out of the cell by ABC transporters (p-glycoproteins) (Sharom, 2011). In the microarray analysis, we found that several genes involved in xenobiotic detoxification, such as CYP and UGT, are significantly upregulated when mekk-3 is knocked down (Table 2). This indicated that on mekk-3 knock-down, similar to conditions in dauer,
the xenobiotic detoxification machinery may be upregulated to clear internally generated toxins for increased longevity.

In *C. elegans*, xenobiotic detoxification genes are regulated by NHR-8, a nuclear hormone receptor of the same family as DAF-12 and NHR-48 (Lindblom et al., 2001; Lindblom & Dodd, 2006). The expression of some xenobiotic detoxification genes was deregulated in nhr-8(ok186) as determined by qRT-PCR (Fig. S7A). So we asked whether mekk-3 RNAI worms require NHR-8 for their long life. We found that in nhr-8 (ok186), knocking down mekk-3 did not result in increased lifespan [MLS of nhr-8(ok186) on control RNAI is 17.53 ± 0.34 days, on mekk-3 RNAI is 12.71 ± 0.30 days; Fig. 5A, Table 1]. Interestingly, although nhr-8 plays a crucial role in mekk-3 knock-down-mediated DR, it appears that eat-2(ad1116) does not require nhr-8 for its increased longevity (Table 1, also see Table S1). The long lifespan on eat-2(ad1116) remains unaffected on nhr-8 RNAI.

In mammals and worms, the CYP genes are regulated by aryl hydrocarbon receptors (AHR) and AHR nuclear translocator (ARNT) (Powell-Coffman et al., 1998). *Caenorhabditis elegans* has one gene each for AHR and ARNT, namely ahr-1 and aha-1, respectively, these proteins regulate the expression of some of the xenobiotic genes that were found to be upregulated on mekk-3 knock-down (Fig. 5B,C). We found that mekk-3 RNAI failed to extend lifespan in ahr-1(ju145), ahr-1(ia3) and aha-1(ok1396) to the same extent as in WT worms (Figs 5B,C and S7D, Table 1, see also Table S1). In fact, mekk-3 RNAI decreased the lifespan of aha-1(ok1396) and as observed above, of nhr-8(ok186). We believe that during mekk-3 knock-down, requirement for xenobiotic detoxification is so enhanced that mutations in key proteins in this pathway may make the worms vulnerable to internally generated toxins and result in suppression of lifespan. It is also possible that these transcription factors have other functions that are required during stresses associated with DR. Further, in ABC transporter (P-glycoproteins) mutants such as pgp-3(ok3091) (Fig. 5D) or pgp-3(pk18) (Fig. S7E), mekk-3 RNAI did not extend lifespan to the same extent as in WT.

The lifespan extension by mekk-3 RNAI requires PHA-4, as shown above. We next asked whether PHA-4 has a role in transcriptional regulation of genes upregulated during this new model of DR. We collated the list of PHA-4 direct targets from modENCODE chromatin immunoprecipitation (ChIP)-sequencing data and compared it with the genes that are upregulated when mekk-3 is knocked down. We found that over 40% of the genes are common (*P* = 1.83e-9, hypergeometric test), pointing to a direct transcriptional role of PHA-4 in mediating this response (Fig. S8A, Table S2). Additionally, genes involved in xenobiotic detoxification are enriched among the common genes (Fig. S8B). Several representative genes involved in xenobiotic detoxification downstream of mekk-3 (such as cyp-32B1, 37B1 as well as ugt-16, 18) are controlled by PHA-4 as revealed by qRT-PCR analysis (Fig. S8B,C). Interestingly, only a few SKN-1 target genes overlap with mekk-3 RNAI-upregulated genes and may explain the partial dependence of skn-1 on mekk-3 knock-down-mediated lifespan extension (Fig. S8D,E; Table S3). Taken together, these data show that activated xenobiotic detoxification system is an important requirement for mekk-3 RNAI-induced DR-like state to extend lifespan, and PHA-4, NHR-8 and AHR-1/AHA-1 are important mediators in this process.

![Fig. 5](image-url) Xenobiotic detoxification is required for mekk-3 knock-down-mediated longevity. (A) mekk-3 RNAI did not extend lifespan of nhr-8(ok186). Mean lifespan (MLS) on control RNAI was 17.53 ± 0.34 days (*n* = 85), while that on mekk-3 RNAI it was 12.71 ± 0.30 days (*n* = 42), *P* = 0.001, by log-rank test. (B) No lifespan extension in ahr-1(ju145) or mekk-3 RNAI on mekk-3 knock-down. Mean lifespan on control RNAI was 16.16 ± 0.45 days (*n* = 49), while that on mekk-3 RNAI it was 14.92 ± 0.38 days (*n* = 51), *P* = 0.0561. (C) mekk-3 RNAI failed to extend lifespan of aha-1(ok1396). Mean lifespan on control RNAI was 16.69 ± 0.33 days (*n* = 141), while that on mekk-3 RNAI it was 12.89 ± 0.34 days (*n* = 148), *P* < 0.0001. (D) No change in lifespan in pgp-3(ok3091) worms on mekk-3 RNAI as compared to control RNAI. Mean lifespan control RNAI was 17.16 ± 0.47 days (*n* = 69), while that on mekk-3 RNAI it was 18.63 ± 0.51 days (*n* = 72), *P* = 0.0915. All lifespans were performed at 20 °C.
**mekk-3 RNAi-induced metabolic reprogramming upregulates the xenobiotic detoxification genes**

Upregulation of the xenobiotic detoxification machinery may either be a direct consequence of mekk-3 RNAi or an indirect effect of the metabolic shift towards beta-oxidation during DR. To differentiate between these two possibilities, we asked whether mekk-3 RNAi leads to upregulation of genes involved in xenobiotic response in a nhr-49 mutant, where beta-oxidation is suppressed. We grew WT and nhr-49 (ok2165) worms on control or mekk-3 RNAi and measured the transcript levels of several phase I and II xenobiotic biotransformation genes. As expected, the levels of these genes were significantly increased in WT worms grown on mekk-3 RNAi (Fig. 6A). Interestingly for all these genes, expression inductions were reduced in nhr-49(ok2165) (Fig. 6A), providing evidence that the metabolic reprogramming is responsible for upregulating the xenobiotic biotransformation system in worms where mekk-3 has been knocked down. Similar results were obtained with nhr-49(nv2041) (Fig. S9). Our data suggest that metabolic reprogramming on mekk-3 knock-down triggers xenobiotic biotransformation gene expression, together leading to long and healthy life.

**Discussion**

In this study, we identified a novel mMEKK3-like kinase in *C. elegans* that we speculate may be an important component of a nutrient signalling pathway. Knocking down mekk-3 leads to phenotypic manifestations and has genetic requirements that are quite similar to DR. Interestingly, the food intake of these worms remain unchanged. However, considering the timing requirements of this gene (Fig. 1D) as well as its epistatic relationship with clk-1 (Fig. S4E), it is possible that mekk-3 is a component of the mitochondrial or related longevity pathways. We show that during this DR-like state, two cellular processes are equally important: (i) metabolic shift towards fatty acid oxidation (ii) leading to an increased level of xenobiotic detoxification (Fig. S8). Together, these two cellular processes create a unique balance in the system that positively affects the lifespan of an organism.

Apart from efficient generation of energy, shifting metabolism from glucose towards lipid oxidation has many other benefits (Mobbs et al., 2007; Guarente, 2008). When glucose is used as an energy source, for every FADH2 molecule, five NADH is generated through glycolysis and TCA cycle. On the other hand, during beta-oxidation, the ratio of NADH: FADH2 is only 2:1. The reducing equivalents generated during glucose or lipid breakdown are oxidized in the mitochondrial ETC. Electrons from NADH are transferred to Complex I, while FADH2 is oxidized at Complex II. Complex I is known to produce larger quantities of ROS during the process of transferring electrons to ubiquinone, compared with Complex II (Mobbs et al., 2007; Guarente, 2008). As relatively more of ROS II is utilized when lipid is oxidized, compared with when glucose is used, increased beta-oxidation generates lower levels of ROS. This is exactly what we observe on mekk-3 knock-down and in eat-2(ad1116). As ROS wrecers havoc at the cellular level and have negative impact on normal tissue functioning (Pamplona & Barja, 2007), metabolic reprogramming during DR or in DR-like states is beneficial to an organism and contributes to its increased longevity.

Lipid degradation, however, has a downside: lipophilic toxins may be produced that accumulate in the system (Lindblom & Dod, 2006). Elevated levels of xenobiotic detoxification enzymes are expressed to counter this issue. In *C. elegans*, such a situation arises in the dauer larvae. During periods of food shortage, worms enter an alternate developmental stage called dauer that have increased fat storage and lipid catabolism to generate energy. During the dauer stage, elevated expression of xenobiotic detoxification genes are observed (Wang & Kim, 2003; McElwee et al., 2004; Lindblom & Dod, 2006). During the mekk-3 knock-down-mediated DR-like state, we also found elevated levels of phase I and phase II detoxification genes. Transcription factors controlling these genes, such as PHA-4/FOXa, NHR-8 and AHR-1, were also found to be important during this process. These facts suggest that lipophilic metabolism and xenobiotic detoxification are equally important: (i) metabolic shift towards fatty acid oxidation (ii) upregulation of genes involved in xenobiotic response. For example, PHA-4, a transcription factor that positively affects the lifespan of an organism (Pamplona & Barja, 2007; Guarente, 2008). When glucose is used as an energy source, electrons from NADH are transferred to Complex I, while FADH2 is oxidized at Complex II. Complex I is known to produce larger quantities of ROS during the process of transferring electrons to ubiquinone, compared with Complex II (Mobbs et al., 2007; Guarente, 2008). As relatively more of ROS II is utilized when lipid is oxidized, compared with when glucose is used, increased beta-oxidation generates lower levels of ROS. This is exactly what we observe on mekk-3 knock-down and in eat-2(ad1116). As ROS wrecers havoc at the cellular level and have negative impact on normal tissue functioning (Pamplona & Barja, 2007), metabolic reprogramming during DR or in DR-like states is beneficial to an organism and contributes to its increased longevity.

Lipid degradation, however, has a downside: lipophilic toxins may be produced that accumulate in the system (Lindblom & Dod, 2006). Elevated levels of xenobiotic detoxification enzymes are expressed to counter this issue. In *C. elegans*, such a situation arises in the dauer larvae. During periods of food shortage, worms enter an alternate developmental stage called dauer that have increased fat storage and lipid catabolism to generate energy. During the dauer stage, elevated expression of xenobiotic detoxification genes are observed (Wang & Kim, 2003; McElwee et al., 2004; Lindblom & Dod, 2006). During the mekk-3 knock-down-mediated DR-like state, we also found elevated levels of phase I and phase II detoxification genes. Transcription factors controlling these genes, such as PHA-4/FOXa, NHR-8 and AHR-1, were also found to be important during this process. These facts suggest that...
during DR or in DR-like states, upregulation of xenobiotic detoxification is essential, supporting lifespan extension by lipid oxidation. In fact, the detoxification genes are not upregulated on mekk-3 knock-down when beta-oxidation is prevented by mutation in nhr-49. Recent studies also reflect similar mechanism in mammals. Calorically restricted mice have been shown to upregulate phase I xenobiotic-metabolizing enzymes (Steinau et al., 2012), pointing at conservation of this response.

In C. elegans, different DR regimes activate alternate pathways and have requirement for different genes. For example, eat-2 mutants and some nongenetic DR models require pha-4 and skn-1 for increased longevity, but do not require daf-16 (Bishop & Guarente, 2007b; Panowski et al., 2007; Park et al., 2010). On the other hand, solid dietary restriction (SDR) regimes require daf-16 (Greer et al., 2007). Interestingly, the mekk-3 knock-down-mediated DR-like state does not require daf-16, similar to eat-2. However, it requires pha-4, but is only partially dependent on skn-1. Additionally, although this new model requires NHR-8 for increased lifespan, eat-2 mutant worms are not affected by nhr-8 knock-down. It is possible that in eat-2 mutants, xenobiotic detoxification genes are controlled entirely by SKN-1 (Oliveira et al., 2009) or PHA-4, while in our model, this process is under shared regulation. These observations point to the intricate nature of nutrient sensing and differential use of signalling cascades to transduce signals downstream of DR.

Over the last few decades, several theories have evolved to explain causes of aging and lifespan extension. Prominent among them are the mitochondrial free radical theory of aging (MFRTA), mitohormesis and gradual ROS response hypothesis (GRRH) (Pampolona & Barja, 2007; Schulz et al., 2007; Hekimi et al., 2011). The key difference between MFRTA and mitohormesis/GRRH is in defining the role of ROS. MFRTA proposes that mitochondrial ROS damage macromolecules to cause aging and consequently lowering ROS may increase lifespan. On the other hand, mitohormesis/GRRH requires a burst of ROS production to prime the system to handle subsequent stresses. We found that on mekk-3 knock-down and in eat-2(ad1116), the worms intrinsically produce lower levels of ROS. At least in the case of mekk-3 knock-down, this may not be due to higher levels of ROS detoxification machinery within the cells. As determined by microarray and real-time PCR analysis, none of the sod genes are significantly upregulated and cellular SOD activity remains unchanged. Although the levels of ctl-1, ctl-2 and ctl-3 were upregulated several folds (data not shown), this may be attributed to increased peroxisomal beta-oxidation. Consequently, the mekk-3 knock-down worms do not show hormesis, similar to eat-2 mutants (Houthooft et al., 2002). Thus, lifespan extension by mekk-3 knock-down supports MFRTA. Similar observations were made in rodents subjected to DR which showed lower levels of mitoROS, lower resistance to oxidative damage and reduced expression of oxidative defence systems (Pampolona & Barja, 2007), suggesting that such effects of DR may be evolutionarily conserved.

mekk-3 regulates lifespan in a tissue nonautonomous manner. In line with its expression pattern, mekk-3 knock-down increases lifespan only when performed in the hypodermis or muscle, but not in the intestine. Thus, MEKK-3 functions in these tissues to regulate metabolism systemically throughout the body, including intestine. Genes expressing in the intestine and neurons have long been known to be major regulators of lifespan and metabolism, especially in case of the IIS pathway (Kenyon, 2010). The cell nonautonomous regulation of longevity by mitochondrial pathway has also been recently described (Durieux et al., 2011). Although the intestine is known as the major metabolic tissue (McGhee, 2007), functioning as adipocytes in worms, the hypodermis is now emerging as an important tissue involved in nutrient signalling. The C. elegans hypodermis also functions in energy storage and during periods of nutrient deprivation or dauer survival, triglycerides are slowly released from the hypodermal layer that ensure long-term survival (Narbonne & Roy, 2009). This process is controlled by the AMPK signalling cascade (Narbonne & Roy, 2009). Recently, miRNA235 has also been shown to function in the hypodermis to control developmental quiescence depending on nutritional status (Hidefumi et al., 2013). Interestingly, the muscle-specific function of mekk-3 mirrors that of AMPK, a well-established energy sensor (Canto & Auwerx, 2011). Pharmaceutical or genetic activation of AMPK in the muscle leads to increased fatty acid oxidation and glucose uptake (Hardie et al., 2006). Thus, it is tempting to speculate that MEKK-3 and AMPK may have overlapping functions in the muscle to regulate metabolism systemically.

MEKK-3 is a negative regulator of lifespan and may constitute a sensory module for nutrient sensing. As it does not express or function in the intestine, it may not be involved directly in the uptake of nutrients. Lowering the levels of MEKK-3 may be speculated to initiate a signalling cascade that signals low nutrient availability, although food intake is normal. This leads to the initiation of a metabolic and physiological reprogramming that benefits the worms in terms of increased lifespan and health span. MEKK-3 is a serine-threonine kinase and has significant homology to mammalian MAPKKK, mMEKK3. It will be interesting to study whether knocking down mMEKK3 can initiate a DR-like state in mammals, without major lifestyle changes.

**Experimental procedures**

Detailed materials and methods are reported as Data S1 (Supporting Information). Unless otherwise mentioned, all strains were maintained at 20 °C using standard C. elegans techniques, and all RNAi experiments were initiated from eggs. Gravid adult worms were bleached, and the eggs were hatched on plates containing respective RNAi bacteria. L4 or young adult worms were transferred to an intermediate RNAi plate for 12 h and then onto RNAi plates overlaid with 5-fluorodeoxyuridine (FUDR) to a final concentration of 0.1 mg mL⁻¹. Worms were scored as dead or alive by tapping them gently with a platinum wire every 2–3 days. Worms that were sick or died from vulval bursting were censored. Statistical analyses for survival were conducted using Mantel–Cox log-rank test using OASIS software available at http://sbi.postech.ac.kr/oasis. Lifespan is expressed as average lifespan ± SEM. Full data and additional biological repeat are reported in Table S1. Fat storage was determined in fixed worms using Nile Red or Oil Red O (Yen et al., 2010). For estimating pharyngeal pumping, 10–15 young adult worms were videographed for 10 s, and number of pumping was counted in the slowed-down video. To quantify food intake, WT L4 larvae grown on control or mekk-3 RNAi were placed on NGM plates seeded with a 250:1 (vol:vol) mixture of HT115 bacteria and Fluoresbrites Multifluorescent microspheres (0.2 μm diameter; Polyscience Inc., Warrington, PA, USA). The worms were allowed to feed for 10 min and then washed twice with M9 buffer and photographed. Fluorescent intensities of worms were measured using NIH imageJ software. Triglyceride was quantified using a kit (Biovision, Milpitas, CA, USA). Autophagic vesicles were estimated using transgenic worms expressing lgg-1::gfp. Microarray analysis was carried out commercially at Genotypic Technology (Bangalore, India) using an Agilent, USA platform. Data were analysed using Genespring software (Agilent, Santa Clara, CA, USA) or CLC Genomics Workbench 4 (CLC Bio, Cambridge, MA, USA), and more than twofold expression changes with P value < 0.05 is reported. The microarray data are available at GEO repository with series record number GSE40252.
Oxidative stress assay was performed in 24-well tissue culture plate containing 1 mL of 100 μM paraquat is reported here. Worms in the paraquat were scored every 2–3 h for survival. Intracellular levels of ROS were determined using 2,7-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Grand Island, NY, USA).

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Author contributions

AM conceived the project, analysed the data and wrote the manuscript. MC, AS and YM performed the experiments, collected and analysed the data. AM is a Ramalingaswami fellow, MC is a DBT JRF, while AS is a CSIR JRF.

Conflict of Interest

The authors declare no conflict of interest.

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The authors declare no conflict of interest.

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Fig. S1 Supporting Information

Fig. S1 (A) Lifespan of eat-2(ad1113) on mekk-3 RNAi. MLS on control RNAi was 24.16 ± 0.48 days (n = 88), on mekk-3 RNAi was 21.66 ± 0.67 days (n = 62), P = 0.0089 by Log rank test. (B) Lifespan of eat-2(ad465) on mekk-3 RNAI. MLS on control RNAI was 28.76 ± 0.76 days (n = 94), on mekk-3RNAI was 25.64 ± 0.43 days (n = 100), P < 0.0001. (C) Oil Red O staining of fat stored in eat-2(ad1116) grown on control or mekk-3 RNAI. Quantification of staining is shown in the right. Error bars indicate SEM; n > 50; Student’s t-test. (D) WT worms grown on mekk-3 RNAI have smaller size compared to control RNAI-grown worms. Error bars indicate SEM; n > 40; Student’s t-test. (E) mekk-3 RNAI failed to extend lifespan of ctk-1(qm30). MLS on control RNAI was 33.04 ± 0.85 days (n = 67), on mekk-3 RNAI was 34.24 ± 0.83 days (n = 76), P = 0.4198. All lifespans were performed at 20 °C.

Fig. S5 (A) WT worms grown on control or mekk-3 RNAI were transferred to liquid DR culture (see Data S1) having different concentrations of HT115 bacteria. The typical bell-shaped curve obtained after exposing WT worms, that were grown on control RNAI, to DR is absent in mekk-3 RNAI-treated worms. Mean lifespan is plotted against decreasing concentration of bacteria; error bars represent SEM. (B) The mekk-3 RNAI is unable to increase the lifespan of WT worms treated with 2-deoxy glucose (DOG) to the extent observed in untreated worms. In presence of 5 mM 2-deoxy glucose, MLS of WT on control RNAI was 23.91 ± 0.63 days (n = 135), on mekk-3 RNAI was 26.59 ± 0.65 days (n = 150) P = 0.0009. The grey lines indicate untreated WT worms grown on control or mekk-3 RNAI. (C) Lifespan of skn-1(zu169) on mekk-3 RNAI. MLS on control RNAI was 17.86 ± 0.49 days (n = 76), on mekk-3 RNAI was 26.39 ± 0.79 days (n = 67) P < 0.0001. The grey lines indicate WT grown on control or mekk-3 RNAI. Lifespans were performed at 20 °C. (D) The mekk-3 RNAI did not further increase the lifespan of glp-1(e2141). MLS on control RNAI was 24.83 ± 0.42 days (n = 120), on mekk-3 RNAI was 25.65 ± 0.55 days (n = 116) P = 0.0003. The grey lines indicate WT grown on control or mekk-3 RNAI. WT and glp-1(e2141) worms were hatched at 25 °C and transferred to 20 °C after 24 h. Lifespans were performed at 20 °C.

Fig. S6 (A) nhr-49(ok2165) worms have higher amount of stored fat as indicated by Oil red O staining. Arrow head indicates the pharynx. Arrows highlight areas showing differences in hypodermal/intestinal fat staining between control and mekk-3 RNAI. Quantification of staining is shown in the right.
right panel. Error bar- SEM; n > 50; Student’s t-test. (B) mekk-3 RNAi was not able to increase lifespan in nhr-49(nr2041) to the same extent as in WT. MLS on control RNAi was 12.91 ± 0.12 (141), on mekk-3 RNAi was 13.67 ± 0.29 (140), P < 0.0006. Lifespans were performed at 20 °C. (C) mekk-3RNAi failed to increase lifespan in daf-22(m30). MLS on control RNAi was 20.34 ± 0.33 (n = 73), on mekk-3 RNAi was 20.59 ± 0.36 (n = 51), P = 0.6539. Lifespans were performed at 20 °C. (D) The expression of sod genes did not increase significantly between control- and mekk-3 RNAi-treated WT worms. The data for each of the sod genes is normalized to its expression in control RNAi-treated worms. Error bars indicate standard deviation over five independent biological replicates. P values- Student’s t-test between control and mekk-3 RNAi-treated worms for each gene. (E) The total SOD activity did not increase when WT worms were grown on mekk-3 RNAi. A daf-2(e1370) mutant had high levels of SOD activity. Graph represents average of 3 biological replicates; *P < 0.01, Student’s t-test between indicated samples. (F) mekk-3 RNAi decreased ROS in WT but failed to decrease it in nhr-49(ok2165). Average of three biological replicates. Error bars indicate standard deviation. Student’s t-test between samples indicated by lines.

**Fig. S7** The expression of several xenobiotic detoxification genes are deregulated in (A) nhr-8(ok186), (B) aha-1(ok1396) and (C) ahr-1(ju145) mutants. Error bars- Standard deviation; P < 0.05, ns- not significant; Student’s t-test between WT and respective mutants for each gene. (D) Lifespan of ahr-1(a3) on mekk-3 RNAi. MLS on control RNAi was 18.43 ± 0.27 days (n = 134), on mekk-3 RNAi was 20.96 ± 0.41 days (n = 95), P < 0.0001 by Log rank test. (E) Lifespan of ppg-3(pk18) on mekk-3 RNAi. MLS on control RNAi was 17.56 ± 0.30 days (n = 81), on mekk-3 RNAi was 19.67 ± 0.32 days (n = 83), P < 0.0001. All lifespans were performed at 20 °C.

**Fig. S8** (A) Venn diagram showing the overlap of PHA-4 direct target genes (ModENCODE) that are upregulated when WT worms are grown on mekk-3 RNAi (overlap 40.8%, P = 1.83e-9, hypergeometric test). See Table S2 for gene-list. (B) Categorization of the PHA-4 target genes that are upregulated on mekk-3 RNAI using DAVID (david.abcc.ncifcrf.gov). (C) Several xenobiotic detoxification genes that are upregulated on mekk-3 RNAI in WT worms are downregulated when pha-4 is also knocked down. Worms were either grown on Control RNAi, mekk-3 RNAI or initiated on mekk-3 RNAI and transferred to pha-4 RNAI post-L4. Error bars- Standard deviation; P < 0.05, ns- not significant; Student’s t-test between WT grown on control RNAI vs. ones grown on mekk-3 or mekk-3 + pha-4 RNAI for each gene. (D) Venn diagram showing the overlap of direct target genes of SKN-1 (ModENCODE) that are upregulated when WT worms are grown on mekk-3 RNAi (overlap 7.5%, P = 0.0086, hypergeometric test). See Table S3A for gene-list. (E) Venn diagram showing the overlap of SKN-1 target genes (from microarray, Oliveira et al., 2009) that are upregulated when WT worms are grown on mekk-3 RNAi (overlap 4.3%, P = 0.00009, hypergeometric test). See Table S3B for gene-list.

**Fig. S9** Transcript levels of select phase I and II xenobiotic detoxification genes were upregulated in WT worms undergoing mekk-3 knock-down-mediated DR (Green bar).

**Fig. S10** Nile Red staining of fixed WT or mutant worms grown on Control or specific RNAI. Left panel shows representative photo of worms while right panel shows quantification of fluorescence.

**Fig. S11** The genes upregulated when WT worms were grown on mekk-3 RNAI were categorized on the basis of GO term using DAVID and REVIGO softwares; details in Data S1.

**Fig. S12** Wild-type worms on Control or mekk-3RNAI as well as eat-2 (ad1116) grown on control RNAi were fed BODIPY containing bacterial feed for 20 min.

**Fig. S13** The mekk-3 RNAI worms have normal bacterial food intake.

**Data S1** Experimental procedures.

**Table S1** Complete lifespan data.

**Table S2** List of PHA-4 target genes that are upregulated when WT is grown on mekk-3 RNAI.

**Table S3** (A) SKN-1 direct target genes (obtained from ModENCODE) that are upregulated in WT grown on mekk-3 RNAI. (B) SKN-1 target genes (determined by microarray; see reference) that are upregulated in WT grown on mekk-3 RNAI.

**Video S1** Confocal images of the head region of a mekk-3::gfp transgenic worm, stacked in Z plane to create a movie.

**Video S2** Confocal images of the tail region of a mekk-3::gfp transgenic worm, stacked in Z plane to create a movie.

**Video S3** Confocal images of the vulval region of a mekk-3::gfp transgenic worm, stacked in Z plane to create a movie.