Metformin promotes lifespan through mitohormesis via the peroxiredoxin PRDX-2.

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The anti-glycemic drug metformin, widely prescribed as first-line treatment of type II diabetes mellitus, has lifespan-extending properties. Precisely how this is achieved, remains unclear. Via a quantitative proteomics approach using the model organism *Caenorhabditis elegans*, we gained molecular understanding of the physiological changes elicited by metformin exposure, including changes in branched-chain amino acid catabolism and cuticle maintenance. We show that metformin extends lifespan through the process of mitohormesis and propose a signaling cascade in which metformin-induced production of reactive oxygen species (ROS) increases overall life expectancy. We further address an important issue in aging research, wherein so far, the key molecular link that translates the ROS signal into a pro-longevity cue remained elusive. We show that this beneficial signal of the mitohormetic pathway is propagated by the peroxiredoxin PRDX-2. Because of its evolutionary conservation, peroxiredoxin signaling might underlie a general principle of pro-longevity signaling.

Aging | Metformin | Peroxiredoxin | Proteomics | Reactive oxygen species

Introduction

Metformin, an anti-glycemic biguanide drug and the most common treatment of type II diabetes mellitus, has life-extending capabilities (1, 2). Several other human diseases, such as cancer (3) and nonalcoholic fatty liver disease (4) are also potentially alleviated by metformin treatment. This suggests that metformin acts on common pathways involved in a spectrum of aging-related disorders. Because of its demonstrated beneficial effect on lifespan in the nematode *Caenorhabditis elegans* (5, 6), and in the rodents *Rattus norvegicus* (1) and *Mus musculus* (2), these models facilitate research into the underlying mode of action.

It has been hypothesized that metformin elicits its beneficial effects by mimicking dietary restriction (DR) (7), a regimen wherein a physiological response is triggered by reducing the uptake of nutritive calories. The physiological response to DR causes lifespan extension and delays age-dependent decline from yeast to primates (8). The idea of similarity sprouts from the observed low blood glucose and insulin levels combined with increased glucose utilization in both calorically restricted and metformin-treated animals (7). In addition, metformin-treated worms show phenotypes similar to DR worms (5), and transcript profiles of metformin-treated and DR mice also overlap significantly (9).

Caution is due, however, in referring to DR, because different methods to induce DR in *C. elegans* act through different genes to elicit corresponding effects on lifespan (10–12). Glucose restriction, a specific type of DR, requires the adenosine monophosphate (AMP)-dependent kinase (AMPK) to extend lifespan. Activation of AMPK leads to increased mitochondrial production of reactive oxygen species (ROS), which induces stress defense and results in a net increase in longevity. This process of lifespan extension based on mitochondrial oxidative stress, is known as mitohormesis (13).

Despite its similarities to DR and its widespread use as an anti-glycemic drug, the actual mode of action of metformin is largely unknown and a subject of much debate. In mammals, metformin is generally believed to act through the activation of AMPK, one of the main regulators of cellular energy homeostasis (2, 14–16), but questions about its direct effects are largely unaddressed.

In order to study the targets of metformin, we performed a proteomic analysis on metformin-treated *C. elegans* and used the results as a framework for follow-up experiments. We observed striking similarities between metformin-treated and glucose-restricted worms and discovered several novel factors involved in mitohormetic regulation of lifespan.

Significance

Recently it has been suggested that metformin, the most commonly used anti-diabetic drug, might also possess general health-promoting properties. Elucidating metformin’s mode of action will vastly increase its application range, and will contribute to healthy aging. We reveal a signaling cascade in which metformin is able to extend lifespan by increasing the production of reactive oxygen species (ROS). This allowed us to further work at the crossroads of human disease and aging research, identifying a key molecule that is able to translate the ROS signal into a pro-longevity cue: an antioxidant peroxiredoxin.

Reserved for Publication Footnotes
Table 1. Functional analysis of differential proteins

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- Functional analysis of differential proteomics data. All enrichment analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (23). 1Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways overrepresented (p < 0.05) in the group of proteins upregulated after metformin treatment. 2Functional clustering of proteins, which are significantly altered after metformin treatment. The reported enrichment score was calculated by DAVID based on the Fisher Exact score of each clustered term. The higher the value, the more enriched the cluster. Only clusters with an enrichment score higher than 1 were reported. Complete information can be found in Dataset S1.

Fig. 1. Metformin treatment does not induce the mitochondria-specific unfolded protein response (UPR\textsuperscript{\textregistered}). Day 1 adult metformin-treated hsp-6::GFP worms show no difference in fluorescence when compared to untreated control worms. hsp-6::GFP worms were exposed to cco-1 RNAi as a positive control. cco-1 encodes a cytochrome c oxidase subunit, an integral part of the mitochondrial electron transport chain (24).

Fig. 2. Metformin increases lifespan according to the principle of hormesis, for which it requires PRDX-2. A Metformin treatment increases metabolic heat production (p < 0.01\*; n = 3 for untreated and n = 4 for treated worms) and respiration (p < 0.05\*; n = 3). Bars represent mean ± SEM. B Metformin induced a significant increase in H\textsubscript{2}O\textsubscript{2} release in day 1 adult worms after both continuous exposure during development (p < 0.05\*; n = 7) and after 24 hours of exposure, starting from the young adult stage (p < 0.05\*; n = 7 for untreated and n = 5 for treated worms). Exposing the worms for 4 hours before measurement did not result in a significant increase (p > 0.05\*; n = 7). Bars represent mean ± SEM. C The antioxidant N-acetylcysteine (NAC) abolishes the lifespan extending effect of metformin (p < 0.001\*\*\*; n ≥ 169 for each curve, see Table S1). D prdx-2 is required for metformin-induced lifespan extension. Metformin treatment significantly reduces lifespan of prdx-2 mutants (p < 0.001\*\*\*; n ≥ 127 for each curve, see Table S1). E Metformin treatment promotes the formation of PRDX-2 disulfide dimers (p < 0.01\*\*), implied to function in signal transduction. Bars represent mean ± SEM (n = 4). F PRDX-2 is required for metformin-induced phosphorylation of the p38 MAP kinase PMK-1. Metformin treatment of wild type worms induced phosphorylation of PMK-1, inferred from a larger band observed on the Western blot. This metformin-mediated induction of PMK-1 phosphorylation is absent in prdx-2 knockout worms. Histone H3 levels were used as a loading control.

Results

Molecular changes in Caenorhabditis elegans upon metformin exposure: a proteome approach

In order to gain a deeper understanding of the physiological changes elicited by metformin exposure, a differential gel-based proteomics experiment was performed. A total of 164 spots with differential abundances were detected, 134 of which could be identified by mass spectrometry. After removal of duplicate iden-
Fig. 3. Metformin inhibits Complex I of the electron transport chain (ETC) in a way distinct from rotenone. A Metformin inhibits complex I but not complex II based respiration. Higher values for O2 flux indicate higher respiration, negative values indicate a small influx of O2, usually due to the injection of a compound. The effect of metformin on mitochondrial respiration was measured by sequentially adding compounds to stimulate different parts of the ETC. Green arrows indicate compounds that were added in both the control and metformin-treated cells; red arrows indicate compounds that were only added in the treated cell. After adding an equal volume of mitochondria (\(i\) start), metformin was added to one of the cells (\(i\) metformin). Subsequently, the complex I substrates pyruvate and malate (\(i\) P+M) were added, followed by the addition of ADP (i ADP), initiating electron transport from complex I. Metformin-treated mitochondria clearly fail to initiate complex I based respiration, thus indicating that metformin inhibits electron transport from complex I in vitro. Finally, the complex II substrate succinate (\(i\) S) was added to the metformin-treated cell, which led to a marked increase in mitochondrial respiration, indicating that metformin does not block electron transport from complex II. Two variations of this experiment were executed to also use the potent complex I inhibitor rotenone as a positive control (Fig. S4A) and to add succinate to the negative control condition as well (Fig. S4B). B Mitochondria treated with metformin produce \(H_2O_2\) at a higher rate than untreated mitochondria (\(p < 0.001***; n = 3\)). Treatment with the complex I inhibitor rotenone had the opposite effect (\(p < 0.001***; n = 3\)).

Fig. 4. Metformin induces the branched-chain amino acid (BCAA) degradation and \(\beta\)-oxidation pathways but the \(\beta\)-oxidation enzyme ACDH-1 is not required for metformin-mediated for longevity. A Metformin treatment stimulates the BCAA degradation pathway (Fig. S5) and in turn reduces the concentration of free BCAAs in \(C.\) elegans (\(p < 0.01**\)). Bars represent mean \(\pm\) SEM (\(n = 6\)). B Metformin-treated worms show reduced fat storage (\(p < 0.001***; n = 30\) for untreated and \(n = 33\) for treated worms), possibly indicating increased flux through the \(\beta\)-oxidation pathway. Bars represent mean \(\pm\) SEM. C Deletion of \(\beta\)-oxidation enzyme \(acdh-1\) results in a proportionally larger effect of metformin on longevity (\(p < 0.001***; n \geq 54\) for each curve, see Table S1).

Fig. 5. Metformin attenuates the morphological decline with aging in \(C.\) elegans A Metformin-treated worms retain a stable volume while control worms older than 6 days start shrinking. B Electron micrograph of the cuticle of a day 9 adult non-treated wild type worm. Some deformations of the cuticle (seen as ‘wrinkling’, marked with an arrow) are starting to manifest. C No structural abnormalities can be seen in a metformin-treated day 9 wild type adult.
To verify whether mitochondrial ROS production was affected by metformin treatment, we measured hydrogen peroxide levels in metformin-treated worms. ROS production was indeed increased (Fig. 2B), further supporting the mitohormesis hypothesis. Induction of ROS production was already clear after 24 hours of exposure to metformin (Fig. 2B). This increase in ROS seems to be an integral part of metformin-induced lifespan extension, as treatment with the potent antioxidants N-acetylcysteine (NAC) (13) and butylated hydroxyanisole (BHA) (26) abolished the positive effect of metformin on lifespan (Fig. 2C and Fig. S2A). Finally, the critical phase for metformin-mediated lifespan extension clearly resembles the critical phase for other mitohormetic stressors (13), as treatment with metformin starting from adulthood onwards or during the first few days of adulthood only, were sufficient to increase lifespan (Fig. S2B). This is opposed to treatment with metformin during larval development only, which had no detectable effect on lifespan (Fig S2B).

**Metformin-mediated lifespan extension requires the peroxiredoxin PRDX-2**

The mitohormetic pathway as seen during glucose restriction is induced when low availability of glucose causes low energy levels, which in turn activates AMPK (13). AMPK activity increases catalysis and respiration, which results in the increased production of ROS and a resultant activation of hormetic protective mechanisms (13). The highest stress in this pathway is the step between the increased ROS production and the induction of stress defense, as no molecule was put forward that might translate the ROS signal into further downstream defense. We therefore set out to reveal this missing link and to further complete the hormetic signaling pathway.

Peroxiredoxins are known hydrogen peroxide scavengers and their oxidized dimeric form is involved in the direct activation of kinases in mammalian cells (27). As the mitochondrial peroxiredoxin PRDX-2 was upregulated during metformin treatment (Dataset S1), this protein is of particular interest as a potential inducer of mitohormesis. Deletion of the prdx-2 gene results in an extreme decrease in lifespan upon metformin treatment (Fig. 2D). Not only did the positive effect of metformin on lifespan disappear, the prdx-2 experimental group collapsed when exposed to metformin. Treatment with NAC partially rescued this deleterious effect, implying that excessive ROS production is at least partly responsible for the detrimental effect of metformin on these mutants (Fig. S2C). In support of these results, we observed increased formation of PRDX-2 dimers after metformin treatment (Fig. 3E, Fig. S3). Since these oxidized dimers are likely involved in cellular signaling (27), we propose that PRDX-2-dependent pro-longevity signaling during the mitohormetic response to metformin treatment.

One of the potential downstream targets of PRDX-2 is the p38 MAP kinase PMK-1, which is involved in the activation of the SKN-1 transcription factor (28). This transcription factor is in turn required for metformin-mediated longevity (5). Western blot analysis revealed a marked increase in phosphorylation of PMK-1 after metformin treatment. In contrast, deletion of prdx-2 resulted in an absence of metformin-induced phosphorylation of PMK-1 (Fig 2F). These data strongly imply that PRDX-2 is required for PMK-1 activation after metformin treatment.

In sum, all these findings subscribe that metformin-mediated lifespan extension in *C. elegans* and that PRDX-2 is an integral part of the mitohormetic pathway.

**Metformin inhibits complex I of the ETC**

Metformin is generally believed to act through inhibition of complex I of the ETC (19–21), although some recent findings cast doubt on this (16, 22). Treatment of *C. elegans* with rotenone, another complex I inhibitor, at a concentration that extends longevity, results in a decrease in total oxygen consumption (25). Our finding that metformin increases respiration in worms there-
fore raises the question whether it is truly capable of inhibiting complex I of the ETC.

We tested whether metformin is able to affect electron flow in mitochondria extracted from *C. elegans* and observed a clear and specific inhibition of electron flow from complex I, while the electron flow from complex II was unaffected (Fig. 3A; Fig. S4A-B). These results, clearly mimicking the inhibitory action of rotenone (Fig. S4A), complement our previous data only if metformin inhibits complex I in a distinct way. To this end, we tested whether metformin and rotenone had different effects on ROS production in mitochondria. At concentrations at which both completely inhibit complex I respiration and after feeding only complex I, metformin increased ROS production while rotenone decreased it (Fig. 3B), implying a fundamental difference between rotenone’s and metformin’s inhibitory action on complex I.

The BCAA degradation pathway is upregulated during metformin treatment

The BCAAs leucine, isoleucine and valine display a certain duality in relation to health and longevity. On one hand, their effectiveness has been noted in the treatment of liver and cardiac diseases (29, 30) and upregulation of BCAAs is one of the metabolic signatures of the long-lived *daf-2* mutant (31, 32). On the other hand, BCAAs have also been causally linked to the development of insulin resistance, type II diabetes (33) and neuropathologies (34). We set out to confirm the marked upregulation of the BCAA degradation pathway (Table 1; Fig. S5) at the level of free BCAAs in metformin-treated worms.

Metformin treatment resulted in a significant decrease (~55%) in the amount of free BCAAs, lending further support to the validity of the proteomics data (Fig. 4A).

**Metabolic flux during metformin treatment**

One of the most striking properties of metformin as an antidiabetic in humans, is its ability to cause weight loss in patients, through, amongst others, activation of the β-oxidation pathway (2, 35, 36). We observed a 4.3-fold upregulation of the mitochondrial acyl-CoA dehydrogenase family member ACDH-1 after metformin treatment (Dataset S1), defining it as the strongest upregulated protein by a margin. ACDH-1 dehydrogenases catalyze the first step in the β-oxidation of fatty acids.

We questioned whether the shift in β-oxidation suggested by ACDH-1 induction truly occurs and whether it is involved in metformin-induced lifespan extension. If so, we would grossly expect overall fat levels to drop in treated worms. Measuring fat content in L4 worms, we found evidence for increased β-oxidation as metformin-treated worms showed significantly lower (~11.4%) fat stores (Fig. 4B). A similar reduction in fat content was found in metformin-treated *C. elegans* using different methods (37).

To our surprise, *acdh-1* knockout worms showed an even stronger metformin-induced lifespan extension than wild type worms (Fig. 4C). This implies a more complex interaction between β-oxidation and metformin (see discussion).

**Metformin-treated worms attenuate age-related morphological decline.**

Aging worms start to show several morphological defects (37) and shrink in size (38) but metformin-treated worms seemed less affected by this phenomenon. After measuring several formalin fixed worms of various ages (Fig. 5A) it became clear that non-treated worms started losing volume after day 6 of adulthood, while metformin-treated worms still retained their normal volume on day 9. Although long-lived mutants are often smaller than their wild type siblings (39), our results fit the recent observations that within isogenic populations, the larger animals are generally the longer-lived ones (38).

Muscle and cuticle are known to show severe morphological defects with increasing age in *C. elegans* (37), making them prime targets to question whether these tissues are better maintained in metformin-treated worms. Our proteomics data already pointed to an increase in muscle mass and several changes in the cytoskeleton (Table 1), including upregulation of intermediate filament proteins (Dataset S1). Electron micrographs of 9-day-old control and metformin-treated worms displayed no difference in body wall muscle volume, but there was a clear difference in cuticle morphology (Fig. 5B-C). While the cuticle of 9-day-old adult non-treated worms started showing signs of age-related “wrinkling” and disorganization, the cuticle of metformin-treated worms resembled that of a young animal. Considering the cuticle’s known role in maintaining the shape and size of nematodes (40), it can be assumed that it is this amelioration of cuticle deterioration that allows metformin-treated worms to retain a healthy, young morphology.

**Discussion**

We profiled the effect of metformin in *C. elegans* using a differential proteomics approach and used the resulting data for further examination of its beneficial effects and mode of action.

We observed many changes in the mitochondrial proteome, which might point towards mitonuclear protein imbalance (41) and an altered mitochondrial metabolism. It is therefore probable for metformin to increase lifespan through both these processes.

Metformin administration during the larval stages had no effect on lifespan, while the critical phase for UPR induction in *C. elegans* is during larval development (24, 42). Because metformin also proved unable to induce the UPR (43), it is not a likely contributor to lifespan extension by metformin in *C. elegans*. Contrary to the UPR (44), the mitohormetic pathway is important for metformin-mediated longevity in this worm.

Hormesis in aging is defined as the process by which a short-term stressor increases the stress response mechanisms of an organism and thereby increases stress resistance and overall life expectancy (43). The mitohormetic pathway, first described in glucose-restricted *C. elegans* (13), states that a low availability of ATP - due to low glycolytic activity - causes activation of AMPK. AMPK in turn promotes general catabolism and mitochondrial respiration, leading to increased production of ROS (the hormic “stressor”), which subsequently act as messengers to activate further stress defenses, prolonging lifespan. This contradicts the classical oxidative stress theory of aging, which postulates that ROS, due to their ability to damage biomolecules, would be the causative factor of aging (44). We were able to fully reproduce the mitohormetic phenomenon in metformin-treated worms and showed that metformin increased respiration, metabolic heat production and ROS generation in *C. elegans*. Inhibition of ROS signaling abolishes the lifespan-extending effects of metformin, proving that metformin-mediated lifespan extension is dependent on the mitohormetic pathway and most closely resembles the *C. elegans* response to glucose restriction.

Though well studied, some major missing links remained in the mitohormetic pathway, in particular how the ROS signal can be translated to increased lifespan. We were able to demonstrate that the *C. elegans* peroxiredoxin PRDX-2, previously shown to be involved in peroxide and heavy metal resistance (45), is of major importance to this function. Peroxiredoxins are a class of antioxidant proteins characterized by their high susceptibility to cysteine oxidation (46, 47). PRDX-2 is a typical 2-Cys peroxiredoxin (45), of which the active, oxidized form exists as a homodimer (48). It has recently been shown that these dimers are subsequently able to oxidize and activate specific substrates, such as the MAP kinase ASK1 (27), thus resolving how these proteins might be able to translate ROS signals into downstream signaling. In the same vein, our experiments revealed increased formation of PRDX-2 dimers after metformin treatment. These
oxidized dimers might activate ASK1’s closest ortholog in *C. elegans*, the MAP kinase NSY-1, which functions upstream in the same signaling pathway as the MAP kinases SEK-1 and PMK-1 (49). This evolutionarily conserved MAP kinase cascade could ultimately activate several downstream targets that mediate stress defense, including SKN-1 (28). We showed that *prdx-2* is required for the downstream activation of PMK-1 in response to metformin exposure, completing the pathway (Fig. 6). Therefore, PRDX-2 may well be the protein responsible for translating oxidative stress into longer lifespan in *C. elegans*. As the longevity-promoting effect of exercise in humans also depends on ROS signaling (50), this pathway may be evolutionarily conserved.

AMPK activation is one of the main factors involved in mitohormetic lifespan extension (13, 26). While metformin is known to activate AMPK, exactly how this occurs remains elusive. The most common hypothesis is that metformin is able to partially inhibit complex I of the mitochondrial ETC, which in turn would lead to energy depletion and activation of AMPK (19–21). Recently, this has been debated (16, 22). One of the main arguments against metformin-mediated inhibition of complex I, is the increased activity of pathways that generate energy after metformin treatment. All these pathways require NAD+ to function, and the increased efflux of NADH from complex I of the ETC would lead to an increased NADH/NAD+ ratio (51) and lowers the activity of the β-oxidation pathway (16). We were able to show that metformin specifically inhibits complex I of the ETC in *vivo*, albeit in a way that seems fundamentally distinct from rotenone: metformin increased mitochondrial ROS production while rotenone decreased it. High concentrations of rotenone reduce the number of electrons that are transferred from NADH to complex I (high NADH/NAD+ ratio), hence less electrons can leak out to form ROS. Our results imply that upon metformin treatment, NADH is still able to transfer its electrons, but these are subsequently lost, resulting in increased ROS production (Fig. 6). Based on these data, we suggest that metformin activates AMPK through inhibition of complex I (Fig. S6), which leads to an increase in respiration and a concomitant upregulation of several catabolic pathways - e.g. β-oxidation, glycolysis, BCAA catabolism and others - to provide the necessary substrate for the ETC. Likely exacerbated by metformin-mediated perturbation of electron transport, the increase in respiration ultimately leads to a mitohormetic increase in ROS production in *vivo* (Fig. 6). As elevated respiration under physiologically normal conditions often leads to a reduction in ROS production (31), this indeed implies a direct involvement of metformin in the observed increase in ROS.

Treatment of *C. elegans* with metformin results in an overall upregulation of several pathways and processes (including glycolysis and the TCA cycle), most of which are involved in catabolism, supporting the shift towards increased respiration.

Two catabolic pathways stood out after metformin treatment: β-oxidation, through the short chain acyl-CoA dehydrogenase ACADH-1, and BCAA degradation. In light of metformin’s inhibitory action on complex I but not complex II of the ETC, the observed increase in β-oxidation seems a logical adaptation as it produces a relatively high amount of the complex II substrate FADH2 compared to other catabolic pathways. This might explain why knocking out *acdh-1* adds to the lifespan increase induced by metformin: high activity of the β-oxidation pathway could temporarily increase ATP levels and inactivate AMPK (Fig. 6; Fig. S6). As such, deletion of *acdh-1* could cause a more stringent activation of the mitohormetic pathway. Further experiments will be needed to fully explore the role of β-oxidation in metformin-induced lifespan extension. Our results suggest that the metformin-mediated increase in β-oxidation is likely a compensatory mechanism that is unrelated to metformin-induced longevity.

As for the BCAA degradation pathway, no clear singular correlation between BCAs and longevity has yet been found in any organism. While some evidence points towards BCAs as a metabolic signature of long life in *C. elegans* insulin receptor mutants (31, 32), other studies have causally linked BCAs to the development of insulin resistance, diabetes (33) and neuropathologies (34). Interestingly, BCAs - leucine in particular - are potent activators of the target of rapamycin (TOR) kinase. Leucine deprivation has previously been associated with reduced TOR signaling (52), which can in turn prolong lifespan and is required for DR-mediated lifespan extension (53). Yet, it remains unclear whether the drop in BCAA levels is necessary for metformin-induced lifespan extension. The reduction in BCAA concentration might result in lower RNA translation into protein, both through inactivation of TOR and a reduction in the amount of substrate necessary for translation (Fig. S6). This in turn may lead to an increase in longevity. There is some precedence for this hypothesis, as amino acid imbalance has been associated with increased longevity in *Drosophila melanogaster* (54), amongst others (55). The recently described bacteria-specific effect of metformin on longevity in *C. elegans* (6) might similarly depend on an amino acid imbalance that occurs through the lowered production of methionine in metformin-treated bacteria (Fig. S6). Our results suggest a hitherto unexplored role for the BCAA degradation pathway in longevity.

Metformin not only increases lifespan, but also healthspan of *C. elegans*: treated worms retain a youthful morphology for a longer time. How metformin is able to attenuate morphological decline of the cuticle in *C. elegans* remains elusive, but the intermediate protein IF2-C (upregulated after metformin treatment, Dataset S1) seems particularly promising as it is required for normal body shape and cuticle strength (56). Integrity of the cuticle and epidermis might play a more important role in longevity than is generally thought. Deterioration in cuticle structure leads to a loss in barrier function, which may be one of the causes of death of older nematodes (37, 40).

In conclusion, this work reveals new insights in the process of aging, and shows that metformin extends lifespan through mitohormesis. A missing link in the mitohormesis pathway in *C. elegans* has now been assigned: the peroxiredoxin PRDX-2, a protein that translates a ROS signal into a pro-longevity cue. Since peroxiredoxin signaling is evolutionary conserved (27), peroxiredoxins might hold a similar function in humans.

### Materials and Methods

#### *C. elegans* strains

The following strains were obtained from the Caenorhabditis Genetics Center (ECC, University of Minnesota): wild type N2, VC289 *prdx-2(gk169)*, VC1011 *acdh-1(ok1489)* and SJ4100 *xch13(hsp-6::GFP)*. G507 *gpl-4(bn2ts) daf-16(mgDf50)* was provided by the Gems lab. Strains were cultivated on standard nematode growth medium (NGM) seeded with *E. coli* OP50. All strains were outcrossed at least four times, with the exception of SJ4100, which was outcrossed three times. The outcrossed *prdx-2(gk169)* and *acdh-1(ok1489)* strains were renamed LSC555 and LSC556 respectively. All experiments were performed at 20°C unless stated otherwise.

#### Sampling for 2D-DIGE

The protein samples for 2D-DIGE were taken from *gpl-4(bn2ts) daf-16(mgDf50)* worms grown in liquid cultures. The *gpl-4(bn2ts)* mutation confers sterility at the permissive temperature of 24°C avoiding contamination with progeny. Preventing germline development also removes abundant contaminating proteins that have no bearing on lifespan, facilitating the analyses. Since *gpl-4* mutations cause a small DAF-16 dependent lifespan increase in *C. elegans* cultured on dead *E. coli* (57), *gpl-4(bn2ts) daf-16(mgDf50)* double mutants were used. This does not interfere with the envisaged results, as lifespan extension due to metformin is independent of DAF-16 (5). Cultivating the worms in liquid cultures allowed full control over metformin dosage while ensuring that all worms were fully fed. Additionally, the high-density samples obtained from liquid cultures ensure high protein concentration in 2D-DIGE experiments.

#### Dataset availability

The raw data from the 2D-DIGE experiments have been submitted to the Sciigo database (Sciigo ID: 8488) and the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession number: GSE39508) [www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/).
Worms were synchronized by isolating eggs from gravid adults through hypotonic shock (38) and a subsequent sucrose density centrifugation to separate eggs from dead worms and bacterial debris. L1 worms were added to Fernbach flasks containing S medium (59) and constantly shaken at 24°C. Flash frozen E. coli K12 bacteria (Artecthon, Lines, Belgium) were used as the bacterial food source. The presence of bacteria was checked twice a day and new K12 bacteria were added accordingly to maintain the cultures at optimal food levels (OD600 = 1.8). The density of bacteria was monitored over several days (i.e., 3000 worms/mL) to prevent large fluctuations in food availability. When worms reached the L4 stage, cultures were supplemented with 2′-deoxy-5-fluorouridine (FUDR, Sigma-Aldrich) at a final concentration of 100 µM to achieve complete sterility, as gip-4[bon2ts]daf-16(mgDf50) worms rarely manage to still produce a few eggs. Once worms reached the adult stage, the test group was exposed to 50 mM metformin starting from the L1 stage. Oxygen activity Monitor (Thermometric, Jena, Germany) was used to monitor the oxygen consumption of synchronized day 1 adult worms were measured using a Clark-type oxygen electrode (see SI Materials and Methods). A Student’s t-test with false positive rate correction (Benjamini-Hochberg method). Complete information regarding lifespan experiments was marked as dead. Animals that crawled off the plate or died of vulval hermaphroditism were recorded and analyzed. Each experiment was performed in at least 12 replicates.

Mitochondria were extracted from worms as previously described (26). Oxygen consumption of the extracted mitochondria was determined using the Amplex Red hydrogen peroxide kit (Invitrogen, Cat #A22188). A Student’s t-test with false positive rate correction (Benjamini-Hochberg method). Complete information regarding lifespan experiments was marked as dead. Animals that crawled off the plate or died of vulval hermaphroditism were recorded and analyzed. Each experiment was performed in at least 12 replicates.

**Measuring mitochondrial respiration**

Mitochondria were extracted from worms as previously described (26). Oxygen consumption of the extracted mitochondria was determined using the Amplex Red hydrogen peroxide kit (Invitrogen, Cat #A22188). A Student’s t-test with false positive rate correction (Benjamini-Hochberg method). Complete information regarding lifespan experiments was marked as dead. Animals that crawled off the plate or died of vulval hermaphroditism were recorded and analyzed. Each experiment was performed in at least 12 replicates.

**Quantifying in vivo hydrogen peroxide production**

Endogenous hydrogen peroxide production was quantified using the Amplex Red hydrogen peroxide kit (Invitrogen, Cat #A22188). A Student’s t-test with false positive rate correction (Benjamini-Hochberg method). Complete information regarding lifespan experiments was marked as dead. Animals that crawled off the plate or died of vulval hermaphroditism were recorded and analyzed. Each experiment was performed in at least 12 replicates.

**Measuring mitochondrial hydrogen peroxide production**

Mitochondrial hydrogen peroxide production was quantified using a protocol based on the Amplex Red hydrogen peroxide kit (Invitrogen, Cat #A22188). A Student’s t-test with false positive rate correction (Benjamini-Hochberg method). Complete information regarding lifespan experiments was marked as dead. Animals that crawled off the plate or died of vulval hermaphroditism were recorded and analyzed. Each experiment was performed in at least 12 replicates.
Electron micrographs
Wild-type worms were synchronized and sterilized as described for the volumes of the bacteria. Extracts were harvested when they reached day 3 of adulthood and prepared for electron microscopy (see SI Materials and Methods).

Quantifying concentration of branched-chain amino acids
Wild-type worms were synchronized and L4 worms were harvested from the plates. BCA concentration was measured using a BCA assay kit (Sigma-Aldrich, Cat# MA0033) according to manufacturer’s instructions. Total protein content of the extracts was quantified using the Qubit Protein Assay (Invitrogen) and used for normalization. A Welch’s t-test was performed to analyze the data.

Statistical analysis
All statistical analyses, apart from the DeCyder and DAVID analysis, were carried out using R (64). All bar graphs show the mean of biologically independent samples, error bars show SEM. P-values < 0.05 were considered significant.

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Methods.
Protein content of the extracts was quantified using the Qubit Protein Assay (Invitrogen) and used for normalization. A Welch’s t-test was performed to analyze the data.