D-Glucosamine supplementation extends life span of nematodes and of ageing mice

Sandra Weimer1,2,*, Josephine Priebs3,*, Doreen Kuhlow2,3, Marco Groth4, Steffen Priebe5, Johannes Mansfeld1,3,6, Troy L. Merry1, Sébastien Dubuis7, Beate Laube1,3, Andreas F. Pfeiffer2, Tim J. Schulz2, Reinhard Guthke5, Matthias Platzer4, Nicola Zamboni7, Kim Zarse1,3 & Michael Ristow1,2,3

D-Glucosamine (GlcN) is a freely available and commonly used dietary supplement potentially promoting cartilage health in humans, which also acts as an inhibitor of glycolysis. Here we show that GlcN, independent of the hexosamine pathway, extends *Caenorhabditis elegans* life span by impairing glucose metabolism that activates AMP-activated protein kinase (AMPK/AAK-2) and increases mitochondrial biogenesis. Consistent with the concept of mitohormesis, GlcN promotes increased formation of mitochondrial reactive oxygen species (ROS) culminating in increased expression of the nematodal *amino acid-transporter 1* (*aat-1*) gene. Ameliorating mitochondrial ROS formation or impairment of *aat-1*-expression abolishes GlcN-mediated life span extension in an NRF2/SKN-1-dependent fashion. Unlike other calorie restriction mimetics, such as 2-deoxyglucose, GlcN extends life span of ageing C57BL/6 mice, which show an induction of mitochondrial biogenesis, lowered blood glucose levels, enhanced expression of several murine amino-acid transporters, as well as increased amino-acid catabolism. Taken together, we provide evidence that GlcN extends life span in evolutionary distinct species by mimicking a low-carbohydrate diet.
GlcN causes an ATP deficit and promotes mitochondrial biogenesis. We next found that exposure to GlcN for 24 h causes a pronounced decrease in nematodal ATP content (Fig. 1c). A decrease in ATP, that is, available energy, typically activates energy sensors such as AMP-activated protein kinase (AMPK, and its regulatory subunit being known as AAK-2 in nematodes) or, indirectly, specific sirtuins (SIR-2.1 being the key isoform in nematodes). Accordingly, we found increased threonine phosphorylation of AAK-2 following exposure to GlcN (Fig. 1d), indicating activation of a C. elegans orthologue of AMPK, while no antibody to detect basal AAK-2 protein expression was available. Consequently, the effect of GlcN on life span was negated in a strain deficient for AAK-2 (Fig. 1e), whereas GlcN still had an effect, albeit reduced, on life span in a strain deficient for SIR-2.1 (Fig. 1f). This indicates that AMPK/AAK-2 activation is required for the life span-extending capabilities of GlcN, whereas SIR-2.1 appears in this regard to be potentially involved, although less essential.

Activation of AMPK is known to promote mitochondrial biogenesis in mammalian tissues. We consistently observed an increase in nematodal content of mitochondrial DNA (mtDNA) (Fig. 1g), reflecting increased mitochondrial mass, that is, increased biogenesis.

GlcN transiently induces mitochondrial reactive oxygen species formation. AMPK activation typically leads to increased mitochondrial respiration as a consequence of increased mitochondrial biogenesis, thereby reflecting increased metabolism of non-glycolytic substrates, namely fatty acids and amino acids. An increase in mitochondrial respiration following addition of GlcN was consistently observed (Fig. 1h). Reactive oxygen species (ROS) are considered necessary by-products of mitochondrial respiration, and increased respiration causes elevated levels of mitochondrial ROS. We therefore quantified ROS formation using two independent methods and found increases in ROS levels after 48 h of GlcN exposure (Fig. 1i,j), which, notably, is in accordance with findings from Arabidopsis thaliana regarding increased ROS levels following GlcN exposure in a hexokinase-dependent manner. However, the ROS levels of C. elegans were found to be decreased 7 days after initiation of GlcN exposure (Fig. 1i,j). We found the activities of ROS defence enzymes, specifically superoxide dismutase (Fig. 1k) and catalase (Fig. 1l), to increase 7 days after the addition of GlcN, suggesting that the mitochondrial ROS signal at 48 h (Fig. 1i,j) induces an adaptive response to promote an endogenous defence mechanism alleviating increased ROS levels at 7 days. We next posited whether this increase in ROS defence capacity could possibly contribute to an increased resistance against paraquat (PQ) stress. GlcN-treated worms survived PQ exposure better and longer than their untreated counterparts (Fig. 1m), indicating that GlcN induces a resistance to stress that may contribute to the extension of life span.

Antioxidants prevent GlcN-mediated life span extension. Mitochondrial ROS signalling in nematodes suggests that a low-dose, transient increase in ROS formation promotes metabolic health and life span, thereby questioning the free radical theory of ageing. To test whether the increase in ROS (Fig. 1i,j) is essential for a GlcN-mediated extension of life span, we repeated the initial life span experiment (Fig. 1b) in the presence of the antioxidants butylated hydroxyl anisole (BHA) and N-acetyl-cystein (NAC), respectively. Although neither BHA (Fig. 2a) nor NAC (Fig. 2b) had a detectable effect on C. elegans life span in the absence of GlcN, the life span-extending capabilities of GlcN were nullified.
in the presence of BHA or NAC (Fig. 2c,d). This indicates that the transient increase in ROS (Fig. 1i,j) is required for the extension of life span caused by GlcN, thus providing additional support for adaptive ROS signalling or mitohormesis or both.

ROS signals are transduced by p38/PMK-1 and NRF-2/SKN-1. Next, we questioned how this essential ROS signal may be sensed and transcriptionally transduced. We initially analysed phosphorylation of a previously established ROS sensor, p38 MAP kinase, which is called PMK-1 in nematodes, and found increased phosphorylation in GlcN-treated worms (Fig. 2e) while an antibody against basal p38 was not available. GlcN consistently failed to extend the life span of nematodes that are deficient for PMK-1 (Fig. 2f), indicating that the activation of p38 is required to extend life span with GlcN. We then tested whether the absence of downstream transcription factors such as DAF-16 or SKN-1, orthologues of mammalian FoxO and NRF-2, respectively, would influence GlcN effects on life span. We observed a marginally significant effect of GlcN on DAF-16-deficient worms (Fig. 2g), whereas the lack of SKN-1 fully negated the effects of GlcN on life span, even resulting in a shortened life span following GlcN treatment (Fig. 2h). This indicates that SKN-1/NRF-2-dependent initiation of transcription is involved in the GlcN-mediated elongation of C. elegans life span.

GlcN activates identical pathways in worms and mammalian cells. The inhibition of glycolysis by DOG feeding markedly

Figure 1 | GlcN induces mitochondrial metabolism and extends C. elegans life span. (a) Glucose oxidation rates in control wild-type (wt) nematodes (grey) and wild-type nematodes exposed to GlcN (red) (*P < 0.001, Student’s t-test, n = 6); colour coding applies to all subsequent panels and figures. (b) Life expectancy of untreated wild-type nematodes and GlcN-treated C. elegans (*P < 0.0001, log-rank test, n = 3). (c) ATP content at different time points in GlcN- and untreated nematodes (*P < 0.01, Student’s t-test, n = 3). (d) Representative western blot of whole-worm lysates in the presence and absence of GlcN in wild-type worms, as well as untreated AAK-2-deficient worms. (e) Life span assay on AAK-2-deficient nematodes in the presence and absence of GlcN (*P = 0.85, log-rank test, n = 3). (f) Life span assay on SIR-2.1-deficient nematodes (*P < 0.05, log-rank test, n = 3). (g) mtDNA content normalized to nuclear DNA content in whole worms in the presence and absence of GlcN (*P < 0.01, Student’s t-test, n = 3). (h) Relative respiration rates of whole worms (*P < 0.05, Student’s t-test, n = 3); colour coding applies to all subsequent panels and figures. (i) Relative superoxide dismutase activities (*P < 0.05, Student’s t-test, n = 3). (j) Relative Amplex Red fluorescence in suspensions of alive nematodes (*P < 0.05, Student’s t-test, n = 3). (k) Relative superoxide dismutase activities (*P < 0.05, Student’s t-test, n = 3). (l) Relative catalase activities (*P < 0.05, Student’s t-test, n = 3); and (m) survival on PQ exposure, all in the presence and absence of GlcN, respectively (*P < 0.01, log-rank test, n = 3). The bars represent the mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.
HepG2 cells, reflecting the findings in nematodes (Figs 1d and 2e), contributing to the extension of life span in nematodes could nematodes (Fig. 1c). We next analysed whether pathways acting as a glycolytic inhibitor4,5 and reflects the findings in not yet been tested in this regard. Despite these discouraging

| Strain, substance and solvent | Maximum life span (d) (± s.d.) | Mean life span (d) (± s.d.) | P-value (versus control, see footnotes) | Number of experiments (n) | Number of nematodes (n) |
|------------------------------|-------------------------------|----------------------------|-----------------------------------------|---------------------------|-------------------------|
| N2 H2O (1)                  | 25.00 ± 0.0                  | 22.01                       |                                         | 1                         | 135                     |
| N2 100 μM GlcN (1)          | 27.00 ± 0.0                  | 23.82                       | <0.00001†                              | 1                         | 138                     |
| N2 H2O (2)                  | 25.00 ± 0.0                  | 21.59                       |                                         | 1                         | 135                     |
| N2 100 μM GlcN (2)          | 27.00 ± 0.0                  | 23.67                       | <0.00001†                              | 1                         | 137                     |
| N2 H2O (3)                  | 25.00 ± 0.0                  | 21.52                       |                                         | 1                         | 150                     |
| N2 100 μM GlcN (3)          | 27.00 ± 0.0                  | 23.14                       | <0.00001†                              | 1                         | 124                     |
| N2 H2O (blinded)            | 24.00 ± 0.0                  | 20.98                       |                                         | 1                         | 107                     |
| N2 100 μM GlcN (blinded)    | 26.00 ± 0.0                  | 22.72                       | <0.00005†                              | 1                         | 126                     |
| N2 H2O (HIT† bacteria)      | 28.00                        | 25.52                       |                                         | 1                         | 131                     |
| N2 100 μM GlcN (HIT† bacteria) | 32.00                        | 27.99                       | <0.00001†                              | 1                         | 138                     |
| N2 H2O                      | 25.00 ± 0.0                  | 21.71 ± 0.3                 |                                         | 3                         | 420                     |
| N2 10 μM GlcN               | 25.00 ± 0.0                  | 22.20 ± 0.3                 | <0.001†                                 | 3                         | 428                     |
| N2 100 μM GlcN              | 27.00 ± 0.0                  | 23.54 ± 0.3                 | <0.00001†                              | 3                         | 399                     |
| N2 1 mM GlcN                | 26.33 ± 1.2                  | 23.33 ± 0.3                 | <0.001†                                 | 3                         | 442                     |
| N2 H2O                      | 25.00 ± 0.0                  | 21.50 ± 0.6                 |                                         | 3                         | 357                     |
| N2 100 μM GlcN              | 27.00 ± 0.0                  | 23.53 ± 0.4                 | <0.0001†                                | 3                         | 408                     |
| N2 1 mM GlcN                | 27.33 ± 0.6                  | 23.82 ± 0.5                 | <0.00001†                              | 3                         | 286                     |
| N2 10 μM GlcN               | 27.00 ± 0.0                  | 24.05 ± 0.2                 | <0.0001†                                | 3                         | 286                     |
| aak-2 (ok524) H2O           | 19.67 ± 1.2                  | 17.63 ± 0.3                 |                                         | 3                         | 397                     |
| sir-2 (ok434) GlcN          | 19.67 ± 1.2                  | 17.60 ± 0.3                 | NS†                                    | 3                         | 397                     |
| sir-2 (ok434) GlcN          | 22.67 ± 0.6                  | 19.50 ± 0.4                 |                                         | 3                         | 294                     |
| sir-2 (ok434) H2O           | 23.00 ± 0.0                  | 20.14 ± 0.4                 | <0.05†                                  | 3                         | 315                     |
| N2 DMSO                     | 23.67 ± 1.15                 | 21.06 ± 0.42                |                                         | 3                         | 348                     |
| N2 GlcN                     | 25.00 ± 0.0                  | 22.53 ± 0.8                 | <0.001†                                 | 3                         | 305                     |
| N2 BHA                      | 23.00 ± 0.0                  | 20.96 ± 0.29                | NS‡                                    | 3                         | 320                     |
| N2 GlcN/BHA                 | 23.00 ± 0.0                  | 20.67 ± 0.16                | NS‡                                    | 3                         | 325                     |
| N2 H2O                      | 24.33 ± 1.2                  | 21.38 ± 0.3                 |                                         | 3                         | 370                     |
| N2 GlcN                     | 25.33 ± 0.6                  | 22.42 ± 0.4                 | <0.00005†                              | 3                         | 338                     |
| N2 NAC                      | 24.67 ± 1.5                  | 21.73 ± 0.4                 | NS†                                    | 3                         | 372                     |
| N2 GlcN/NAC                 | 24.33 ± 1.2                  | 21.05 ± 0.4                 | NS†                                    | 3                         | 346                     |
| pmk-1 (km25) H2O            | 24.00 ± 0.0                  | 20.46 ± 0.3                 |                                         | 6                         | 456                     |
| pmk-1 (km25) GlcN           | 24.00 ± 0.0                  | 20.76 ± 0.3                 | NS**                                   | 6                         | 388                     |
| daf-16 (mu86) H2O           | 21.00 ± 0.0                  | 18.27 ± 0.3                 |                                         | 3                         | 443                     |
| daf-16 (mu86) GlcN          | 21.00 ± 0.0                  | 19.01 ± 0.3                 | <0.05†                                  | 3                         | 431                     |
| skn-1 (zu135) H2O           | 19.67 ± 1.2                  | 17.34 ± 0.5                 |                                         | 3                         | 200                     |
| skn-1 (zu135) GlcN          | 19.00 ± 0.0                  | 16.64 ± 0.5                 | NS†                                    | 3                         | 190                     |
| F2IDS1 RNAi/H2O             | 30.0                         | 24.46                      |                                         | 1                         | 184                     |
| F2IDS1 RNAi/100 μM GlcN     | 35.0                         | 28.24                      | <0.0001†                                | 1                         | 178                     |
| aat-1 RNAi/H2O              | 20.00 ± 0.4                  | 21.78 ± 0.3                 |                                         | 3                         | 214                     |
| aat-1 RNAi/100 μM GlcN      | 20.00 ± 0.4                  | 21.59 ± 0.3                 | NS†††                                   | 3                         | 225                     |

NS, not significant.

*75th percentile;
†HIT, heat-inactivated;
‡Controls: N2 H2O;
§N2 H2O HIT bacteria;
∥aak-2 (ok524) H2O;
*skn-1 (zu135) H2O;
#N2 DMSO;
**pmk-1 (km25) H2O;
††def-16 (mu86) H2O;
†††def-16 (mu86) GlcN;
‡‡sir-2 (ok434) H2O;
§§F2IDS1 RNAi/H2O;
|||pmk-1 RNAi/H2O;

reduces rodent life span19 while other glycolytic inhibitors have not yet been tested in this regard. Despite these discouraging findings, we here have tested the effects of GlcN treatment on HepG2 human hepatoma cells and found that GlcN reduces ATP content in such cells (Fig. 3a), which is consistent with GlcN acting as a glycolytic inhibitor4,5 and reflects the findings in nematodes (Fig. 1c). We next analysed whether pathways contributing to the extension of life span in nematodes could be similarly activated in hepatic cells. We found that GlcN treatment increases Thr172-phosphorylation of AMPK (Fig. 3b) as well as Thr180/Tyr182-phosphorylation of p38 (Fig. 3b) in HepG2 cells, reflecting the findings in nematodes (Figs 1d and 2e, respectively).

GlcN supplementation extends life span of ageing mice. Based on these promising ex vivo observations, we then chronically exposed C57BL/6NRj mice of both sexes, starting at an age of 100 weeks, to GlcN. As the prominent finding of the current study, we observed that GlcN increased the life span of aged mice (Fig. 3c). Both log-rank as well as Cox regression analyses indicated significant differences between controls and GlcN-treated rodents (log-rank: P = 0.002; Cox regression: P = 0.01). When applying log-rank statistics to both sexes in separate analyses, it appeared that the response was more pronounced in females (Fig. 3d) than in males (Fig. 3e). Nevertheless, the interaction term for ‘treatment by sex’ within Cox Regression analyses turned out to be insignificant (P = 0.716), unambiguously indicating that
GlcN promotes the life span of both females and males, independent of sex. Calculation of maximum life span was performed by applying both the Fisher’s exact test and the Z-pooled exact unconditional test. Both tests indicated that maximum life span was extended by GlcN treatment (Fisher’s P = 0.0143 for 90th percentile, and Z-pooled P = 0.01255).

**GlcN affects glucose metabolism and mitochondrial biogenesis.**

We found that food uptake was unaffected not only by the GlcN application but also in regard to the aforementioned interaction term applying two-way analysis of variance (ANOVA) (Fig. 4a and Supplementary Fig. 2a,b). Not surprisingly, GlcN consumption increased blood plasma levels of the compound to the pharmacologically achievable concentrations of ~2 μM (Fig. 4b and Supplementary Fig. 2c,d), as analysed by HPLC. When quantifying the GlcN metabolite GlcN-6-phosphate, which acts as the competitive inhibitor of glycolysis, by mass spectroscopy, we observed an increase following GlcN consumption (Fig. 4c). The interaction term rejected a sex-specific effect (Supplementary Fig. 2e,f). No differences in murine body mass or body composition were observed before or after treatment with GlcN (Fig. 4d–f and Supplementary Fig. 2g–l).

Treatment of *C. elegans* with GlcN increased nematodal mitochondrial DNA content (Fig. 1g). Similarly, we observed an increase in mtDNA content in liver specimen from GlcN-treated mice (Fig. 4g). With the interaction term ‘treatment by sex’ gaining significance (F[1,21] = 11.389, P = 0.003, two-way ANOVA), this effect appears mainly attributable to females (Supplementary Fig. 2m,n). Energy expenditure was then quantified by indirect calorimetry. Although no effect of GlcN feeding on the combined group was detected (Fig. 4h), a trend emerged where female mice appeared to respond to GlcN differently than males (Fig. 4i, right pair of bars). No sex-specific effects were observed applying two-way ANOVA statistics for the interaction term ‘treatment by sex’ (Supplementary Fig. 3a,b). Nevertheless, there were no observable differences in glucose tolerance tests (Supplementary Fig. 3c–h). As previously shown, the administration of high-dose GlcN to model systems may cause insulin resistance. Accordingly, insulin tolerance tests in...
**Figure 3 | GlcN promotes hepatic energy depletion and increases life span in ageing mice.** (a) ATP content of HepG2 cells exposed to GlcN for 30 min (bars represent mean ± s.d.; ***P<0.001 versus unexposed; Student’s t-test, n=8). (b) Representative western blots of HepG2 cells following exposure to GlcN for indicated durations using indicated primary antibodies. (c-e) Survival curves of C57BL/6NRj mice on a GlcN-containing diet starting at an age of 100 weeks (red) in comparison with control mice. (c) Survival of combined male and female C57BL/6NRj mice (log-rank: P=0.002; Cox regression: P=0.01; n=74 control mice and n=72 mice on GlcN-containing diet). (d) Survival of female C57BL/6NRj mice (log-rank: P=0.007; n=37 control mice and n=38 mice on GlcN-containing diet). (e) Survival of male C57BL/6NRj mice (log-rank: P=0.097; n=37 control mice and n=34 mice on GlcN-containing diet); P-values that were obtained using Cox regression analyses (including interaction term for 'treatment by sex') are given in black font, P-values that were calculated using the log-rank test are given in blue font. Controls are always depicted in black or grey colour, whereas GlcN-treatment is depicted in red.

**Figure 4 | Metabolic consequences of GlcN supplementation.** (a) Food uptake of C57BL/6-NRj mice (both sexes) chronically exposed to GlcN (red) and respective controls (grey). (b) Plasma levels of GlcN in mice (both sexes) on a GlcN-containing diet in comparison with control mice (F(1,33) = 27.67, P<0.001, n=18 control mice and n=19 GlcN-fed mice). (c) Hepatic levels of GlcN-6-phosphate (F(1,16) = 8.74, P<0.01, n=10 control mice and n=10 GlcN-fed mice). (d-f) Body mass and body composition parameters in such mice. (g) Relative mtDNA content in liver specimen (F(1,21) = 5.05, P<0.05, n=12 control mice and n=13 GlcN-fed mice). (h) Energy expenditure normalized to metabolic body mass of such mice; calculated means for every hour during day, grey area reflects dark phase of the light cycle. (i) Random fed (F(1,36) = 4.49, P<0.05, n=20 control mice and n=20 GlcN-fed mice) as well as fasting blood glucose levels in such mice. Controls are always depicted in black and grey colour, whereas GlcN-treatment is depicted in red. The bars represent the mean ± s.d. *P<0.05, **P<0.01, ***P<0.001 versus control; two-way ANOVA.
GlcN acts independent of the hexosamine pathway. As GlcN-feeding caused increases in GlcN plasma levels (Fig. 4b) and GlcN-6-phosphate concentrations in liver specimen (Fig. 4c), we next tested whether, in addition to inhibiting glycolysis\(^4,5\), feeding caused increases in GlcN plasma levels (Fig. 4b) and (Supplementary Fig. 3x–z). There were no observable differences for serum levels of triglycerides (Supplementary Fig. 3o–q), free fatty acids (Supplementary Fig. 3r–t), total cholesterol (Supplementary Fig. 3l–n). There were no observable differences corresponding areas under the curve were different by trend only at specific time points (Supplementary Fig. 3i–k), whereas the inducible enzyme GlcN-6-phosphate-N-acetyltransferase (EC 2.3.1.4) (Fig. 5a) that acetylates the GlcN-6-phosphate. Unfortunately, at least two independent genes in worms (Worm Base accession codes B0024.12 and T23G11.2) encode this enzyme\(^35\) (Fig. 5a), precluding an RNAi-based knockdown approach and appropriate mutants being unavailable. We chose alternatively to impair expression F21D5.1, the only C. elegans orthologue of mammalian phospho-acetyl-GlcN-mutase (E.C. 5.4.2.3) (Fig. 5a), with RNAi. Such treatment had no effect on the life span-extending capabilities of GlcN treatment in nematodes (Fig. 5b), indicating that inhibition of glycolysis, rather than increased hexosamine metabolism, is responsible for the phenotype observed.

GlcN induces expression of amino-acid transporters in both species. To potentially identify further downstream mechanistic pathways aside from AMPK activation and increased adaptive mtROS signalling via activation of p38 (Figs 1d,e; 2e,f and 3b), we next performed RNA next-generation sequencing analyses on whole-worm extracts as well as liver specimen from mice. In the latter, 231 gene transcripts were found to be induced by long-term exposure to GlcN in comparison with control mice (Fig. 6a and Supplementary Data 1), whereas in C. elegans, 1,221 transcripts were found to be upregulated by GlcN (Fig. 6a and Supplementary Data 2). Performing a Venn analysis on these genes for both species, 14 genes were found to be similarly upregulated in both mice and worms (Fig. 6a) based on orthology search (Supplementary Data 3). Out of these, Slc7a11, Slc7a8 and Slc13a3 (Supplementary Data 1) are amino-acid transporters in mice, whereas aat-1 is a nematodal amino-acid transporter (Supplementary Data 2), all of which were induced by GlcN in the respective species (Fig. 6a).

We next determined in silico analyses of putative promoter elements in the genes upregulated in C. elegans (Fig. 6a and Supplementary Data 2). We identified a putative SKN-1-binding site in the promoter of the aat-1 gene (Fig. 6b) consistent with the life span-shortening effects of GlcN in skn-1 nematodes (Fig. 2h). Moreover, 36.3% of all GlcN-dependent genes in C. elegans carry a putative SKN-1-binding element (Fig. 6c), reiteratively consistent with the experimental data (Fig. 2h).

Then, expression of aat-1 mRNA in skn-1 nematodes in the presence and absence of GlcN was determined. Unlike in wild-type worms treated with GlcN deficiency for SKN-1 halted the induction of aat-1 expression (Supplementary Data 2), insinuating that increased amino-acid uptake mediates the life span-extending effects of GlcN in a SKN-1-dependent manner.

To test this, we impaired aat-1 expression by RNAi, and observed that the life span-extending effect of GlcN was fully eradicated in these nematodes (Fig. 6d), evidencing that increased amino-acid transport as mediated by AAT-1 is required for GlcN-mediated extension of life span.

GlcN causes increased catabolism of amino acids. We then determined serum concentrations of urea as a possible indicator for amino-acid turnover in mice, but observed no difference (Supplementary Fig. 4d–f). This result is possibly because of effective excretion of any excess urea via the kidneys.

Finally, we analysed the branched-chain amino acid (BCAA) catabolism\(^36,37\) in liver samples from GlcN-treated mice. Unlike most other amino acids, BCAAs are subject to unidirectional, that is, irreversible catabolism, and individual catabolites can therefore be used to estimate turnover rates. We observed an increase in L-leucine/L-isoleucine catabolites in liver samples of mice (Fig. 6e–g and Supplementary Fig. 4g–l), indicating that increased amino-acid turnover, following chronic GlcN exposure in nematodes (Fig. 6d) and mice (Fig. 6e–g and Supplementary Fig. 4g–l) is linked to life span extension.

Figure 5 | GlcN extends life span independent of the hexosamine pathway. (a) Schematic overview on initial enzymatic steps of GlcN metabolism (so-called hexosamine pathway), and the corresponding C. elegans orthologues. (b) Life span analysis in C. elegans treated with RNAi against F21D5.1 (phospho-acetyl-α-glucosamine-mutase) in the presence (red) and absence of GlcN (P<0.0001, log-rank test, n=1).
The current findings indicate that GlcN at pharmacologically relevant concentrations is capable of extending life span in *C. elegans* and ageing mice. This appears to be a result of decreased glycolysis and a compensatory increase of amino-acid turnover. Although it should be noted that GlcN may impact on an observational and uncontrolled basis, it has been repeatedly suggested that supplementation with GlcN is safe for human use even at high doses, making it readily available for interventions to extend human healthspan particularly because, on an observational and uncontrolled basis, it has been repeatedly suggested that supplementation with GlcN may decrease overall mortality in humans49,50.

Unlike for DOG and most other life span-extending compounds, extensive published evidence indicates that GlcN is safe for human use even at high doses, making it readily available for interventions to extend human healthspan particularly because, on an observational and uncontrolled basis, it has been repeatedly suggested that supplementation with GlcN may decrease overall mortality in humans49,50.

**Note added in proof:** While this publication was in the press, Denzel and co-workers published findings on the role of N-acetyl-glucosamine (GlcNAc) supplementation in *C. elegans* lifespan extension, suggesting a different mechanism. This appears to depend on the hexosamine pathway which, however, is unlike to contribute to the phenotype observed in our study (Fig. 5 and Supplementary Fig. 4a–c), indicating that GlcN and GlcNAc promote longevity independently51.

### Methods

**Chemicals.** All chemicals were obtained from Sigma-Aldrich (Munich, Germany) unless stated otherwise.

**Statistical analyses.** Data are expressed as means ± s.d. unless otherwise indicated. Statistical analyses for all *C. elegans* data except life span and stress resistance
assays were performed by Student’s t-test (unpaired, two-tailed) after testing for equal distribution of the data and equal variances within the data set. For comparing significant distributions between different groups in the life span assays, statistical calculations were performed using the log-rank test. Statistical analyses for all murine data except life span analyses were performed by Student’s t-test (unpaired, two-tailed). Mortality rates during the murine survival study were assessed by using log-rank and Cox regression tests to compare survival using SPSS Version 20 (IBM, Armonk, NY, USA). Cox regression was performed including the ‘treatment by sex’ interaction to test for potential sex-specific interactions. In parallel, sex-specific analyses were performed using Student’s t-test (unpaired, two-tailed). As a substrate, labelled glucose was added to a final concentration of 17.1 mM, 50 mM and 4 mM to yield powder. Guanidinium-hydrochloric acid (Guanidium HCl) (4 M) was prepared, heated to 100 °C and then mixed with the frozen powder to destroy ATPase activity and to further lyse samples. The mixture was boiled for 15 min at 100 °C with a subsequent centrifugation step (30 min at 13,200 g and 4 °C). The supernatant was diluted with ddH2O 1:200 and analysed using a commercially available kit (CellTiter Glo; Promega, Fitchburg, WI, USA) according to the manufacturer’s instructions. For normalization of the luminescence signal, protein was determined as described below.

Immunoblotting. Frozen worm pellets were grilled in a nitrogen-chilled mortar and suspended in phosphate buffer containing protease and phosphatase inhibitors (Complete protease inhibitor cocktail (Roche, Penzberg, Germany), Pierce iPerfect Proteinase Inhibitor Cocktail, Thermo Scientific). Absorbance was measured in a microplate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany).

Nematodal quantification of mitochondrial ROS formation. Before ROS measurement Mitotracker Red CM-H2X ROS (Invitrogen, Carlsbad, CA, USA) incubation plates were prepared as follows: for each treatment, 500 μl heat-inactivated OP50 (65 °C and 30 min) were mixed with 100 μl Mitotracker Red CM-H2X stock solution (100 μM) and spotted on a large NGM agar plate that was allowed to dry for ~20 min. Nematodes were incubated without GlcN, then washed off the plates with S-Buffer and then allowed to settle by gravitation to remove offspring. Worms were washed two additional times with S-Buffer and centrifuged (300 g, 30 s). The worm pellet was transferred to freshly prepared Mitotracker Red CM-H2X solution and incubated for 2 h at 20 °C. To remove excess dye from the gut, worms were transferred to NGM agar plates with the corresponding compound or, as a positive control, to plates containing 1 μM rotenone for 1 h at 20 °C. Aliquots of 100 μl worm suspension were distributed into 96-well Fluorotrac plates (Greiner Bio-One, Frickenhausen, Germany). Fluorescence intensity was measured in a microplate reader (FLUOstar Optima) using well-scanning mode (excitation wavelength (ex): 570 nm, emission wavelength (em): 610 nm). To normalize fluorescence signal, the remaining worm suspension was used for protein determination.

Amplex Red-based quantification of supernatant hydrogen peroxide. Worms were removed from plates with 50 mM sodium-phosphate buffer, pH 7.4, washed twice and transferred into an upright Plexiglas cylinder (1.5 ml volume) with continuous stirring at low speed (100 r.p.m.) at 20 °C. First, determination of fluorescence was done without horse radish peroxidase only in the presence of 1 μM Amplex Red (Invitrogen) to detect possible unspecific increase in fluorescence (which was not observed). Next, 0.01 U ml−1 horse radish peroxidase was added and changes of fluorescence were recorded with a fluorescence detector (FL402 ProLine, ION, Berlin, Germany) for at least 15 min at excitation and emission wavelengths of 571 and 585 nm, respectively. Subsequently, worms were removed and collected for protein determination to normalize fluorescence values.

Cell culture. HepG2 hepatoma cells were obtained from LGC Standards (Wesel, Germany) and were grown in RPMI medium containing 10% heat-inactivated fetal bovine serum at 37 °C in 5% CO2/95% O2. Cells were serum starved for 5 h and incubated with or without 5 mM GlcN for the indicated time before collection.
Superoxide dismutase and catalase activity assays. To determine antioxidant enzyme activities (superoxide dismutase (SOD), catalase (CAT)) nematodes were harvested and washed with ice-cold buffer. The frozen pellet was grinded in a nitrogen chilled mortar together with 200 μl 50 mM phosphate buffer plus 1 mM EDTA and sonicated threefold. Lysate was cleared by centrifugation for 15 min at 12,000 g and 4 °C. Supernatant was used for the subsequent measurement of cat- alase or superoxide dismutase activity as well as for a protein quantification to normalize enzyme activities. Determination of catalase activity was performed like previously described with minor changes50. Briefly, the diluted (25 mM potassium phosphate buffer plus 1 mM EDTA plus 0.1% BSA, pH 7.5) sample supernatant was mixed with assay buffer (100 mM potassium phosphate buffer, pH 7) and methanol (VWR, Darmstadt, Germany). Hydrogen peroxide (Applichem, 30%) was added and incubated for 20 min under continuous shaking at 20 °C. Reaction was terminated by addition of potassium hydroxide (Applichem, 10 M) and xanthine oxidase (Sigma-Aldrich; 192 mM in 0.5 M potassium hydroxide) was added to oxidize the Peroxidase-formaldehyde complex and incubated for further 5 min before the absorbance was measured at 540 nm. SOD activity was quantified using a method described earlier51. Sample supernatant was incubated with WST-1 working solution (Tris–HCl, pH 8, diethylenetriamine-penta-acetic acid (Sigma-Aldrich; 150 μM), hypoxanthine (Applichem, 100 μM), WST-1 (180 μM)) and xanthine oxidase (Sigma-Aldrich; 240 μM ml⁻¹) for 20 min at 37 °C. Then, absorbance was measured at 450 nm.

PQ stress assay. N2 nematodes at an adult age of 6 days were transferred manually to fresh NGM plates containing 10 mM PQ (Acros Organics, Geel, Belgium) covered with heat-inactivated OP50 (30 min at 65 °C in a water bath) and attended by daily determination of the survival rate until all nematodes were died. PQ survival rate was calculated as the number of dead nematodes divided by the number of wild-type nematodes on the same plate. The PQ survival rate was then used to determine the PQ resistance index (PQRI) for each strain as a phenotypic measure of PQ resistance, which was calculated as the log survival rate of the wild-type strain divided by that of the test strain.

Murine breeding and housing conditions. C57BL/6JR mice were bred in our own facilities based on founders from Janvier Sas (Le Genest Saint Isle, France); it should be noted that these mice lack the nicotineamide nucleotide transhydrogenase mutation found in C57BL/6 J-derived strains52. Animals were studied starting at an age of 100 weeks. In mice we use to produce mice is very common for ethical and regulatory reasons. In addition, there is a need to produce mice that are free of any contaminating organisms such as bacteria and viruses. This is done by breeding mice in a specific pathogen-free (SPF) environment and using negative pressure rooms for housing. The SPF environment provides a controlled environment that is free of potential pathogens. The negative pressure rooms help to prevent the entry of any contaminants into the room. By maintaining these conditions, we can ensure that we are able to produce healthy and disease-free mice for our research.

Murine body mass and body composition analyses. Body mass was quantified using a graded scale. Body composition was measured by use of quantitative nuclear magnetic resonance technique (Echo MRI-100 Body Composition Analyzer, Echo Medical Systems, Houston, USA) as described53. Blood sampling. Blood samples, collected in tubes containing 21 U lithium heparin and centrifuged for 10 min at 4 °C and 8,000 r.p.m. (6,800 g), were obtained both in the fed state as well as after mice were fasted 16 h overnight by using lancets for submandibular bleeding (Goldenrod Animal Lancet, Medipoint, Allensford, NY, USA).

GlCN plasma concentrations. Plasma was derivatized with AccQ-Fluor Reagent Kit (Waters, Milford, MA, USA) according to the manufacturer’s instructions. HPLC was performed on Nexera sytem equipped with a Shimadzu, Kyoto, Japan) Chromatographic separation was performed on Reprospher 100 C18-DE 1.8 μm, 50 × 2 mm (Dr Maisch GmbH, Ammerbuch-Entringen, Germany) at 45 °C. Elution was performed using acetonitrile and sodium acetate buffer, pH 5.25, at flow rate of 0.8 ml min⁻¹ using a gradient. Detection was carried out using excitation wavelength of 250 nm and emission wavelength of 395 nm.

Glucose tolerance tests. Glucose tolerance tests were performed by intraper- itoneal glucose injection (50 g/kg, Merck, Darmstadt, Germany) after mice were fasted 16 h overnight. Plasma was collected before and 10, 30, 60 and 120 min after administering of glucose and immediately frozen at −80 °C for measurement of glucose and insulin54.

Insulin tolerance tests. Mice were subjected to an insulin tolerance test by intraperitoneal injection of 1.5 U kg⁻¹ of human recombinant insulin (Insulan Rapid, Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany). Glucose was determined from a tail vein blood before and 15, 30, 45, 60, 75, and 90 min after insulin injection by a glucometer (Contour, Bayer AG, Leverkusen, Germany).

Respiratory quotient and total energy expenditure. Total energy expenditure and respiratory quotients were determined by indirect calorimetry at 22 °C for 24 h with an open-circuit calorimeter system (TSE PhenoMaster System, Inc., Midland, MI, USA). Rates of oxygen consumption (VO2) and carbon dioxide production (VCO2) were recorded for a 24-h period after mice were allowed to acclimatize to the system for a period of 2 days. The air-tight respiratory cages were measured with a flow rate of about 0.38 l min⁻¹. VO2 and VCO2 were recorded for 15 min in 16-min intervals for each animal, so that three or four data points were obtained every other hour. TEE (kcal h⁻¹) was calculated with the equation TEE = 16.17 VO2 + 5.03 VCO2 − 5.98 N, where N is excreted nitrogen and was assumed to be 0.1 g per day. Total energy expenditure was normalized to 24 h and metabolic body mass.

Determination of plasma parameters. Determination of glucose, triglycerides, alanine-aminotransferase, free fatty acids and total cholesterol in plasma was performed using an automated analyser (Cobas Mira S, Hoffmann-La Roche, Basel, Switzerland) with the appropriate commercially available reagent kits (Glucose HK CP, triglycerides, ALT CP, cholesterol CP, ABX, Montpellier, France; and NEFA HR, Wako, Neuss, Germany).

Quantification of GlCN-6-phosphate. Quantification of GlCN-6-phosphate and its metabolites was performed on an Agilent 6550 QTOF instrument by flow injection analysis time-of-flight mass spectrometry55. All samples were injected in duplicates. Ions were annotated based on their accurate mass and the Kyoto Encyclopedia of Genes and Genomes (KEGG) hsa reference list allowing a tolerance of 0.001 Da. Unknown ions and those annotated as adducts were discarded. This resulted in a total of 472 putatively annotated ions with unique m/z.

Targeted analysis of BCAA catabolism. To assess activation of catabolic pathways of BCAAs, we quantified levels of methyl-branched CoA metabolites, crotonyl-CoA and succinate on a Thermo Quantum Ultra instrument by targeted ion pairing-liquid chromatography tandem mass spectrometry using multiple reaction monitoring36,37.

Quantification of mtDNA. Total DNA was isolated by standard proteinase K and phenol–chloroform methods. mtDNA copy number level was analysed by quantitative real-time PCR using Viia 7 (Applied Biosystems). The amount of 0.4 ng of total DNA was used as a template for the amplification of mtDNA in mouse or C. elegans. Level of primers against mouse mtDNA (5’-AGAACACCT GTCTGACCCAC-3’ and 5’-TTGCTTGGCAAGATTTACC-3’) were normal- ized against nuclear 18SrRNA gene (5’-AACCTTGGATGTTGATCGCC-3’ and 5’- CCTGATGATGATGATGATCC-3’) in the same approach was used for analysis of C. elegans samples by using primer for mtDNA (5’-CTTFTATTCT ATATAGGGCTTC-3’ and 5’-AAAAAGAAATCTCCGTAGCAAG-3’) normalized against nuclear 18SrRNA homologue (5’-GGGAAAGATTTGGG AAGAA-3’ and 5’-ATCCGGAGATGCGGCGT-3’).

Extraction of RNA. Total RNA was isolated using QIAzol (Qiagen, Hilden, Germany) based on the phenol–chloroform extraction method. Afterwards, the RNA was quantified photometrically with a NanoDrop 1000 (PeqLab, Erlangen, Germany) and stored at −80 °C until use.

Next-generation sequencing (RNAseq). Total RNA was isolated for degraga- tion using Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). For library preparation an amount of 2 μg of total RNA per sample was processed using Illumina’s TruSeq RNA Sample Prep Kit (Illumina; San Diego; CA, USA) following the manufacturer’s instruction. Each library includes its own index sequence to allow multiplexing. The libraries were sequenced using v3 sequencing chemistry and a HiSeq2000 (Illumina) in a single read approach with 50 cycles resulting in reads with a length of 50 nucleotides. Libraries were sequenced in a multiplex manner pooling four libraries per lane. Sequencing ends up with ~30–40 Mio reads per sample. Sequence data were extracted in FastQ format and used for mapping approach.

Quantification of atf-1 mRNA. Total RNA was isolated and reverse-transcribed to first-strand cDNA using high-capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to manufacturer’s protocols. Quantitative real-
time PCR was performed using optical 384-well plates, SYBR Select Master Mix and ViiA 7 (Applied Biosystems). All samples were measured in triplicates, and non-template controls were used to confirm specificity. Expression of act-1 (F27C8.1) was quantified using specific primers 5′-ACCGAGCTTGGTCTCC TTTT-3′ and 5′-TTTGGGTCTGCAACTCTCCT-3′, and normalized against a housekeeping gene (r07G3.1) using primers 5′-CTGCTGACAGGAGAT TACC-3′ and 5′-CTGGACATCTCGGAAATAAGG-3′.

Bioinformatics of RNA expression data. All reads were mapped against the respective genomic sequences (ce10 for C. elegans; mm10 for Mus musculus) using TopHat 1.4.1. Only uniquely mappable reads were regarded. For counting the reads per gene (raw counts) the Python package HTSeq (http://www.huber.eimbl.de/ users/anders/HTSeq/doc/overview.html) was used in mode ‘unique’ together with gene annotation for all RefSeq genes downloaded from the UCSC website. Raw counts for the genes were analysed using the R Statistical Computing Environment89 and edger94. The latter provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg’s approach95 for controlling the false discovery rate. If false discovery rate values were <0.05, genes were assigned as differentially expressed. For the comparison of orthologous differentially expressed genes in C. elegans and M. musculus, the R package orthology was applied77.

Promoter analyses. The search for SKN-1 transcription factor binding sites for each gene was done within the proximal promoter region 2 kb upstream of the predicted start codon. Therefore, a FASTA file containing the promoter regions for all genes was created using WormMart. Next, the remaining sequence file was scanned for one or more matches to the position-specific scoring matrices of SKN-1 using the matrix scan function of the pattern-matching programme regulatory signals database68. The threshold false discovery rate values were set to 0.0001.

References
1. McLalndon, T. E., LaValley, M. P., Gulin, J. P. & Felson, D. T. Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis. JAMA 283, 1469–1475 (2000).
2. Reginster, J. Y. et al. Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 357, 251–256 (2001).
3. Wandell, S. et al. Effects of glucosamine, chondroitin, or placebo in patients with osteoarthritis of hip or knee: network meta-analysis. BMJ 341, c4675 (2010).
4. Silverman, J. L. Glucosamine Inhibition of (I-14C)glucose oxidation as measured by rat adipose tissue in vitro. Biochim. Biophys. Acta. 78, 94–100 (1963).
5. Oguchi, M., Sato, M., Miyatake, Y. & Akamatsu, N. Studies on the metabolism of glucosamine; oxidation activity for c-glucosamine in yeast by glucose in rat liver and its possible identity with glucosamine. J. Biochem. 82, 559–567 (1977).
6. Marshall, S., Bacote, V. & Traxinger, R. R. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. J. Biol. Chem. 266, 4706–4712 (1991).
7. Robinson, K. A., Sens, D. A. & Buse, M. G. Pre-exposure to glucosamine induces insulin resistance of glucose transport and glycogen synthesis in isolated rat skeletal muscles. Study of mechanisms in muscle and in rat-1 fibroblasts overexpressing the human insulin receptor. Diabetes 42, 1333–1346 (1993).
8. Patti, M. E., Virkamaki, A., Landaker, E. J., Kahn, C. R. & Yki-Jarvinen, H. Activation of the hexosamine pathway by glucosamine in vivo induces insulin resistance of early postreceptor insulin signaling events in skeletal muscle. Diabetes 48, 1562–1571 (1999).
9. Marzio, T. et al. Effects of glucosamine infusion on insulin secretion and glucose metabolism in normal and diabetic individuals. Diabetes Metab. Res. Rev. 27, 14–27 (2011).
10. Anderson, J. W., Nicolosi, R. J. & Borzelleca, J. F. Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. Food. Chem. Toxicol. 43, 187–201 (2005).
11. Hamilton, B. et al. A systematic RNAi screen for longevity genes in C. elegans. Genes Dev. 19, 1544–1555 (2005).
12. Hanzen, M., Hsu, A., Dillin, A. & Kenyon, C. New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a Caenorhabditis elegans genomic RNAi screen. PLoS Genet. 1, e17 (2005).
13. Schulte, T. J. et al. Glucosamine restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab. 6, 280–293 (2007).
14. Scholterrer, A. et al. C. elegans as model for the study of high glucose mediated lifespan reduction. Diabetes 58, 2450–2456 (2009).
15. Minor, R. et al. Chronic ingestion of 2-deoxy-d-glucose induces cardiac vacuolization and increases mortality in rats. Toxicol. Appl. Pharmacol. 243, 332–339 (2010).
16. Ingram, D. K. & Roth, G. S. Glycolytic inhibition as a strategy for developing calorie restriction mimetics. Exp. Gerontol. 46, 148–154 (2011).
17. Appfeld, J., O’Connor, G., McDonagh, T., DiStefano, P. S. & Curtis, A. M. AMP-activated protein kinase aak-2 links energy levels and insulin-like signals to lifespan in C. elegans. Genes Dev. 18, 3004–3009 (2004).
18. Hardie, D. G. AMP-activated protein kinase— an energy sensor that regulates all aspects of cell function. Genes Dev. 25, 1895–1908 (2011).
19. Duke, H. W. et al. Glucosamine causes overproduction of reactive oxygen species, leading to repression of hypoxycytol elongation through a hexokinase-mediated mechanism in Arabidopsis. J. Plant Physiol. 166, 203–212 (2009).
20. Yang, W. & Hekimi, S. A mitochondrial superoxide signal triggers increased longevity in Caenorhabditis elegans. PLoS Biol. 8, e1000556 (2010).
21. Pan, Y., Schroeder, E. A., Ocampo, A., Barrientos, A. & Shadel, G. S. Regulation of yeast chronological life span by TORC1 via adaptive mitochondrial ROS signaling. Cell Metab. 13, 668–678 (2011).
22. Sema, L. A. & Chandel, N. S. Physiological roles of mitochondrial reactive oxygen species. Mol. Cell 48, 158–167 (2012).
23. Yun, J. & Finkel, T. Mitohormesis. Cell Metab. doi:10.1016/j.cmet.2014.01.011 (in the press).
24. Ristow, M. & Schmeisser, K. Mitohormesis: Promoting health and lifespan by increased levels of reactive oxygen species (ROS). Dis. Model. Mech. 7, 335–339 (2014).
25. Majoros, R. ’O-GlcNAc code’. Nucleic Acids Res. 42, D789–D793 (2014).
26. Buescher, J. M., Mocco, S., Sauer, U. & Zamboni, N. Ultrafast performance liquid chromatography-tandem mass spectrometry method for fast and robust quantification of amino and aromatic metabolites. Anal. Chem. 82, 4043–4052 (2010).
27. Schmeisser, S. et al. Neuronal ROS signaling rather than AMPK/sirtuin-mediated energy sensing links dietary restriction to lifespan extension. Mol. Med. 2, 92–102 (2013).
28. Wang, C., Li, Q., Redden, D. T., Weindruch, R. & Allison, D. B. Statistical methods for testing effects on ‘maximum lifespan’. Mech. Ageing. Dev. 125, 629–632 (2004).
29. Love, D. C. & Hanover, J. A. The hexosamine signaling pathway: deciphering the ‘O-GlcNAc code’. Sci. STKE 2005, re13 (in the press).
30. Harris, T. W. et al. WormBase 2014: new views of curated biology. Nucleic Acids Res. 42, D789–D793 (2014).
31. Jovell, J. M., Mocco, S., Sauer, U. & Zamboni, N. Ultrafast performance liquid chromatography-tandem mass spectrometry method for fast and robust quantification of amino and aromatic metabolites. Anal. Chem. 82, 4043–4052 (2010).
32. Zimmerman, M., Thormann, V., Sauer, U. & Zamboni, N. Nontargeted profiling of coenzyme A thioesters in biological samples by tandem mass spectrometry. Anal. Chem. 82, 8284–8290 (2010).
33. Blackwell, T. K., Bowerman, R., Priess, J. R. & Weintraub, H. Formation of a monomeric DNA binding domain by Skn-1 bZIP and homeodomain elements. Science 266, 621–628 (1994).
34. An, J. H. & Blackwell, T. K. SKN-1 links C. elegans mesendodermal specification to a conserved oxidative stress response. Genes Dev. 17, 1882–1893 (2003).
35. Wang, J. et al. RNAi screening implicates a SKN-1-dependent transcriptional response in stress resistance and longevity deriving from translation inhibition. PLoS Genet. 6, e1001048 (2010).
36. Schmeisser, K. et al. Role of sirtuins in lifespan regulation is linked to methylation of nicotinamide. Nat. Chem. Biol. 9, 693–700 (2013).
37. Dunker, D. et al. Mitochondria mediates links low-dose arsenite exposure to lifespan extension. Aging Cell 12, 508–517 (2013).
38. Quastel, J. H. & Cantero, A. Inhibition of tumour growth by D-glucosamine. Nature 171, 252–254 (1953).
44. Oh, H. J. et al. d-glucosamine inhibits proliferation of human cancer cells through inhibition of p70S6K. *Biochem. Biophys. Res. Commun.* **360**, 840–845 (2007).

45. Kim, D. S. et al. Glucosamine is an effective chemo-sensitizer via transglutaminase 2 inhibition. *Cancer Lett.* **273**, 243–249 (2009).

46. Hwang, M. S. & Baek, W. K. Glucosamine induces autophagic cell death through the stimulation of ER stress in human glioma cancer cells. *Biochem. Biophys. Res. Commun.* **399**, 111–116 (2010).

47. Liu, B. Q. et al. Glucosamine induces cell death via proteasome inhibition in human ALVA41 prostate cancer cell. *Exp. Mol. Med.* **43**, 487–493 (2011).

48. Jung, C. W. et al. Anti-cancer properties of glucosamine-hydrochloride in YD-8 human oral cancer cells: Induction of the caspase-dependent apoptosis and down-regulation of HIF-1α. *Toxicol. In Vitro* **26**, 42–50 (2012).

49. Pocobelli, G. et al. Total mortality risk in relation to use of less-common dietary supplements. *Am. J. Clin. Nutr.* **91**, 1791–1800 (2010).

50. Bell, G. A., Kantor, E. D., Lampe, J. W., Shen, D. D. & White, E. Use of glucosamine and chondroitin in relation to mortality. *Eur. J. Epidemiol.* **27**, 593–603 (2012).

51. Denzel, M. S. et al. Hexosamine pathway metabolites enhance protein quality control and prolong life. *Cell* **156**, 1167–1178 (2014).

52. Brenner, S. The genetics of Caenorhabditis elegans. *Genetics* **77**, 71–94 (1974).

53. Ristow, M. et al. Frataxin activates mitochondrial energy conversion and oxidative phosphorylation. *Proc. Natl Acad. Sci. USA* **97**, 12239–12243 (2000).

54. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254 (1976).

55. Smith, P. K. et al. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85 (1985).

56. Johansson, L. H. & Borg, L. A. A spectrophotometric method for determination of catalase activity in small tissue samples. *Anal. Biochem.* **174**, 331–336 (1988).

57. Peskin, A. V. & Winterbourn, C. C. A microtitre plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). *Clin. Chim. Acta.* **293**, 157–166 (2000).

58. Mekada, K. et al. Genetic differences among C57BL/6 substrains. *Exp. Anim.* **58**, 141–149 (2009).

59. Kuhlow, D. et al. Measurement of protein using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization in rat plasma: application to a model study. *Anal. Biochem.* **319**, 7074–7080 (2003).

60. Wang, X. et al. Optimizing high-performance liquid chromatography method for quantification of glucosamine using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization in rat plasma: application to a pharmacokinetic study. *Biomed. Chromatogr.* **22**, 1265–1271 (2008).

61. Ristow, M. et al. Frataxin-deficiency in pancreatic islets causes diabetes due to loss of beta-cell mass. *J. Clin. Invest.* **112**, 527–534 (2003).

62. Fuhrer, T., Heer, D., Begemann, B. & Zamboni, N. High-throughput, accurate mass metabolome profiling of cellular extracts by flow injection-time-of-flight mass spectrometry. *Anal. Chem.* **83**, 7074–7080 (2011).

63. R Development Core Team. R: A language and environment for statistical computing (2008).

64. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2010).

65. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Statist. Soc. B* **57**, 289–300 (1995).

66. Priebe, S. & Mentel, U. in BIOINFORMATICS 2013 - International Conference on Bioinformatics Models, Methods and Algorithms. (eds Fernandes, P., Sole-Casals, J., Fred, L. N. A. & Gamboa, H.) 105–110.

67. Turatsinze, J. V., Thomas-Chollier, M., Defrance, M. & van Helden, J. Using RSAT to scan genome sequences for transcription factor binding sites and cis-regulatory modules. *Nat. Protoc.* **3**, 1578–1588 (2008).

68. Wingender, E. The TRANSFAC project as an example of framework technology that supports the analysis of genomic regulation. *Brief. Bioinform.* **9**, 326–332 (2008).

Acknowledgements

*C. elegans* strains used in this work were provided by the Caenorhabditis Genetics Center (University of Minnesota, USA), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The excellent technical assistance of Inonne Heine, Annett Müller, Susann Richter and Waltraud Scheiding, the excellent secretarial assistance of Mandy Schalowski and Rebeka Eckstein, as well as language editing by Kim Krakorz are gratefully acknowledged. Funding for this project was denied by the German Research Association (Deutsche Forschungsgemeinschaft, DFG), grant application number R1 1976/3-1. This work is in part supported by the research programme of the Jena Centre for Systems Biology of Ageing (JenAge) funded by the German Ministry for Education and Research (Bundesministerium für Bildung und Forschung; support code BMBF 0315581).

Author contributions

S.W., J.P., D.K. and M.R. designed, performed and evaluated all experiments with the following exceptions: K.Z. and J.M. did additional and independent life span experiments in *C. elegans*; K.Z. performed bacterial growth assays; M.G. and M.P. performed next-generation sequencing analysis of mRNAs, whereas sample preparation, RNA extraction and quality control were done by S.W. and J.P.; bio-informatical evaluation was done by S.P., J.M. and R.G. Promoter analysis was done by J.M. B.L. and K.Z. performed GlcN plasma analysis; S.D. and N.Z. performed all mass spectroscopy data; T.L.M. did all tissue culture experiments; A.F.P. and T.J.S were involved in the study design and contributed several assays; the entire work was supervised by M.R.; the figures were assembled and the manuscript was written by S.W., J.P. and M.R. All authors discussed and commented on the manuscript.

Additional information

Accession numbers: The deep sequencing data have been deposited in NCBI Gene Expression Omnibus under accession code GSE54853.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Weimer, S. et al. d-Glucosamine supplementation extends life span of nematodes and of ageing mice. *Nat. Commun.* 5:3563 doi: 10.1038/ncomms4563 (2014).

This work is licensed under a Creative Commons Attribution 3.0 Unported License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/3.0/