

Citrulline drives age-related lipid deposition for healthspan

Received: 7 November 2025

Accepted: 2 July 2026

Published online: 14 July 2026



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Too much or too less lipid deposition increases mortality, while in contrast, modest lipid deposition during aging is crucial for healthspan. How animals determine the aging state and then promote appropriate lipid deposition for lifespan benefits are largely unknown. In this study, we identified citrulline as a key metabolite driving aging-related lipid deposition for healthspan in *Caenorhabditis elegans*. Citrulline deficiency reduced aging-related lipid accumulation and shortened lifespan, an effect reversible by dietary supplementation. Mechanistically, during aging, the transcription factor MXL-3 is activated to upregulate the expression of *pyr-1*, encoding ornithine transcarbamylase (OTC), for the production of citrulline, which then activates the lipogenic enzymes DGAT-2 and MBOA-2 to consequently promote lipid synthesis and deposition for lifespan extension. Collectively, we uncover a MXL-3-citrulline-lipogenesis axis to ensure healthspan, providing distinct insights into metabolic aging.

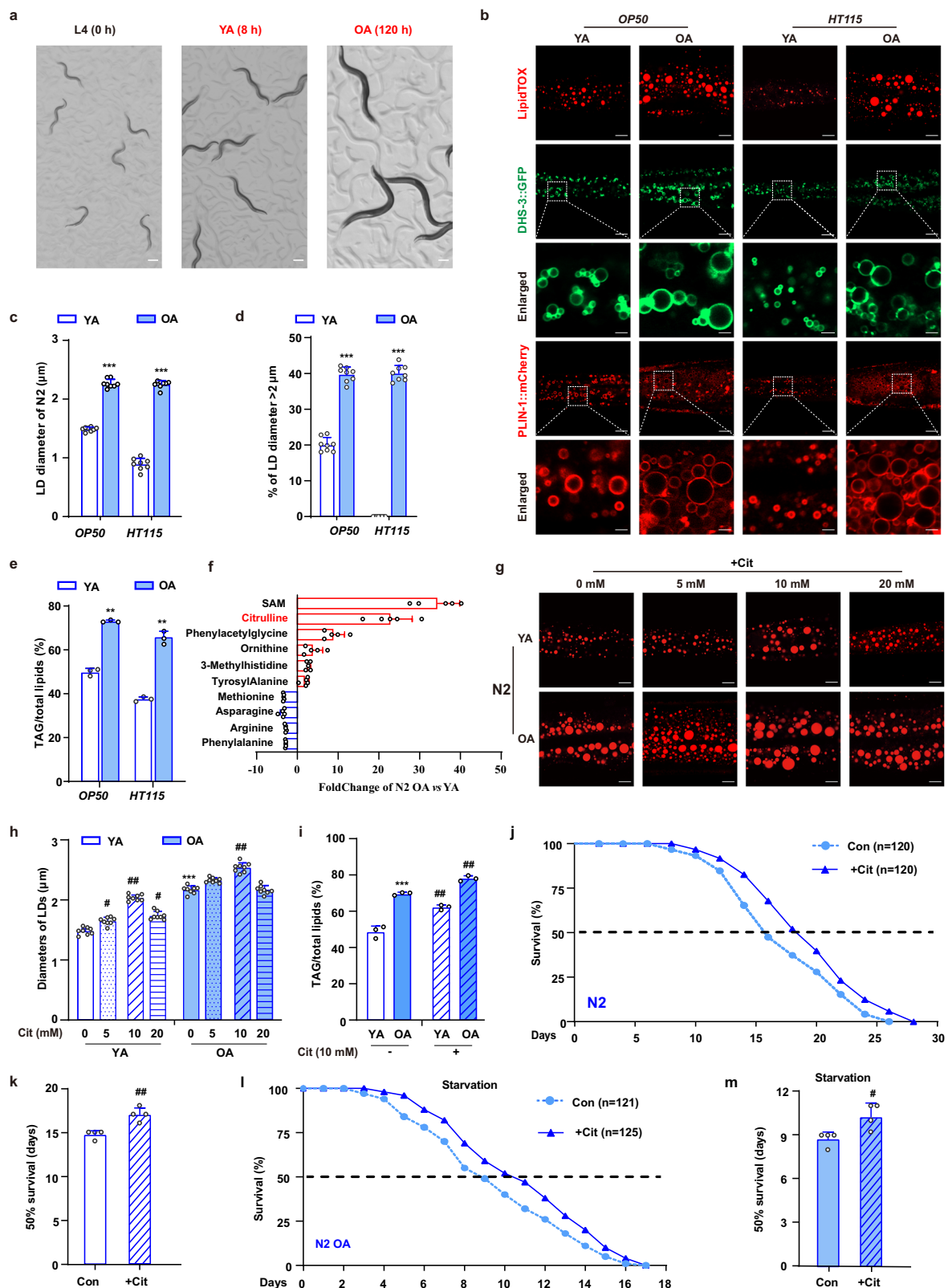
Aging is accompanied by a decline in metabolic flexibility, which induces fat storage¹, reciprocally promoting aging processes^{2,3}. Adipose tissue, a central hub of energy storage and metabolic regulation, is among the earliest and most prominently aging tissues⁴. The excessive accumulation of fat ultimately leads to increased vulnerability to age-related diseases^{5–8}, such as metabolic diseases, including type 2 diabetes, atherosclerosis, Alzheimer's disease, cancer, etc., which ultimately shorten the lifespan. As well, body fat loss is also harmful to health, and is even more serious than obesity⁹. Lipodystrophies or loss of adipose tissue may reduce hormone(s) secretion by adipose tissue, leading to metabolic abnormalities, which associate with insulin resistance, cardiomyopathy, developmental delay, and progeroid syndromes¹⁰. Transgenic mice lacking white adipose tissue are growth delayed and die prematurely¹¹. Consistently, a systematic review on the relationship between body mass index (BMI) and risks of all-cause mortality and disability showed increased risks of mortality and disability with the underweight (BMI < 18), as well as the obese

(BMI > 30), especially in the elderly^{12,13}. Thus, too much or too little fat storage may increase mortality and shorten lifespan.

In contrast, population cohort studies have reported that modest lipid deposition is associated with a longevity benefit, especially in elder populations^{14–19}. Notably, in a 20-year cohort study, the elderly (> 65 years old) with a BMI of 24–30 (overweight or mildly obese) had the lowest risk of death in all-cause mortality risk¹⁵. A recent study indicated that the adverse association of obesity diminishes with age, ultimately culminating in a protective effect from a higher BMI in elders over 80 years old¹⁸. Studies encompassing over 880,000 individuals demonstrated that overweight is associated with a better prognosis and reduced all-cause mortality for cardiovascular disease patients^{16–22}. In a study of dietary restriction in diversity outbred mice, the mice that retained more weight and adiposity had longer lifespans, especially for older mice²³. For migratory animals²⁴, hibernating bears²⁵, and fat-tailed sheep²⁶, high levels of body fat was beneficial for survival in unfavorable environments. Collectively, these studies reveal

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a positive correlation between lifespan extension and modest lipid deposition, which may be called healthy fat. However, whether modest lipid deposition can indeed slow aging and what the regulatory mechanism is remains unknown.

A recent study using multi-omics analyses showed substantial changes in metabolic and aging biomarkers during the two critical transition periods of 40 and 60 years old²⁷, which increase the risk of

lipid deposition²⁸. It remains unknown how animals at these stages sense what signal to regulate lipid deposition for healthspan. The model organism *C. elegans* has been long and widely used for lifespan studies. Using a targeted metabolomic approach, we found that the amino acid citrulline was significantly upregulated in older worms, and that the transcription factor MXL-3 functions to regulate the synthesis of citrulline, which then activates lipogenesis via the lipogenic

Fig. 1 | Citrulline drives aging-related lipid deposition for healthspan.

a Representative images of *C. elegans* at larval stage 4 (L4); young adult (YA), and old adult (OA). Scale bar, 200 μ m. **b** Lipid droplets (LDs) of YA and OA wild-type (N2) worms visualized by LipidTOX, DHS-3::GFP or PLIN-1::mCherry. Scale bar, 2 μ m (enlarged), 10 μ m (others). **c, d** Quantification of LD diameter (**c**) and percentage of LDs > 2 μ m (**d**) from (**b**). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). **e** Percentage of TAG in total lipids (TAG + PL) by TLC/GC. Data are presented as the mean $\pm$ SD, $n = 3$ biological replicates. ** $P < 0.01$ (YA vs. OA, Student's t test). **f** Significantly changed amino acids or derivatives during aging analyzed by metabolomics. SAM: S-Adenosylmethionine. Data are presented as the mean $\pm$ SD, $n = 5$ biological replicates. **g** LipidTOX staining of worms supplemented with citrulline (Cit) at different concentrations. Scale bar, 10 μ m. **h** Quantification of LD diameter from

(**g**). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. ** $P < 0.001$ (YA vs. OA, Student's t test). # $P < 0.05$, ## $P < 0.01$ (Cit vs. control, Student's t test). **i** Percentage of TAG in total lipids (TAG + PL) by TLC/GC. Data are presented as the mean $\pm$ SD, $n = 3$ biological replicates. *** $P < 0.001$ (YA vs. OA, Student's t test). ## $P < 0.01$ (Cit vs. control, Student's t test). **j** Survival curves on NGM plates with or without 10 mM citrulline (Cit). (monitored from L4). $n = 120$ worms. **k** 50% survival time from (**j**). Data are presented as the mean $\pm$ SD, $n = 4$ biological replicates. ## $P < 0.01$ (Cit vs. control, Student's t test). **l**, Survival curves of OA worms under starvation conditions (M9) with or without 10 mM citrulline. $n =$ total worms scored. **m** 50% starvation survival time from (**l**). Data are presented as the mean $\pm$ SD, $n = 4$ biological replicates. * $P < 0.05$ (Cit vs. control, Student's t test). Source data with detailed p -values are provided as a Source Data file.

enzymes DGAT-2 and MBOA-2, consequently triggering aging-related lipid deposition in lipid droplets for lifespan extension.

Results

Citrulline coordinately promotes lipid remodeling and lifespan extension in *C. elegans*

In humans, during aging, metabolism is drastically changed at the 40-year transition²⁷, which is related to obesity risks²⁸. In brief, the life cycle of *C. elegans* goes through four larval stages (L1–L4) and two adult stages. Young adult (YA) worms lay eggs for about 5 days, and then transition into the old adult (OA) stage until death (Fig. 1a). The passage to the OA stage in worms may mimic the 40-year transition in humans. Compared to the YA worms, the OA worms displayed a significantly increased size of lipid droplets (LDs) indicated by LipidTOX staining as well as two LD markers DHS-3::GFP *{ldrls1 [dhs-3p::dhs-3::gfp +unc-76(+)]}* and PLIN-1::mCherry *{ldrls2 [plin-1p::plin-1::mCherry+unc-76(+)]}* (Fig. 1b–d)^{29,30}. Quantification of lipids by thin-layer chromatography/gas chromatography (TLC/GC) further confirmed that OA worms store higher levels of triglycerides (TAGs) than YA worms (Fig. 1e). Notably, this age-related increased lipid accumulation occurred even in worms fed on a low-fat diet, *E. coli HT115* (Fig. 1b–e)³¹, suggesting an intrinsic metabolic remodeling rather than dietary effects. Collectively, these data demonstrated an age-related lipid remodeling characterized by LD expansion and TAG accumulation.

To explore what drives age-related lipid remodeling, quantitative metabolomics was performed (Fig. 1f). In total, 156 metabolites were identified having significantly different amounts between YA and OA worms (Supplementary Fig. 1a,b and Supplementary Data 1). Of which, 63 amino acids and derivatives were enriched at OA stages (Fig. 1f and Supplementary Fig. 1b). Since S-Adenosylmethionine (SAM) and citrulline were the top two metabolites with the highest increased levels in OA worms (Fig. 1f), and methionine and arginine were previously shown to extend lifespan in *C. elegans*³², we therefore focused on these metabolites. We hypothesized that the age-related lipid accumulation in OA worms may be due to an increase or decrease of a specific metabolite, and thus dietary supplementation of such a metabolite should correspondingly promote lipid accumulation in YA worms or alleviate lipid accumulation in OA worms, respectively; while its reduction should lead to opposite effects.

Although the levels of methionine and arginine were decreased in OA worms (Fig. 1f), dietary supplementation of either had no effect at all on lipid accumulation in both YA and OA worms (Supplementary Fig. 1c, d), suggesting that the increased lipid deposition in OA worms may not be due to reduced levels of methionine and arginine. In contrast, dietary supplementation of either SAM (Supplementary Fig. 1c, d) or citrulline (Fig. 1g, h) could promote lipid accumulation in both YA and OA worms, whereas dietary ornithine supplementation had no effects (Supplementary Fig. 1c, d) in spite of its upregulation in OA worms (Fig. 1f). Interestingly, deficiency of SAM by knockdown or knockout of *sams-1* (S-adenosyl methionine synthetase) has been shown to extend lifespan^{33,34}, but increase lipid droplet

accumulation^{35–37} in *C. elegans*, suggesting that both an excess and deficiency of SAM can lead to lipid accumulation, therefore we excluded SAM.

Next, we analyzed published data regarding citrulline in humans and found that compared to young people, the level of serum/plasma citrulline was significantly increased in elderly people (Supplementary Data 2)^{38–50}, implying a potential role of citrulline related to aging, whereas its role linking lipid metabolism and lifespan was unknown. Thus, we focused on citrulline. Because a 10 mM supplement gave a significant increase of LD size and TAG level in both YA and OA worms (Fig. 1g–i) but did not affect development (Supplementary Fig. 1e,f), we used this concentration for all of the following experiments to test its effect on lifespan. Dietary supplementation of citrulline significantly increased the level of citrulline in both YA and OA worms (Supplementary Fig. 1g). Remarkably, it promoted lifespan extension in worms under both normal culture and starvation conditions, in which the 50% survival rate was significantly increased from 14.5 ± 0.5 to 17.0 ± 0.8 days, and 7.3 ± 0.5 to 8.3 ± 0.5 days (Fig. 1j–m), respectively. Taken together, these data suggest that citrulline is a newly identified metabolite in coordinately regulating lipid remodeling and lifespan extension.

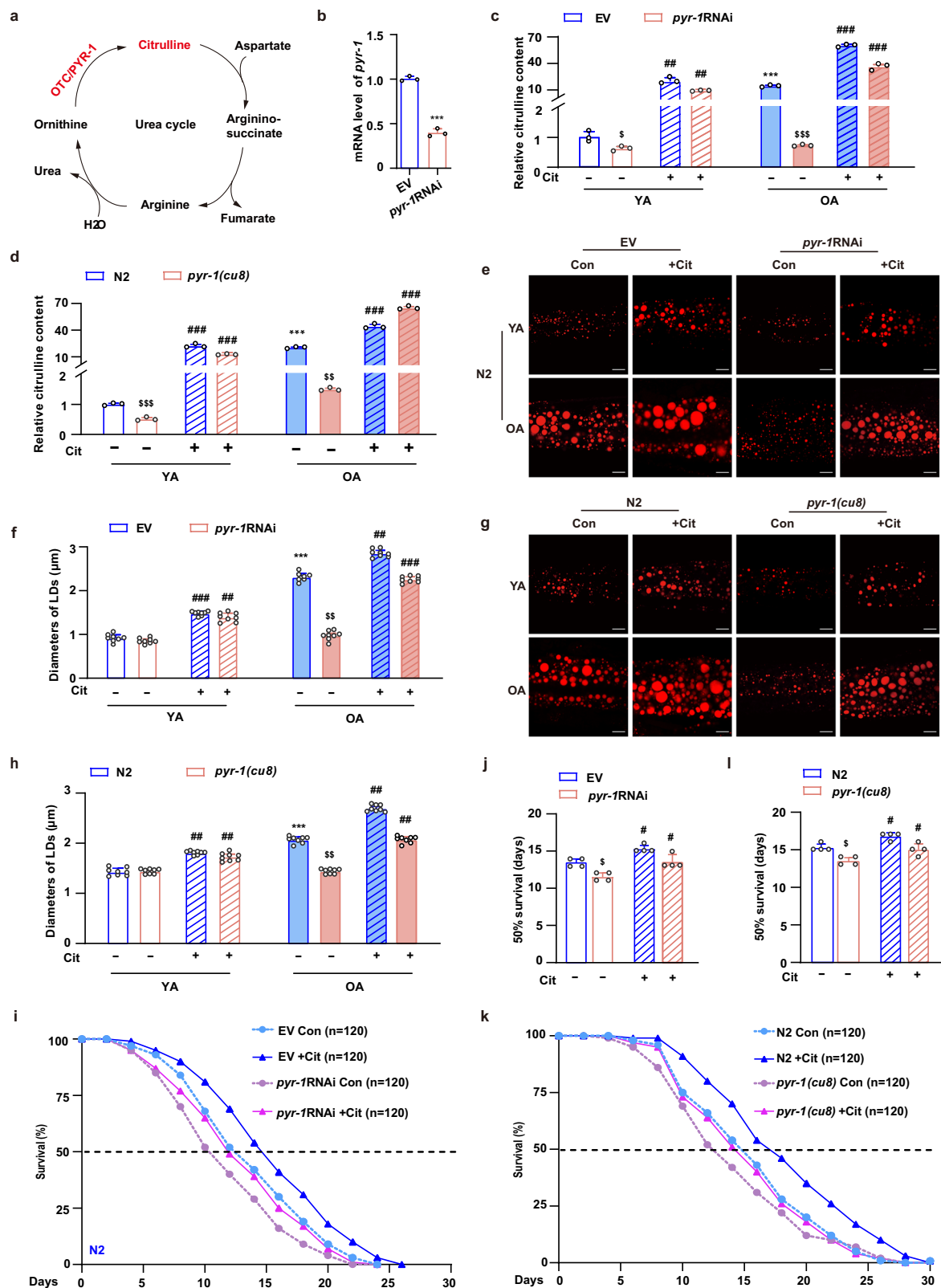
Genetic disruption of citrulline biosynthesis attenuates lipid remodeling and lifespan extension

Citrulline is synthesized through ornithine transcarbamylase (OTC) in the urea cycle⁵¹ (Fig. 2a), while whether citrulline biosynthesis is present in *C. elegans* is unknown. PYR-1 (Fig. 2a) was identified as the single homolog in *C. elegans* through a BLAST search in Wormbase (www.wormbase.org) using human OTC (P00480). RNAi-mediated knockdown of *pyr-1* significantly reduced the mRNA expression of *pyr-1* (Fig. 2b), and also the level of citrulline in OA worms (Fig. 2c), as did in a loss-of-function mutant, *pyr-1(cu8)*, in OA worms (Fig. 2d), suggesting that PYR-1/OTC is involved in citrulline biosynthesis and that this pathway is also conserved in *C. elegans*.

Although *pyr-1* RNAi had no obvious effect on lipid accumulation in YA worms, it completely suppressed the age-related LD enlargement and lipid accumulation in OA worms, which could be fully rescued by citrulline supplementation (Fig. 2e, f). Consistently, the *pyr-1(cu8)* mutant worms showed similar phenotypes as *pyr-1* RNAi worms (Fig. 2g, h), suggesting that PYR-1 is indeed involved in citrulline biosynthesis. More importantly, dysfunction of PYR-1 by either RNAi or mutation consistently led to shorter lifespan than the control (EV or N2, respectively), which could be fully recovered by citrulline supplementation under both normal culture and starvation conditions (Fig. 2i–l). Collectively, these findings further reinforce that citrulline is the driver to remodel lipid metabolism for lifespan extension.

MXL-3 regulates citrulline to remodel lipid metabolism for lifespan extension

To explore how citrulline regulates lipid remodeling for lifespan extension, a transcriptomics analysis was carried out between YA



worms and OA worms. Since OA worms displayed increased lipid accumulation, we therefore focused on genes involved in lipid metabolism. A comparative analysis revealed that the expression of lipogenic genes was upregulated, such as *pod-2*, *fasn-1*, *dgat-2*, and *mboa-2*, while the expression of lipolytic/oxidation genes, such as *atgl-1* and *acox-1.3*, was downregulated in OA worms (Fig. 3a and Supplementary Data 3). These results were further confirmed by quantitative PCR

(qPCR) (Supplementary Fig. 2a). We and other groups previously reported several transcription factors, including *nhr-49*^{52,53}, *nhr-80*^{54,55}, *pha-4*⁵⁶, *daf-16*⁵⁷, and *mxl-3*^{58,59} as being involved in lipid metabolism in *C. elegans*, and their transcriptional expression was also upregulated in OA worms (Fig. 3b). Activated NHR-80 and NHR-49, mediated through the lipid oleoylethanolamide (OEA) and dihomog- γ -linolenic acid (DGLA), promote longevity^{53,60}. DAF-16 is involved in insulin/IGF-1

Fig. 2 | Dysfunction of citrulline biosynthesis impairs age-related lipid deposition and healthspan. **a** Urea cycle pathway. OTC: ornithine transcarbamylase. **b** Relative mRNA level of *pyr-1* in indicated worms. Data are presented as the mean $\pm$ SD, $n = 3$ biological replicates. *** $P < 0.001$ (YA vs. OA, Student's t test). **c, d** Relative citrulline levels in YA and OA worms. Data are presented as the mean $\pm$ SD, $n = 3$ biological replicates. *** $P < 0.001$ (YA vs. OA, Student's t test). *** $P < 0.001$ (Cit vs. control, Student's t test). $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.001$ (*pyr-1* vs. wild type, Student's t test). **e, g** LipidTOX staining of fixed worms. Scale bar, 10 μ m. **f, h** Quantification of LD diameter from **(e)** and **(g)**, respectively. Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$

signals and other signals to regulate longevity and lipid metabolism^{61,62}. PHA-4 mediates diet-restriction-induced longevity⁶³. Thus, we initially examined these factors in response to citrulline and aging-related lipid remodeling.

Similar to wild-type N2 worms, the loss-of-function mutations of *nhr-49(nr2041)*, *nhr-80(tm1011)*, *pha-4(zu225)*, *hlh-30(tm1978)*, and *daf-16(mu86)* worms still showed significantly increased lipid accumulation at the OA stage compared to the YA stage (Supplementary Fig. 2b, c), suggesting that these factors may not be involved in aging-related lipid deposition. The insulin/IGF-1 signaling and diet restriction pathways are conserved from *C. elegans* to mammals to regulate longevity. Unlike the N2 worms (Figs. 1j, 2i), either *pyr-1* RNAi or dietary citrulline could not affect the lifespan of *daf-2(e1370)* worms (Supplementary Fig. 2d), a mutation of the insulin/IGF-1 receptor⁶⁴. In addition, dietary citrulline could not extend the lifespan of *eat-2(ad465)* worms (Supplementary Fig. 2e), a genetic model for dietary restriction⁶⁵. Therefore, citrulline-triggered lipid accumulation for lifespan extension may be independent of these signaling pathways.

In contrast, although the LD size was indistinguishable between N2 worms and the *mxl-3(ok1947)* mutant worms at the YA stage, the lipid accumulation of *mxl-3(ok1947)* worms was still unchanged at the OA stage, unlike the N2 worms (Fig. 3c, d). To test the role of MXL-3 in aging-related lipid accumulation, an *mxl-3(full)::gfp* construct was generated and injected to N2 and *mxl-3(ok1947)* worms. This construct had no effect on lipid accumulation in YA worms in either wild type (N2) or the *mxl-3(ok1947)* background, whereas it could rescue the lipid accumulation of *mxl-3(ok1947)* OA worms, similar to N2 worms (Fig. 3c, d). Thus, these results suggest that MXL-3 is the factor involved in aging-related lipid remodeling.

Next, we asked whether MXL-3 regulates citrulline to remodel lipid metabolism and lifespan extension. Similar to the lipid phenotypes, the level of citrulline was apparently unchanged in both the YA and OA *mxl-3(ok1947)* mutant worms, unlike the N2 worms (Fig. 3e). In addition, qPCR analysis showed a reduced level of *pyr-1* mRNA in *mxl-3(ok1947)* mutant worms compared to N2 worms at OA stage (Supplementary Fig. 3a). We then generated a construct (*pyr-1p::gfp*) using the promoter of *pyr-1* fused with GFP (green fluorescent protein) (Supplementary Fig. 3b) and injected it into N2 and *mxl-3(ok1947)* worms. Similar to the qPCR results, the relative fluorescence intensity of *PYR-1p::GFP* was also reduced in OA *mxl-3(ok1947)* mutant worms (Supplementary Fig. 3c, d). These results suggested that MXL-3 may regulate *PYR-1* expression to affect citrulline level. Furthermore, dietary supplementation of citrulline greatly increased the level of citrulline in *mxl-3(ok1947)* worms (Fig. 3f), while also simultaneously restoring their lipid accumulation back to wild type, as indicated by LipidTOX staining and DHS-3::GFP (Fig. 3g, h) as well as PLIN-1::mCherry (Supplementary Fig. 3e, f). Thus, these results support that MXL-3 regulates citrulline production to remodel aging-related lipid metabolism.

At the OA stage, compared to the N2 worms, the *mxl-3(ok1947)* worms have a lower citrulline level and lipid accumulation, raising the question as to whether MXL-3 has a role in citrulline-regulated lifespan extension. Distinguished from a previous report⁵⁸, the lifespan of *mxl-3(ok1947)* worms was obviously shorter than the N2 worms under both

(YA vs. OA, Student's t test). $^{##}P < 0.01$, $^{###}P < 0.001$ (Cit vs. control, Student's t test). $^{**}P < 0.01$ (*pyr-1* vs. wild type, Student's t test). **i** Survival curves on RNAi plates with or without 10 mM citrulline (Cit). $n = 120$ worms. **j** 50% survival time from **(i)**. Data are presented as the mean $\pm$ SD, $n = 4$ biological replicates. $^{*}P < 0.05$ (Cit vs. control, Student's t test). $^{*}P < 0.05$ (*pyr-1* vs. wild type, Student's t test). **k** Survival curves on NGM plates with or without 10 mM citrulline (Cit) (monitored from L4). $n = 120$ worms. **l** 50% starvation survival time from **(k)**. Data are presented as the mean $\pm$ SD. $n = 4$ biological replicates. $^{*}P < 0.05$ (Cit vs. control, Student's t test). $^{*}P < 0.05$ (*pyr-1* vs. wild type, Student's t test). Source data with detailed p -values are provided as a Source Data file.

our normal culture and starvation conditions (Fig. 3i–l). Consistently, dietary supplementation of citrulline extended the lifespan of N2 worms, and more importantly, it rescued the shorter lifespan of *mxl-3(ok1947)* worms back to normal under both conditions (Fig. 3i–l). Collectively, these lines of evidence suggest that MXL-3 regulates citrulline-triggered lipid accumulation for lifespan extension.

Aging triggers MXL-3 expression and lysosomal translocation

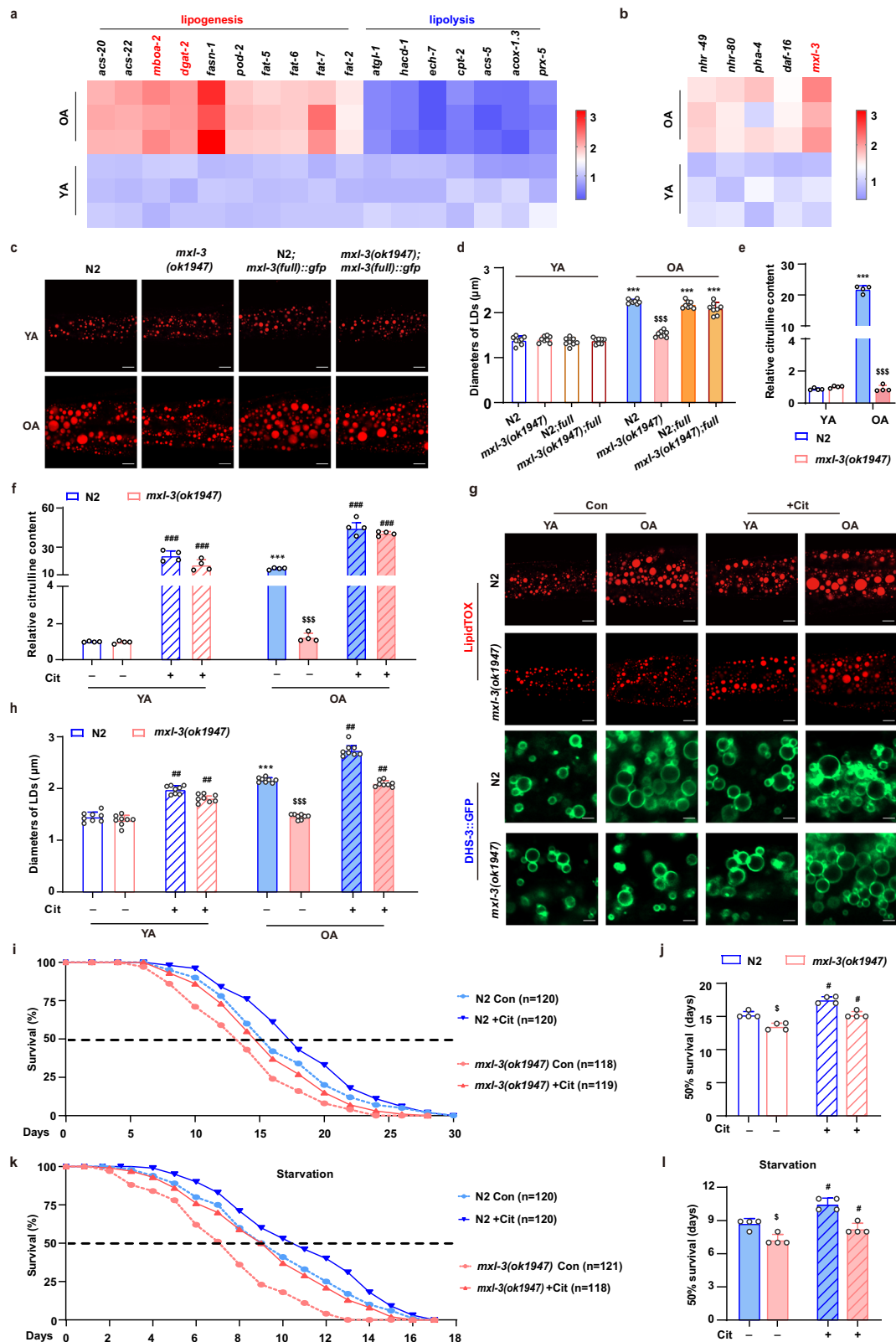
As mentioned above, *mxl-3* transcription was upregulated in OA worms (Fig. 3b and Supplementary Fig. 2a). GFP fluorescence and western blot analysis consistently showed an increased expression of MXL-3::GFP {*wgl5745 [mxl-3::TY1::EGFP::3 × FLAG + unc-119(+)]*} in OA worms compared to the YA worms under control and citrulline treatment (Fig. 4a–d). The MXL-3::GFP was mainly expressed in the nucleus, but also in the cytosol and less in lysosomes in YA worms. Intriguingly, during aging, MXL-3::GFP displayed a pronounced translocation to lysosomes, which could be further enhanced by citrulline treatment (Fig. 4e–g).

To explore the lysosome translocation of MXL-3, we generated other transgenic worm strains, MXL-3(Full)::GFP {*unc-119(ed9);kunEx521[mxl-3p::mxl-3(full)::6 × his::gfp] + unc-119(+)*} and MXL-3(Δ LLS)::GFP {*unc-119(ed9);kunEx510[mxl-3p::mxl-3(Δ lls)::6 × his::gfp] + unc-119(+)*}⁶⁶, lacking the lysosomal localization signal (DMYLL), in the N2 and *mxl-3(ok1947)* mutant background (Fig. 4h). Unlike the MXL-3(Full)::GFP, the MXL-3(Δ LLS)::GFP not only lost the ability to localize to the lysosome (Fig. 4i), but also more importantly, was unable to rescue the lipid phenotype of *mxl-3(ok1947)* mutant worms at the OA stage (Fig. 4j, k), suggesting that this motif (LLS) is required for its roles in both aging-related lipid accumulation and lysosomal localization.

Amino acids 228–235 determines the function of MXL-3 in regulating aging-related lipid deposition for lifespan extension

As mentioned above, since aging-induced citrulline accumulation could promote MXL-3 lysosomal translocation, we therefore asked whether citrulline could bind to MXL-3 reciprocally. The AlphaFold program was applied for structure prediction of MXL-3, and it was able to identify potential binding sites for the substrate citrulline. A number of the top-ranked binding sites were subsequently selected for functional validation based on the binding energies and corresponding hydrogen bond distances of these potential interacting amino acids (AAs) (Fig. 5a, b and Supplementary Fig. 4a). Meanwhile, the AlphaFold structure prediction showed that the MXL-3 is predicted to be a transcription factor located in the cytoplasm and nucleus, containing a confirmed N-terminal lysosomal localization sequence (LLS) (AA7–11) (Fig. 4h) and a basic helix-loop-helix (bHLH) domain (AA48–116) (Fig. 5c).

To test the functions of these potential binding sites and their response to citrulline, several transgenic worms were generated with corresponding deletions of these amino acids (Fig. 5c). In the N2 background, the expression of MXL-3(Full)::GFP was mostly translocated to lysosomes during aging or under citrulline treatment. Deletion constructs, such as MXL-3(Δ 54–80)::GFP {*unc-119(ed9);kunEx522[mxl-3p::mxl-3(Δ 54–80)::6 × his::gfp] + unc-119(+)*}



, MXL-3(Δ 117-125)::GFP *{unc-119(ed9);kunEx523[mxl-3p::mxl-3(Δ 117-125)::6 $\times$ his::gfp] + unc-119(+)}*, and MXL-3(Δ 138-160)::GFP *{unc-119(ed9);kunEx524[mxl-3p::mxl-3(Δ 138-160)::6 $\times$ his::gfp] + unc-119(+)}*, all responded to aging and citrulline treatment similar to the MXL-3(Full)::GFP in terms of lipid accumulation and lysosome localization (Supplementary Fig. 4b–d), suggesting that these amino acids are unnecessary for its function and response to citrulline.

In contrast, the MXL-3(Δ 228-235)::GFP *{unc-119(ed9);kunEx525[mxl-3p::mxl-3(Δ 228-235)::6 $\times$ his::gfp] + unc-119(+)}*, with the five potential binding residues D228, Q229, A232, K234, I235 deleted, but still containing the lysosomal localization motif, was retained mainly in the nucleus in OA worms or under citrulline treatment (Fig. 5d, e), suggesting that it lost the ability to respond to citrulline for lysosomal translocation. Next, MXL-3(Δ 228-235)::GFP was then crossed into the

Fig. 3 | MXL-3 mediates citrulline triggered aging-related lipid deposition and healthspan. **a, b** The levels of lipogenesis/lipolysis genes (**a**) and lipid metabolism regulating transcription factors (**b**) in OA and YA worms. **c** LipidTOX staining of fixed worms. Scale bar, 10 μ m. **d** Quantification of LD diameter from (**c**). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). **** $P < 0.001$ (mxl-3 vs. wild type, Student's t test). **e** Relative citrulline levels in YA and OA worms analyzed by metabolomics. Data are presented as the mean $\pm$ SD, $n = 4$ biological replicates. *** $P < 0.001$ (YA vs. OA, Student's t test). **** $P < 0.001$ (mxl-3 vs. wild type, Student's t test). **f** Relative citrulline level in worms analyzed by LC/MS. Data are presented as the mean $\pm$ SD, $n = 4$ biological replicates. *** $P < 0.001$ (YA vs. OA, Student's t test). **** $P < 0.001$ (Cit vs. control, Student's t test). **** $P < 0.001$ (mxl-3 vs. wild type, Student's t test). **g** LDs in worms visualized by LipidTOX and DHS-3::GFP. Scale bar, 10 μ m (LipidTOX),

2 μ m (DHS-3::GFP). **h** Quantification of LD diameter from (**g**). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). ** $P < 0.01$ (Cit vs. control, Student's t test). **** $P < 0.001$ (mxl-3 vs. wild type, Student's t test). **i** Survival curves on NGM plates with or without 10 mM citrulline (Cit) (monitored from L4). n = total worms scored. **j** 50% survival time from (**i**). Data are presented as the mean $\pm$ SD, $n = 4$ biological replicates. * $P < 0.05$ (Cit vs. control, Student's t test). * $P < 0.05$ (mxl-3 vs. wild type, Student's t test). **k** Survival curves of OA worms under starvation conditions (M9) with or without 10 mM citrulline. n = total worms scored. **l** 50% starvation survival time from (**k**). Data are presented as the mean $\pm$ SD, $n = 4$ biological replicates. * $P < 0.05$ (Cit vs. control, Student's t test). * $P < 0.05$ (mxl-3 vs. wild type, Student's t test). Source data with detailed p -values are provided as a Source Data file.

mxl-3(ok1947) mutant worms. Unlike MXL-3(Full)::GFP, the MXL-3(Δ 228–235)::GFP protein failed to rescue the lipid accumulation (Fig. 5f, g) and lifespan (Fig. 5h) phenotypes of the *mxl-3(ok1947)* worms. Moreover, unlike other constructs, MXL-3(Δ 228–235)::GFP could not restore the expression of *pyr-1* in *mxl-3(ok1947)* OA worms as well (Supplementary Fig. 4e). Collectively, these lines of evidence demonstrate that amino acids 228–235 of MXL-3 are indispensable for its function in regulating citrulline-triggered lipid deposition for lifespan extension.

Next, to test whether MXL-3 can directly bind to citrulline, a microscale thermophoresis (MST) assay was performed. Two constructs, the wild-type MXL-3 and truncated MXL-3 (Δ 228–235 AA) fused with His tag (Fig. 5i), were generated and then expressed in the mammalian 293 F cell line. MST analysis showed that the purified MXL-3 protein displayed binding activity to citrulline, while the truncated MXL-3 (Δ 228–235 AA) protein completely lost the binding activity (Fig. 5j). However, citrulline supplementation still promoted lipid accumulation in *mxl-3(ok1947);mxl-3(Δ 228–235)::gfp* worms, similar to *mxl-3(ok1947)* worms (Supplementary Fig. 4f, g), further strengthening that MXL-3 acts upstream of citrulline to regulate aging-related lipid deposition, while the binding of MXL-3 and citrulline may be an accompanying phenomenon and is not required for citrulline-mediated lipid accumulation.

Citrulline activates DGAT-2/MBOA-2 mediated lipogenesis for lifespan extension

Since increased citrulline led to lipid accumulation, we next asked how it remodeled lipid metabolism. The RNA-seq data revealed that OA worms displayed upregulated lipogenesis (Fig. 3a). Diacylglycerol acyltransferase (DGAT) is a key enzyme in triglyceride synthesis, catalyzing the covalent binding of diacylglycerol (DAG) and fatty acid acyl-CoA to form triglycerides⁶⁷. In *C. elegans*, several genes, including *dgat-2* and *mboa-1* to *mboa-7* are the homologs of mammalian DGAT^{55,56,68,69}. Mutants of these genes were used to examine their function in response to aging and citrulline. Similar to the N2 worms, the single mutant worms of each gene still showed increased lipid accumulation at the OA stage (Fig. 6a–c and Supplementary Fig. 5a, b). In contrast, the aging-induced lipid accumulation was absent in the *dgat-2(kun140);mboa-2(gk870525)* double mutant worms (Fig. 6a–c).

We next generated a transgenic MBOA-2::GFP worm [*unc-119(ed9);kunEx520[mboa-2::6 $\times$ his::gfp] + unc-119(+)*]. It showed an increased GFP fluorescence and protein level under OA and citrulline treatment, as did GFP::DGAT-2 worms [*hjsi56 [vha-6p::3xFLAG::TEV::GFP::dgat-2::let-858 3'UTR]*] (Supplementary Fig. 5c–f)⁶⁸. These results are consistent with the upregulated mRNA level of both *dgat-2* and *mboa-2* in OA worms (Fig. 3a and Supplementary Fig. 2a). RNAi knockdown of either *dgat-2* or *mboa-2* significantly repressed the fluorescence expression of GFP::DGAT-2 or MBOA-2::GFP, respectively (Supplementary Fig. 5g, h). The *dgat-2* RNAi had no effect on the aging-related lipid accumulation in other *mboa-2* mutant worms (Supplementary Fig. 5a, b). Similar to the *mboa-2(gk870525)* mutant (Fig. 6a–c), *mboa-2* RNAi repressed the lipid accumulation in all three *dgat-2* mutant worms at the

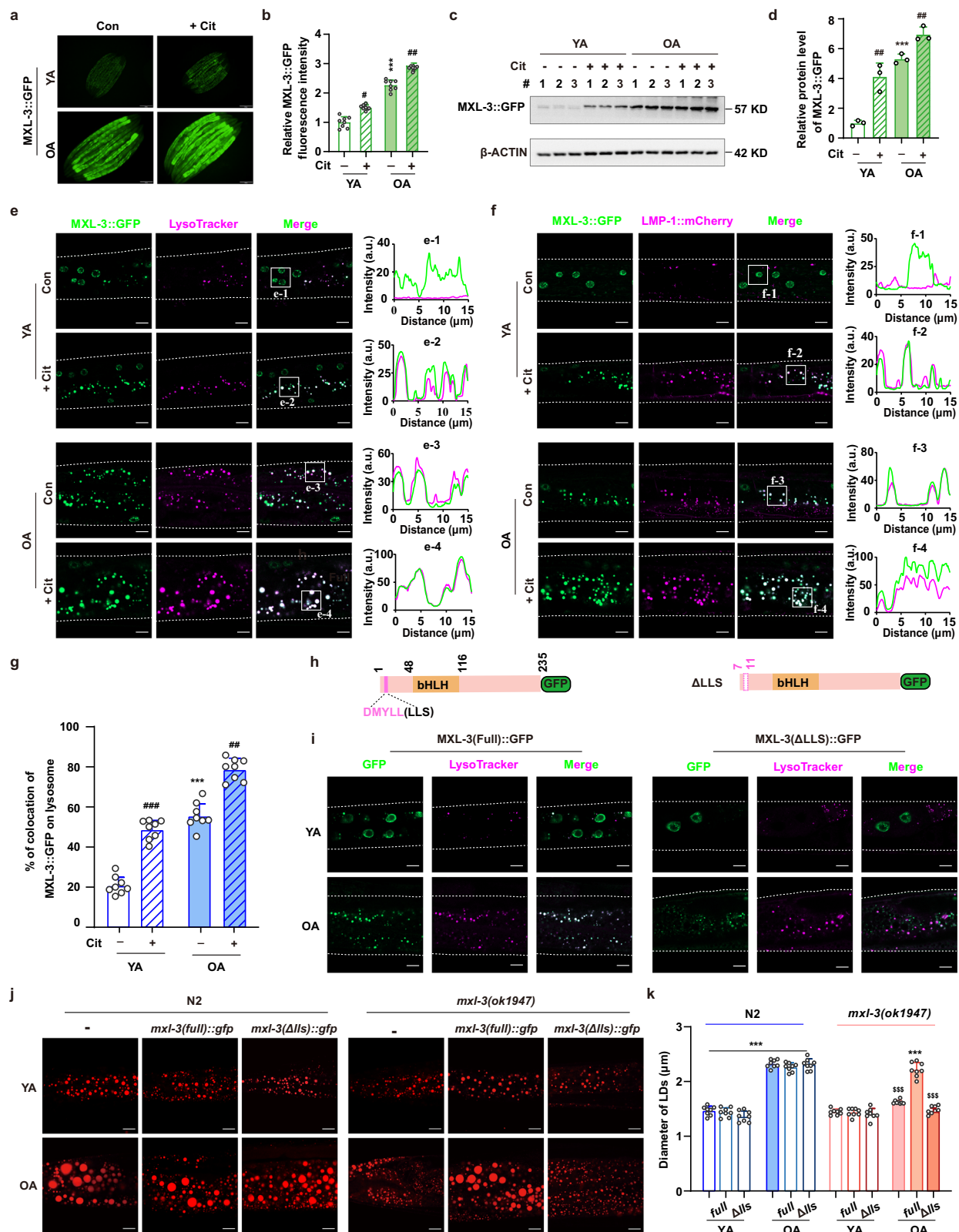
OA stage (Supplementary Fig. 5i, j). Furthermore, we generated two constructs, *dgat-2p::dgat-2::his::gfp* and *mboa-2p::mboa-2::his::gfp* (Supplementary Fig. 5k), and individually injected into N2 and *dgat-2(kun140);mboa-2(gk870525)* mutant worms. Both constructs could successfully rescue the lipid accumulation of *dgat-2(kun140);mboa-2(gk870525)* mutant worms at both the YA and OA stage, although they did not affect the lipid accumulation in N2 worms (Fig. 6d, e). More importantly, they completely recovered the reduced lifespan of *dgat-2(kun140);mboa-2(gk870525)* mutant worms back to the same as N2 (Fig. 6f, g). Taken together, these lines of evidence support a role of DGAT-2/MBOA-2 mediating lipogenesis in aging-related lipid accumulation and lifespan extension.

Next, the *dgat-2(kun140);mboa-2(gk870525)* double mutant worms, along with the wild type N2 worms, were subjected to dietary citrulline treatment. Remarkably, supplemented citrulline completely failed to increase the lipid accumulation (Fig. 6h–j) and to rescue the lifespan (Fig. 6k–n) in *dgat-2(kun140);mboa-2(gk870525)* worms. This result is completely different from the *mxl-3(ok1947)* worms (Fig. 3g–l), even though all these mutant worms had similar phenotypes with resistance to age-induced lipid accumulation and a shortened lifespan. Collectively, these results further reinforce that DGAT-2/MBOA-2 mediated lipogenesis is necessary and essential for citrulline-induced lipid accumulation and lifespan extension during aging.

MXL-3-citrulline axis regulates the expression and lysosomal localization of DGAT-2/MBOA-2

In *C. elegans*, DGAT-2 is known as an endoplasmic reticulum (ER) or LD-associated protein for lipid synthesis^{68,70}, but the subcellular localization of MBOA-2 remains poorly characterized. The colocalization of MBOA-2::GFP with the ER marker mCherry::HDEL [*hjsi158 [vha-6p::sel-1(1-79)::mCherry::hdel::let-858 3'UTR]*], LD marker PLIN-1::mCherry, and lysosomal dye LysoTracker Red, revealed that it was localized to these intracellular organelles (Supplementary Fig. 6). Interestingly, the fluorescence of MBOA-2::GFP exhibited increased lysosomal translocation at the OA stage (Fig. 7a, b and Supplementary Fig. 6), which was further greatly enhanced by citrulline treatment (Fig. 7a, b). A similar phenomenon was seen with GFP::DGAT-2 to different extents (Fig. 7a, b). These results suggest that aging or citrulline treatment promotes lysosomal translocation of both MBOA-2 and DGAT-2, similar to MXL-3.

Next, to test the relationship between MXL-3 and DGAT-2/MBOA-2, MBOA-2::GFP and GFP::DGAT-2 was individually crossed into the *mxl-3(ok1947)* mutant. The fluorescence intensity of MBOA-2::GFP and GFP::DGAT-2 (Supplementary Fig. 7a–d) and especially lysosomal translocation (Fig. 7c–e), were significantly reduced in the *mxl-3(ok1947)* mutant compared to wild type N2 at the OA stage. These effects could be recovered by citrulline treatment (Fig. 7c–e), suggesting that MXL-3 acts upstream to regulate the expression and lysosomal translocation of MBOA-2 and DGAT-2. Similar effects were observed under RNAi treatment of *pyr-1*, a downstream effector of



MXL-3 (Supplementary Fig. 8). Moreover, a triple mutant *mxl-3(ok1947);dgat-2(kun140);mboa-2(gk870525)* was generated. It failed to recover the lipid accumulation at the OA stages under citrulline treatment, a result similar to the double mutants *dgat-2(kun140);mboa-2(gk870525)*, but different from the single *mxl-3(ok1947)* mutant (Fig. 7f, g), suggesting that DGAT-2/MBOA-2 mediated lipogenesis is necessary for MXL-3-citrulline promoting age-related lipid

accumulation. Collectively, these data further support an MXL-3-citrulline-DGAT-2/MBOA-2 axis regulating lipid remodeling during aging (Fig. 8).

Discussion

Either excessive lipid deposition or a lipid deficiency increases the risk of mortality. Studies on human and animals revealed that maintaining

Fig. 4 | MXL-3 responds to citrulline to translocate to lysosomes for lipid deposition and healthspan. **a, b** Fluorescence intensity (**a**) and quantification (**b**) of MXL-3::GFP. Scale bar, 200 μ m. Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). ** $P < 0.05$, # $P < 0.01$ (Cit vs. control, Student's t test). **c** Western blot of MXL-3::GFP with anti-GFP antibody, $n = 3$ three biological replicates (#1-3). β -ACTIN was used as a loading control. **d** Quantitative analysis of MXL-3::GFP level from (**c**). Data are presented as the mean $\pm$ SD, $n = 3$ biological replicates (#1-3). *** $P < 0.001$ (YA vs. OA, Student's t test). ** $P < 0.01$ (Cit vs. control, Student's t test). **e, f** Left: Localization of MXL-3::GFP relative to lysosomes stained by LysoTracker (**e**) or marked by LMP-1::mCherry (**f**). Scale bar, 10 μ m. Right: Intensity profiles of labeled scans from high-magnification regions, e-1-4 and f-1-4, showing the localization of MXL-3::GFP

with lysosomes as marked on the left. **g** Quantification of MXL-3::GFP co-localization with lysosomes from (**e**). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). ** $P < 0.01$, *** $P < 0.001$ (Cit vs. control, Student's t test). **h** Schematic of full-length MXL-3(Full)::GFP, and lysosomal localization signal (LLS) deletion MXL-3(Δ LLS)::GFP. The location of the LLS and bHLH domain is indicated. **i** Localization of full-length/LLS-deletion MXL-3::GFP with respect to lysosomes stained by LysoTracker. Scale bar, 10 μ m. **j** LipidTOX staining of fixed worms. Scale bar, 10 μ m. **k** Quantification of LD diameter from (**j**). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). \$\$\$ $P < 0.001$ (Indicated strain vs. wild type, Student's t test).

proper physiological lipid accumulation may confer health benefits and aid recovery in the elderly^{13-17,19-23}. Our work in *C. elegans* confirmed that an increase of lipid deposition during aging is essential and necessary to ensure healthspan. Moreover, we show that citrulline, a urea cycle metabolite, triggers aging-related lipid remodeling through the lipogenic enzymes DGAT-2 and MBOA-2 to ensure healthspan in *C. elegans*, though the underlying mechanism for this requires further investigation. Aging activates the transcription factor MXL-3 expression, which then upregulates the ornithine transcarbamylase PYR-1 expression and citrulline biosynthesis for lipid deposition and healthspan. Depletion of lipid deposition by mutation of genes involving in citrulline biosynthesis, such as PYR-1 and its regulator MXL-3, reduces worm lifespan under both normal and starvation conditions. As well, restoring lipid deposition by dietary supplementation of citrulline rescues their lifespan. This effect is completely abolished in worms with a dysfunction of both lipogenic enzymes DGAT-2 and MBOA-2, underscoring that lipid deposition is essential for its pro-longevity effects. Thus, we uncover citrulline as the specific metabolite driving DGAT-2/MBOA-2-mediated lipogenesis in regulating aging-related lipid deposition for healthspan (Fig. 8). To the best of our knowledge, this study may be the first using the model organism *C. elegans* to show that aging-related lipid deposition is healthy fat, which is indeed necessary and beneficial for healthspan.

Metabolites play pivotal roles in aging by modulating energy metabolism, oxidative stress, and inflammation, among other processes. Through targeted metabolomics, we found that SAM and citrulline displayed as the top two hits with the highest increased level in OA worms compared to YA worms. Current studies have reported a complicated role of SAM in lipid metabolism and lifespan. Lower SAM synthesis can be beneficial for longevity in *C. elegans*^{33,34}; but reversely, shorten lifespan in *Drosophila*⁷¹. Moreover, reduction of SAM synthesis also leads to increased lipid accumulation in *C. elegans*, mouse liver, and human cells^{35,72}. Conversely, in mice, genetic knock-out of the glycine N-methyltransferase gene results in an increase of SAM and hepatic steatosis, fibrosis, and hepatocellular carcinoma (HCC)^{72,73}. Consistently, our work showed that dietary supplementation of SAM could promote lipid accumulation in both YA and OA worms. Thus, both a deficiency and an excess of SAM can lead to lipid accumulation, and the distinct roles of SAM in lipid metabolism and lifespan extension may be context-dependent.

Citrulline, a non-proteinogenic amino acid, functions as a metabolite linking lipid accumulation for lifespan extension. Aging triggers the accumulation of citrulline, which is essential and necessary to maintain lifespan in *C. elegans*. Studies in rodent models have documented an age-related decline of citrulline levels in mice^{74,75} and rats, with notable reductions in specific brain regions such as the vestibular nucleus (VNC) and cerebellum (CE) when comparing young (4 months) versus old (24 months) animals^{76,77}. In contrast, human data have consistently revealed a positive correlation between circulating citrulline levels and advancing age^{40,45,47,78,79}. This trend is further corroborated by multiple large-scale studies^{42,48-50}, including evidence of elevated citrulline in longevity cohorts^{38,39,41,44,46}. Why there is a

discrepancy between rodents and humans is unknown, and may be due to the difference of species or the specific organs and tissues investigated. Nevertheless, our findings in *C. elegans* demonstrate a similar age-dependent increase in citrulline, which aligns more closely with human epidemiological data instead of the rodent models. This conserved pattern underscores the utility of *C. elegans* as an experimental model for uncovering the mechanisms of citrulline in aging.

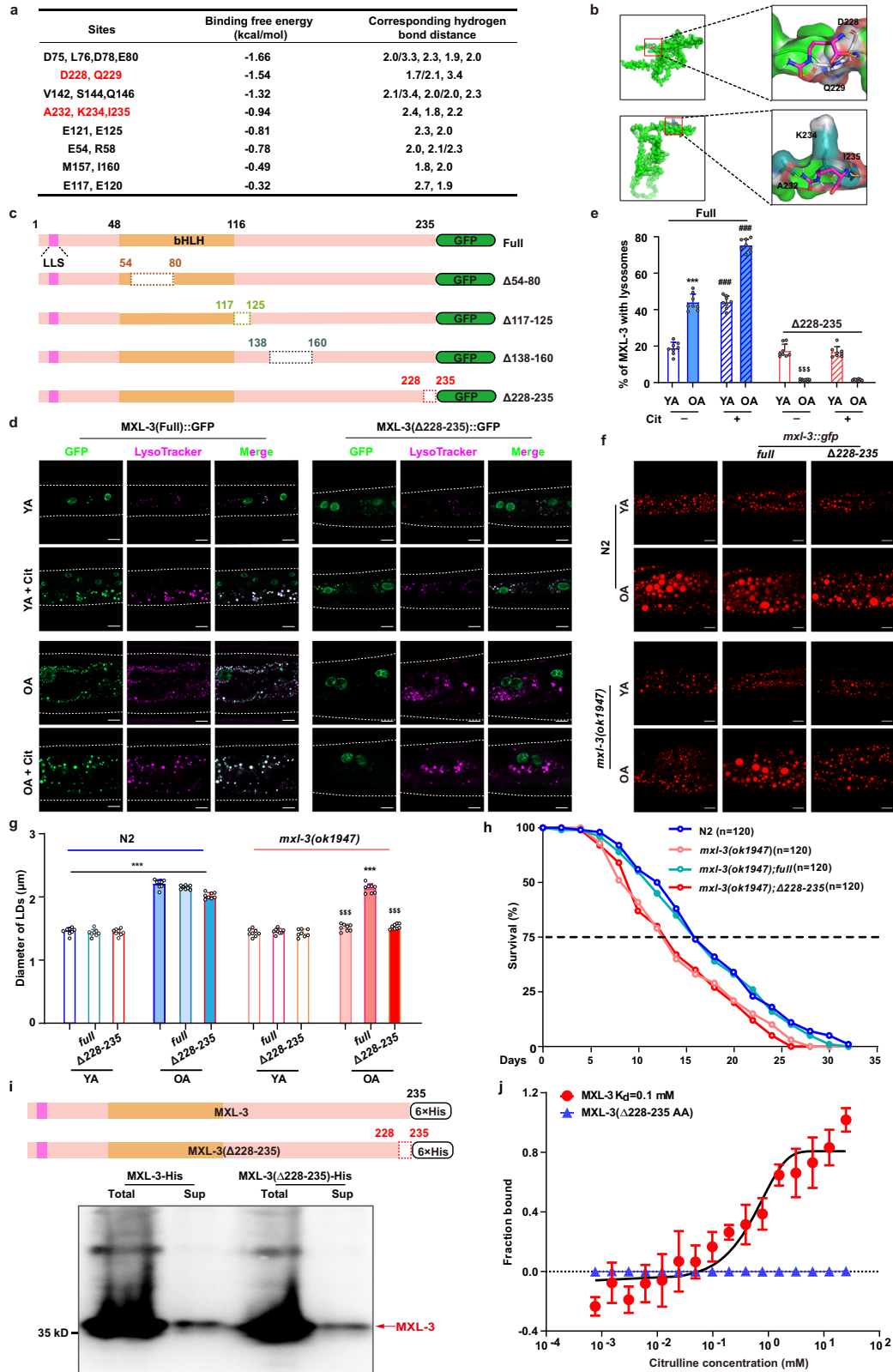
The lysosome integrates nutrient, energy, and lipid signals to centrally govern longevity⁸⁰. In particular, lysosomes act as the mTORC1 activation site for amino acid sensing, as well as being associated with the AMPK platform for glucose and energy monitoring⁸¹⁻⁸³. Intriguingly, during aging, MXL-3 undergoes a striking lysosomal translocation, a process enhanced by citrulline. Five amino acids (DMYLL) in the N-terminus of MXL-3 are required for its lysosomal localization. Deletion of this lysosomal localization signal not only impaired its localization to lysosomes but, more importantly, failed to rescue the lipid deposition in OA worms. Likewise, the region of AA228-235 is essential for functions of MXL-3, including regulating lysosomal translocation, *pyr-1* expression, lipid deposition, and lifespan; so does the binding to citrulline, though it may be an accompanying phenomenon. Moreover, MXL-3, which may function through PYR-1-mediated citrulline synthesis, regulates age-dependent lysosomal localization of DGAT-2 and MBOA-2, which may challenge these enzymes as ER or lipid droplet-associated proteins. These observations expand our knowledge regarding lysosomes functioning as the central hub in regulating metabolism and lifespan.

Oral citrulline supplementation has been demonstrated with compelling benefits. Primarily functioning as a superior precursor to arginine, it effectively elevates plasma arginine levels and enhances nitric oxide (NO) production, thereby supporting vascular endothelial function and hemodynamics, which is a key concern in aging populations⁸⁴⁻⁸⁷. In addition, it improves body composition and attenuates age-related metabolic dysfunction⁸⁸. It also has been shown to regulate muscle protein metabolism by restoring nitrogen balance and enhancing protein synthesis in malnourished aged rats⁸⁹⁻⁹¹, and it ameliorates cognitive deficits in aging models⁹². Furthermore, citrulline exhibits antioxidant properties by acting as a potent hydroxyl radical scavenger, thereby reducing oxidative stress associated with aging and physical exertion^{93,94}. More importantly, oral L-citrulline supplementation is generally well-tolerated across a wide range of doses, with no serious adverse effects reported in both human and animal models^{95,96}, thus making it a compelling nutritional intervention for mitigating aging.

Methods

C. elegans strain maintenance and RNAi

Worms were grown on NGM plates seeded with *Escherichia coli* OP50 or HT115. All experiments were conducted at 20 °C. Strains were obtained from the Caenorhabditis Genetic Center or kindly provided by the Pingsheng Liu lab (Institute of Biophysics, Beijing, China), Xiaochen Wang lab (Southern University of Science and Technology,



Shenzhen, China) and Ho Yi Mak lab (Hong Kong University of Science and Technology, Hong Kong, China). *C. elegans* strains used in this study are listed in Supplementary Data 4. RNAi was performed using the feeding method. *E. coli* HT115 carrying the L4440 plasmid as an empty vector or double-stranded RNA-expressing plasmids targeting the genes of interest were purchased from the Ahringer *C. elegans* RNAi library. RNAi bacteria were cultured overnight at 37 °C in LB

medium containing 100 μg/mL ampicillin, and then seeded onto the NGM supplemented with 100 μg/mL ampicillin and 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). Synchronized L1 worms were transferred onto plate seeded with the indicated bacteria, until they reached young adult (YA) or old adult (OA) stages, when they were collected for further analyses. For all RNAi experiments, worms were fed on *E. coli* HT115.

Fig. 5 | The AA228-235 motif in MXL-3 determines its citrulline binding and function in lipid deposition and healthspan. **a** The potential binding sites of MXL-3 with citrulline, along with corresponding binding energies and hydrogen bond distances were determined. **b** Molecular docking analysis of MXL-3 and citrulline predicting the binding pockets and key interactions. **c** Schematic of full-length MXL-3(Full)::GFP, truncated MXL-3(Δ 54-80)::GFP, MXL-3(Δ 117-125)::GFP, MXL-3(Δ 138-160)::GFP, and MXL-3(Δ 228-235)::GFP proteins. **d** Localization of full-length and Δ 228-235 MXL-3::GFP with respect to lysosome stained by LysoTracker. Scale bar, 10 μ m. **e** Quantification of MXL-3::GFP co-localization with lysosomes from (d). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). *** $P < 0.001$ (Cit vs. control, Student's t test). *** $P < 0.001$ (MXL-3(Δ 228-235)::GFP vs. MXL-3(Full)::GFP, Student's t test).

test). **f** LipidTOX staining of fixed worms. Scale bar, 10 μ m. **g** Quantification of LD diameter from (f). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). *** $P < 0.001$ (Indicated strain vs. wild type, Student's t test). **h** Survival curves on NGM plates (monitored from L4). $n = 120$ worms. **i** Diagram of the constructs MXL-3 full-length (MXL-3) and truncated (MXL-3(Δ 228-235 AA)) proteins fused with a His tag, and western blot analysis with anti-His antibody of proteins expressed in mammalian 293 F cells. Total: Total cell lysate; Sup: Soluble supernatant. **j** The binding curves of citrulline with purified full-length MXL-3 and the truncated MXL-3 (Δ 228-235 AA) proteins measured by MST-Nanotemper. Data are presented as the mean $\pm$ SD, $n = 4$ biological replicates.

Generation of transgenic worms

The constructs for the generation of transgenic worms are diagrammed in the indicated corresponding figures. In general, constructed plasmids were injected into *EG4322* (ttTi5605; *unc-119[ed9]*) following the MOS (Minos 1) protocol⁹⁷, or a specifically indicated worm strain background. The sequence information of primers used in this study is listed in Supplementary Data 5.

LipidTOX staining of fixed worms

About 500 YA or OA worms were collected and suspended in 1 mL of water on ice, and then 50 μ L of freshly prepared 10% PFA (paraformaldehyde) solution was added and mixed. Worms were immediately frozen briefly in liquid nitrogen, subjected to two incomplete freeze/thaw cycles, and washed with M9 buffer three times to remove the PFA solution. LipidTOX (1:2000, H34475, Life Technologies) was added to the worms and incubated for 1 hour at room temperature⁷⁰. Next, worms were washed three times with M9 buffer and placed onto 2% agarose pads for microscopic observation and photography. Images were obtained with a Zeiss LSM-800 Meta laser scanning microscope with a 63 $\times$ oil immersion lens in a multi-track mode using a single or dual excitation, which were used to measure the diameter of the LDs with LSM Image software (Carl Zeiss). Three or four biological replicates were performed. In each biological replicate, >100 individual worms were observed for phenotype determination, and eight representative worms were measured for the mean diameter of LDs in each worm strain.

LysoTracker staining

LysoTracker Red DND-99 (L7528, Invitrogen) was used to assess lysosome biosynthesis. *C. elegans* were grown up to YA or OA worms on the indicated bacteria-seeded plates. Then, worms were transferred to a seeded plate containing LysoTracker Red (diluted in M9 1:500) or M9 (control), and cultured for 4 h in the dark prior to observation. Finally, worms were washed three times with M9 buffer and mounted onto 2% agarose pads for microscopic observation and photography with a high-resolution laser confocal microscope (LSM800, Carl ZEISS) with a Plan-Apochromat 63 $\times$ /1.4 Oil DIC objective. Image processing was performed with ZEN software, black edition (Carl Zeiss).

Analysis of TAG

About 50,000 adult worms were harvested. Then the total lipid was extracted and separated by TLC on silica gel plates (1.05721.0001, Supelco) using a hexane:diethyl ether:acetic acid (80:20:2) solvent system⁹⁸. Then, the individual TAG and PL (phospholipid) bands were scraped from the plates, and methylated with methanol containing 2.5% H₂SO₄ for 2 h. Finally, the methylated fatty acids were analyzed using an Agilent 8890 A gas chromatograph (GC) equipped with a 30 m $\times$ 0.25 mm $\times$ 0.25 μ m DBWAX column (Agilent), with nitrogen as the carrier gas at 1.4 mL/min, and a flame ionization detector. The column temperature programming started at 120 $^{\circ}$ C held for 1 min and heated up to 190 $^{\circ}$ C at a rate of 20 $^{\circ}$ C/min. The column temperature

was held for 10 min at 190 $^{\circ}$ C, and then increased to 200 $^{\circ}$ C (2 $^{\circ}$ C/min) and kept for 3 min. The column was then increased to 220 $^{\circ}$ C (2 $^{\circ}$ C/min) and kept for 3 min. GLC NESTLE 37 MIX (CDAA-M-252795-DZ, ANPEL Prep, China) was used as a fatty acid standard to determine specific fatty acids. TAG content was calculated as the percentage of TAG-derived fatty acids relative to total lipids (TAG + PL). Each sample was analyzed in triplicate, with 3-4 biological replicates for each group.

mRNA isolation, qPCR, and RNA-Seq

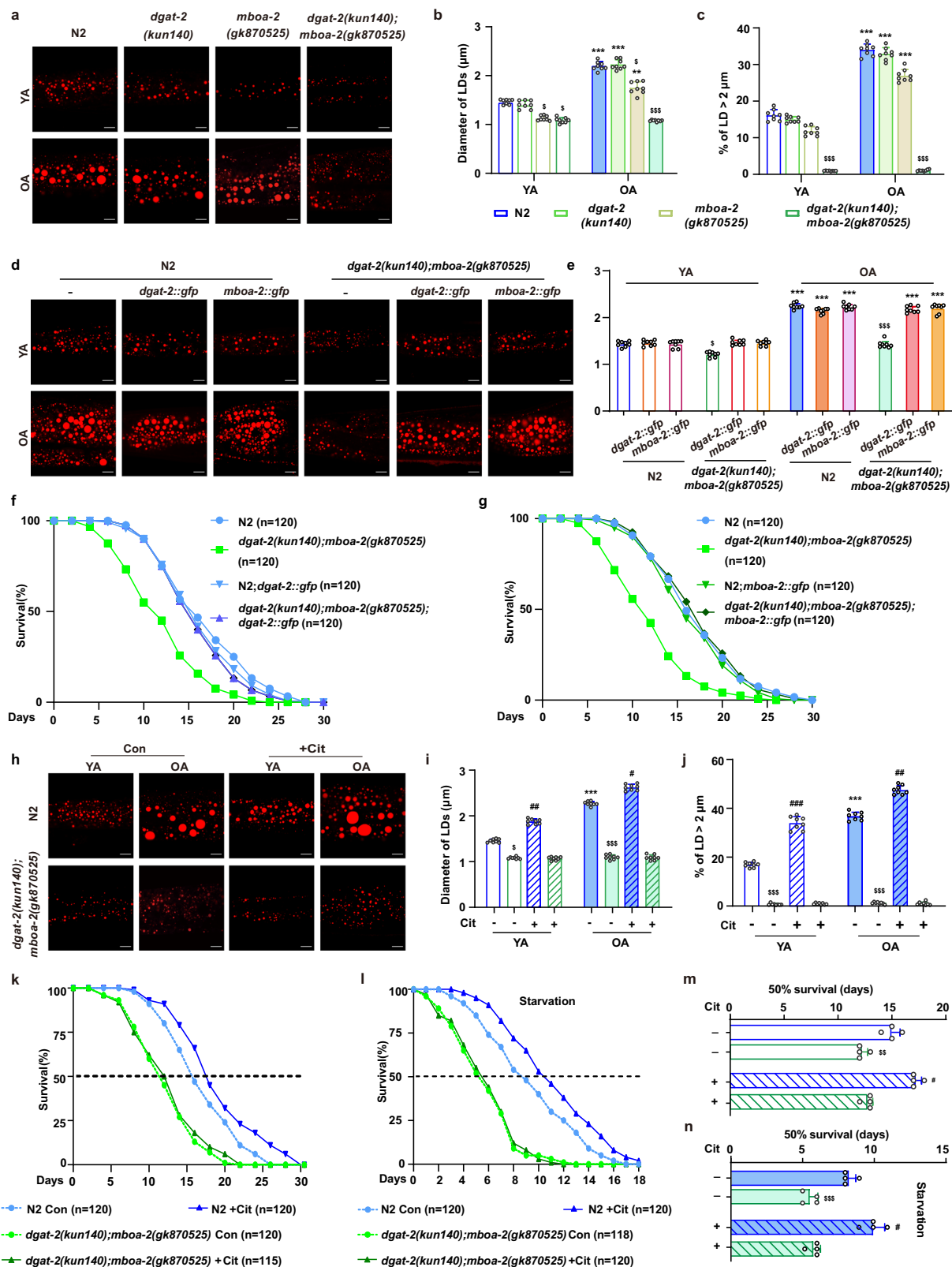
About 2000 YA and OA worms were harvest in RNAiso plus (9109, Takara), and the mRNA of worms was isolated. mRNA was reversely transcribed to cDNA using the PrimeScript RT reagent kit (RR047A, Takara Bio, Inc.). For qPCR, mRNA levels were quantified in triplicate with four biological replicates using SYBR Green Master Mix (Applied Biosystems) on an ABI 7900HT system. Relative expression was calculated via the $\Delta\Delta$ Ct method, with *tbb-2* as the reference gene⁷⁰. Primer sequences for the indicated genes are listed in Supplementary Data 5. For RNA sequencing, 1.5 μ g total RNA was used to prepare libraries (Novogene) and sequenced on an Illumina HiSeq 4000 platform. Sequencing libraries were generated using NEBNextUltraTM RNA Library Prep Kit for Illumina (NEB) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample.

Visualization of fluorescence

For fluorescence observation, YA and OA worms were washed and mounted on agarose pads containing 10 mM levamisole for immobilization. Then, worms were imaged using widefield microscopy (BX53, Olympus) or a high-resolution laser confocal microscope with Airyscan (Zeiss, LSM-800).

Western blotting analysis

YA and OA worms were collected in M9 buffer and washed several times on ice, then quickly frozen with liquid nitrogen and stored at -80° C for later use. Worms were lysed using 100 μ L RIPA (P0013, Beyotime) supplemented with protease inhibitors cocktail (P8340, Sigma) and phenylmethylsulfonyl fluoride (PMSF) (10837091001, Roche) added to each worm sample, followed by homogenization at 4 $^{\circ}$ C⁵⁶. Protein concentrations were determined by BCA assay (23235, Thermo Fisher). Samples were denatured in 2 $\times$ SDS buffer (100 mM Tris-HCl [pH6.8], 20% glycerol, 4% SDS, 0.2% bromophenol blue, 3% DTT) at 95 $^{\circ}$ C for 5 min, equal amounts of protein samples were loaded and separated by 10–12% SDS-PAGE, and transferred to PVDF membranes at 0.25 A for 100 min on ice. The membranes were blocked in 5% fat-free milk (A600669, Sangon Biotiotech), then incubated with anti-GFP (1:1000, ab290, Abcam), anti- β -ACTIN (1:1000, A5441, Sigma), and anti-His-tag (1:10000, 66005-1-Ig, Proteintech) antibodies at 4 $^{\circ}$ C overnight. The membranes were washed and incubated with goat-anti-rabbit (1:5000, 31460, Thermo Fisher) or goat-anti-mouse IgG (1:5000, 31430, Thermo Fisher). Signal was detected using SageCapture systems (Sage) and analyzed with LANE ID software (Sage).



Dietary supplementation of citrulline

Citrulline (C9530, Solarbio) was dissolved in ddH₂O and filter-sterilized, and then added to NGM at the indicated concentrations. For standard assays, 10 mM citrulline-containing plates were seeded with *OP50* or *HT115* bacteria, dried for 2–3 days, then synchronized L1 worms were transferred to the NGM plates supplemented with or

without citrulline. The worms were harvested at the designed stage for LipidTOX staining or fluorescence expression analysis.

Metabolomic analysis

Approximately 100,000 YA and OA worms were harvested with ddH₂O and allowed to settle on ice. The worms were then rinsed three times to

Fig. 6 | Citrulline activates DGAT2/MBOA2 to promote lipid deposition for healthspan. **a** LipidTOX staining of fixed worms. Scale bar, 10 μ m. **b, c** Quantification of LD diameter (**b**) and percentage of LDs > 2 μ m (**c**) from (**a**). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). $^{\#}P < 0.05$, $^{##}P < 0.01$ (Cit vs. control, Student's t test). $^{***}P < 0.001$ (*dgat-2;mboa-2* vs. wild type, Student's t test). **d** LipidTOX staining of fixed worms. Scale bar, 10 μ m. **e** Quantification of LD diameter from (**d**). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). $^{\#}P < 0.05$, $^{***}P < 0.001$ (*dgat-2;mboa-2* vs. wild type, Student's t test). **f, g** Survival curves on NGM plates (monitored from L4). $n = 120$ worms. **h** LipidTOX staining of

fixed worms. Scale bar, 10 μ m. **i, j** Quantification of LD diameter (**i**) and percentage of LDs > 2 μ m (**j**) from (**h**). Data are presented as the mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). $^{\#}P < 0.05$, $^{##}P < 0.01$ (Cit vs. control, Student's t test). $^{***}P < 0.001$ (*dgat-2;mboa-2* vs. wild type, Student's t test). **k** Survival curves on NGM plates with or without 10 mM citrulline (Cit). (monitored from L4). $n =$ total worms scored. **l** Survival curves of OA worms under starvation conditions (M9) with or without 10 mM citrulline. $n =$ total worms scored. **m, n** 50% normal survival time from (**k**) and (**l**). Data are presented as the mean $\pm$ SD, $n = 4$ biological replicates. $^{\#}P < 0.05$ (Cit vs. control, Student's t test). $^{**}P < 0.01$, $^{***}P < 0.001$ (*dgat-2;mboa-2* vs. wild type, Student's t test).

thoroughly remove bacterial residue. After settling, the supernatant was removed, and the samples were flash-frozen in liquid nitrogen before storage at -80°C . The samples were thawed on ice, and cold methanol:acetonitrile:H₂O (2:2:1) was added, then the worms were homogenized by sonication. The mix was allowed to settle at -20°C for 10 min. The homogenates were then centrifuged at $14,000 \times g$ for 15 min at 4°C . The supernatants were transferred to a new tube and dried. The dried samples were resolved with acetonitrile:H₂O (1:1), vortexed, and centrifuged at $14,000 \times g$ for 15 min at 4°C . The supernatants, filtrated with a 0.2 μ m filter, were used for the measurements. A two- microliters sample was analyzed using an Agilent 1290 Infinity LC ultra-high performance liquid chromatography (UHPLC) system equipped with HILIC and C18 columns. For the HILIC column, the column temperature was maintained at 35°C , the flow rate was 0.3 mL/min. The mobile phase consisted of A (2 mM ammonium formate, 10% acetonitrile in water) and B (0.4% formic acid in acetonitrile). The gradient elution program of mobile phase B was as follows: 0–1.0 min, 85%; 1.0–3.0 min, linearly decreased from 85% to 80%; 3.0–4.0 min, 80%; 4.0–6.0 min, linearly decreased from 80% to 70%; 6.0–10.0 min, linearly decreased from 70% to 50%; 10–15.5 min, held at 50%; 15.5–15.6 min, linearly increased from 50% to 85%; 15.6–23 min, held at 85%. For the C18 column, the column temperature was set to 40°C , the flow rate was 0.4 mL/min. The mobile phase consisted of A (5 mM ammonium acetate) and B (99.5% acetonitrile). The gradient elution program of mobile phase B was as follows: 0–5 min, linearly increased from 5% to 60%; 5–11 min, linearly increased from 60% to 100%; 11–13 min, held at 100%; 13–13.1 min, linearly decreased from 100% to 5%; 13.1–16 min, held at 5%. The sample temperature was maintained at 4°C throughout the experiment. Mass spectrometry analysis was performed using an AB6500 + QTRAP mass spectrometer (AB SCIEX). The electrospray ionization (ESI) source conditions were as follows: source temperature: 580°C , Ion Source Gas1 (GS1): 45, Ion Source Gas2 (GS2): 60, Curtain Gas (CUR): 35, IonSpray Voltage (IS): +4500 V or -4500 V in positive or negative modes, respectively. The multiple reaction monitoring (MRM) mode was used for detection. The raw MRM data were processed using MultiQuant or Analyst software for peak integration to obtain the peak area ratios of each analyte to the internal standard. Concentrations were calculated based on standard curves. Data were analyzed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Five biological replicates were collected for each group.

Citrulline measurement

Approximately 100,000 YA and 50,000 OA worms were harvested and extracted with methanol:acetonitrile:H₂O (2:2:1). After centrifugation, the supernatant was dried and resolved with acetonitrile:H₂O (1:9), vortexed, and centrifuged for at $14,000 g$ for 15 min at 4°C . The supernatants, filtrated using a 0.2 μ m filter, were used for the measurements. Four biological repeats were collected for each group. A five-microliter sample was analyzed using an ultra-performance chromatography-tandem mass spectrometry (UPLC-MS/MS) platform. The separation was performed on a UPLC BEH HILIC column (2.1 $\times$ 50 mm, 1.7 μ m, Waters, USA) with gradient elution, and the column temperature was set to 40°C . Mobile phase A was 10 mM aqueous ammonium

acetate, and mobile phase B was acetonitrile. The flow rate was 0.4 mL/min. The gradient program of mobile phase B was as follows: 0–0.5 min, 90%; 0.5–2 min, linearly decreased from 90% to 60%; 2–4.0 min, 60%; 4–4.1 min, linearly increased from 60% to 90%; 4.1–6.0 min, held at 90%. The sample temperature was maintained at 25°C throughout the experiment. The Agilent 1290-6460 C UPLC-MS/MS system (Agilent, USA) was operated in positive electrospray ionization (ESI⁺) mode. The instrument parameters were set as follows: gas temperature 300°C , gas flow rate 5 L/min (99.5% nitrogen), sheath gas temperature 250°C , capillary voltage 3500 V, nozzle voltage 500 V. Citrulline was determined in multiple reaction monitoring (MRM) mode⁹⁹. The MRM ion transitions for citrulline were: Cit 176 $\rightarrow$ 113 (quantitative ion, CE = 16 V) and 176 $\rightarrow$ 70.1 (qualitative ion, CE = 24 V) (Supplementary Data 6). The Agilent MassHunter Qualitative Analysis software version B.07.01 (Agilent Technologies, Palo Alto, CA, USA) was used for UPLC-MS/MS data analyses. The linearity of the standard curves was evaluated for each metabolite using seven calibration points. The concentrations were normalized to total protein content (determined using the BCA assay). Data were analyzed using GraphPad Prism 8.

Survival rate experiment

For normal lifespan analysis, approximately 120 synchronized L4 worms were transferred to NGM plates and designated as day 0 (D0), the following days were designated as day 1 (D1), day 2 (D2), and so on. Worms were transferred to fresh plates every 2 days until reproduction ceased. Survival was monitored on alternate days until the last worm died. Three biological replicates were performed for each worm strain. Data were analyzed using GraphPad Prism.

For worm survival under starvation, approximately 120 OA worms were cultured on NGM plates with or without 10 mM citrulline, transferred to M9 buffer with or without citrulline, and cultured at 20°C . The number of living and dead worms was counted daily until the last worm died. Data were analyzed using GraphPad Prism.

Protein-small molecule docking experiment

A molecular docking study was conducted to evaluate the binding affinity of core ligand compounds toward target proteins. The molecular structure of citrulline (CID: 9750) was obtained in Mol2 format from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). Conformational optimization was performed using Chem 3D software, followed by charge assignment with AutoDockTools 1.5.7^{100–102}. Hydrogenation and torsion angle adjustment were applied to convert the citrulline structure to pdbqt format. The predicted structure of protein MXL-3 (UniProt ID: Q18711) was acquired from the UniProt database (<https://www.uniprot.org>). The protein underwent hydrogenation and removal of water molecules, and was subsequently converted into pdbqt format. Molecular docking between the protein and ligand was performed using AutoDockTools 1.5.7, and the results were visualized with PyMOL software.

Microscale thermophoresis (MST) assay

To evaluate the binding capacity of citrulline to both the full-length MXL-3 protein and the MXL-3 (Δ 228–235 AA) deletion protein, we

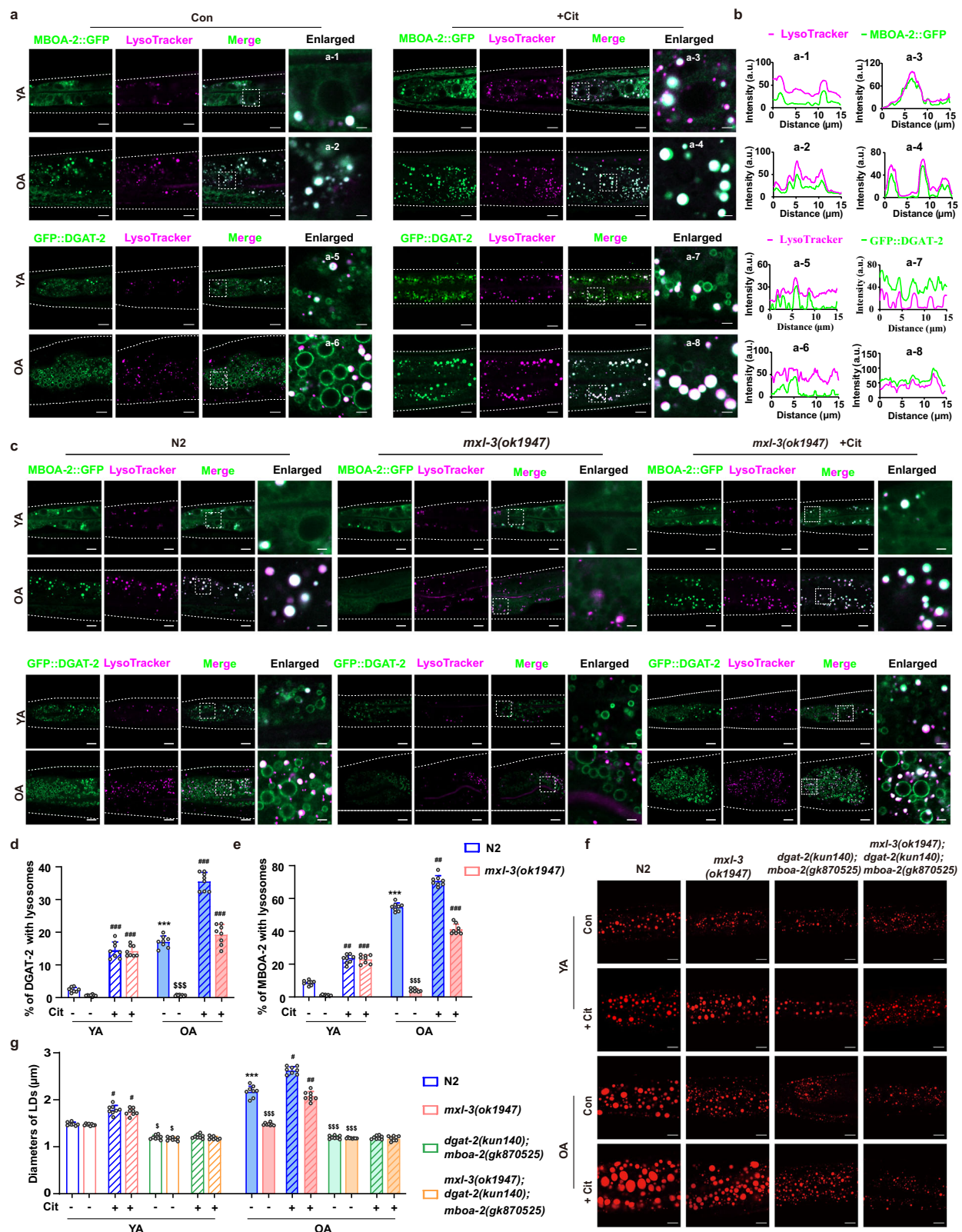


Fig. 7 | MXL-3 regulates the lysosomal localization and function of DGAT-2/MBOA-2. **a, c** Localization of MBOA-2::GFP or GFP::DGAT-2 relative to lysosomes stained by LysoTracker in worms treated with or without citrulline (Cit). Scale bar, 2 μ m (enlarged), 10 μ m (others). **b** Intensity profiles of labeled scans from high-magnification regions, A-1-4 and A-5-8 show the localization of MBOA-2::GFP or GFP::DGAT-2 with LysoTracker from (a). **d, e** Quantification of GFP::DGAT-2 (d) or MBOA-2::GFP (e) co-localization with lysosomes from (c). Data are presented as the

mean $\pm$ SD, $n = 8$ representative animals per group. *** $P < 0.001$ (YA vs. OA, Student's t test). ** $P < 0.01$, *** $P < 0.001$ (Cit vs. control, Student's t test). **** $P < 0.0001$ (*mxl-3* vs. wild type, Student's t test). **** $P < 0.0001$ (Cit vs. control, Student's t test). * $P < 0.05$, ** $P < 0.01$ (Cit vs. control, Student's t test). * $P < 0.05$, ** $P < 0.01$ (Indicated mutant vs. wild type, Student's t test).

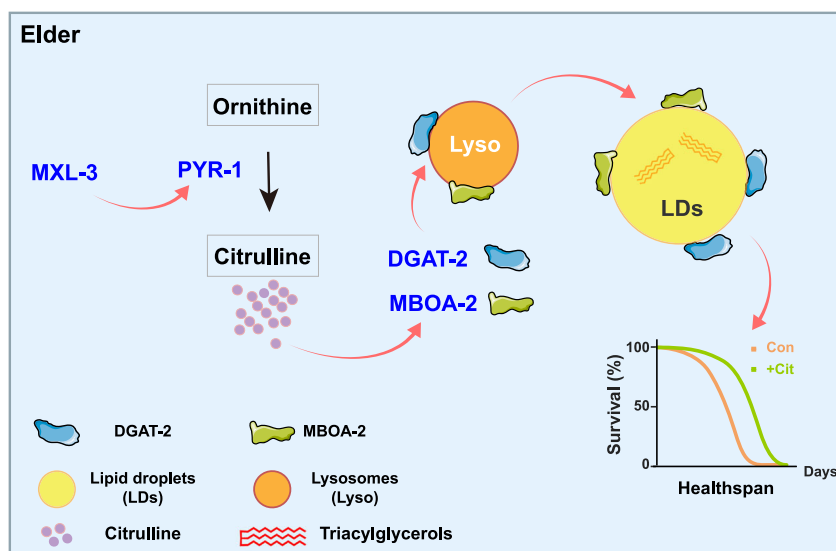


Fig. 8 | A proposed model for how the MXL-3-citrulline-DGAT-2/MBOA-2 axis regulates lipid metabolism for healthspan in *C. elegans*. During aging, the transcription factor MXL-3 is activated, and then upregulates the expression of

PYR-1 for the biosynthesis of citrulline, which further triggers the lysosomal translocation of the lipogenic enzymes DGAT-2 and MBOA-2, thereby promoting triacylglycerol (TAG) synthesis and accumulation to ensure healthspan.

employed the mammalian cell expression system (HEK293F cells) for protein expression and purification as described¹⁰³. Firstly, the sequences of the full-length *C. elegans* MXL-3 protein and the truncated MXL-3 ($\Delta 228$ –235 AA) protein were cloned into the pEZT-his vector and expressed using the BacMam system. The baculovirus was generated in Sf9 cells for viral amplification, then infected HEK293F cells for protein expression and purification.

Subsequently, 200 nM of each purified protein was labeled with a fluorescent dye using the RED-Tris-NTA protein labeling kit (MO-L018, NanoTemper Technologies). Then, the labeled proteins were mixed with various concentrations of citrulline and incubated. Finally, the mixtures were loaded into the MST-Nanotemper instrument for measurement, and the binding data were recorded and analyzed using the affinity analysis software (NanoTemper Technologies) to determine the dissociation constants.

Data analysis

All data are presented as mean $\pm$ SD. Statistical significance was determined using Student's *t* test (two tails) as appropriate for each dataset. Three or four biological replicates were performed for each experiment. Eight representative worms were measured for the mean diameter of LDs for each worm strain. All graphs were generated using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA), and figures were assembled in Adobe Illustrator 2023.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The raw metabolomics data have been deposited in the NIH Common Fund's National Metabolomics Data Repository (NMDR) Metabolomics Workbench under study ID ST004933 and can be accessed via the project <https://doi.org/10.21228/M8Q27R>. The dataset is accessible via the following link <https://dev.metabolomicsworkbench.org:22222/data/DRCCMetadata.php?Mode=Study&StudyID=ST004933&Access=JlpR5250>. All other data generated in this study are provided in the Supplementary Information / Source Data file. Any additional

information is available from the corresponding authors upon reasonable request. Source data are provided in this paper.

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Acknowledgements

We thank Dr. Leonard Krall for the English copyediting. We also thank Shanghai Applied Protein Technology Co., Ltd. (Shanghai, China) and Metabo-Profile (Shanghai, China) for their advice and assistance with metabolomics detection and analysis.

Author contributions

Author contributions: B.L. and C.X.L. conceived and designed the experiments. B.L., C.X.L., and J.J.Z. wrote the paper. C.X.L., Y.L.W. and X.M.X. performed most of the experiments and data analysis. C.B.L. and Y.M.H. provided some technical methods. X.J.Z., Z.Z., J.L.T., L.F., Y.L., Q.M.Z. and J.L. provided guidance for some experiments and contributed reagents/materials/analysis tools. B.L. initiated the project. All authors reviewed the manuscript.

Funding

This work was supported by the Ministry of Science and Technology of the People's Republic of China (2024YFA1802800 to B.L.), Yunnan Provincial Science and Technology Project at Southwest United Graduate School (202302AO370004 to B.L.), the Noncommunicable Chronic Diseases-National Science and Technology Major Project (2024ZD0523100 and 2024ZD0523103 to J.J.Z.), the Yunnan Fundamental Research Projects (202401AS070146 to J.J.Z., 202301BF070001-023 to B.L., 202301AT070123 to Y.L.W.), the National Natural Science Foundation of China (32360161 to J.J.Z., 32371232 to B.L., 32160155 to Y.L.W., 82360726 to X.J.Z., 82300972 to Y.M.H.), and the Yunnan Xingdian Talent Support Program to B.L. and J.J. Z.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41467-026-75498-4>.

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Peer review information *Nature Communications* thanks Daniel Ortega-Cuellar, Xinyu Mei and the other anonymous reviewer for their contribution to the peer review of this work. A peer review file is available.

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