Figures and figure supplements

Metformin extends C. elegans lifespan through lysosomal pathway

Jie Chen et al
Figure 1. Metformin coordinates mTORC1 and AMPK on purified lysosome. (A) HEK293T cells stably expressing LAMP1-RFP-FLAG were mechanically broken. (B) Lysosomes were purified through immunoprecipitation (Organelle markers: LAMP2, lysosome; EEA1, early endosome; Prohibitin, mitochondria; PDI, ER; Histone H3, nucleus). (C) Lysosomal accumulation of purified Myc-raptor upon amino acids stimulation. (D–E) Lysosomal disassociation of Myc-raptor (D), lysosomal accumulation of Myc-AXIN/LKB1 and phosphorylation of AMPK (E) upon Concanamycin A (Con A) or Metformin (Met) treatment. (F–H) Quantifications of immunoblots in (C–E). Immunoblots of Myc-Raptor, Myc-AXIN and Myc-LKB1 were normalized to that of LAMP-RFP-FLAG, and immunoblots of p-AMPK were normalized to that of AMPK. Relative intensities of three independent biological replicates are shown as mean ± SEM. ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001.

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Figure 1—figure supplement 1. Metformin inhibits mTORC1 independent of AMPK. (A) AMPKα1/2 double knockout cells stably expressing LAMP1-RFP-FLAG were mechanically broken. (B) Lysosomes were purified through immunoprecipitation (Organelle markers: LAMP2, lysosome; EEA1, early endosome; Prohibitin, mitochondria; PDI, ER; Histone H3, nucleus). (C) Lysosomal accumulation of purified Myc-raptor upon amino acids stimulation. (D–E) Lysosomal disassociation of Myc-raptor (D) and lysosomal accumulation of Myc-AXIN/LKB1 (E) upon Concanamycin A (Con A) or Metformin (Met) treatment. (F–H) Quantifications of immunoblots in (C–E). Immunoblots of Myc-Raptor, Myc-AXIN and Myc-LKB1 were normalized to that of LAMP-RFP-FLAG, and immunoblots of p-AMPK were normalized to that of AMPK. Relative intensities of three independent biological replicates are shown as mean ± SEM. ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001.

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Figure 1—figure supplement 2. Purified Myc-LKB1 forms a complex with endogenous STRAD and MO25. DOI: https://doi.org/10.7554/eLife.31268.005
Figure 1—figure supplement 3. Metformin targets lysosome in C. elegans. (A) Representative fluorescent images of mitochondrial stress response reporter strain hsp-6p::gfp cultured under normal condition, or in the presence of metformin or rotenone. (B) Quantification of fluorescent images in (A). Mean ± SEM of 3 independent biological replicates are shown (sample size: ≥40 worms). (C) Representative fluorescent images of Magic Red Cathepsin assay in worms administrated with or without metformin. (D) Quantification of fluorescent images in (C). Mean ± SEM of 3 independent biological replicates are shown (sample size: ≥40 worms). Scale bar: 100 um. ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001.
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Figure 2. Metformin inhibits TORC1 pathway in C. elegans. (A) Representative western blotting of RSKS-1 phosphorylation in the presence or absence of Metformin. (B) Quantification of immunoblots in (A): Relative intensities of 3 independent biological replicates are shown as mean ± SEM. (C) Representative fluorescent images of HLH-30 nuclei localization in the presence or absence of metformin. (D) Percentage of worms with HLH-30 nuclear localization in (C) was quantified. Mean ± SEM of 3 independent biological replicates are shown (sample size: ≥40 worms). (E) Q-PCR analysis of HLH-30 target genes in the presence or absence of metformin. ~300 worms were pooled in each sample. Data from three independent biological replicates are shown as mean ± SEM. ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001.

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Figure 2—figure supplement 1. RSKS-1, C.elegans homolog of S6K1 is phosphorylated at T389 residue. (A) T389 of S6K1 is highly conserved among species. A ClustW of S6K1 protein sequence in different species was plotted by MEGA6. (B) rsks-1 RNAi reduces RSKS-1 phosphorylation level in wild type animals.

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Figure 2—figure supplement 2. Metformin inhibits TORC1 independent of AMPK in *C. elegans*. (A) Representative fluorescent images of HLH-30 nuclei localization in wild type or *aak-2* mutant animals in the presence or absence of metformin. (B) Percentage of worms with HLH-30 nuclear localization in (A) was quantified. Mean ± SEM of 3 independent biological replicates are shown (sample size: ≥40 worms). (C) Q-PCR analysis of HLH-30 target genes in *aak-2* mutants with or without metformin treatment. ~300 worms were pooled in each sample. Data from three independent biological replicates are shown as mean ± SEM. ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001. DOI: https://doi.org/10.7554/eLife.31268.009

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Figure 3. Metformin extends healthspan partially due to TORC1 inhibition. (A) Lifespan analysis of control worms or daf-15 heterozygous mutants in the presence or absence of metformin. (B) Representative images of Oil-Red-O (ORO) staining of control worms or daf-15 heterozygous mutants in the presence or absence of metformin. (C) Quantification of (B). Error bars represent mean ± SEM of 3 independent biological replicates (sample size: \( n \geq 40 \) worms). (D–E) Locomotion (D) and age pigments (E) were measured in control worms or daf-15 heterozygous mutants in the presence or absence of metformin. Mean ± SEM of 3 independent biological replicates are shown (sample size: \( n \geq 20 \) worms for locomotion assay; \( n \geq 40 \) worms for age pigments assay). (F) Representative fluorescent images of HLH-30 nuclei localization in control worms or daf-15 heterozygous mutants in the presence or absence of metformin. Arrows indicate nuclear localized HLH-30::GFP. (G) Quantification of (F). Percentage of unc-24/+; HLH-30::GFP or unc-24/daf-15; HLH-30::GFP worms with nuclear accumulation of HLH-30 were counted. Error bars represent mean ± SEM of 3 independent biological replicates. (sample size: \( n \geq 40 \) worms) (H) Representative western blotting of AAK-2 phosphorylation in the presence or absence of metformin. (I) Quantification of (H). ~300 worms were pooled in each protein sample. Error bars represent mean ± SEM of 3 independent biological replicates. ns, no significant difference; *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).

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**Figure 3—figure supplement 1.** Deletion of daf-15 suppresses TORC1 activity. (A) Generation of daf-15 heterozygous mutants and its corresponding control animals. (B) Representative immunoblottings of RSKS-1 phosphorylation in N2 or daf-15 heterozygous mutants. (C) Quantification of immunoblots in (B). Relative intensities of 3 independent biological replicates are shown as mean ± SEM. (D) Schematic representation of experimental design for the detection of healthspan parameters. ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001.

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Figure 3—figure supplement 2. Metformin promotes longevity through a pathway additive to TORC1 inhibition. (A–C) Lifespan analysis of hlih-30, pha-4 or skn-1 loss-of-function mutants in the presence or absence of metformin.

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**Figure 4.** Metformin extends lifespan through v-ATPase-Ragulator-dependent activation of AMPK. (A) A scheme depicting genes within *C. elegans* TORC1 and AMPK pathways. (B–C) Lifespan analysis of wild type, vha-3 (B), or lmtr-3 (C) animals in the presence or absence of metformin. (D) Representative fluorescent images of HLH-30 nuclear localization in worms administered with control, vha-3 or lmtr-3 RNAi. (E) Quantification of (D), percentage of worms with HLH-30 nuclear accumulation. Mean ± SEM of 3 independent biological replicates are shown (sample size: n ≥ 40 worms). (F) Q-PCR analysis of HLH-30 target genes in worms administered with control, vha-3 or lmtr-3 RNAi. ~300 worms were collected for each mRNA sample. Data from 3 independent biological replicates are shown as mean ± SEM. (G) Representative immunoblots of RSKS-1 phosphorylation in worms administered with control, vha-3 or lmtr-3 RNAi. (H) Quantification of (G). ~300 worms were pooled in each protein sample. Relative intensities of 3 independent biological replicates are shown as mean ± SEM. (I) Representative immunoblots of AAK-2 phosphorylation in wild type, vha-3 or lmtr-3 mutants, in the presence or absence of metformin. (J) Quantification of (I). ~300 worms were collected in each protein sample. Relative intensities of 3 independent biological replicates are shown as mean ± SEM. ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001.

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Figure 4—figure supplement 1. Genotyping of v-ATPase and Ragulator mutants. (A) Schemes depicting deletion and PCR primer positions within each mutant. (B) Primer sequences for genotyping. (C) PCR results of genotyping.

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Figure 4—figure supplement 2. Lysosomal localization of C. elegans v-ATPase-Ragulator proteins. (A–D) Representative fluorescent images to test co-localization of VHA-3, VHA-12, LMTR-2 or LMTR-3 with LMP-1. L1 worms were cultured in the presence or absence of metformin until L4 stage. Scale bar: 10 μm.

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**Figure 4—figure supplement 3.** Mutation of v-ATPase or Ragulator perturbs lysosomal function in *C. elegans*. (A) Representative fluorescent images of Magic Red Cathepsin assay in wild-type, *vha-3*, *vha-12*, *lmtr-2*, or *lmtr-3* loss-of-function mutants, and *vha-3*, *vha-12*, *lmtr-2*, or *lmtr-3* loss-of-function mutants injected with the corresponding rescue plasmid. Scale bar: 100 um. (B) Quantification of (A). Error bars represent mean ± SEM of 3 independent biological replicates (sample size: n ≥ 40). ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001. DOI: https://doi.org/10.7554/eLife.31268.016
Figure 4—figure supplement 4. Metformin extends lifespan through v-ATPase-Ragulator-dependent AMPK activation. (A–B) Lifespan assays of wild type, vha-12 (A), or lmtr-2 mutants (B) in the presence or absence of metformin. (C–D) Knockdown efficiency of vha-3 (C) or vha-12 RNAi (D). (E) Representative immunoblots of AAK-2 phosphorylation in N2, vha-12, or lmtr-2 mutants treated with or without metformin. (F) Quantification of (E). ~300 worms were pooled in each protein sample. p-AAK-2 intensities were normalized to that of tubulin, and relative intensities of 3 independent biological replicates are shown as mean ± SEM. (G) Censored worms due to vulva blasting were counted during lifespan analysis of N2, lmtr-3 or lmtr-2 worms in the presence or absence of metformin. Error bars represent mean ± SEM of two independent biological replicates (sample size: ~100 N2 worms; ~250 lmtr-2/3 mutants). ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001.

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Figure 5. Metformin extends lifespan through axl-1-dependent activation of AMPK. (A) Representative fluorescent images to test co-localization of AXL-1 with LAMP1. HEK293T cells stably expressing AXL-1-EGFP and LAMP1-RFP-FLAG were cultured in the presence or absence of 2 mM metformin for 12 hr. Scale bar: 10 um. (B) Representative fluorescent images of Magic Red Cathepsin assay in wild type worms or axl-1 mutants. Scale bar: 100 um. Error bars represent mean ± SEM of 3 independent biological replicates (sample size: n ≥ 40). (C) Representative immunoblots of AAK-2 phosphorylation in wild type or axl-1 mutants with or without metformin treatment. (D) Quantification of (C). ~300 worms were pooled in each protein sample. Relative intensities of 3 independent biological replicates are shown as mean ± SEM. (E–F) Lifespan analysis of wild type, axl-1 (E), or par-4 mutants (F) in the presence or absence of metformin. ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001.

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Figure 6. Metformin attenuates age-related fitness decline through lysosome-dependent activation of AMPK. Neutral fat deposition (A–B), locomotion (C–F) and age pigments (G) were measured in wild-type worms, or vha-3, lmtr-3 or axl-1 mutants in the presence or absence of metformin. Error bars represent mean ± SEM of 3 independent biological replicates. (sample size: n ≥ 20 worms for locomotion assay; n ≥ 40 worms for ORO staining or age pigments assay). (H) Metformin may target v-ATPase-Ragulator complex and promote longevity through coordination of Ce.TORC1 and Ce.AMPK.

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Figure 6—figure supplement 1. Proposed model for metformin’s action. Under normal condition, v-ATPase is in an active conformation, which mediates lysosomal amino acid signaling to recruit and activate TORC1. Metformin treatment may target v-ATPase and change its conformation, Figure 6—figure supplement 1 continued on next page.
leading to the dissociation and inactivation of TORC1. Meanwhile, v-ATPase-Regulator may act as a platform for the docking of AXIN/LKB1 and subsequent activation of AMPK.

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