1 **TITLE: Organelle proteomic profiling reveals lysosomal heterogeneity in association with**

2 **longevity**

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ABSTRACT

28   Lysosomes are active sites to integrate cellular metabolism and signal transduction. A collection

29   of proteins associated with the lysosome mediate these metabolic and signaling functions. Both

30   lysosomal metabolism and lysosomal signaling have been linked to longevity regulation;

31   however, how lysosomes adjust their protein composition to accommodate this regulation

32   remains unclear. Using deep proteomic profiling, we systemically profiled lysosome-associated

33   proteins linked with four different longevity mechanisms. We discovered the lysosomal

34   recruitment of AMPK and nucleoporin proteins and their requirements for longevity in response

35   to increased lysosomal lipolysis. Through comparative proteomic analyses of lysosomes from

36   different tissues and labeled with different markers, we further elucidated lysosomal

37   heterogeneity across tissues as well as the increased enrichment of the Ragulator complex on

38   Cystinosin positive lysosomes. Together, this work uncovers lysosomal proteome heterogeneity

39   across multiple scales and provides resources for understanding the contribution of lysosomal

40   protein dynamics to signal transduction, organelle crosstalk and organism longevity.

41

INTRODUCTION

43

44   Lysosomes are membrane-bound organelles specialized to constitute an acidic environment in

45   the cytosol. Lysosomes carry many proteins that are essential for maintaining lysosomal

46   activities and mediating lysosomal regulatory effects. Inside the lysosomal lumen, a series of

47   acidic hydrolases, including lipases, proteases, glucosidases, acid phosphatases, nuclease and

48   sulfatases, are responsible for the degradation and recycling of extracellular and intracellular

49   materials delivered through endocytic, phagocytotic and autophagic processes (Appelqvist et al.,

50   2013; Ballabio & Bonifacino, 2020; Lawrence & Zoncu, 2019). Additionally, on the lysosomal

51   membrane, a group of integral transmembrane proteins play crucial roles in the maintenance of

52   luminal acidic pH and ion homeostasis, the control of lysosomal membrane potential and export

53   of metabolic products, as well as the regulation of organelle interaction and signal transduction

54   (Ballabio & Bonifacino, 2020; Lawrence & Zoncu, 2019). For example, the lysosomal vacuolar-

55   type H^+-ATPase (v-ATPase) on the membrane is the primary driver for the active accumulation

56   of protons in the lysosomal lumen, which also requires a neutralizing ion movement mediated by

57   ion channels and transporters (Graves et al., 2008; Nicoli et al., 2019). In addition, v-ATPase

58   coordinates with lysosomal amino acid transporter SLC38A9 and lysosomal cholesterol exporter

59   NPC1 in regulating the activation of mechanistic/mammalian target of rapamycin complex I

60   (mTORC1) by amino acid and lipid cues (Castellano et al., 2017; Wang et al., 2015). The

61   recruitment of mTORC1 to the lysosome is mediated by RagA/B and RagC/D GTPase

62   heterodimers that are associated with the scaffold protein complex Ragulator tethered on the

63   lysosomal membrane (de Araujo et al., 2017). Through interacting with Axin, Ragulator also

64   mediates the activation of AMP-activated protein kinase (AMPK) on the lysosomal surface

65   (Zhang et al., 2014). Furthermore, lysosomes are not static, isolated organelles, instead they are

66   highly mobile vesicles that undergo frequent movements in both anterograde (nucleus-to-

67   periphery) and retrograde (periphery-to-nucleus) directions and form dynamic interactions with

68   other organelles including endosomes, autophagosomes, endoplasmic reticulum and

69   mitochondria (Ballabio & Bonifacino, 2020; Pu et al., 2016). These trafficking and interaction

70   processes are mediated by lysosomal integral transmembrane proteins as well as diverse proteins

71   that are recruited to lysosomes in response to different extracellular and intracellular inputs

72   (Ballabio & Bonifacino, 2020; Pu et al., 2016).

73

74   Lysosomes control numerous cellular processes, and dysfunction of lysosomes has been linked

75   with various diseases, such as lysosomal storage disorders (Ballabio & Gieselmann, 2009; Platt

76   et al., 2012), Alzheimer's disease (Nixon & Cataldo, 2006), Parkinson disease (Navarro-Romero

77   et al., 2020) and some types of cancer (Davidson & Vander Heiden, 2017; Fehrenbacher &

78   Jaattela, 2005). Emerging evidence also suggests that lysosome functions as a central regulator

79   of organism longevity, through its involvement in autophagy and its modulation of metabolic

80   signaling pathways. The induction of autophagic flux has been observed in multiple pro-

81   longevity states, and is required for the pro-longevity effects caused by those genetic, dietary and

82   pharmacological interventions, such as reduced insulin/IGF-1 signaling, caloric restriction, and

83   spermidine treatment (Hansen et al., 2018). On the other hand, lysosomes are now recognized as

84   the key platform to modulate the activities of mTORC1 and AMPK signaling, two well-

85   characterized longevity regulating pathways (Savini et al., 2019). In addition, our studies have

86   discovered lysosomal lipid messenger pathways that are induced by a lysosomal acid lipase

87   LIPL-4 and promote longevity via both cell-autonomous and cell-nonautonomous signaling

88   mechanisms (Folick et al., 2015; Ramachandran et al., 2019; Savini et al., 2022; Wang et al.,

89   2008). Given the importance of lysosomes in regulating longevity, it will be crucial to

90   understand how changes in the lysosomal protein composition are associated with longevity

91   regulation.

92

93   To systemically profile the protein composition of lysosomes, methods have been developed to

94   purify lysosomes using gradient centrifugation (Gao et al., 2017; Lubke et al., 2009; Markmann

95   et al., 2017; Schroder et al., 2010). More recently, a lysosome immunoprecipitation method,

96   which uses anti-HA (human influenza virus hemagglutinin) antibody conjugated magnetic beads

97   to immuno-purify lysosomes from mammalian cells expressing transmembrane protein 192

98   (TMEM192) fused with three tandem HA (3×HA) epitopes, has further improved the specificity

99   and speed of lysosomal isolation (Abu-Remaileh et al., 2017). This rapid isolation method has

100   facilitated follow-up mass spectrometry (MS)-based proteomics as well as metabolomics

101   analyses (Abu-Remaileh et al., 2017; Eapen et al., 2021; Laqtom et al., 2022).

102

103   In the present study, we have applied an immunoprecipitation-based method for rapid isolation

104   of lysosomes from live adult *C. elegans* using transgenic strains expressing lysosomal membrane

105   proteins tagged with 3×HA (Lyso-Tag). We then conducted large-scale proteomic profiling using

106   isolated lysosomes and remaining non-lysosomal fractions, to determine the enrichment of each

107   identified protein on the lysosome. Based on these analyses, we have defined a lysosome-

108   enriched proteome and compared it between wild-type and long-lived worms, revealing

109   lysosomal protein composition changes associated with longevity. We have also generated

110   transgenic strains expressing Lyso-Tag specifically in four major somatic tissues, the hypodermis

111   (epidermis), muscle, intestine (digestive tract/fat tissue/liver) and neurons, leading to the

112   discovery of lysosomal proteome heterogeneity in different tissues. Furthermore, by comparing

113   the lysosome-enriched proteome with LAMP1/LMP-1 Lyso-Tag and the one with

114   Cystinosin/CTNS-1 Lyso-Tag, we discovered that the Ragulator complex and other mTORC1

115   regulators exhibit increased enrichments on lysosomes containing the cysteine transporter

116   Cystinosin.

117

118   **RESULTS**

119

120   **Map lysosome-enriched proteome systemically in *C. elegans***

121

122   To comprehensively reveal proteins that are enriched at the lysosome, we have applied rapid

123   lysosome immunoprecipitation followed by MS-based proteomic profiling (Lyso-IP) (Figure 1A).

124   We first generated a transgenic strain overexpressing the lysosome-associated membrane protein,

125   LMP-1 (Eskelinen, 2006) fused to both 3×HA and RFP (LMP-1 *LysoTg*) under the whole-body

126   *sur-5* promoter. Fluorescence imaging of RFP confirmed the lysosomal localization of the LMP-

127   1 fusion protein in live organisms and made it possible to follow purified lysosomes *in vitro*

128   (Figure 1B). The presence of transgenes does not affect worms' developmental timing and

129   lifespan (Figure 1-figure supplement 1 A, B). The 3×HA epitope tag is used to purify lysosomes

130   from homogenized worm lysate via immunoprecipitation using anti-HA antibody-conjugated

131   magnetic beads (Figure 1A). In general, about 160,000 worms at day-1 adulthood were harvested

132   and homogenized. Upon centrifugation to remove debris and nuclei, 3×HA-tagged lysosomes

133   were immunoprecipitated and separated from other cellular content (flow-through controls,

134   Figure 1A). The whole process from harvesting worms to purified lysosomes takes around 25

135   minutes. Many purified lysosomes were able to take up LysoTracker probes and exhibit positive

136   fluorescence signals, indicating that they remain intact with an acidic pH, while there are also

137   some broken lysosomes losing LysoTracker staining (Figure 1C). When blotting with antibodies

138   against different organelle markers, we found that the purified lysosomes show no or nearly no

139   protein markers of other organelles, including HSP-60 (mitochondria heat shock protein) (Hartl

140   et al., 1992; Mayer, 2010), CYP-33E1 (Endoplasmic Reticulum (ER) cytochrome P450) (Brown

141   & Black, 1989), SQV-8 (Golgi glucuronosyltransferase) (Hadwiger et al., 2010) and beta-actin

142   (cytoskeleton) (Figure 1D), while the flow-through controls show these protein markers but

143   nearly no lysosomal protein marker LMP-1 (Figure 1D). Together, these results demonstrate the

144   efficacy of the Lyso-IP approach to enrich lysosomal proteins.

145

146   Next, we conducted proteomic profiling of purified lysosomes with their paired flow-through

147   controls (Figure 1A). The correlation analysis shows good reproducibility among four

148   independent biological replicates (Figure 1-figure supplement 1C), and the PCA analysis shows

149   a clear separation between Lyso-IP replicates and flow-through controls (Figure 1E). In parallel,

150   we also conducted immunoprecipitation using homogenized lysate from wild-type (WT) worms

151   that do not carry a Lyso-Tag and then analyzed proteomic profiles of three independent samples

152   as non-tag controls (Figure 1A).

153

154   Based on these proteomic data, we used three criteria to define lysosome-enriched proteins: first,

155   their levels in the purified lysosomes are 10-fold or higher than those in the flow-through

156   controls (Figure 2A); second, their enrichments can be repeated in all biological replicates

157   (Figure 2A); and lastly, their enrichments over non-tag controls are more than 2-fold (Figure 2B).

158   Together, 216 lysosome-enriched candidates were identified from more than 6000 detected

159   proteins, and 178 candidates have mammalian homologs (Figure 2-figure supplement 1,

160   Supplementary File 1). This lysosome-enriched proteome consists of 83 membrane transporters

161   and channels, 47 enzymes, 26 signaling factors, 12 structural components, and 6 involved in

162   vesicle trafficking (Figure 2C). These include known lysosomal proteins, such as various

163   lysosomal Cathepsins that catalyze protein degradation (Turk et al., 2012), lysosomal specific

164   ARL8 GTPase that mediates lysosome trafficking (Nakae et al., 2010), and subunits of

165   lysosomal v-ATPase that pumps protons into the lysosomal lumen to maintain an acidic pH

166   (Forgac, 2007) (Supplementary File 1).

167

168   Lysosomal v-ATPase consists of both V0 and V1 domains that are associated with the lysosomal

169   membrane and face the cytosol, respectively (Figure 2D). Reversible dissociation of the V1 and

170   V0 domains responds to nutritional signals and plays a crucial role in the regulation of the

171   lysosomal v-ATPase activity (Kane, 1995; McGuire & Forgac, 2018; Ratto et al., 2022; Stransky

172   & Forgac, 2015). Except for VHA-18 (V1 H subunit), we were able to detect all other subunits

173   of lysosomal v-ATPase, including VHA-5, 6, 7 and UNC-32 (V0 a subunits), VHA-1, 2, 3 and 4

174   (V0 c subunits), VHA-16 (V0 d subunit), VHA-17 (V0 e subunit), VHA-13 (V1 A subunit),

175   VHA-12 (V1 B subunit), VHA-11 (V1 C subunit), VHA-14 (V1 D subunit), VHA-8 (V1 E

176   subunit), VHA-9 (V1 F subunit), VHA-10 (V1 G subunit), and VHA-15 (V1 H subunit), and

177   also two v-ATPase transporting accessory proteins, VHA-19 and VHA-20 (Figure 2D,

178   Supplementary File 1). Among the V0 domain subunits, VHA-4, 5, 6, 7 and 16 and UNC-32 are

179   enriched over 10-fold in all four replicates, VHA-1, 2 and 3 are enriched over 10-fold in three

180   replicates and over 5-fold in one replicate, and the low abundant VHA-17 was only detected in

181   two replicates, with more than 10-fold enrichments in both (Figure 2D). The VHA-19

182   transporting accessory protein is enriched over 10-fold in two replicates and less than 5-fold in

183   two replicates (Figure 2D). In contrast, for the subunits of the V1 domain and the VHA-20

184   transporting accessory protein, they show no enrichment in the purified lysosomes compared to

185   the flow-through controls (Figure 2D). These results suggest that the free form of the V1 domain

186   and the associated form bound with the V0 domain at lysosomes both exist under well-fed

187   condition in wild-type worms.

188

189   In addition to 30.7% of proteins with known lysosome/endosome localization, the lysosome-

190   enriched proteome includes a small portion of proteins localized to other cellular organelles,

191   ER/Golgi (6.0%), mitochondria (1.4%), peroxisome (0.4%), lipid droplet (0.9%), and synaptic

192   vesicle (1.8%) (Figure 2E). On the other hand, there is a large portion of proteins with annotated

193   plasma membrane localization (24.8%) (Figure 2E). Many of these plasma membrane proteins

194   are receptors that are known to be subject to endocytosis and subsequent recycling lysosomal

195   degradation, such as INA-1/integrin alpha-6 (De Franceschi et al., 2015), VER-3/vascular

196   endothelial growth factor receptor (Ewan et al., 2006), PTC-1/protein patched receptor (Gallet &

197   Therond, 2005), and IGLR-2/leucine-rich repeat-containing G-protein coupled receptor (Snyder

198   et al., 2013) (Supplementary File 1) (Braulke & Bonifacino, 2009). We also identified proteins

199   involved in the endocytosis process, including low-density lipoprotein receptor-related proteins,

200   LRP-1 (Grant & Hirsh, 1999) and arrestin domain-containing proteins, ARRD-13 and ARRD-18

201   (Kang et al., 2014) (Supplementary File 1) that mediate the internalization of plasma membrane

202   receptors (Ma et al., 2002). Thus, the lysosome-enriched proteome also reveals membrane

203   receptor proteins that undergo recycling through the endo-lysosomal system.

204

205   **Profile lysosome-enriched proteome heterogeneity among different tissues**

206

207   Lysosomes are known as a heterogeneous population of vesicles, differing in their size, shape,

208   pH and cellular distribution. They broadly exist in all tissues of an organism and play diverse

209   roles in a tissue-specific manner. To examine how lysosome-enriched proteomes exhibit

210   heterogeneity among different tissues, we have generated four transgenic strains that overexpress

211   LMP-1 Lyso-Tag specifically in neurons, muscle, intestine, and hypodermis using tissue-specific

212   promoters, *unc-119*, *myo-3*, *ges-1,* and *col-12*, respectively (Figure 3A). Using these transgenic

213   strains, we purified lysosomes in a tissue-specific manner and conducted proteomic profiling.

214   The correlation analysis shows good reproducibility among three independent biological

215   replicates (Figure 3-figure supplement 1 A-D).

216

217   Unlike the whole-body Lyso-IP, the flow-through samples from tissue-specific Lyso-IP contain

218   not only non-lysosomal fractions from the targeted tissue but also lysosomes from non-targeted

219   tissues. Thus, these flow-through samples cannot be simply used as controls to determine the

220   enrichment of proteins at the lysosome in the targeted tissue. To assess tissue-specific changes,

221   we have normalized the level of each identified protein to the level of LMP-1 in the same

222   replicate, and then compared the normalized ratio between the whole-body Lyso-IP and the

223   tissue-specific Lyso-IP (Figure 3B-E, Supplementary File 2). We found that among the 216

224   proteins identified from the whole-body Lyso-IP, 85 of them show comparable ratios between

225   the whole-body Lyso-IP and the four tissue-specific Lyso-IPs (Figure 3F, Group I), suggesting

226   relative homogenous lysosomal enrichments of these proteins among different tissues. Nine of

227   them were completely absent in the tissue-specific Lyso-IP, which may be related to their low

228   abundance (Figure 3F, Group IV).

229

230   Furthermore, there are 122 proteins that exhibited significant differences in their enrichments

231   between the whole-body Lyso-IP and the tissue-specific Lyso-IPs ($p<0.05$), 56 of them (Group

232   II) showing an increase in the tissue-specific Lyso-IPs while the other 66 (Group III) showing a

233   decrease (Figure 3F). One of the candidates in Group II is Y58A7A.1, a copper uptake

234   transporter, that shows a higher ratio in the hypodermis (Figure 3F). Copper transporters are

235   crucial players in various biological processes and copper dysfunction contributes to oxidative

236   stress, impaired respiration and enzymic activities, and disease progression. To validate whether

237   Y58A7A.1 is a copper transporter specifically localized at the lysosome in the hypodermis, we

238   generated a CRISPR knock-in line where the endogenous Y58A7A.1 is tagged with

239   mNeonGreen. Using this line, we confirmed the hypodermis-specific lysosomal localization of

240   Y58A7A.1 (Figure 3-figure supplement 1 E).

241

242   These results show that the lysosomal proteome exhibits heterogeneity among different tissues

243   within the organism, which may be related to the metabolic status in each tissue and,

244   consequently, contribute to the specific activities and signaling effects of the tissue. Our studies

245   provide a list of candidates for further investigation into the tissue-specific regulation of

246   lysosomal metabolism and signaling.

247

248   **Lysosome-enriched proteome alterations associate with different pro-longevity mechanisms**

249

250   Considering the emerging role of lysosomes as a cellular hub to integrate protein signals and

251   regulate longevity, we next examined whether the protein composition of lysosomes exhibits

252   heterogeneity in association with different longevity mechanisms. To this end, we crossed LMP-

253   1 *LysoTg* with four different long-lived strains: the *lipl-4* transgenic strain (*lipl-4 Tg*) that

254   constitutively expresses a lysosomal acid lipase (Wang et al., 2008), the loss-of-function mutant

255   of *daf-2* (*daf-2(lf)*) that encodes the insulin/IGF-1 receptor (Kenyon et al., 1993; Martins et al.,

256   2016), the loss-of-function mutant of *isp-1* (*isp-1(lf)*) that reduces mitochondrial electron

257   transport chain complex III activity (Feng et al., 2001), and the *glp-1* loss-of-function mutant

258   (*glp-1(lf)*) that has a defective germline at 25 °C non-permissive temperature (Berman & Kenyon,

259   2006) (Figure 4A). We then conducted Lyso-IP proteomic analyses and compared lysosome-

260   enriched proteomes between wild-type and long-lived strains. The correlation analysis shows

261   good reproducibility among three independent biological replicates (Figure 4-figure supplement

262   1 A-D), and the PCA analysis shows a clear separation between Lyso-IP replicates and flow-

263   through controls (Figure 4-figure supplement 1 E, F).

264

265   In the *lipl-4 Tg* worms, we have identified 449 lysosome-enriched proteins (Supplementary File

266   3), and 176 of them overlap with the candidates from WT worms (Figure 4B). Thus, 82% of

267   proteins enriched on WT lysosomes are also enriched on *lipl-4 Tg* lysosomes; however, 61% of

268   proteins enriched on *lipl-4 Tg* lysosomes are absent in WT lysosomes (Figure 4B). In parallel,

269   259 lysosome-enriched proteins were identified in the *daf-2(lf)* mutant using LMP-1 Lyso-IP

270   (Supplementary File 4), 147 of them overlapping with the LMP-1 Lyso-IP candidates from WT

271   worms, 197 of them overlapping with the LMP-1 Lyso-IP candidates from the *lipl-4 Tg* worms,

272   and 55 unique to the *daf-2(lf)* mutant (Figure 4C). In the *isp-1(lf)* mutant, we identified 177

273   lysosome-enriched proteins (Supplementary File 5). Among them, 26 candidates are unique to

274   the *isp-1(lf)* mutant, while 107, 135 and 126 candidates overlap with those in the WT, *lipl-4 Tg*

275   and *daf-2(lf)* worms, respectively (Figure 4C). Meanwhile, 200 lysosome-enriched proteins were

276   identified in the *glp-1(lf)* mutant (Supplementary File 6). When compared to the control worms

277   growing at the same 25 °C temperature (Supplementary File 7), 43 were unique to the *glp-1(lf)*

278   mutant, while 157 overlapped with WT candidates (Figure 4-figure supplement 1 G). Overall,

279   there is only one lysosome-enriched candidate shared among all four long-lived strains but

280   absent from the WT lysosome-enriched proteome (Figure 4-figure supplement 1 H), suggesting

281   that distinct pro-longevity mechanisms influence the protein composition of the lysosome in their

282   specific ways. Furthermore, for the long-lived *daf-2(lf), isp-1(lf)* and *glp-1(lf)* worms, the

283   overlaps of their lysosome-enriched proteome with the WT are 57%, 60% and 78.5% (Figure 4C,

284   Figure 4-figure supplement 1 G), respectively. These percentages are higher than the 39%

285   overlap observed between the long-lived *lipl-4 Tg* worms and the WT (Figure 4B). These results

286   support that increased lysosomal lipolysis leads to bigger changes on lysosomal protein

287   composition than other pro-longevity mechanisms.

288

289   In the *lipl-4 Tg, daf-2(lf),* and the *glp-1(lf)* lysosome-enriched proteomes, we found the

290   enrichment of autophagosome proteins and proteins that mediate the fusion between

291   autophagosomes and lysosomes, including ATG-9/ATG9A (*lipl-4 Tg, daf-2(lf),* and *glp-1(lf)*),

292   SQST-1/SQSTM1 (*daf-2(lf)* and *glp-1(lf)*), EPG-7/RB1CC1 (*lipl-4 Tg*), VAMP-7/VAMP8 (*lipl-*

293   *4 Tg*) and Y75B8A.24/PI4KIIα (*lipl-4 Tg*) (Figure 5A), which is consistent with the previously

294   reported induction of autophagy in these long-lived conditions (Lapierre et al., 2011; Nakamura

295   & Yoshimori, 2018);.

296

297   In addition, we found that the Ragulator complex, LMTR-2/LAMTOR2, LMTR-3/LAMTOR3

298   and LMTR-5/LAMTOR5, that serves as a scaffold for the activation of mTORC1 and AMPK

299   (Zhang et al., 2014), shows a higher enrichment at the lysosome from the *lipl-4 Tg* worms than

300   WT (Figure 5A). However, such increased enrichments were not detected in the lysosome from

301   the *daf-2(lf), isp-1(lf)* or *glp-1(lf)* mutant (Figure 5A). It is known that the Ragulator complex

302   mediates the lysosomal activation of AMPK (Zhang et al., 2014). There are two homologs of

303   AMPK catalytic units, AAK-1 and AAK-2 in *C. elegans.* We found that AAK-2 is enriched

304   more than 10-fold in the Lyso-IP samples compared to the flow-through controls from the *lipl-4*

305   *Tg* worms, but it is only detected in the flow-through controls from WT worms (Figure 5B).

306   Likely due to its low abundance, AAK-1 was detected twice in the Lyso-IP samples from the

307   *lipl-4 Tg* worms but once only in the flow-through sample from WT worms (Figure 5B). On the

308   other hand, AAK-1 and AAK-2 were not present in the lysosome-enriched proteome from the

309   *daf-2(lf), isp-1(lf)* or *glp-1(lf)* mutant (Figure 5B). These results suggest that AMPK is

310   specifically recruited to the lysosomal surface in the *lipl-4 Tg* worms, which may contribute to

311   the pro-longevity effect. To test this idea, we inactivated AMPK using the *aak-2* loss-of-function

312   mutant together with the *aak-1* RNA interference (RNAi) knockdown. We found that the AMPK

313   inactivation reduces the lifespans of the *lipl-4 Tg* and WT worms by 29% and 17%, respectively,

314   and suppresses the lifespan extension caused by *lipl-4 Tg* from 72% to 48% (Figure 5C,

315   Supplementary File 8). Thus, *aak-1* and *aak-2* are partially responsible for the lifespan extension

316   caused by *lipl-4 Tg*. For the AMPK catalytic subunits, It is known that the activation of AMPK

317   displays high spatial specificity in the cell when responding to different upstream stimuli (Khan

318   & Frigo, 2017). In *C. elegans,* it was previously shown that AAK-2 mediates the longevity effect

319   conferred by the *daf-2(lf)* mutant (Apfeld et al., 2004). Our results indicate that this regulation

320   might not be associated with the lysosomal activation of AMPK, and the spatial specificity of

321　　AMPK activation at different subcellular compartments may be linked with different longevity

322　　mechanisms.

323

324　　Moreover, compared to WT lysosomes, the enrichment of lysosomal v-ATPase is higher in *lipl-4*

325　　*Tg* lysosomes, especially for the V0 subunits, VHA-1, 2, 3 the V1 subunits, VHA-11 and VHA-

326　　15, and the v-ATPase transporting accessory proteins, VHA-19 and VHA-20 (Figure 5A). There

327　　are also 13 lysosomal channels/transporters, including T14B4.3/ATP6AP2, the proton-

328　　translocating ATPases required for the v-ATPase-mediated lysosomal acidification (Cruciat et al.,

329　　2010) and CLH-6/CLCN7, the H(+)/Cl(-) exchange transporter mediating the acidification of the

330　　lysosome (Graves et al., 2008; Nicoli et al., 2019), and 16 lysosomal hydrolases that are

331　　specifically associated with the *lipl-4 Tg* lysosomes (Figure 5A). However, none of these

332　　components exhibit increased enrichments in the lysosome from the *daf-2(lf), isp-1(lf)* or *glp-1(lf)*

333　　mutant (Figure 5A). Together, these results suggest that the proportion of mature acidic

334　　lysosomes may be increased in the *lipl-4 Tg* worms*,* which may lead to increased autophagy, the

335　　lysosomal activation of AMPK, and consequently the induction of longevity.

336

337　　**Enhanced lysosome-nucleus proximity mediates longevity responding to lysosomal lipolysis**

338

339　　It is known that the luminal pH of lysosomes is affected by their cellular position, with

340　　perinuclear lysosomes being more acidic (Johnson et al., 2016). In the cell, mobile lysosomes

341　　can change their distribution along the perinuclear-peripheral axis in response to different

342　　nutrient signals and metabolic status (Ballabio & Bonifacino, 2020; Pu et al., 2016). Interestingly,

343　　when analyzing the LMP-1 lysosome-enriched proteome in the *lipl-4 Tg* worms, we found an

344   enrichment of nucleus-localized proteins (Figure 6A), including two nucleoporin proteins NPP-

345   6/Nup160 and NPP-15/Nup133 in the Nup160 complex that localizes at the basket side of the

346   nuclear pore (Figure 6B) (Vasu et al., 2001). Such enrichment of nucleoporin proteins was not

347   found in the LMP-1 lysosome-enriched proteome of the *daf-2(lf), isp-1(lf)* or *glp-1(lf)* long-lived

348   mutant. We thus hypothesize that LIPL-4-induced lysosomal lipolysis may increase the

349   proximity between lysosomes and the nucleus, accompanied by an increase in lysosomal acidity.

350

351   To test this hypothesis, we imaged lysosomal positions in intestinal cells where *lipl-4* is

352   expressed. Using a dual reporter strain expressing both lysosomal LMP-1::RFP fusion and

353   nucleus-localized GFP, we found that lysosomes exhibit a dispersed pattern in the intestinal cell

354   of WT worms (Figure 6C). However, in the *lipl-4 Tg* worms, lysosomes are clustered in the

355   perinuclear region (Figure 6C), supporting the hypothesis that the proximity between lysosomes

356   and the nucleus is increased. To quantitatively measure this change in lysosomal positioning, we

357   analyzed the RFP fluorescent signal distribution in intestinal cells (Figure 6-figure supplement 1

358   A). We found the perinuclear and peripheral distribution of lysosomes in the *lipl-4 Tg* worms is

359   significantly increased and decreased, respectively, compared to WT worms (*p<0.01*, Figure 6D,

360   Figure 6-figure supplement 1 B). In contrast, such perinuclear clustering is not observed in

361   intestinal cells of the *daf-2(lf)* mutant (Figure 6E, 6F, Figure 6-figure supplement 1 C).

362

363   Moreover, we found that the RNAi knockdown of *npp-6* suppresses the lifespan extension in the

364   *lipl-4 Tg* worms (Figure 6G, 6H) but does not affect the lifespan extension in the *daf-2(lf)*

365   (Figure 6I, 6J) or the *isp-1(lf)* mutant (Figure 6-figure supplement 1 D, E). These results suggest

366   that the nucleoporin protein NPP-6 is specifically involved in the regulation of lysosomal LIPL-

367   4-induced longevity. Given the importance of nucleoporin in nuclear transport, we further test

368   whether nuclear import and/or export may play a role in regulating *lipl-4 Tg* induced longevity.

369   To this end, we knocked down *xpo-1* and *ima-3*, which encodes Exportin-1 and Importin-α,

370   mediating nuclear export and import, respectively, by RNAi. We found that the RNAi

371   inactivation of *ima-3,* but not *xpo-1* suppresses the lifespan extension caused by *lipl-4 Tg* (Figure

372   6-figure supplement 1 F-H and Supplementary File 8). These results suggest that the increased

373   proximity between lysosomes and the nucleus may facilitate the nuclear import of *lipl-4 Tg-*

374   induced lysosomal retrograde signals to promote longevity.

375

376   **Cystinosin positive mature lysosomes enrich specific lysosomal proteins**

377

378   The analysis of the candidates specifically detected in the *lipl-4 Tg* worms suggests that the

379   proportion of mature lysosomes may affect lysosomal protein composition. Although LMP-1 is a

380   well-established lysosomal protein marker and highly abundant on the lysosomal surface, it can

381   be also detected in late endosomes and sometimes in early endocytic compartments. With the

382   hope to profile proteins enriched in mature lysosomes, we chose CTNS-1, the *C. elegans*

383   lysosomal cystine transporter Cystinosin that is a well-established marker of mature lysosomes

384   (Gahl et al., 1982; Jonas et al., 1982; Kalatzis et al., 2001). Using CRISPR knock-in lines with

385   endogenous CTNS-1 and LMP-1 tagged with wrmScarlet and mNeonGreen, respectively, we

386   found that CTNS-1 and LMP-1 signals show only partial overlap in the intestine, muscle,

387   hypodermis and neurons (Figure 7-figure supplement 1). We then generated a transgenic strain

388   expressing CTNS-1 tagged with both 3×HA and RFP (CTNS-1 *lysoTg*). Fluorescence imaging of

389   RFP confirmed the lysosomal localization of the CTNS-1 fusion protein in live organisms

390   (Figure 7A). Using this transgenic strain, we followed the same Lyso-IP and MS profiling

391   pipeline. The correlation analysis shows good reproducibility among three independent

392   biological replicates (Figure 7-figure supplement 2 A), and the PCA analysis indicates a clear

393   separation between Lyso-IP samples and flow-through controls (Figure 7-figure supplement 2 B).

394   Using the same selection criteria, we identified 293 candidates whose levels are enriched at least

395   10-fold in the purified lysosomes than those in the flow-through controls among all independent

396   biological replicates and show over 2-fold enrichment compared to the non-tag controls

397   (Supplementary File 9). There are 95 lysosome-enriched proteins shared between the LMP-1 and

398   the CTNS-1 Lyso-IP proteomic profiling datasets (Figure 7B, Supplementary File 9), and 47 of

399   these shared proteins are annotated with lysosomal localization (Supplementary File 9). We have

400   also crossed the CTNS-1 *lysoTg* strain with the *lipl-4 Tg*, *daf-2(lf),* and *glp-1(lf)* worms and then

401   conducted Lyso-IP proteomic profiling. However, the pull-down efficiency was very low in

402   these long-lived worms, which prevented us from identifying proteins unique to CTNS-1 Lyso-

403   IP in those conditions.

404

405   In WT worms, the proportions of the identified proteins with different categories of subcellular

406   annotation are comparable between LMP-1 and CTNS-1 Lyso-IP conditions (Figure 7C, 2E),

407   and for proteins with lysosomal annotation, the proportion is 25% and 28% in LMP-1 and

408   CTNS-1 Lyso-IP, respectively (Figure 7D). However, among the 121 proteins only identified in

409   LMP-1 Lyso-IP, there are only 8 with lysosomal annotation (7%); while for the 198 proteins

410   only identified in CTNS-1 Lyso-IP, 35 are with lysosomal annotation and the proportion remains

411   as 18% (Figure 7D).

412

413   Among the lysosomal proteins that are unique to CTNS-1 Lyso-IP, there are autophagosome

414   proteins and proteins that mediate the fusion between autophagosomes and lysosomes, including

415   ATG-9/ATG9A (Popovic & Dikic, 2014), C33A11.2/DRAM2 (Crighton et al., 2006), EPG-

416   7/RB1CC1 (Nishimura et al., 2013), and VAMP-7/VAMP8 (Diao et al., 2015; Itakura et al.,

417   2012) (Figure 7E). Furthermore, the Ragulator complex components LMTR-2/3/5, the lysosomal

418   amino acid transporter F13H10.3/SLC38A9 and the lysosomal calcium channel CUP-5/TRPML1

419   that regulate mTORC1 signaling (Li et al., 2016; Rebsamen et al., 2015; Wang et al., 2015;

420   Wyant et al., 2017) exhibited a higher enrichment in the lysosome purified from WT worms

421   using CTNS-1 Lyso-IP than using LMP-1 Lyso-IP (Figure 7E). To further confirm the increased

422   enrichment of mTORC1 signaling components with CTNS-1 lysosomes, we generated a

423   CRISPR knock-in line with endogenous LMTR-3 tagged with wrmScarlet to visualize its

424   subcellular localization. After crossing this line with LMP-1::mNeonGreen and CTNS-1::

425   mNeonGreen knock-in lines, we found that LMTR-3 shows a complete overlap with CTNS-1 in

426   the intestine, muscle and hypodermis (Figure 7F, Figure 7-figure supplement 2 C), but it only

427   shows a partial overlap with LMP-1 in the intestine, muscle and hypodermis (Figure 7F, Figure

428   7-figure supplement 2 D).

429

430   Furthermore, when systemically examining 85 lysosome-related proteins that were previously

431   annotated in *C. elegans* based on sequence homology (Sun et al., 2020), we found that 63 were

432   detected in the proteomic profiling, while 22 were not detected likely due to their low abundance

433   (Figure 7G). Many lysosomal hydrolases exhibit increased enrichments with CTNS-1 Lyso-IP.

434   Interestingly, similar increased enrichments of these candidates were also observed in the LMP-1

435   Lyso-IP result using the *lipl-4 Tg* worms (Figure 7E, 7G). These results further support that the

436   long-lived *lipl-4 Tg* worms carry more acidic lysosomes. The enrichment of cysteine proteases

437   including CPR-6, LGMN-1, CPL-1, CPZ-1 and TAG-196 is consistent with that CTNS-1 is

438   located at mature lysosomes as a cysteine transporter (Gahl et al., 1982; Jonas et al., 1982;

439   Kalatzis et al., 2001). Together, we found that lysosome-enriched proteomes identified from both

440   LMP-1 and CTNS-1 Lyso-IP consist of well-characterized lysosomal enzymes and integral

441   membrane proteins as well as proteins that contribute to lysosomal signaling, dynamics and

442   contact with other cellular compartments. Besides many known lysosomal proteins, various

443   proteins that are not previously linked with lysosomes are now identified through these

444   systematic analyses.

445

446   **Lysosome-enriched proteins regulate different lysosomal activities**

447

448   To understand the role of these newly identified lysosome-enriched proteins in regulating

449   lysosomal functions, we have examined their effects on lysosomes using an RNAi screen based

450   on LysoSensor fluorescence intensity. We focused on 95 lysosome-enriched proteins shared

451   between LMP-1 and CTNS-1 Lyso-IPs and knocked down their coding genes by RNAi, and then

452   used LysoSensor probes to stain lysosomes. From screening these 95 candidates (Supplementary

453   File 10), we have identified five genes whose inactivation cause changes in LysoSensor signal

454   intensity, and four of them have human homologs, including two lysosomal v-ATPase subunits,

455   UNC-32/ATP6V0A and VHA-5/ATP6V0A, the lysosomal amino acid transporter SLC-

456   36.2/SLC36A1 (SLC36A4), and a transmembrane protein R144.6/TMEM144 (Supplementary

457   File 10, Figure 8A-E, Figure 8-figure supplement 1). We further examined their effects on the

458   lysosomal number, size, and pH. We found that the RNAi knockdown of the two lysosomal v-

459 ATPase subunits, UNC-32 and VHA-5, lead to decreased lysosomal numbers (Figure 8F), but an

460 increase in the lysosomal size (Figure 8G). We also used fluorescence lifetime microscopy to

461 measure the fluorescence lifetime of LysoSensor, which is negatively correlated with pH (Deng

462 et al., 2023; Lin et al., 2001). Unexpectedly, we found that the RNAi knockdown of *unc-32*

463 increases the fluorescence lifetime of LysoSensor, indicating a decrease in lysosomal pH (Figure

464 8H). We think this decrease is an attempt to compensate for the 2.5-fold reduction in the total

465 number of lysosomes. Overall, *unc-32* inactivation compromises lysosomal v-ATPase and leads

466 to a defect in lysosomal maturation. On the other hand, the RNAi knockdown of R144.6 did not

467 affect lysosomal number or size but increased lysosomal pH (Figure 8F-H).

468

469 Unlike well-known lysosomal proteins, UNC-32, VHA-5 and SLC-36.2, the subcellular

470 localization of the R144.6 protein remains unknown. R144.6 is a predicted carbohydrate

471 transporter, and structural simulation using AlphaFold2 suggested it as a solute carrier family

472 (SLC) transporter (Figure 8I). We generated a CRISPR knock-in line in which the endogenous

473 R144.6 protein is fused with mNeonGreen and then stained these worms with LysoTracker Red

474 to mark lysosomes. In the hypodermis, we found that mNeonGreen and LysoTracker Red signals

475 overlap, confirming the lysosomal localization of this newly identified transmembrane protein

476 from Lyso-IP (Figure 8J). On the other hand, its expression was not detected in the muscle, and

477 in the intestine, its mNeonGreen signals did not overlap with LysoTracker Red, which is

478 consistent with tissue-specific Lyso-IP analyses. R144.6 was enriched in the hypodermis-specific

479 Lyso-IP at a similar level as in the whole-body Lyso-IP; however, it was not detected in the

480 muscle- or intestine-specific Lyso-IP. These results further support the enrichment specificity of

481   proteins at the lysosome in different tissues as well as the power of the Lyso-IP proteomic

482   profiling in discovering new lysosomal proteins with functional significance.

483

484   **DISCUSSION**

485

486   Our studies reveal the heterogeneity of lysosomal protein composition that is associated with

487   lysosomal status, tissue specificity, and organism longevity. Through systematic profiling of

488   lysosome-enriched proteins under different conditions, we confirmed the induction of lysosome-

489   related autophagy by different longevity-promoting pathways, unveiled increased cellular

490   interaction between lysosomes and the nucleus upon the induction of lysosomal lipolysis and its

491   contribution to longevity regulation, and underlined the importance of the spatial control of

492   AMPK activation in regulating longevity. Our work provides not only methods for future studies

493   to profile the dynamics of the lysosomal proteome in response to diverse physiological inputs,

494   but also resources for understanding the vital contribution of these dynamics in modulating

495   signal transduction, organelle crosstalk and organism longevity.

496

497   These proteomic studies can provide hits for changes in the interaction between lysosomes and

498   other organelles under different conditions. One example is the lysosome-nucleus interaction. In

499   the Lyso-IP fraction from WT worms, we did not detect any proteins with sole nuclear

500   localization; however, in the Lyso-IP fraction from the *lipl-4 Tg* or *daf-2(lf)* mutant worms,

501   nuclear proteins were identified and the percentage of the increase over WT is significantly

502   higher in the *lipl-4 Tg* worms ($p<0.05$, Figure 6A, Figure 6-figure supplement 1 I). Based on this

503   finding, we discovered the previously unknown perinuclear accumulation of lysosomes in the

504   *lipl-4 Tg* worms (Figure 6C, 6D) and further confirmed its importance for longevity regulation

505   (Figure 6G, 6H). It has been shown previously that perinuclear lysosomes are more acidic than

506   peripheral lysosomes (Johnson et al., 2016; Webb et al., 2021). Thus, the increase in perinuclear

507   lysosomes may be associated with the increased proportion of mature lysosomes in the *lipl-4 Tg*

508   worms, which is supported by the increased enrichments of lysosomal v-ATPase,

509   channels/transporters and hydrolases (Figure 5A). This increased distribution of lysosomes

510   toward the perinuclear region could facilitate proteins and metabolites transporting from the

511   lysosome to the nucleus through the nuclear pore and in turn their signaling effects. However,

512   whether this perinuclear distribution of lysosomes is associated with an increase in the direct

513   contact between lysosomes and the nucleus remains to be determined using technologies with

514   higher spatial resolution such as electron microscopy imaging. On the other hand, we did not

515   detect perinuclear accumulation of lysosomes in the *daf-2(lf)* mutant worms by cellular imaging,

516   and the nuclear proteins detected through LMP-1 Lyso-IP from the *daf-2(lf)* mutant worms are

517   mainly involved in RNA splicing. In yeast cells, defects in pre-mRNA processing have been

518   associated with nucleophagy (Leger-Silvestre et al., 2005) that involves SQSTM1 and lysosomes

519   (Ivanov et al., 2013; Mijaljica & Devenish, 2013). We thus speculate that the increased

520   enrichment of nuclear proteins in the *daf-2(lf)* mutant worms may be associated with the

521   induction of nucleophagy but not changes in lysosomal positioning.

522

523   mTORC1 and AMPK are key metabolic checkpoints that regulate anabolic and catabolic

524   processes in mutually opposing ways. In sensing the lack of nutrients, AMPK signals activate the

525   catabolic process while inhibiting the anabolic one. On the other hand, responding to nutrient

526   availability, mTORC1 activation upregulates anabolic metabolism and promotes cell growth.

527   Intriguingly, it is now known that both mTORC1 and AMPK are recruited to the lysosomal

528   surface for activation, which requires the scaffold Ragulator complex that consists of LAMTOR

529   subunits. We found that the Ragulator complex (LMTR-2, 3 and 5) shows a higher enrichment in

530   the lysosome purified from WT worms using CTNS-1 Lyso-IP than using LMP-1 Lyso-IP

531   (Figure 7E), and cellular imaging of endogenous LMTR-3 confirmed its much higher overlap

532   with CTNS-1-positive lysosomes than with LMP-1-positive lysosomes (Figure 7F, Figure 7-

533   figure supplement 2 C, D). These results suggest a predominant association of the Ragulator

534   complex with mature lysosomes, which could in turn determine the preference of mTORC1 and

535   AMPK activation at the lysosomal surface. Alternatively, it would be also possible that the

536   Ragulator complex carries a preference toward CTNS-1/Cystinosin-containing lysosomes, which

537   would infer the interaction between lysosomal cysteine metabolism and mTORC1 signaling.

538   Interestingly, previous studies show that Cystinosin co-immunoprecipitates with the Ragulator

539   complex in mammalian cells (Andrzejewska et al., 2016), and in *Drosophila,* cysteine efflux

540   from the lysosome via Cystinosin antagonizes mTORC1 signaling and upregulates the

541   tricarboxylic acid cycle (Jouandin et al., 2022). Whether this inhibitory effect of lysosomal

542   cysteine on mTORC1 is related to the preferential interaction between the Ragulator complex

543   and Cystinosin would be an interesting question for future studies.

544

545   Both mTORC1 and AMPK have been implicated in the regulation of longevity across different

546   species, being intertwined with other longevity regulatory mechanisms (Savini et al., 2019). In

547   the long-lived *lipl-4 Tg* worms, the lysosomal enrichment of the Ragulator complex is increased

548   with LMP-1 Lyso-IP, which may be a result of the increased proportion of mature lysosomes

549   upon the induction of lysosomal lipolysis. At the same time, we could not rule out the possibility

550   that the increased enrichment of the Ragulator complex is a result of the induced level of

551   lysosomal CTNS-1/Cystinosin in the *lipl-4 Tg* worms. We found that with LMP-1 Lyso-IP, the

552   level of the CTNS-1/Cystinosin transporter is increased in the *lipl-4 Tg* worms, together with the

553   increase of several cysteine cathepsins (Figure 7G). Our previous studies found that

554   mitochondrial β-oxidation is increased in the *lipl-4 Tg* worms, leading to decreased triglyceride

555   storage (Ramachandran et al., 2019). The *lipl-4 Tg* worms also show induced autophagy (Mak et

556   al., 2020). These phenotypes are the same as those observed in fruit flies with Cystinosin

557   overexpression (Jouandin et al., 2022). Considering the inhibitory effect of Cystinosin on

558   mTORC1 in fruit flies, the induction of Cystinosin in the *lipl-4 Tg* worms might reduce

559   mTORC1 signaling. In supporting this idea, our unpublished study shows that *lipl-4 Tg* does not

560   further enhance the lifespan extension of the *raga-1* mutant that has reduced mTORC1 signaling.

561   Furthermore, our study reveals the lysosomal enrichment of AMPK in the *lipl-4 Tg* worms, and

562   its requirement for the longevity effect (Figure 5B, 5C). On the other hand, the involvement of

563   lysosomal mTORC1 and AMPK signaling in regulating the longevity effect was not identified in

564   the *daf-2(lf), isp-1(lf)* or *glp-1(lf)* mutant. Organelle-specific signaling regulation of longevity

565   would be interesting topics for future studies.

566

567   **MATERIALS AND METHODS**

568

569   *C. elegans* **strains and maintenance**

570   The following strains were used in this study: N2, CB1370 *daf-2(e1370),* RB754 *aak-2(ok524)*,

571   *unc-76(e911)*, MCW953 *nre-1(hd20);lin-15b(hd126)*, MCW14 *raxIs3 [ges-1p::lipl-4::SL2GFP]*,

572   MCW859 *raxIs103[sur-5p:lmp-1::RFP-3×HA;unc-76(+)]* (*sur-5* promoter for whole-body

573   overexpression), MCW935 *daf-2(e1370);raxIs103[sur-5p:lmp-1::RFP-3×HA;unc-76(+)]*,

574   MCW923 *raxIs3[ges-1p::lipl-4::SL2GFP];raxIs103[sur-5p:lmp-1::RFP-3×HA;unc-76(+)]*,

575   *MCW861 unc-76(e911);raxEx311[Pmyo-3:lmp-1::RFP-3×HA;unc-76(+)]* (*myo-3* promoter for

576   muscle overexpression), MCW924 *unc-76(e911); raxEx346[Pcol-12:lmp-1::RFP-3×HA;unc-*

577   *76(+)]* (*col-12* promoter for hypodermis overexpression), MCW862 *unc-*

578   *76(e911);raxEx312[Punc-119:lmp-1::RFP-3×HA;unc-76(+)]* (*unc-119* promoter for neuron

579   overexpression), MCW914 *unc-76(e911);raxEx341[Pges-1:lmp-1::RFP-3×HA;unc-76(+)]*

580   (*ges-1* promoter for intestine overexpression), MCW934 *raxIs118[sur-5p:ctns-1::RFP-*

581   *3×HA;unc-76(+)]*.The strains *Y58A7A.1(syb7950[Y58A7A.1::mNeonGreen])*,

582   *R144.6(syb4893[R144.6::mNeonGreen], lmp-1(syb4827[lmp-1::mNeonGreen]), ctns-*

583   *1(syb5019[ctns-1::wrmscarlet]), ctns-1(syb4805[ctns-1::mNeonGreen]), and lmtr-*

584   *3(syb8005[wrmscarlet::lmtr-3])* were generated via CRISPR/Cas9 genome editing by

585   SunyBiotech (Fuzhou, China). The strains N2, CB1370, and RB754 were obtained from

586   *Caenorhabditis* Genetics Center (CGC). The strain *unc-76(e911)* was obtained from Dr. Zheng

587   Zhou's Lab. Other strains were generated in our lab.

588

589   *C. elegans* strains were maintained at 20˚C on standard NGM agar plates seeded with OP50

590   *E.coli* (HT115 *E. coli* for RNAi experiments) using standard protocols (Stiernagle, 2006) and

591   kept at least three generations without starvation before experiments.

592

593   **Molecular cloning and generating transgenics**

594   All the expression constructs were generated using the Multisite Gateway System (Invitrogen) as

595   previously described (Mutlu et al., 2020). The *lmp-1* and *ctns-1* coding sequences were PCR-

596 amplified from *C. elegans* cDNA then inframe fused with RFP-3×HA, and all promoters were

597 PCR-amplified from *C. elegans* genomic DNA.

598

599 Transgenic strains were generated by microinjecting the day-1-adult germline of *unc-76(e911)*

600 worms with DNA mixture containing expression construct and *unc-76(+)* rescuing plasmid. For

601 integration strains, the stable extrachromosomal arrays were integrated with gamma irradiation

602 (4500 rads for 5.9 minutes) and backcrossing to wild-type N2 at least 8 times.

603

604 **Lysosome immunoprecipitation (Lyso-IP)**

605 Lyso-IP is based on the method used in mammalian cells (Abu-Remaileh et al., 2017). Briefly,

606 transgenic strains stably expressing C-terminal RFP- and 3×HA-tagged lysosomal membrane

607 protein LMP-1 or CTNS-1 under whole-body *Psur-5* or tissue-specific promoters were generated.

608 Around 160,000 day-1-adult worms per genotype were collected, washed 3 times with M9 buffer

609 then washed 1 time with ice-cold KPBS buffer (136 mM KCl, 10 mM KH_2PO_4). Worms in 2 ml

610 ice-cold KPBS were quickly homogenized with Dounce homogenizer (Sigma cat. # D9063) on

611 ice until no visible animals were seen under the microscope. The lysate was centrifuged at 1000

612 g for 3 min at 4 ˚C to remove debris and then the supernatant was incubated with anti-HA

613 magnetic beads (Thermo Fisher Scientific, cat. # 88837, washed 3 times with ice-cold KPBS

614 buffer before use. Each IP needs a 160 ul of beads.) for 6 minutes at 20 ˚C with rotation. The

615 bound beads and flowthrough were separated using a magnetic stand. The bound bead fraction

616 was washed 4 times with ice-cold KPBS. The bound bead and flowthrough fractions were both

617 used for LC/MS-based proteomics analyses. In order to finish processing all samples as quickly

618 as possible, no more than 3 samples were processed in parallel.

619

LC/MS-based proteomic analyses

621 The bound beads after washing were directly eluted in 100 µl of 5% SDS buffer and trypsin

622 digestion was carried out using S-Trap™ (Protifi, NY) as per manufacturer's protocol. For the

623 flow-through sample after IP, 100 µl sample was diluted in 5% SDS buffer and trypsin digestion

624 was carried out using S-Trap™. The peptide concentration was measured using the Pierce™

625 Quantitative Colorimetric Peptide Assay (Thermo Scientific cat. # 23275). The digested peptides

626 were subjected to simple C18 clean-up using a C18 disk plug (3M Empore C18) and dried in a

627 speed vac. 1 µg of the peptide was used for LC-MS/MS analysis which was carried out using a

628 nano-LC 1200 system (Thermo Fisher Scientific, San Jose, CA) coupled to Orbitrap Fusion™

629 Lumos ETD mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The peptides were

630 loaded on a two-column setup using a pre-column trap of 2 cm × 100 µm size (Reprosil-Pur

631 Basic C18 1.9 µm, Dr. Maisch GmbH, Germany) and a 5 cm × 75 µm analytical column

632 (Reprosil-Pur Basic C18 1.9 µm, Dr. Maisch GmbH, Germany) with a 75 min gradient of 5-28%

633 acetonitrile/0.1% formic acid at a flow rate of 750 nl/min. The eluted peptides were directly

634 electro-sprayed into a mass spectrometer operated in the data-dependent acquisition (DDA)

635 mode. The full MS scan was acquired in Orbitrap in the range of 300-1400 m/z at 120,000

636 resolution followed by top 30 MS2 in Ion Trap (AGC 5000, MaxIT 35 ms, HCD 28% collision

637 energy) with 15 sec dynamic exclusion time.

638

639 The raw files were searched using the Mascot algorithm (Mascot 2.4, Matrix Science) against the

640 *Caenorhabditis elegans* NCBI refseq protein database in the Proteome Discoverer (PD 2.1,

641 Thermo Fisher) interface. The precursor mass tolerance was set to 20 ppm, fragment mass

642   tolerance to 0.5 Da, maximum of two missed cleavage was allowed. Dynamic modification of

643   oxidation on methionine, protein N-terminal Acetylation and deamidation (N/Q) was allowed.

644   Assigned peptides are filtered with a 1% FDR using Percolator validation based on q-value, and

645   the Peptide Spectrum Matches (PSMs) output from PD2.5 will be used to group peptides onto

646   gene levels using the 'gpGrouper' algorithm (Saltzman et al., 2018). This in-house program uses

647   a universal peptide grouping logic to accurately allocate and provide MS1 based quantification

648   across multiple gene products. Gene-protein products (GPs) quantification will be performed

649   using the label-free, intensity-based absolute quantification (iBAQ). iBAQ-based fraction of total

650   values (iFOT) was calculated by dividing the iBAQ for each gene product by the total species

651   iBAQ to normalize sample amount variation.

652

653   **Antibodies**

654   Anti-*C. elegans* LMP-1, HSP-60, CYP-33, and SVQ-8 monoclonal antibodies were purchased

655   from Developmental Studies Hybridoma Bank (DSHB). Those antibodies were originally

656   generated by Dr. Michael L. Nonet's lab (Hadwiger et al., 2010). Anti-β-actin antibody (C4) was

657   purchased from Santa Cruz (sc-47778).

658

659   **Microscopy imaging**

660   <u>Regular microscopy</u>

661   Tissue-specific lyso-tag expression example images (Figure 3A) were captured using Leica

662   DMi8 THUNDER Imaging Systems using 20× objective. The images that show the

663   colocalization between CRISPR knock-in lines LMP-1::mNeonGreen and CTNS-1::wrmScarlet

664   were captured by Zeiss LSM 980 with Airyscan. The images that show the colocalization

665   between wrmScarlet::LMTR-3 and LMP-1::mNeonGreen/CTNS-1::mNeonGreen were taken

666   using Nikon CSU-W1 spinning disk confocal microscopy system. Other microscopy images

667   were captured using an Olympus FV3000 confocal microscopy system using 60× or 20×

668   objective. *C. elegans* were anesthetized in 1% sodium azide in M9 buffer and placed on a 2%

669   agarose pad sandwiched between the glass microscopic slide and coverslip.

670

671   <u>Fluorescence lifetime microscopy</u>

672   L1 RNAi sensitive *nre-1(hd20);lin-15b(hd126)* worms were seeded on 3.5cm RNAi plates and

673   raised at 20°C for two days, and then around 20 worms each well were transferred to the 3.5cm

674   RNAi plates containing RNAi bacteria and 0.5 μM of LysoSensor Green DND-189

675   (Invitrogen™ L7535) and raised for 18h (in dark) at 20°C. The worms were imaged using ISS

676   Q2 Time-resolved Laser Scanning Confocal Nanoscope. The laser excitation wavelength was set

677   at 476nm and the 500-633nm emission filter was used to detect the LysoSenor Green signal. The

678   first pair of intestinal cells of each worm was imaged. The lifetimes of LysoSensor-containing

679   puncta were measured using ISS VistaVision software. The pH of the lysosome was then

680   calculated based on the LysoSensor lifetime-pH calibration curve (Deng et al., 2023).

681

682   **LysoSensor RNAi screen**

683   The primary screen was performed on 95 lysosomal-enriched candidates shared between the

684   LMP-1 and the CTNS-1 Lyso-IP proteomic profiling datasets. Each RNAi bacteria clone was

685   seeded onto 12-well RNAi plates containing 1 mM IPTG and allowed to dry. The dried plates

686   were then incubated at room temperature overnight to induce dsRNA expression. Synchronized

687   L1 *nre-1(hd20);lin-15b(hd126)* worms were seeded on 12-well RNAi plates and raised at 20 °C

688   for two days, and then around 30 worms each well were transferred to the RNAi plates

689   containing RNAi bacteria and 0.5 μM of LysoSensor Green DND-189. After 18 hours,

690   LysoSensor signals were examined by the naked eyes using a Nikon SMZ18 fluorescence stereo

691   microscope. The candidates with obvious LysoSensor alteration were selected for the secondary

692   LysosSensor RNAi screen. In the secondary screen, worms stained using LysoSensor Green

693   DND-189 were imaged by the Olympus FV3000 confocal microscopy system. The changes in

694   LysoSensor signals in the first pair of intestine cells were quantified by ImageJ (including

695   intensity, size, and number).

696

697   **Lysosome distribution quantification**

698   The quantification method was modified from previous publications on lysosomal distribution in

699   mammalian cell lines (Johnson et al., 2016; Willett et al., 2017). Images were first captured using

700   Olympus Fluoview software and imported into Matlab by the Bio-Formats tool (Linkert et al.,

701   2010). Next, the cell membrane and nuclear membrane are outlined manually. The algorithm

702   (code included in Supplementary materials) determines the geometric center of the nucleus and

703   radiates at all angles to locate line segments between the nuclear and cell membrane. The line

704   segments are then evenly divided and circled to segment the cytosol into different regions. We

705   calculate: 1. The mean RFP fluorescence distribution across regions (normalized to the mean

706   intensity of the whole cell to avoid variations of RFP expression across cells) (Figure 6D, 6F); 2.

707   The cumulative intensity distribution from the most perinuclear region to the most peripheral

708   region (normalized to the overall intensity of the whole cell) (Figure 6-figure supplement 1 B, C).

709   The algorithm requires a convex shape of the cell, and most of the gut cells imaged meet this

710   need.

711

Lifespan assays

Worms were synchronized by bleach-based egg preparation and subsequent starvation in M9

buffer for over 24 hours. Synchronized L1 worms were placed on the plates, and animals were

synchronized again by manual picking at mid L4 stage and marked as Day 0. Approximately 90-

120 worms were placed in three parallel plates for each condition. Worms are hence observed

and transferred to freshly made RNAi plates every other day. Animals are categorized as alive,

dead (cessation of movement in response to platinum wire probing) or censored. The statistical

analyses were performed with the SPSS23 Kaplan-Meier survival function and the log-rank test.

GraphPad Prism 9 was used to graph the results.

Structural stimulation by AlphaFold2

The structure of R144.6 (UniProt: Q10000) was predicted by AlphaFold2 and downloaded from

The AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/) (Jumper et al., 2021;

Varadi et al., 2022). The molecular graphics of the R144.6 structure were performed with UCSF

Chimera (Pettersen et al., 2004).

Statistical methods

Principal components analysis (PCA) was performed by R package Factextra (Le et al., 2008)

with the normalized iFOT abundance of proteins detected as the input. The Pearson correlation

matrices and coefficient (r) among replicates were generated by GraphPad Prism 9. The unpaired,

two-tailed t-tests for the multiple comparisons were used to calculate the p-values in the

correlation analyses by GraphPad Prism 9.

734

Data availability

The mass spectrometry data for protein identification have been deposited via the MASSIVE

repository (MSV000090909) to the Proteome X change Consortium

(http://proteomecentral.proteomexchange.org) with the dataset identifier PXD038865.

Analysis code for Figure 6D, 6F and Figure 6-figure supplement 1 is included in the Source

Code File 1.

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1055

1056 **FIGURE LEGENDS**

1057

1058 **Figure 1. Rapid lysosome isolation coupled with proteomic profiling**

1059 (**A**) Schematic of the workflow for immunoprecipitation-based lysosome purification (Lyso-IP)

1060 and mass-spectrometry based proteomic profiling to identify lysosome-enriched proteomes in *C.*

1061 *elegans.*

1062 (**B**) Example images of transgenic strains carrying LMP-1 Lyso-Tag (LMP-1::RFP-3×HA) with

1063 LysoTracker staining to mark lysosomes *in vivo*. Scale bar=5 μm.

1064 (**C**) Example images of beads carrying purified lysosomes from Lyso-IP with LysoTracker

1065 staining to mark intact lysosomes *in vitro.* Scale bar=5 μm.

1066 (**D**) Western blot for protein markers of different subcellular compartments using purified

1067 lysosomes (Lyso-IP), paired non-lysosomal fractions (Flow-through) or Pellet.

1068 (**E**) PCA analysis of four independent biological replicates of Lyso-IP and Flow-through samples.
1069

1070 **Figure 2. Systematic view of lysosome-enriched proteome**

1071 (**A**) Scatter plots showing candidate selection from four independent biological replicates in

1072 proteomics analyses. Proteins with at least 10-fold higher levels in Lyso-IP samples than in flow-

1073 through (FT) controls are highlighted with different colors based on repeated times in four

1074 replicates.

1075 (**B**) Scatter plot showing candidate selection with normalization to non-tagged controls using

1076 wild-type worms. 216 proteins with over 2-fold higher levels in Lyso-IP samples than in non-

1077 tagged controls are highlighted in red.

1078 (**C**) Pie chart showing molecular function categories of lysosome-enriched proteins.

1079 (**D**) The lysosomal enrichment ratio (Lyso-IP vs FT) for each subunit of lysosomal vacuolar

1080 ATPase (v-ATPase) in four independent replicates is shown. Inserted scheme showing lysosomal

1081 V-ATPase assembly.

1082 (**E**) Pie chart showing subcellular location categories of lysosome-enriched proteins.

1083

1084 **Figure 3. Lysosomal proteome heterogeneity across tissues**

1085 (**A**) Example images of transgenic strains carrying Lyso-Tag (LMP-1::RFP-3×HA) driven by

1086 four different tissue-specific promoters. Scale bar=20 μm.

1087 (**B-E**) Scatter plot showing the relative enrichment ratio for each of 216 lysosome-enriched

1088 proteins identified from whole-body LMP-1 Lyso-IP in comparison with tissue-specific LMP-1

1089 Lyso-IPs, hypodermis (**B**), muscle (**C**), intestine (**D**) and neuron (**E**). X axis, enrichment ratio

1090 tissue-specific vs. whole-body; Y axis, normalized protein abundance over LMP-1; each dot

1091 represents the average of three replicates.

1092 (**F**) Heatmap showing the relative enrichment of 216 lysosome-enriched proteins identified from

1093 whole-body LMP-1 Lyso-IP in comparison with tissue-specific LMP-1 Lyso-IPs. Group I,

1094 comparable ratios between whole-body and tissue-specific Lyso-IPs; Group II, increase in tissue-

1095 specific Lyso-IPs (*p<0.05* by student's t-test); Group III, decrease in tissue-specific Lyso-IPs

1096 (*p<0.05* by student's t-test); Group IV, absent in tissue-specific IPs.

1097

1098 **Figure 4. Lysosomal proteome in different pro-longevity models**

1099 (**A**) Scheme showing four different longevity regulatory mechanisms used in this study. Loss-of-

1100 function mutants (lf) of *isp-1, daf-2,* and *glp-1* reduce mitochondrial electron transport chain

1101 (ETC) complex III, insulin/IGF-1 signaling, and germline stem cell proliferation, respectively,

1102 leading to lifespan extension; while increasing lysosomal lipolysis by *lipl-4* transgenic

1103 overexpression (*lipl-4 Tg*) promotes longevity.

1104 (**B**) Venn diagram showing the overlap between the lysosome-enriched proteomes from wild-

1105 type (WT) and *lipl-4 Tg* worms.

1106　(**C**) Upset graph showing the distribution and overlap of lysosome-enriched proteins across the

1107　four pro-longevity models. Inserted Venn diagram showing the overlaps between the lysosome-

1108　enriched proteomes of WT worms and the long-lived *daf-2(lf)* and *isp-1(lf)* mutants.

1109

1110　**Figure 5. Increased enrichment of lysosomal proteins upon lysosomal lipolysis**

1111　(**A**) Normalized protein levels (z-score across samples) of autophagy-related components,

1112　mTORC1 signaling factors, lysosomal v-ATPase V0, V1, and transporting accessory (TA)

1113　subunits, lysosomal hydrolases and transporter proteins from LMP-1 Lyso-IP proteomic analyses

1114　of WT, *lipl-4 Tg, daf-2(lf))*, *isp-1(lf)* worms grown at 20°C and WT and *glp-1(lf)* worms grown

1115　at 25°C.

1116　(**B**) The lysosomal enrichment ratio (Lyso-IP vs FT) for two homologs of AMPK catalytic

1117　subunits, AAK-1 and AAK-2 in WT, *lipl-4 Tg*, *daf-2(lf), isp-1(lf) and glp-1(lf)* worms.

1118　(**C**) Reduction of AMPK using the loss-of-function mutant of *aak-2, aak-2(lf)* together with *aak-*

1119　*1* RNAi knockdown decreases lifespan by 17% and 29% in the WT and *lipl-4 Tg* background,

1120　respectively. As a result, the lifespan extension caused by *lipl-4 Tg* is reduced from 72% to 48%.

1121　*** *p<0.001* by Log-rank test. The lifespan data are also in Supplementary File 8.

1122

1123　**Figure 6. Enhanced lysosome-nucleus proximity contributing to longevity**

1124　(**A**) The percentage of proteins with different subcellular localization is compared between

1125　lysosome-enriched proteomes from WT and *lipl-4 Tg* worms. * *p=0.019* by 2-sample test for

1126　equality of proportions.

1127　(**B**) Heatmap showing the average levels of nucleoporin proteins NPP-6 and NPP-15 in Lyso-IP

1128　(IP) and flow-through (FT) samples from WT, *lipl-4 Tg, daf-2(lf),* and *isp-1(lf)* worms.

1129   **(C, E)** Representative images of intestinal cells in WT, *lipl-4 Tg* (**C**), and *daf-2(lf)* (**E**) worms

1130   carrying LMP-1::RFP-3×HA and nucleus-enriched GFP, showing the accumulation of

1131   lysosomes around the perinuclear region in the *lipl-4 Tg* but not *daf-2(lf)* worms. Dashed lines

1132   circle intestinal cells and n marks the nucleus. Scale bar=20 μm.

1133   **(D, F)** Line graph showing the spatial distribution of lysosomes from the nuclear to peripheral

1134   region quantified by normalized regional RFP fluorescence signals in intestinal cells of WT, *lipl-*

1135   *4 Tg* (**D**), and *daf-2(lf)* (**F**) worms. N =50 WT /33 *lipl-4 Tg*, 33 WT/ 28 *daf-2(lf)*. Data are

1136   represented as mean ± SD. *p* values for (D) (from left to right): 1.23×10^{-7}, 2.25×10^{-5}, 0.00322,

1137   0.368, 0.273, 0.0447, 0.00268, 1.20×10^{-5}; *p* values for (F) (from left to right): 0.633, 0.0211,

1138   0.00259, 0.0359, 0.767, 0.151, 0.106, 0.0671.

1139   **(G-H)** *lipl-4 Tg* worms show lifespan extension compared to WT worms (**G**), which is fully

1140   suppressed by RNAi knockdown of *npp-6* (**H**). ****p<0.001,* n.s. *p>0.05* by Log-rank test.

1141   **(I-J)** *daf-2(lf)* worms show lifespan extension compared to WT worms (**I**), which is not affected

1142   by *npp-6* RNAi knockdown (**J**). ****p<0.001* by Log-rank test.

1143   The lifespan data are also in Supplementary File 8.

1144

1145   **Figure 7. Lysosome-enriched proteome identified with Cystinosin**

1146   **(A)** Example images of transgenic strains carrying CTNS-1 Lyso-Tag (CTNS-1::RFP-3×HA)

1147   with LysoTracker staining to mark lysosomes *in vivo*. Scale bar=5 μm.

1148   **(B)** Venn diagram showing the overlap between lysosome-enriched proteomes using LMP-1

1149   Lyso-IP and CTNS-1 Lyso-IP.

1150   **(C)** Pie chart showing subcellular location categories of lysosome-enriched proteins.

1151   **(D)** The proportion of candidates with lysosomal localization annotation in different candidate

1152   groups. "LMP-1 all" and "CTNS-1 all", all candidates from LMP-1 Lyso-IP and CTNS-1 Lyso-

1153   IP, respectively; "LMP-1 only" and "CTNS-1 only", candidates only identified from LMP-1

1154   Lyso-IP or CTNS-1 Lyso-IP, respectively.

1155   **(E)** Normalized protein levels (z-score across samples) of autophagy-related components and

1156   mTORC1 signaling factors from CTNS-1 Lyso-IP proteomic analyses of WT worms and LMP-1

1157   Lyso-IP proteomic analyses of WT and *lipl-4 Tg* worms.

1158   **(F)** Representative muscle images in the wrmScarlet::LMTR-3 knock-in line crossed with either

1159   LMP-1::mNeonGreen knock-in line or CTNS-1::mNeonGreen knock-in line. Scale bar=20 μm.

1160   **(G)** Normalized protein levels (z-score across samples) of previously annotated lysosomal

1161   proteins from LMP-1 Lyso-IP proteomic analyses of WT and *lipl-4 Tg* worms and CTNS-1

1162   Lyso-IP proteomic analyses of WT worms.

1163

1164   **Figure 8. Lysosome-enriched proteins regulating lysosomal functions**

1165   (**A-E**) Confocal fluorescence microscopy images of intestinal cells in worms stained with

1166   LysoSensor DND-189 and treated with *empty vector* (**A**), *slc36.2* RNAi (**B**), *R144.6* RNAi (**C**),

1167   *vha-5* RNAi (**D**) and *unc-32* RNAi (**E**). Scale bar=50 μm.

1168   **(F, G)** RNAi knockdown of *unc-32* or *vha-5* decreases the lysosome number *(****p< 0.0001)* (**F**)

1169   but increases the lysosome size *(****p< 0.0001, *** p<0.001)* (**G**). The average lysosome

1170   number and size per pair of intestinal cells were quantified. Data are shown as mean ± standard

1171   deviation (SD). Student *t*-test (unpaired, two-tailed) was performed between the *empty vector*

1172   and RNAi-treated groups. At least three independent experiments with ~ 10 worms in each were

1173   performed for each condition. n.s. *p>0.05,*

1174 (**H**) RNAi knockdown of *R144.6* and *unc-32 (***p< 0.001)* increase and decrease lysosomal pH,

1175 respectively. Lysosomal pH was calculated based on LysoSensor's lifetime measured by

1176 Fluorescence Lifetime Microscopy. Data are shown as mean ± SD. Student t-test (unpaired,

1177 two-tailed) was performed between the *empty vector* and RNAi-treated groups. Two independent

1178 experiments with at least 5 worms in each were performed in *R144.6* RNAi and *unc-32* RNAi

1179 conditions. The *vha-5* and *slc36.2* RNAi knockdown did not show significant changes in one

1180 replicate and were not retested with another replicate. n.s. *p>0.05*.

1181 (**I**) The structure of the R144.6 protein predicted by AlphaFold2 supports it as a solute carrier

1182 family transporter.

1183 (**J**) Confocal fluorescence microscopy images show that mNeonGreen signals from

1184 endogenously tagged R144.6 colocalize with LysoTracker Red signals in the hypodermis. Scale

1185 bar=10 μm.

1186
1187 **Figure 1-figure supplement 1. Analysis of *LysoTg* lines and Lyso-IP profiling in wild-type**

1188 **worms**

1189 (**A**) Developmental timing of WT and transgenic strains expressing LMP-1 and CTNS-1 Lyso-

1190 Tag (LMP-1 and CTNS-1 *LysoTg*). n.s. *p>0.05* by Chi-squared test.

1191 (**B**) Lifespan of WT, LMP-1 *LysoTg,* and CTNS-1 *LysoTg* worms. The lifespan data are also in

1192 Supplementary File 8.

1193 (**C**) Correlation analysis of four independent biological replicates of Lyso-IP (IP) and Flow-

1194 through (FT) samples from proteomics analyses.

1195 **Figure 2-figure supplement 1. Pie chart showing the proportion of LMP-1 Lyso-IP**

1196 **candidates from WT worms with mammalian homologs.**

1197

1198 **Figure 3-figure supplement 1. Tissue-specific Lyso-IPs and candidate imaging**

1199 **(A-D)** Pearson Correlation matrices of tissue-specific lyso-IP (IP) samples and flow-through (FT)

1200 samples show the correlation among three different replicates. **(A)** Hypodermis, **(B)** Muscle, **(C)**

1201 Intestine, **(D)** Neuron.

1202 **(E)** Representative images showing colocalization of Y58A7A.1::mNeonGreen and LysoTracker

1203 Red in the hypodermis. Scale bar=20 μm.

1204

1205 **Figure 4-figure supplement 1. Lyso-IP analyses from different long-lived strains.**

1206 (**A-D)** Correlation analysis of three independent biological replicates of Lyso-IP (IP) and Flow-

1207 through (FT) from proteomics analyses of the long-lived *lipl-4* transgenic strain (*lipl-4 Tg*, **A**),

1208 the *daf-2* loss-of-function mutant (*daf-2(lf)*), **B**), the *isp-1* loss-of-function mutant (*isp-1(lf)*), **C**)

1209 and *glp-1* loss-of-function mutant grown in 25°C (*glp-1(lf)*) 25°C, **D**).

1210 **(E)** PCA analysis of Lyso-IP replicates (IP) and flow-through controls (FT) in LMP-1 Lyso-IP of

1211 WT, *lipl-4 Tg, daf-2(lf),* and *isp-1(lf)* worms.

1212 **(F)** PCA analysis of Lyso-IP replicates (IP) and flow-through controls (FT) in LMP-1 Lyso-IP of

1213 WT and *glp-1(lf)* worms grown at 25°C.

1214 (**G**) Venn diagram showing the overlap between the lysosome-enriched proteomes from WT and

1215 *glp-1(lf)* worms grown at 25°C.

1216 (**H**) Upset graph showing the overlap of lysosome-enriched proteins present in the long-lived

1217 worms but absent from WT worms.

1218

1219 **Figure 6-figure supplement 1. Lysosomal positioning in longevity regulation**

1220 (**A**) Summary of the method flow for quantifying the lysosomal distribution in intestinal cells of

1221 *C. elegans*. Scale bar=10 μm.

1222 (**B, C**) Curve graph showing the normalized accumulated intensity of lysosomal signals from the

1223 nuclear to the peripheral region in WT, *lipl-4 Tg* (**B**), and *daf-2(lf)* (**C**) animals. *p<0.05*;

1224 ***p<0.01*, ****p<0.001*, *****p<0.0001*, n.s. *p>0.05* by Student's t-test (unpaired, two-tailed) for

1225 each region. N =50 WT /33 *lipl-4 Tg*, 33 WT/ 28 *daf-2(lf)*. Data are represented as mean ± SD. *p*

1226 values for (B) (from left to right): 2.65×10^{-8}, 3.19×10^{-8}, 7.93×10^{-8}, 3.62×10^{-7}, 4.79×10^{-6}, 2.98

1227 $\times 10^{-5}$, 4.41×10^{-5}; *p* values for (C) (from left to right): 0.357, 0.0529, 0.00611, 0.00246, 0.00985,

1228 0.0261, 0.0423.

1229 (**D-E**) *isp-1(lf)* worms show lifespan extension compared to WT worms (**D**), which is not

1230 affected by RNAi knockdown of *npp-6* (**E**). ****p<0.001* by Log-rank test.

1231 (**F-H**) *lipl-4 Tg* worms show lifespan extension compared to WT worms (**F**), which is not

1232 affected by *xpo-1* RNAi knockdown (**G**) and is partially suppressed by RNAi knockdown of

1233 *ima-3* (**H**). ****p<0.001* by Log-rank test.

1234 The lifespan data are also in Supplementary File 8.

1235 (**I**). The percentage of proteins with different subcellular localization is compared between

1236 lysosome-enriched proteomes from WT and *daf-2* worms.

1237

1238 **Figure 7-figure supplement 1. The colocalization between LMP-1::mNeonGreen and**

1239 **CTNS-1::wrmScarlet in different tissues**

1240 Representative images of knock-in lines with both LMP-1::mNeonGreen and CTNS-

1241 1::wrmScarlet show partial colocalization between LMP-1 and CTNS-1 signals in different

1242 tissues. Scale bar=20 μm.

1243

1244 **Figure 7-figure supplement 2. CTNS-1 Lyso-IPs and LMTR-3 imaging analyses**

1245 (**A**) Correlation analysis of three independent biological replicates of CTNS-1 Lyso-IP (IP) and

1246 Flow-through (FT).

1247 (**B**) PCA analysis of three independent biological replicates of CTNS-1 Lyso-IP (IP) and Flow-

1248 through (FT).

1249 (**C, D**) Example images of knock-in lines with wrmScarlet::LMTR-3 and CTNS-1::mNeonGreen

1250 (**C**) and LMP-1::mNeonGreen (**D**) in hypodermis and intestine. Scale bar=20 μm.

1251

1252 **Figure 8-figure supplement 1. LysoSensor intensity quantification in five candidates**

1253 The LysoSensor signals are visualized by confocal fluorescence microscopy in *empty vector* (**A**),

1254 *slc36.2* RNAi (**B**), *R144.6* RNAi (**C**), *vha-5* RNAi (**D**), and *unc-32* RNAi (**E**) conditions. Scale

1255 bar=50 μm. The relative LysoSensor changes were quantified in (**F**). ~ 10 worms were quantified

1256 in each condition. Data are shown as mean ± SD. Student t-test (unpaired, two-tailed) was

1257 performed between the *empty vector* and RNAi-treated groups. *(**p< 0.01, ****p< 0.0001).*

1258

1259 **Supplementary File 1. Lysosome-enriched proteins identified from LMP-1 Lyso-IP using**

1260 **WT worms**

1261 **Supplementary File 2. Lysosome-enriched proteome exhibits tissue-specificity**

1262 **Supplementary File 3. Lysosome-enriched proteins identified from LMP-1 Lyso-IP using**

1263 *lipl-4 Tg* **worms**

1264 **Supplementary File 4. Lysosome-enriched proteins identified from LMP-1 Lyso-IP using**

1265 *daf-2(lf)* **mutant**

1266   **Supplementary File 5. Lysosome-enriched proteins identified from LMP-1 Lyso-IP using**

1267   ***isp-1(lf)* mutant**

1268   **Supplementary File 6. Lysosome-enriched proteins identified from LMP-1 Lyso-IP using**

1269   ***glp-1(lf)* mutant in 25°C**

1270   **Supplementary File 7. Lysosome-enriched proteins identified from LMP-1 Lyso-IP using**

1271   **WT worms in 25°C**

1272   **Supplementary File 8. Summary of lifespan analyses**

1273   **Supplementary File 9. Lysosome-enriched proteins identified from CTNS-1 Lyso-IP using**

1274   **WT worms**

1275   **Supplementary File 10. LysoSensor screening of lysosome-enriched proteins shared**

1276   **between LMP-1 and the CTNS-1 Lyso-Ips**

1277   **Source Code File 1. Matlab code for lysosome distribution quantification**

A

Lyso-Tag (HA) — Harvest → Homogenize → Lysate → Centrifuge → Pellet / Supernatant → anti-HA IP → Flow-through / Lyso-IP → LC/MS profiling → Data analyses

WT — Harvest → Homogenize → Lysate → Centrifuge → Pellet / Supernatant → anti-HA IP → WT control → LC/MS profiling → Data analyses

Flow-through / Lyso-IP / WT control

B Worms carrying LMP-1::RFP::HA
Lysotracker | LMP-1-RFP | Merge

C Beads carrying purified lysosomes
Lysotracker | LMP-1-RFP | Merge

D
Lyso-IP | Flow-through | Pellet

anti-LMP-1 — Lysosome
anti-HSP-60 — Mitochondria
anti-β-actin — Cytosol
anti-CYP-33 — ER
anti-SVQ-8 — Golgi

E
Lyso-IP
Flow-through
PC2 (15.5%)
PC1 (31.2%)
#1 #2 #3 #4

A

Repeated times in replicates
● 1/4 ● 2/4 ● 3/4 ● 4/4

Replicate #1
Replicate #2
Replicate #3
Replicate #4

Lyso-IP (Log$_{10}$)
Flow-through (Log$_{10}$)

B

● 216 candidates

Lyso-IP (Log$_2$)
Ctrl IP (Log$_2$)

C

■ Channel/Transporter
■ Enzyme
■ Signaling
■ Structure
■ Vesicle Trafficking
■ Other/Unknown

E

■ Lysosome/Endosome
■ Plasma membrane
■ Cytoplasm
■ ER/Golgi
■ Extracellular
■ Synaptic vesicle
■ Mitochondria
■ Lipid droplet
■ Peroxisome
■ Unknown

D

Replicate ■ #1 ■ #2 ■ #3 ■ #4

Fold Change (Lyso-LP vs. FT)

VHA-4 VHA-5 VHA-6 VHA-7 VHA-16 UNC-32 VHA-1 VHA-2 VHA-3 VHA-17
V-ATPase V0 domain subunits

VHA-13 VHA-12 VHA-11 VHA-14 VHA-8 VHA-9 VHA-10 VHA-15
V-ATPase V1 domain subunits

VHA-19 VHA-20
V-ATPase transporting acccesory

A: VHA-13
B: VHA-12
C: VHA-11
D: VHA-14
E: VHA-8
F: VHA-9
G: VHA-10
H: VHA-15

a: VHA-5,6,7, UNC-32
c: VHA-1,2,3,4
d: VHA-16
e: VHA-17

AC45: VHA-19
Atp6ap2: VHA-20

A

Hypodermis
col-12p::Lyso-Tag

Muscle
myo-3p::Lyso-Tag

Intestine
ges-1p::Lyso-Tag

Neuron
unc-119p::Lyso-Tag

B Hypodermis-specific vs. Whole-body

- Increase (*p<0.05*)
- Decrease (*p<0.05*)
- No change (*p>0.05*)

Target protein/LMP-1 (Log_{10})

Hypodermis-specific/Whole-body (Log_{10})

C Muscle-specific vs. Whole-body

- Increase (*p<0.05*)
- Decrease (*p<0.05*)
- No change (*p>0.05*)

Target protein/LMP-1 (Log_{10})

Muscle-specific/Whole-body (Log_{10})

D Intestine-specific vs. Whole-body

- Increase (*p<0.05*)
- Decrease (*p<0.05*)
- No change (*p>0.05*)

Target protein/LMP-1 (Log_{10})

Intestine-specific/Whole-body (Log_{10})

E Neuron-specific vs. Whole-body

- Increase (p<0.05)
- Decrease (p<0.05)
- No change (p>0.05)

Target protein/LMP-1 (Log_{10})

Neuron-specific/Whole-body (Log_{10})

F

Whole-body Hypodermis Muscle Intestine Neuron

I

II

Y58A7A.1

III

IV

10 8 6 4 2 0

A

Autophagy: ATG-9, C33A11.2, EPG-7, VAMP-7, Y75B8A.24, SQST-1

Ragulator: LMTR-2, LMTR-3, LMTR-5

V-ATPase V0: VHA-4, VHA-5, VHA-6, VHA-7, VHA-16, UNC-32, VHA-1, VHA-2, VHA-3, VHA-17

V1: VHA-13, VHA-12, VHA-11, VHA-14, VHA-8, VHA-9, VHA-10, VHA-15

TA: VHA-19, VHA-20

Hydrolase: BGAL-1, Y105E8B.9, CPR-6, F37H8.5, C29E4.10, IMP-1, LGMN-1, GBA-3, LIPL-7, ACP-5, PCP-1, Y55D5A.3, ASM-1, F02E9.7, PHO-1, Y71H2AM.2, CTSA-3.1

Transporter: HAF-8, CTNS-1, F27D9.2, CUP-5, CUP-15, SMF-1, UNC-1, CATP-6, CATP-7, CHUP-1, T14B4.3, CLH-6, F13H10.3

Columns: FT #1, FT #2, FT #3, IP #1, IP #2, IP #3 — *glp-1(lf)* LMP-1 (25°C); FT #1, FT #2, FT #3, IP #1, IP #2, IP #3 — *WT* LMP-1 (25°C); FT #1, FT #2, FT #3, IP #1, IP #2, IP #3 — *isp-1(lf)* LMP-1; FT #1, FT #2, FT #3, IP #1, IP #2, IP #3 — *daf-2(lf)* LMP-1; FT #1, FT #2, FT #3, FT #4, IP #1, IP #2, IP #3, IP #4 — *WT* LMP-1; FT #1, FT #2, FT #3, IP #1, IP #2, IP #3 — *lipl-4 Tg* LMP-1

Color scale: -4, -2, 0, 2, 4

B

Lysosome Enrichment (IP/FT, Log$_{10}$); AAK-2, AAK-1; x-axis: *WT*, *lipl-4 Tg*, *daf-2(lf)*, *glp-1(lf)*, *isp-1(lf)*

C

Survival % vs Age [days]

- *WT EV* (black) | 72% *** | 17% ***
- *lipl-4 Tg EV* (magenta)
- *aak-2(lf) aak-1 RNAi* (blue) | 48% *** | 29% ***
- *lipl-4 Tg; aak-2(lf) aak-1 RNAi* (purple)

A Worms carrying CTNS-1::RFP::HA
Lysotracker | CTNS-1-RFP | Merge

B CTNS-1 198 | 95 | LAMP-1 121

C
- Lysosome/Endosome
- Plasma Membrane
- Cytoplasm
- ER/Golgi
- Extracellular
- Synaptic Vesicle
- Lipid Droplet
- Peroxisome
- Cytoskeleton
- Nucleus
- Unknown

D Lysosomal annotation (%)
LMP-1 all | CTNS-1 all | LMP-1 only | CTNS-1 only

E
ATG-9, C33A11.2, EPG-7, VAMP-7 — Autophagy
LMTR-2, LMTR-3, LMTR-5, F13H10.3, CUP-5 — mTORC1
FT #1, FT #2, FT #3, FT #4, IP #1, IP #2, IP #3, IP #4 — WT LMP-1
FT #1, FT #2, FT #3, IP #1, IP #2, IP #3 — WT CTNS-1
FT #1, FT #2, FT #3, IP #1, IP #2, IP #3 — lipl-4 Tg LMP-1

F
LMP-1-mNG | wrmSC-LMTR-3 | Merge — Muscle
CTNS-1-mNG | wrmSC-LMTR-3 | Merge

G
SCAV-3, LMP-1, LMP-2, CUP-5.1, SLC-36.2, Y51F10.4, LAAT-1, CTNS-1, F13H10.3.1 — Transmembrane
VHA-1, VHA-2, VHA-3, VHA-5, VHA-6, VHA-7, VHA-16, VHA-17, VHA-19, UNC-32, VHA-8, VHA-9, VHA-10, VHA-11, VHA-12, VHA-13, VHA-14, VHA-15 — v-ATPase
CTSA-4.1, CTSA-1.1, CTSA-2, Y16B4A.2, CTSA-3.1, ASP-1, ASP-3, ASP-4, ASP-13, CPR-1, CPR-3, CPR-4, CPR-5, CPR-6, LGMN-1, CPL-1, CPZ-1, TAG-196 — Cathepsin protease
ASM-1, SUL-2, HEX-1, HEX-2, HEX-3, HEX-5, GBA-1, GBA-3, GBA-4, PHO-1, Y105E8B.9, M05B5.4, LIPL-5, LIPL-7 — Non-protease hydrolase
FT #1, FT #2, FT #3, FT #4, IP #1, IP #2, IP #3, IP #4 — WT LMP-1
FT #1, FT #2, FT #3, IP #1, IP #2, IP #3 — WT CTNS-1
FT #1, FT #2, FT #3, IP #1, IP #2, IP #3 — lipl-4 Tg LMP-1

LysoSensor Green

A — *Empty vector*
B — *slc-36.2 RNAi*
C — *R144.6 RNAi*
D — *vha-5 RNAi*
E — *unc-32 RNAi*

F

Relative changes (lysosome number)

n.s.
****

EV R144.6 slc36.2 unc-32 vha-5

G

Relative changes (lysosome size)

n.s.
**** ***

EV R144.6 slc36.2 unc-32 vha-5

H

Lysosomal pH

*** n.s.
*** n.s.

EV R144.6 slc36.2 unc-32 vha-5

I

Cytoplasma

N   C

Lumen

J

R144.6::mNeonGreen | LysoTracker | Merged

A

L1-L3 / early L4 (36 hours)

L1-L3 / early L4 / middle L4 / late L4 (48 hours)

L3 / lateL4 / young adult / adult (60 hours)

n.s.

WT / LMP-1 LysoTg / CTNS-1 LysoTg

B

WT / LMP-1 LysoTg / CTNS-1 LysoTg

C

WT Lyso-IP

	IP-1	IP-2	IP-3	IP-4
IP-1	1.00	0.72	0.74	0.80
IP-2	0.72	1.00	0.95	0.92
IP-3	0.74	0.95	1.00	0.95
IP-4	0.80	0.92	0.95	1.00

	FT-1	FT-2	FT-3	FT-4
FT-1	1.00	0.81	0.60	0.88
FT-2	0.81	1.00	0.75	0.74
FT-3	0.60	0.75	1.00	0.50
FT-4	0.88	0.74	0.50	1.00

A *col-12p::Lyso-Tag Tg*

	IP_1	IP_2	IP_3		FT_1	FT_2	FT_3
IP_1	1.00	0.97	0.94	FT_1	1.00	0.92	0.85
IP_2	0.97	1.00	0.93	FT_2	0.92	1.00	0.96
IP_3	0.94	0.93	1.00	FT_3	0.85	0.96	1.00

B *myo-3p::Lyso-Tag Tg*

	IP_1	IP_2	IP_3		FT_1	FT_2	FT_3
IP_1	1.00	0.88	0.91	FT_1	1.00	0.96	0.77
IP_2	0.88	1.00	0.97	FT_2	0.96	1.00	0.78
IP_3	0.91	0.97	1.00	FT_3	0.77	0.78	1.00

C *ges-1p::Lyso-Tag Tg*

	IP_1	IP_2	IP_3		FT_1	FT_2	FT_3
IP_1	1.00	0.90	0.90	FT_1	1.00	0.91	0.65
IP_2	0.90	1.00	0.96	FT_2	0.91	1.00	0.64
IP_3	0.90	0.96	1.00	FT_3	0.65	0.64	1.00

D *unc-119::Lyso-Tag Tg*

	IP_1	IP_2	IP_3		FT_1	FT_2	FT_3
IP_1	1.00	0.98	0.62	FT_1	1.00	0.98	0.79
IP_2	0.98	1.00	0.62	FT_2	0.98	1.00	0.72
IP_3	0.62	0.62	1.00	FT_3	0.79	0.72	1.00

E

Y58A7A.1::mNeonGreen **LysoTracker Red** **Merge**

A Outline nuclear membrane → Outline plasma membrane → Regions divided

B

Cumulative area fluorescence

- WT
- lipl-4 Tg

*** *** *** *** *** *** *** ***

Nuclear ——————— Peripheral

C

Cumulative area fluorescence

- WT
- daf-2(lf)

** ** ** * *

Nuclear ——————— Peripheral

D

Survival %

- WT
- isp-1(lf) ***

vector ctrl

Age [days]

E

Survival %

- WT
- isp-1(lf) ***

npp-6 RNAi

Age [days]

F

% Survival

- WT
- lipl-4 Tg ***

vector ctrl

Age [days]

G

% Survival

- WT
- lipl-4 Tg ***

xpo-1 RNAi

Age [days]

H

% Survival

- WT
- lipl-4 Tg ***

ima-3 RNAi

Age [days]
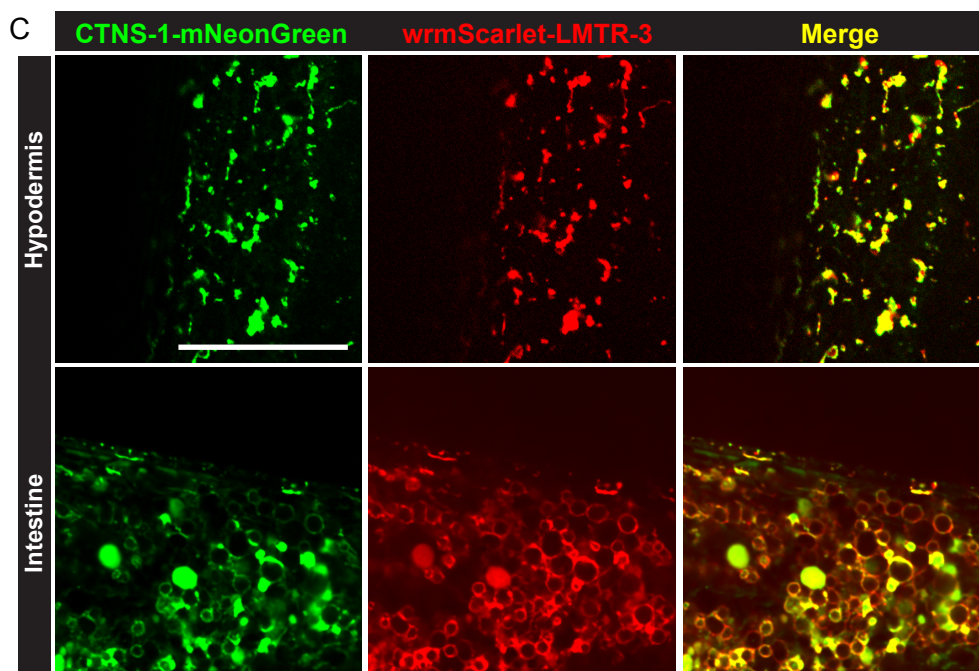
I

- WT
- daf-2(lf)

Unknown
Nucleus
Cytoskeleton
Lipid Droplet
Peroxisome
Mitochondria
Synaptic Vesicle
Extracellular
ER/Golgi
Cytoplasm
Plasma Membrane
Lysosome/Endosome

Percentage of total

LMP-1-mNeonGreen	CTNS-1-wrmScarlet	Merge

Hypodermis

Muscle

Intestine

Neuron

A

WT CTNS-1 Lyso-IP

	IP-1	IP-2	IP-3
IP-1	1.00	0.81	0.86
IP-2	0.81	1.00	0.89
IP-3	0.86	0.89	1.00

	FT-1	FT-2	FT-3
FT-1	1.00	0.98	0.89
FT-2	0.98	1.00	0.89
FT-3	0.89	0.89	1.00

B

CTNS-1 Lyso-IP

WT, FT
WT, IP

PC2 (20.2%)

PC1 (46.7%)

C

CTNS-1-mNeonGreen — wrmScarlet-LMTR-3 — Merge

Hypodermis

Intestine

D

LMP-1-mNeonGreen — wrmScarlet-LMTR-3 — Merge

Hypodermis

Intestine

LysoSensor Green

A *Empty vector*

B *slc-36.2 RNAi*

C *R144.6 RNAi*

D *vha-5 RNAi*

E *unc-32 RNAi*

F